BASIC CELL AND MOLECULAR BIOLOGY



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Gerald Bergtrom

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This text was compiled on 08/19/2026

TABLE OF CONTENTS

Licensing

1: Cell Tour, Life's Properties and Evolution, Studying Cells

1.1: Introduction

1.2: Scientific Method – The Practice of Science

1.3: Domains of Life

1.4: Tour of the Eukaryotic Cell

1.5: How We Know the Functions of Cellular Organelles and Structures- Cell Fractionation

1.6: The Origins, Evolution, Speciation, Diversity and Unity of Life

1.7: Microscopy Reveals Life's Diversity of Structure and Form

1.8: Key Words and Terms

2: Basic Chemistry, Organic Chemistry and Biochemistry

2.1: Introduction

2.2: Atoms and Basic Chemistry

2.3: Chemical bonds

2.4: A Close Look at Water Chemistry

2.5: Some Basic Biochemistry- Carbon, Monomers, Polymers and the Synthesis and Degradation of Macromolecules

2.6: Key Words and Terms

3: Details of Protein Structure

3.1: Introduction

3.2: Levels (Orders) of Protein Structure

3.3: Changes in Protein Shape Can Cause Disease

3.4: Quaternary Structure

3.5: Some Proteins are Chemically Modified and Others Require Prosthetic Groups to be Biologically Active

3.6: Protein Domains, Motifs, and Folds in Protein Structure

3.7: Proteins, Genes and Evolution- How Many Proteins are We?

3.8: View 3D Animated Images of Proteins in the NCBI Database

3.9: Key Words and Terms

4: Bioenergetics

4.1: Introduction

4.2: Kinds of Energy

4.3: Deriving Simple Energy Relationships

4.4: Key Words and Terms

5: Enzyme Catalysis and Kinetics

5.1: Introduction

5.2: Enzymes

5.3: Key Words and Terms

6: Glycolysis, the Krebs Cycle and the Atkins Diet

6.1: Introduction

6.2: Glycolysis (from the Greek glyco (sugar) lysis (Separation), or Sugar Breakdown

6.3: Some Details of Glycolysis

6.4: Gluconeogenesis

6.5: The Atkins Diet and Gluconeogenesis

6.6: The Krebs/TCA/Citric acid cycle

6.7: Key Words and Terms

7: Electron Transport, Oxidative Phosphorylation, and Photosynthesis

7.1: Introduction

7.2: The Electron Transport Chain (ETC)

7.3: Oxidative Phosphorylation

7.4: Photosynthesis

7.5: More Thoughts on the Mechanisms and Evolution of Respiration and Photosynthesis

7.6: Key Words and Terms

8: DNA, Chromosomes and Chromatin

8.1: Introduction

8.2: The Stuff of Genes

8.3: DNA Structure

8.4: Genes and Chromatin in Eukaryotes

8.5: Structure and Organization of DNA in Bacteria

8.6: Key Words and Terms

9: Details of DNA Replication and Repair

9.1: Introduction

9.2: DNA Replication

9.3: DNA Repair

9.4: Key Words and Terms

10: Transcription and RNA Processing

10.1: Introduction

10.2: Overview of Transcription

10.3: Details of Transcription

10.4: Details of Eukaryotic mRNA Processing

10.5: Ribosomal RNA Processing in Eukaryotic Nuclei

10.6: tRNA Processing in Eukaryotic Nuclei

10.7: RNA and Ribosome Export from the Nucleus

10.8: Key Words and Terms

11: The Genetic Code and Translation

11.1: Introduction

11.2: An Overview of the Genetic Code

11.3: Gene and Protein Colinearity and Triplet Codons
11.4: Translation
11.5: Key Words and Terms

12: Regulation of Transcription and Epigenetic Inheritance
12.1: Introduction
12.2: Gene Regulation in Prokaryotes
12.3: The Problem with Unregulated (Housekeeping) Genes in All Cells
12.4: Gene Regulation in Eukaryotes
12.5: Epigenetics
12.6: Key Words and Terms

13: Post Transcriptional Regulation of Gene Expression
13.1: Introduction
13.2: Post-transcriptional Control of Gene Expression
13.3: Eukaryotic Regulation of Translation
13.4: Key Words and Terms

14: Repetitive DNA - A Eukaryotic Genomic Phenomenon
14.1: Introduction
14.2: The Complexity of Genomic DNA
14.3: The 'Jumping Genes' of Maize
14.4: Transposons Since McClintock
14.5: On the Evolution of Transposons, Genes, and Genomes
14.6: Roles of Transposition in Evolution and Diversity
14.7: Coping with the Dangers of Rampant Transposition
14.8: Key Words and Terms

15: DNA Technologies
15.1: Overview
15.2: Make and Screen a cDNA Library
15.3: DNA Sequencing
15.4: Genomic Libraries
15.5: The Polymerase Chain Reaction (PCR)
15.6: Genomic Approaches- The DNA Microarray
15.7: Ome-Sweet Ome
15.8: From Genetic Engineering and Genetic Modification
15.9: Key Words and Terms

16: Membrane Structure
16.1: Overview
16.2: Plasma Membrane Structure
16.3: Membrane Proteins
16.4: How Membrane Proteins are Held in Membranes

16.5: A Diversity of Membrane Protein Functions
16.6: Glycoproteins
16.7: Glycolipids
16.8: Glycoproteins and Human Health
16.9: Key Words and Terms

17: Membrane Function

17.1: Introduction
17.2: Membrane Transport
17.3: Ligand and Voltage Gated Channels in Neurotransmission
17.4: Endocytosis and Exocytosis
17.5: Directing the Traffic of Proteins in Cells
17.6: How Cells are Held Together and How they Communicate
17.7: 17.7 Signal Transduction
17.8: Key Words and Terms

18: The Cytoskeleton and Cell Motility

18.1: Introduction
18.2: Cytoskeletal Components
18.3: The Molecular Structure and Sub-Cellular Organization of Cytoskeletal Components
18.4: Key Words and Terms

19: Cell Division and the Cell Cycle

19.1: Introduction
19.2: Bacterial Cell Division and the Eukaryotic Cell Cycle
19.3: Regulation of the Cell Cycle
19.4: When Cells Die
19.5: Disruption of the Cell Cycle Checkpoints Can Cause Cancer
19.6: Key Words and Terms

20: The Origins of Life

20.1: Introduction
20.2: Thinking about Life's Origins- A Short Summary of a Long History
20.3: Formation of Organic Molecules in an Earthly Reducing Atmosphere
20.4: Origins of Organic Molecules in a Non-Reducing Atmosphere
20.5: Origins of Life Chemistries in an RNA World
20.6: Molecules Talk- Selecting Molecular Communication and Complexity
20.7: A Summary and Some Conclusions
20.8: Key Words and Terms

Index

Glossary

Glossary

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1: CELL TOUR, LIFE'S PROPERTIES AND EVOLUTION, STUDYING CELLS

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast *hypotheses* and *theories* and place them and other elements of the scientific enterprise into their place in the cycle of the *scientific method*.
2. compare and contrast structures common to and that distinguish *prokaryotes*, *eukaryotes* and *archaea*, and groups within these *domains*.
3. articulate the function of different cellular substructures.
4. explain how *prokaryotes* and *eukaryotes* accomplish the same functions, i.e. have the same *properties of life*, even though prokaryotes lack most of the structures.
5. outline a procedure to study a specific cell *organelle* or other substructure.
6. describe how the different structures (particularly in eukaryotic cells) relate/interact with each other to accomplish specific functions.
7. describe some structural and functional features that distinguish prokaryotes (eubacteria), eukaryotes and archaea.
8. place cellular organelles and other substructures in their evolutionary context, i.e., describe their origins and the selective pressures that led to their *evolution*.
9. distinguish between the random nature of *mutation* and *natural selection* in evolution
10. relate archaea to other life forms and speculate on their origins in evolution.
11. suggest why evolution leads to more complex ways of sustaining life,
12. explain how *fungi* are more like animals than plants.

1.1: Introduction

1.2: Scientific Method – The Practice of Science

1.3: Domains of Life

1.4: Tour of the Eukaryotic Cell

1.5: How We Know the Functions of Cellular Organelles and Structures- Cell Fractionation

1.6: The Origins, Evolution, Speciation, Diversity and Unity of Life

1.7: Microscopy Reveals Life's Diversity of Structure and Form

1.8: Key Words and Terms

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1.1: Introduction

You will read in this book about experiments that revealed secrets of cell and molecular biology, many of which earned their researchers Nobel and other prizes. But let's begin here with a *Tale of Roberts*, two among many giants of science in the renaissance and age of enlightenment whose seminal studies came too early to win a Nobel Prize.

One of these, **Robert Boyle**, was born in 1627 to wealthy, aristocrat parents. In his teens, after the customary *Grand Tour* of renaissance Europe (Greece, Italy...) and the death of his father, he returned to England in 1644, heir to great wealth. In the mid-1650s he moved from his estates to Oxford where he set about studying physics and chemistry. He built a laboratory with his own money in order to do experiments on the behavior of gasses under pressure, and with a little help, discovered *Boyle's Law*, confirming that the gasses obey mathematical rules. He is also credited with showing that light and sound could travel through a vacuum, that something in air enables combustion, that sound travels through air in waves, that heat and particulate motion were related, and that the practice of alchemy was bogus! In fact, Boyle pretty much converted alchemy to chemistry by doing *chemical analysis*, a term he coined. As a chemist, he also rejected the old Greek concept of earth, air, fire and water elements. Instead, he defined elements as we still do today: the element is the smallest component of a substance that cannot be further chemically subdivided. He did this a century before Antoine Lavoisier listed and define the first elements! Based on his physical studies and chemical analysis, Boyle even believed that the indivisible unit of elements were atoms, and that the behavior of elements could be explained by the motion of atoms. Boyle later codified in print the scientific method that made him a successful experimental scientist.

The second of our renaissance Roberts was **Robert Hooke**, born in 1635. In contrast to Boyle parents, Hooke's were of modest means. They managed nonetheless to nurture their son's interest in things mechanical. While he never took the Grand Tour, he learned well and began studies of chemistry and astronomy at Christ Church College, Oxford in 1653. To earn a living, he took a position as Robert Boyle's assistant. It was with Hooke's assistance that Boyle did the experiments leading to the formulation of Boyle's Law. While at Oxford, he made friends and useful connections. One friend was the architect Christopher Wren. In 1662, Boyle, a founding member of the Royal Society of London, supported Hooke to become the society's *curator of experiments*. However, to support himself, Hooke hired on as professor of geometry at Gresham College (London). After "the great fire" of London in 1666, Hooke, as city surveyor and builder, participated with Christopher Wren in the design and reconstruction of the city. Always interested in things mechanical, he also studied the elastic property of springs. This led him to *Hooke's Law*, which said that the force required to compress a spring was proportional to the length the spring was compressed. In later years these studies led Hooke to imagine how a coil spring might be used (instead of a pendulum) to regulate a clock. While he never invented such a clock, he was appointed to a Royal Commission to find the first reliable method to determine longitude at sea. He must have been gratified to know that the solution to accurate determination of longitude at sea turned out to involve a coil- spring clock! Along the way in his 'practical' studies, he also looked at little things, publishing his observations in *Micrographia* in 1665. Therein, he described microscopic structures of animal parts and even snowflakes. He also described fossils as having once been alive, and compared structures in thin slices of cork that he saw in his microscope to monk's cells (rooms, chambers) in a monastery. Hooke is best remembered for his law of elasticity, and of course, for coining the word *cell*, which we now understand as the smallest unit of living things.

Now fast-forward almost 200 years to observations of plant and animal cells early in the 19th century. Many of these studies revealed common structural features including a nucleus, a boundary wall and a common organization of cells into groups to form multicellular structures of plants and animals and even lower life forms. These studies led to the first two precepts of ***Cell Theory***:

Cell Theory

1. Cells are the basic unit of living things;
2. Cells can have an independent existence.

Later in the century when Louis Pasteur disproved notions of *spontaneous generation*, and German histologists observed *mitosis* and *meiosis* (the underlying events of cell division in eukaryotes) a third precept rounded out Cell Theory: They reproduce.

cell theory

3. Cells come from pre-existing cells.

We begin this chapter with a reminder of the ***scientific method***, that way of thinking about our world that emerged formally in the 17th century. Then we take a tour of the cell, reminding ourselves of basic structures and organelles. After the 'tour', we consider the ***origin of life*** from a common ancestral cell and the subsequent ***evolution*** of cellular complexity and the incredible diversity of life forms. Finally, we consider some of the *method*s we use to study cells. Since cells are small, several techniques of microscopy, cell dissection and functional/biochemical analysis are described to illustrate how we come to understand cell function.

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1.2: Scientific Method – The Practice of Science

For an amusing look at how scientists think, check out *The Pleasure of Finding Things Out: The Best Short Works of Richard Feynman (*1999, New York, Harper Collins). Here we focus on the essentials of the scientific method originally inspired by Robert Boyle, and then look at how science is practiced today. Scientific method refers to a standardized protocol for observing, asking questions about, and investigating natural phenomena. Simply put, it says look/listen, infer a cause and test your inference. As captured by the Oxford English Dictionary, the essential inviolable commonality of all scientific practice is that it relies on "systematic observation, measurement, and experiment, and the formulation, testing and modification of hypotheses."

A. The Method

Adherence to the method is not strict, and may sometimes breach adherence to protocol! In the end, scientific method in actual practice recognizes human biases and prejudices and allows deviations from the protocol. Nevertheless, an understanding of scientific method will guides the prudent investigator to balance personal bias against the leaps of intuition that successful science requires. The practice of scientific method by most scientists would indeed be considered a success by almost any measure. Science "as a way of knowing" the world around us constantly tests, confirms, rejects and ultimately reveals new knowledge, integrating that knowledge into our worldview.

Here in the usual order are the key elements of the scientific method:

1. *Observe* natural phenomena (includes reading the science of others).
2. Infer and propose an ***hypothesis*** (explanation) based on objectivity and reason. Hypotheses are declarative sentences that sound like a fact, but aren't! Good hypotheses are testable, easily turned into *if/then (predictive) yes-or-no* questions.
3. Design an experiment to test the hypothesis: results must be measurable evidence for or against the hypothesis.
4. Perform the experiment and then observe, measure, collect data and test for statistical validity (where applicable). Then, repeat the experiment.
5. Consider how your data supports or does not support your hypothesis and then integrate your experimental results with earlier hypotheses and prior knowledge.

But, how do *theories* and *laws* fit into the scientific method?

A scientific ***theory***, contrary to what many people think, is not a guess. Rather, a theory is a statement well supported by experimental evidence and widely accepted by the scientific community. One of the most enduring, tested theories is of course the theory of evolution. Among scientists, theories might be thought of as 'fact' in common parlance, but we recognize that they are still subject to testing and, modification, and may even be overturned. While some of Darwin's notions have been modified over time, in this case, those modifications have only strengthened our understanding that species diversity is the result of natural selection. You can check out some of Darwin's own work (1859, 1860; *The Origin of Species*] at Origin of Species. For more recent commentary on the evolutionary underpinnings of science, check out Dobzhansky T (1973, *Nothing in biology makes sense except in the light of evolution*. Am. Biol. Teach. 35:125-129) and Gould, S.J. (2002, *The Structure of Evolutionary Theory*. Boston, Harvard University Press).

A **scientific *Law*** is thought of as universal and even closer to 'fact' than a theory! Scientific laws are most common in math and physics. In life sciences, we recognize Mendel's *Law of Segregation* and *Law of Independent Assortment* as much in his honor as for their universal and enduring explanation of genetic inheritance in living things. But Laws are not facts*!* Laws too, are always subject to experimental test.

Astrophysicists are actively testing universally accepted laws of physics. Strictly speaking, even Mendel's *Law of Independent Assortment* should not be called a law. Indeed, it is not true as he stated it! Check the Mendelian Genetics section of an introductory textbook to see how chromosomal crossing over violates this law.

In describing how we do science, the Wikipedia entry states: "the goal of a scientific inquiry is to obtain knowledge in the form of testable explanations (hypotheses) that can predict the results of future experiments. This allows scientists to gain an understanding of reality, and later use that understanding to intervene in its causal mechanisms (such as to cure disease)." The

better an hypothesis is at making predictions, the more useful it is, and the more likely it is to be correct. In the last analysis, think of Hypotheses as *educated guesses* and think of Theories and/or Laws as one or more experimentally supported hypothesis that everyone agrees should serve as *guideposts* to help us evaluate new observations and hypotheses.

A good hypothesis is a rational guess that explains scientific observations or experimental measurements. Therefore by definition, hypotheses are testable based on predictions based on logic. Additional observation can refine or change the original hypothesis, and/or lead to new hypothesis whose predictive value can also be tested. If you get the impression that scientific discovery is a cyclic process, that's the point! Exploring scientific questions reveals more questions than answers!

We now recognize that a key component of the scientific method is the requirement that the work of the scientist be disseminated by publication! In this way, shared data and experimental methods can be repeated and evaluated by other scientists.

B. Origins of the Scientific Method

Long before the word *scientist* began to define someone who investigated natural phenomena beyond simple observation (i.e., by doing experiments), philosophers developed formal rules of *deductive* and *inferential logic* to try to understand nature, humanity's relationship to nature, and the relationship of humans to each other. In fact, Boyle was not alone in doing experimental science. We therefore owe the logical underpinnings of science to *philosophers* who came up with systems of *deductive* and *inductive logic* so integral to the scientific method. The scientific method grew from those beginnings, along with increasing empirical observation and experimentation. We recognize these origins when we award the Ph.D. (*Doctor of Philosophy),* our highest academic degree! We are about to learn about the life of cells, their structure and function, and their classification, or grouping based on those structures and functions. Everything we know about life comes from applying the principles of scientific method.

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1.3: Domains of Life

We believe with good reason that all life on earth evolved from a common ancestral cell that existed soon after the origins of life on our planet. Too long ago, not all life was divided into two groups: the true bacteria and everything else!

Now we group life into one of three ***domains***:

> They are among the first descendants of that common ancestral cell. They lack nuclei (*pro* meaning *before* and *karyon* meaning *kernel*, or *nucleus*). They include *bacteria* and *cyanobacteria* (blue-green algae).

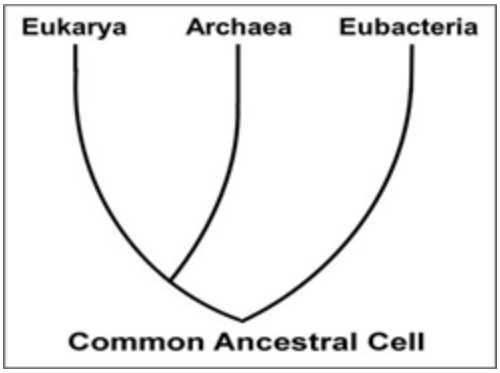
> Include all higher life forms, characterized by cells with true nuclei (Eu, true; *karyon*, *nucleus*)

> (meaning "old" bacteria) include many ***extremophile*** bacteria ('lovers' of life at extreme high temperatures, salinity, etc.). Originally classified as ancient prokaryotes, *Archaebacteria* were shown by 1990 to be separate from prokaryotes and eukaryotes, a third domain of life.

The archaea are found in such inhospitable environments as boiling hot springs or arctic ice, although some also live in conditions that are more temperate. Carl Woese compared the DNA sequences of genes for ribosomal RNAs in normal bacteria and extremophiles. Based on sequence similarities and differences, he concluded that the latter are in fact a domain separate from the rest of the bacteria as well as from eukaryotes. For a review, see (Woese, C. 2004; *A new biology for a new century*. Microbiol. Mol. Biol. Rev. 68:173-186)

The three domains of life (**Archaea**, **Eubacteria** and ***Eukarya***) quickly supplanted the older division of living things into Five Kingdoms, the *Monera (prokaryotes), Protista, Fungi, Plants, and Animals (all eukaryotes!)*. In a final surprise, the sequences of archaebacterial genes clearly indicate a common ancestry of archaea and eukarya.

Thus, Archaea are *not* true bacteria! They share genes and proteins as well as metabolic pathways found in eukaryotes but *not* in bacteria, supporting their close evolutionary relationship to eukaryotes. That they also contain genes and proteins as well as metabolic pathways unique to the group is further testimony to their domain status. Understanding that all living organisms belong to one of three domains has dramatically changing our understanding of evolution. The evolution of the three domains is illustrated below.

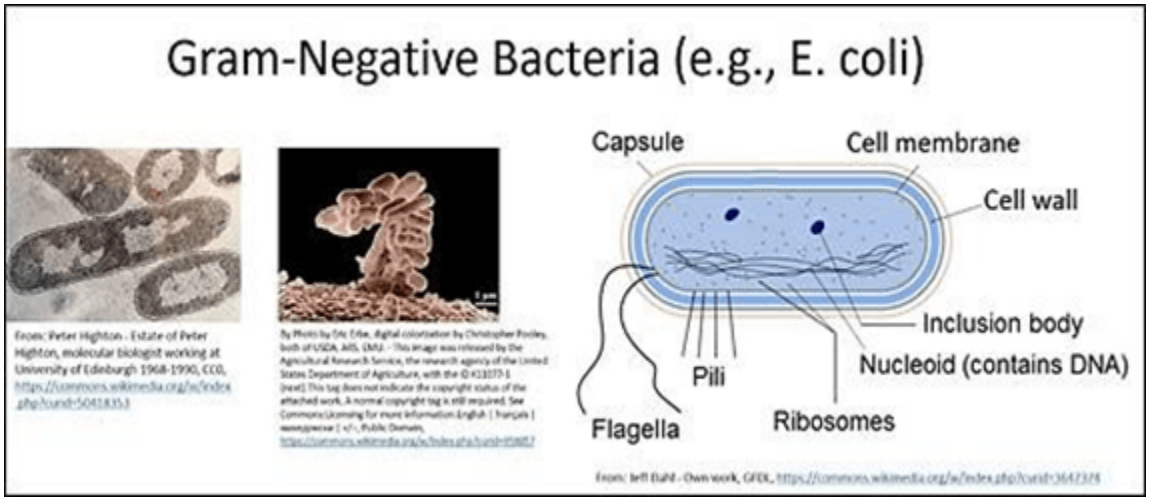


A. The Prokaryotes (Eubacteria = *Bacteria* and C*yanobacteria*)

Prokaryotic cells lack a nucleus and other organelles such as mitochondria, chloroplasts, endoplasmic reticulum, and assorted eukaryotic vesicles and internal membranes. Bacteria do contain *bacterial microcompartments* (BMCs), but these are made up entirely of protein and are ***not*** surrounded by a phospholipid membrane.

These function for example in CO2 fixation to sequester metabolites toxic to the cells. Click Bacterial Organelles for more

information. Bacteria are typically unicellular, although a few (like some cyanobacteria) live colonial lives at least some of the time. Transmission and scanning electron micrographs of rod-shaped bacteria are shown in the example below at the left. A diagram of bacterial structure is also shown (right).

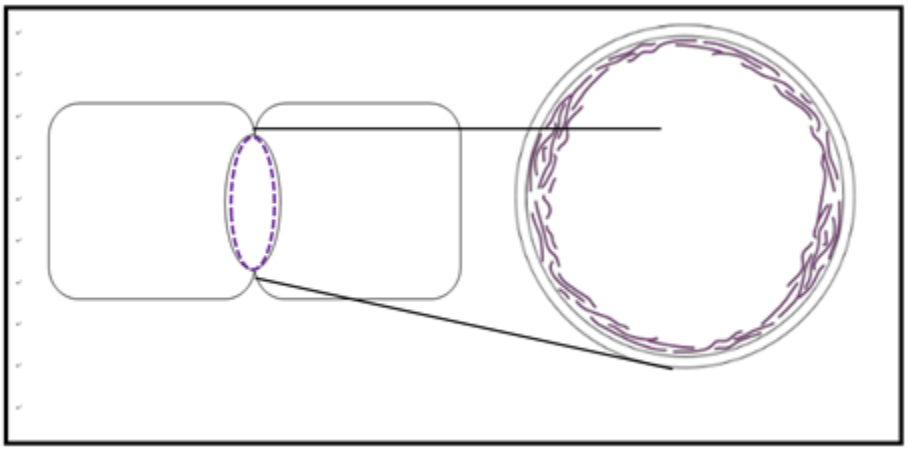


1. Bacterial Reproduction

Without the compartments afforded by the internal membrane systems common to eukaryotic cells, intracellular chemistries, from DNA replication, transcription, translation, and all the metabolic biochemistry of life, happen in the cytoplasm of the cell. DNA is a circular double helix that duplicates as the cell grows. While not enclosed in a nucleus, bacterial DNA is concentrated in a region of the cell called the ***nucleoid***. When not crowded at high density, bacteria replicate their DNA throughout the life of the cell, dividing by ***binary fission***. The result is the equal partition of duplicated bacterial *"chromosomes"* into new cells. The bacterial chromosome is essentially naked DNA, unassociated with proteins.

2. Cell Motility and the Possibility of a Cytoskeleton

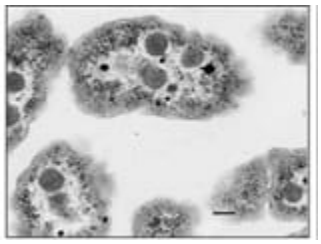
Movement of bacteria is typically by *chemotaxis*, a response to environmental chemicals. Some may respond to other stimuli such as light (*phototaxy*). They can move to or away from nutrients, noxious/toxic substances, light, etc., and achieve motility in several ways. For example, many move using flagella made up largely of the protein ***flagellin***. Flagellin is absent from eukaryotic cells. On the other hand, the cytoplasm of eukaryotic cells is organized by a complex cytoskeleton of rods and tubes made of *actin* and *tubulin* proteins. Prokaryotes were long thought to lack these or similar cytoskeletal components. However, two bacterial genes that encode proteins homologous to eukaryotic actin and tubulin were recently discovered. The *MreB* protein forms a *cortical ring* in bacteria undergoing *binary fission*, similar to the actin cortical ring that pinches dividing eukaryotic cells during *cytokinesis* (the actual division of a single cell into two smaller daughter cells). This is modeled below in the cross-section (right) near the middle of a dividing bacterium (left).



The *FtsZ* gene encodes a homolog of tubulin proteins. It seems that together with flagellin, the MreB and FtsZ proteins may be part of a primitive prokaryotic *cytoskeleton* involved in cell structure and motility.

3. Some Bacteria Have Internal Membranes

While bacteria lack organelles (the membrane-bound structures of eukaryotic cells), internal membranes in some bacteria form as inward extensions (*invaginations*) of plasma membrane. Some of these capture energy from sunlight (photosynthesis) or from inorganic molecules (*chemolithotrophy*). *Carboxysomes* are membrane bound photosynthetic vesicles in which CO2 is fixed (reduced) in cyanobacteria (shown below).



CC-BY; From: en.Wikipedia.org/wiki/File:Carboxysomes_EM.jpg

Photosynthetic bacteria have less elaborate internal membrane systems.

4. Bacterial Ribosomes Do the Same Thing as Eukaryotic Ribosomes... and Look Like Them!

Ribosomes are the protein synthesizing machines of life. *Ribosomes* of prokaryotes are smaller than those of eukaryotes, but are able to translate eukaryotic messenger RNA (mRNA) *in vitro*. Underlying this common basic function is the fact that the ribosomal RNAs of all species share base sequence and structural similarities indicating a long evolutionary relationship. Recall similarities revealed the closer relationship of archaea to eukarya than prokarya.

Clearly, the prokarya (Eubacteria) are a diverse group of organisms, occupying almost every wet, dry, hot or cold nook and cranny of our planet. Despite this diversity, all prokaryotic cells share many structural and functional metabolic properties with each other... and with the archaea and eukaryotes! As we have seen with ribosomes, shared structural and functional properties support the common ancestry of all life. Finally, we not only share common ancestry with prokaryotes, we even share living arrangements with them. Our gut bacteria represent up to 10X more cells than our own! Read more at The NIH Human Microbiome Project. Also check out the following link for A Relationship Between Microbiomes, Diet and Disease**.**

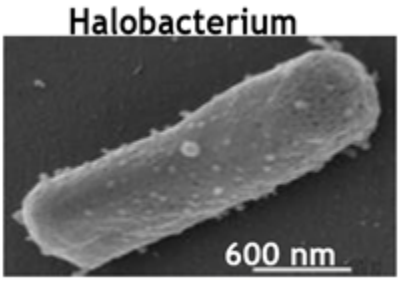
B. The Archaebacteria (Archaea)

Allessandro Volta, a physicist who gave his name to the Volt, discovered methane producing bacteria (*methanogens*) way back in 1776! He found them living in the extreme environment at the bottom of Lago Maggiore, a lake shared by Italy and Switzerland. These unusual bacteria are *cheomoautotrophs* that get energy from H2 and CO2 and also generate methane gas in the process. It was not until the 1960s that Thomas Brock (from the University of Wisconsin-Madison) discovered *thermophilic* bacteria living at temperatures approaching 100oC in Yellowstone National Park in Wyoming. Organisms living in any extreme environment were soon nicknamed *extremophiles*. One of the thermophilic bacteria, now called *Thermus aquaticus*, became the source of *Taq* polymerase, the heat-stable DNA polymerase that made the *polymerase chain reaction* (PCR) a household name in labs around the world!

Extremophile and “normal” bacteria are similar in size and shape(s) and lack nuclei. This initially suggested that most extremophiles were prokaryotes. But as Carl Woese demonstrated, it is the archaea and eukarya that share a more recent common ancestry! While some bacteria and eukaryotes can live in extreme environments, the archaea include the most diverse extremophiles. Here are some examples of extremophiles:

- *Acidophiles*: grow at acidic (low) pH.
- *Alkaliphiles*: grow at high pH.

• *Halophiles*: require high salt concentrations; see *Halobacterium salinarium* below.



CC-BY; Adapted from: https://openi.nlm.nih.gov/imgs/512/185/3495301/PMC3495301_gb0010-0424-f2.png

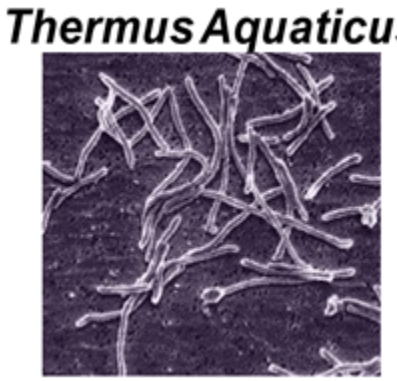
• *Methanogens*: produce methane; a cross section of *Methanosarcina acetivorans* is shown above (right). Note the absence of significant internal structure.

• *Barophiles*: grow best at high hydrostatic pressure.

• *Psychrophiles*: grow best at temperature 15 °C or lower.

• *Xerophiles*: growth at very low water activity (drought or near drought conditions).

• *Thermophiles* and *hyperthermophiles*: organisms that grow best at 40°C or higher, or 80°C or higher, respectively. *Pyrolobus fumarii*, a hyperthermophile, can live at a temperature 113°C. Another thermophile *Thermus aquaticus,* noted for its role in developing the polymerase chain reaction, is shown below.



CC-0 (public domain) Adapted From: https://upload.wikimedia.org/wikipedia/commons/4/48/Thermus aquaticus.JPG

• *Toxicolerants*: grow in the presence of high levels of damaging elements (e.g., pools of benzene, nuclear waste).

Archaea were originally seen as oddities of life, thriving in unfriendly environments. They also include organisms living in less extreme environments, including soils, marshes and even in the human colon. They are also abundant in the oceans where they are a major part of plankton, participating in the carbon and nitrogen cycles. In the guts of cows, humans and other mammals, methanogens facilitate digestion, generating methane gas in the process. In fact, cows have even been cited as a major cause of global warming because of their prodigious methane emissions! On the plus side, methanogenic Archaea are being exploited to create biogas and to treat sewage. Other extremophiles are the source of enzymes that function at high temperatures or in organic solvents. As already noted, some of these have become part of the biotechnology toolbox.

C. The Eukaryotes

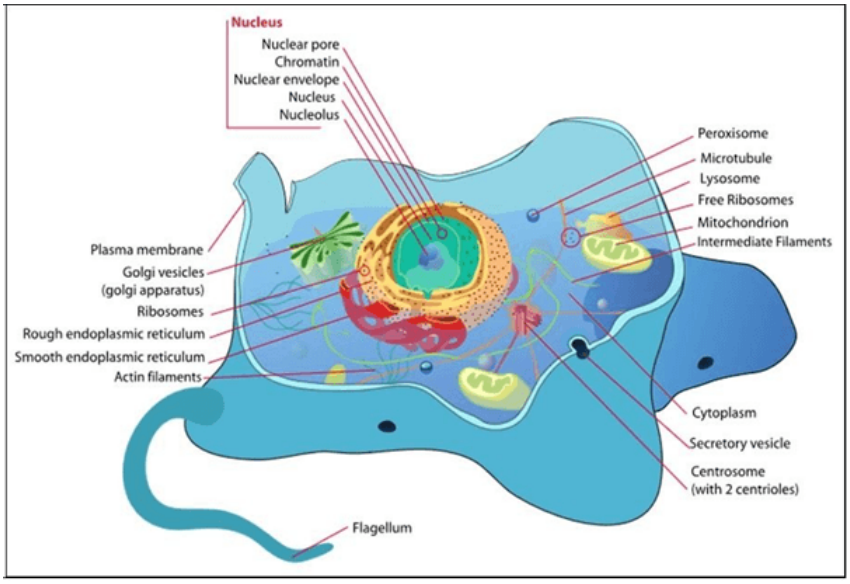
1. Large Compartmentalized Cells

The volume of a typical eukaryotic cell is some 1000 times that of a typical bacterial cell. Eukaryotic life would not even have been possible if not for a division of labor of eukaryotic cells among different *organelles* (membrane-bound structures). Imagine

a bacterium as a 100 square foot room (the size of a small bedroom, or a large walk-in closet!) with one door. Now imagine a room 1000 times as big. That is, imagine a 100,000 square foot 'room'. You would expect many smaller rooms inside such a large space, each with its own door(s). The eukaryotic cell is a lot like that large space, with lots of interior "rooms" (i.e., organelles) with their own entryways and exits. The smaller prokaryotic "room" has a much larger plasma membrane *surface area/volume ratio* than a typical eukaryotic cell, enabling required environmental chemicals to enter and quickly diffuse throughout the cytoplasm of the bacterial cell. Chemical communication between parts of a small cell is therefore rapid. In contrast, the communication over a larger expanse of cytoplasm inside a eukaryotic cell requires the coordinated activities of subcellular compartments. Such communication might be relatively slow. In fact, eukaryotic cells have lower rates of metabolism, growth and reproduction than do prokaryotic cells. The existence of large cells required the evolution of a *division of labor* supported by *compartmentalization*.

2. Animal and Plant Cell Structure Overview

Typical animal and plant cells with their organelles and other structures are illustrated below.



A plasma (cell) membrane surrounds all cells. A cell wall further surrounds prokaryotic, algal, fungal and plant cells, creating rigid structure around the cell membrane and supporting cell shape. Bacterial cell walls are composed of ***peptidoglycan***, long polysaccharide chains attached to polypeptide (amino acid) chains. ***Cellulose***, ***hemicellulose***, and ***pectin*** are major polysaccharides of the plant cell wall. Fungal cells contain a wall, whose principal component is ***chitin***. Chitin is the same material that makes up the exoskeleton or arthropods (including insects and lobsters!). Fungi, more closely related to animal than plant cells, are a curious beast for a number of reasons! For one thing, the organization of fungi and fungal cells is somewhat less defined than animal cells. Structures between cells called *septa* separate fungal *hyphae*, allow passage of cytoplasm and even organelles between cells. Some primitive fungi have few or no septa, in effect creating *coenocytes*, which are single giant cells, with multiple nuclei.

We end this look at the domains of life by noting that, while eukaryotes are a tiny minority of all living species, "their collective worldwide biomass is estimated at about equal to that of prokaryotes" (Wikipedia). On the other hand, our bodies contain 10 times as many microbial cells as human cells! In fact, it is becoming increasingly clear that a human owes as much of its existence to its microbiota (see above) as it does to its human cells. Keeping in mind that plants and animal cells share many internal structures and organelles that perform the same or similar functions, let's look at them and briefly describe their functions.

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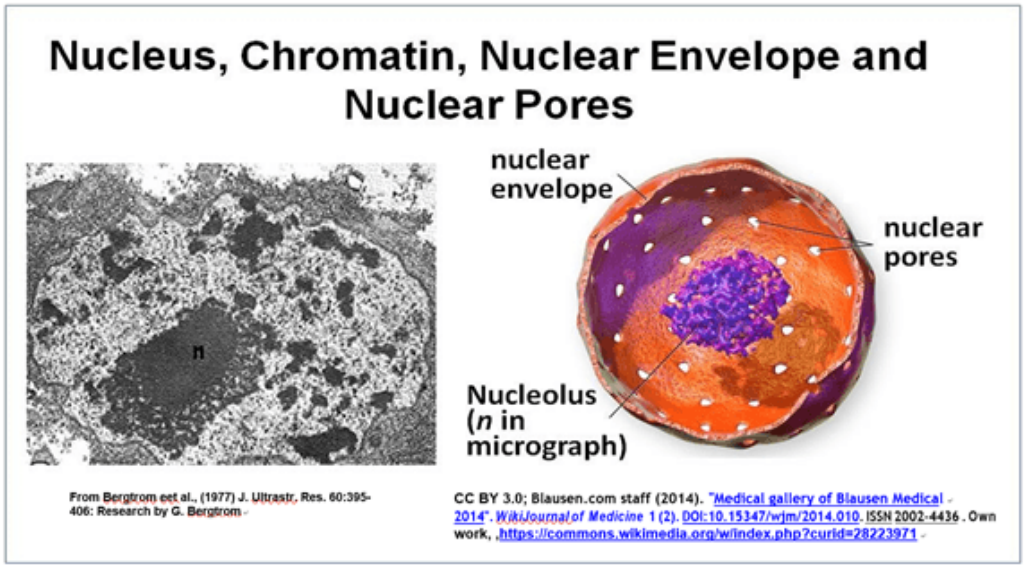
1.4: Tour of the Eukaryotic Cell

A. The Nucleus

The nucleus separates the genetic blueprint, i.e., DNA from the cell cytoplasm. Although the eukaryotic nucleus breaks down during mitosis and meiosis as chromosomes form and cells divide, it spends most of its time in ***interphase***, the time between cell divisions. This is where the status of genes (and therefore of the proteins produced in the cell) is regulated. *rRNA*, *tRNA* and *mRNA* are transcribed from genes, processed in the nucleus, and exported to the cytoplasm through *nuclear pores*. Some other RNAs remain in the nucleus, typically participating in the regulation of gene activity. In all organisms, dividing cells must produce and partition copies of their duplicated genetic material equally between new daughter cells. Let's look first at the structural organization of the nucleus, and then at its role in the genetics of the cell and of the whole organism.

1. Structure of the Interphase Nucleus

The nucleus is the largest organelle in the cell. A typical electron microscope image of a nucleus, the largest eukaryotic organelle in a cell, is shown below.



This cross-section of an interphase nucleus reveals its double membrane, or ***nuclear envelope***. The outer membrane of the nuclear envelope is continuous with the *RER* (rough endoplasmic reticulum). Thus, the lumen of the RER iscontinuous with the space separating the nuclear envelope membranes. The electron micrograph also shows a prominent ***nucleolus*** (labeled **n**) and a darkly granular RER surrounding the nucleus. Zoom in on the micrograph; you may see the double membrane of the nuclear envelope. You can also make out ribosomes (small granules) bound to both the RER and the outer nuclear membrane. Nuclear envelope ***pores*** (illustrated in the cartoon at the right) allow large molecules and even particles to move in and out of the nucleus across both membranes.

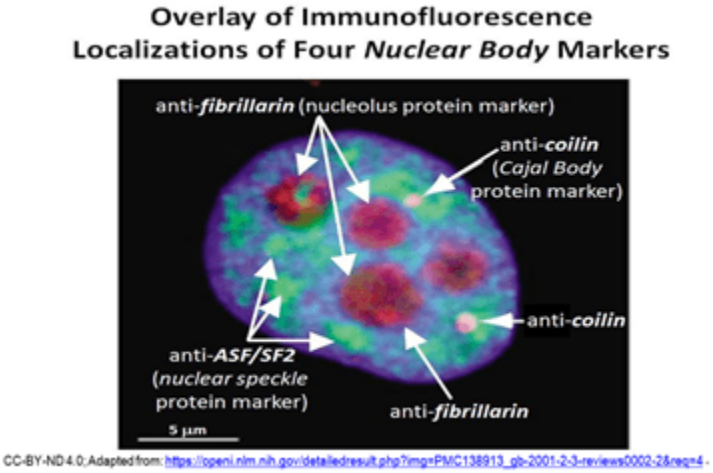
* 1.4 The Nucleus

The nucleus is *not* an unorganized space surrounded by the nuclear envelope, as seems to appear in the transmission electron micrographs. The nucleolus is just the largest of several nuclear inclusions that seem to segregate nuclear functions.

Santiago Ramón y Cajal reported more structures in the nuclei of neurons more than 100 years ago, drawing his observations before modern photomicrographic technology became widely available. See what he saw at Cajal's Nuclear Bodies, including the nucleolus and what came to be known as ***Cajal bodies*** (*CBs*). As we saw earlier, ***Ramón y Cajal*** shared the Nobel Prize in Physiology or Medicine 1906 with *Camillo Golgi* for their studies of nerve cell structure. Check out a gallery of Cajal's hand-drawn

micrographs of brain nerve cells in Cajal's Beautiful Brain Cells.

Later seen in an electron microscope, *CB*s look like coils of tangled thread, and were thus called ***coiled bodies*** (conveniently, also CBs). Other nuclear bodies since identified include ***Gems***, ***PML bodies***, nuclear speckles (or ***splicing speckles***), ***histone locus bodies*** (*HLB*s) **...,** and more! Different nuclear bodies turn out to be associated with specific proteins. The localization of specific proteins to different nuclear bodies can be seen in the immunofluorescence micrograph below.



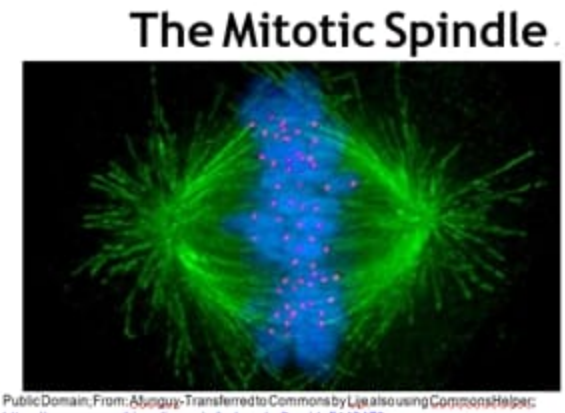
CC-BY-ND 4.0; Adapted from: https://openi.nlm.nih.gov/detailedresult.php?img=PMC138913_gb-2001-2-3-reviews0002-2&req=4 .

Nucleoli contain ***fibrillarin*** proteins and stain red because they have been treated with red-fluorescence-tagged ***antifibrillarin*** antibodies. CBs contain the protein ***coilin***. They fluoresce pink because the nuclei were treated with fluorescence- tagged ***anticoilin*** antibodies. Green-fluorescent antibodies to the **ASF/SF2** protein localize to nuclear speckles. As part of, or included in a nuclear matrix, nuclear bodies organize and regulate different aspects of nuclear activity and molecular function. The different nuclear bodies perform specific functions and interact with each other and with proteins DNA and RNA to do so. We will revisit some nuclear bodies in their working context in later chapters.

2. Every Cell (i.e., Every Nucleus) of an Organism Contains the Same Genes

We read earlier that bacteria are busy doubling and partitioning their naked DNA chromosomes at the same time as they grow and divide by binary fission. In eukaryotic cells, a ***cell cycle*** divides life into discrete consecutive events. During most of the cell cycle, cells are in interphase and DNA is wrapped up in proteins in a structure called ***chromatin***. It is not merely the DNA, but chromatin that must be duplicated when cells reproduce. Duplication of DNA involves rearranging chromatin proteins. This occurs before cell division (***mitosis*** and ***cytokinesis***). As the time of cell division nears, chromatin associates with even more proteins, condensing to form *chromosome*s, while the nuclear envelope dissolves.

You may recall that every somatic cell of an organism contains paired *homologous chromosome*s, and therefore two copies of every gene an organism owns. On the other hand, sperm and eggs contain one of each pair of chromosomes, and thus one copy of each gene. Whether by mitosis or meiosis, cytokinesis separates duplicated chromosomes to *daughter* cells. In the fluorescence micrograph of a cell in the *metaphase* stage of *mitosis* (below), the chromosomes (blue) are just about to be pulled apart by microtubules of the spindle apparatus (green).



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As the chromosomes separate and daughter cells form, nuclei reappear and chromosomes de-condense. These events mark the major visible difference between cell division in bacteria and eukaryotes. Cytokinesis begins near the end of mitosis. S*exual reproduction*, a key characteristic of eukaryotes, involves ***meiosis*** rather than mitosis. The mechanism of *meiosis*, the division of *germ cells* leading to production of sperm and eggs, is similar to mitosis except that the ultimate daughter cells have just one each of the parental chromosomes, eventually to become the gametes (eggs or sperm).

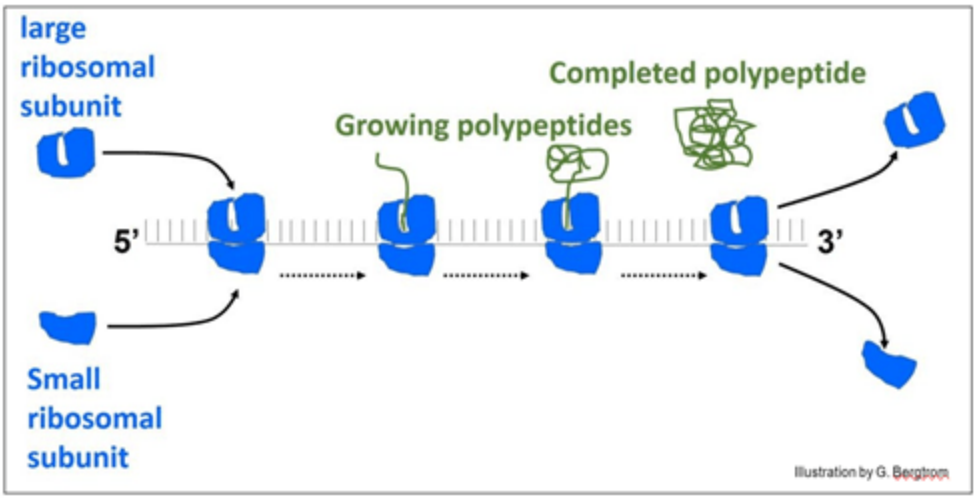
A key take-home message here is that every cell in a multicellular organism, whether egg, sperm or somatic, contains the same genome (genes) in its nucleus. This was understood since mitosis and meiosis were first described in the late 19th century. However, it was finally demonstrated in 1962, when John Gurdon and Shinya Yamanaka transplanted nuclei from the intestinal cells the frog *Xenopus laevis* into enucleated eggs (eggs from which its own nucleus had been removed). These 'eggs' grew and developed into normal tadpoles, proving that no genes are lost during development, but just expressed differentially.

We will revisit animal cloning later in this book. But for now, it's sufficient to know that Molly the cloned frog was followed in 1996 by Dolly, the first cloned sheep, and then other animals, all cloned from enucleated eggs transplanted with differentiated cell nuclei. Click Cloning Cuarteterra for the 60 *Minutes* story of the cloning of *Cuarteterra*, a champion polo mare whose clones are also champions! For their first animal cloning experiments, Gurdon and Yamanaka shared the 2012 Nobel Prize form Physiology or medicine.

B. Ribosomes

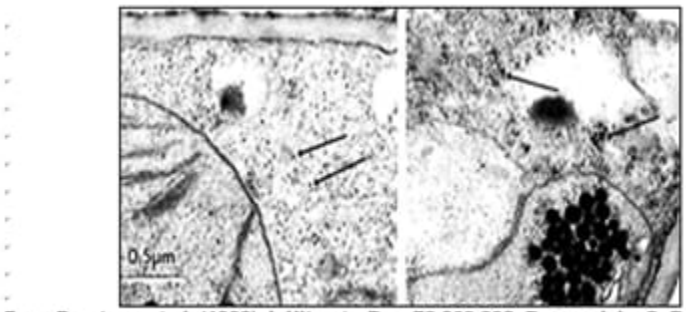
On the other end of the size spectrum, ribosomes are evolutionarily conserved protein synthesizing machines in all cells. They consist of a large and a small subunit, each made up of multiple proteins and one or more molecules of ribosomal RNA (rRNA). Ribosomes bind to messenger RNA (mRNA) molecules, moving along the mRNA as they translate 3-base code words (codons) to link amino acids into polypeptides. Multiple ribosomes can move along the same mRNA, becoming a polyribosome, simultaneously translating the same polypeptide encoded by the mRNA. The granular appearance of cytoplasm in electron micrographs is largely due to the ubiquitous distribution of ribosomal subunits and polysomes in cells.

The illustration below shows a 'string' of ribosomes, the ***polyribosome*** or ***polysome*** for short.



In the illustration, ribosomes assemble at the left of the messenger RNA (mRNA) to form the polysome. When they reach the end of the message, the ribosomes disassemble from the RNA and release the finished polypeptide.

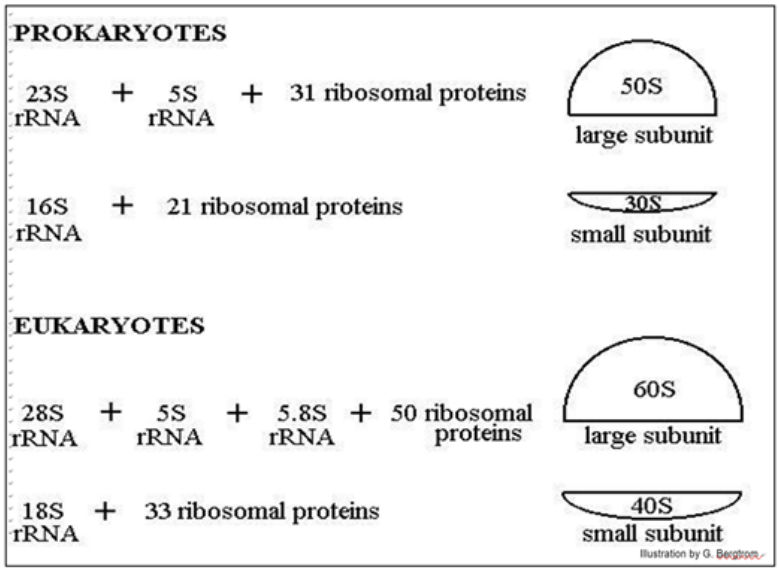
In an electron micrograph of leaf cells from a quiescent desiccated dessert plant, *Selaginella lepidophylla*, you can make out randomly distributed ribosomes and ribosomal subunits (arrows, below left). In cells from a fully hydrated plant, you can see ***polysomes*** as more organized strings of ribosomes (arrows, below right).



From Bergtrom et al. (1982) J. Ultrastr. Res. 78:269-282. Research by G. Bergtrom

Eukaryotic and prokaryotic ribosomes differ in the number of RNA and proteins in their large and small subunits, and thus in their overall size. Isolated ribosomes centrifuged in a sucrose density gradient move at a rate based on their size (or more specifically, their *mass*).

The illustration below shows the difference in ribosomal 'size', their protein composition and the number and sizes of their ribosomal RNAs.



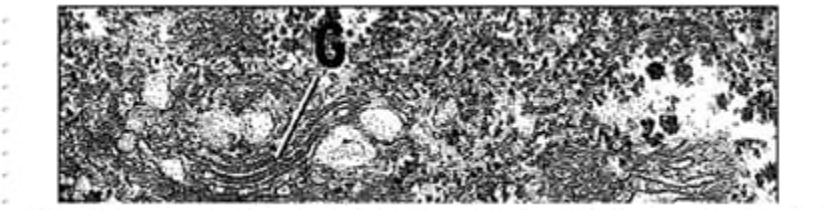
The position of ribosomal subunits in the gradient is represented by an ***S value,*** after *Svedborg*, who first used sucrose density gradients to separate macromolecules and particles by mass. Note that the ribosomal RNAs themselves also separate on sucrose density gradients by size, hence their different S values.

101 Ribosomes & Polysomes

C. Internal membranes and the Endomembrane System

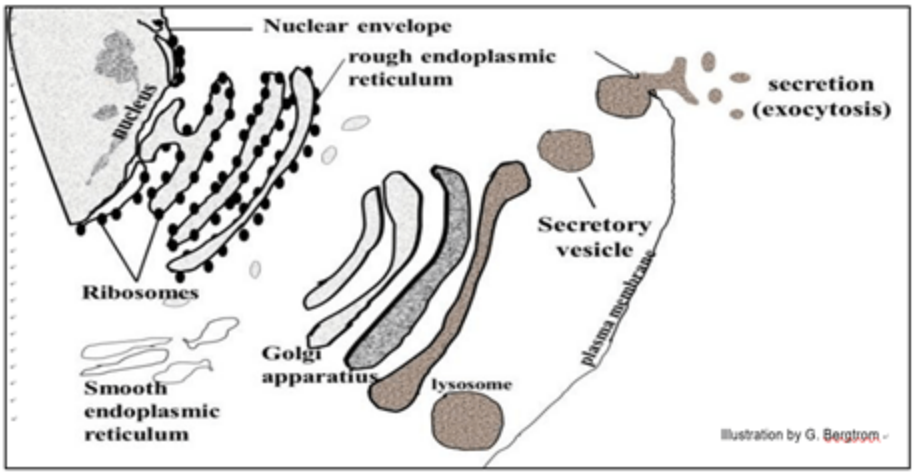
Microscopists of the 19th century saw many of these structures using the art of histology, staining cells to increase the visual contrast between cell parts. One of these, ***Camillo Golgi***, an early neurobiologist, developed a silver (black) stain that first detected a network of vesicles we now call Golgi bodies (***Golgi vesicles***) in nerve cells. For their discoveries in cellular neuroscience, Golgi and ***Santiago Ramón y Cajal*** shared the 1906 Nobel prize for Medicine or Physiology.

Many ***vesicles*** and ***vacuoles*** in cells, including Golgi vesicles, are part of the ***endomembrane system.*** Proteins synthesized on the ribosomes of the **RER (*rough endoplasmic reticulum*)** can enter the interior space (*lumen*) or can become part of the RER membrane itself. Production of ***RER***, ***SER*** (***smooth endoplasmic reticulum***), ***Golgi bodies***, ***lysosomes***, ***microbodies*** and other vesicular membranes, as well as their protein content all begin in the RER. The RER and protein contents bud into *transport vesicles* that fuse with *Golgi Vesicles* **(**G in the electron micrograph below).



Adapted from Bergtrom and Robinson (1977) J. Ultrastr. Res. 60:395-405. Research by G. Bergtrom

In their journey through the endomembrane system, *packaged proteins* undergo stepwise modifications (maturation) before becoming biologically active (below).



10.2 Golgi Vesicles & the Endomembrane System

Some proteins made in the endomembrane system are secreted by ***exocytosis***. Others end up in organelles like ***lysosomes*** that contain hydrolytic enzymes. These enzymes are activated when the lysosomes fuse with other organelles destined for degradation. ***Food vacuoles*** form when a plasma membrane *invaginates,* engulfing food particles. They then fuse with lysosomes to digest the engulfed nutrients.

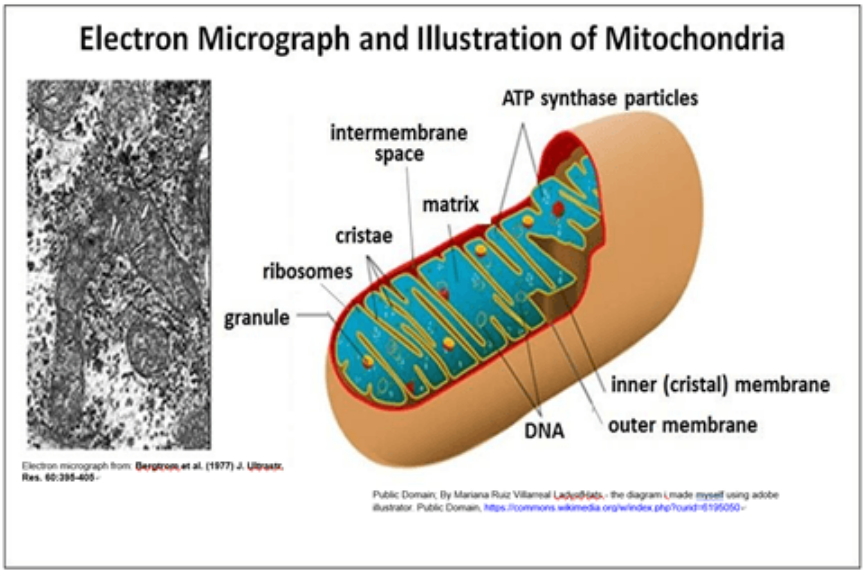
Autophagosomes are small vesicles that surround and eventually encapsulate tired organelles (for example, worn out mitochondria), eventually merging with lysosomes whose enzymes degrade their contents. In 2016, Yoshinori Ohsumi earned the Nobel Prize in Physiology and Medicine for nearly 30 years of research unraveling the cell and molecular biology of autophagy. ***Microbodies*** are a class of vesicles smaller than lysosomes, but formed by a similar process. Among them are peroxisomes that break down toxic peroxides formed as a by-product of cellular biochemistry. Some vesicles emerging from the RER will become part of the SER, which has several different functions (e.g., alcohol detoxification in liver cells).

10.3 Smooth Endoplasmic Reticulum

Other organelles include the ***contractile vacuoles*** of freshwater protozoa that expel excess water that enters cells by osmosis. Some protozoa have *extrusomes*, vacuoles that release chemicals or structures that deter predators or enable prey capture. A large aqueous central vacuole dominates the volume of many higher plant cells. When filled with water, they will push all other structures against the plasma membrane. In a properly watered plant, this water-filled vacuole exerts osmotic pressure that among other things, keeps plant leaves from wilting and stems upright.

D. Mitochondria and Plastids

Nearly all eukaryotic cells contain *mitochondria,* shown below.

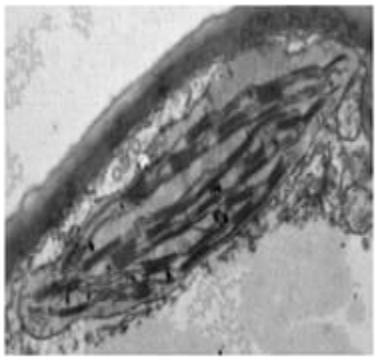


A double membrane surrounds the mitochondrion. Each contains and replicates its own DNA containing genes encoding some mitochondrial proteins. Note that the surface area of the inner mitochondrial membrane is increased by being folded into ***cristae***, which are sites of ***cellular respiration*** (aerobic nutrient oxidation).

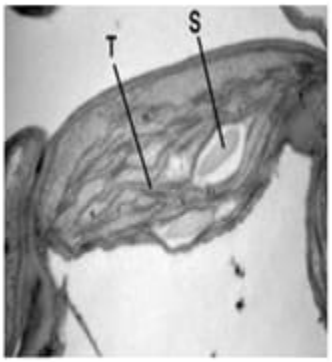
Earlier, we speculated eukaryotic organelles that could have originated within bacteria. Mitochondria most likely evolved from a complete aerobic bacterium (or proto- bacterium) that was engulfed by a primitive eukaryotic cell. The bacterium escaped destruction, becoming an *endosymbiont* in the host cell cytoplasm. Lynn Margulis first proposed the ***Endosymbiotic Theory*** (Margulis, L. [*Sagan, L*], *1967. On the origin of mitosing cells*. Journal of Theoretical Biology **14** (3): 225–274; available at: Margulis L. Endosymbiotic theory). Margulis proposed that chloroplasts also started as ***endosymbionts***. Like mitochondria, the plastids of plants and some algae have their own DNA, most likely originating as cyanobacteria engulfed by primitive eukaryotic cells. Living in symbiosis with the rest of the cell, they would eventually evolve into plastids, including chloroplasts. Detailed evidence for the *Endosymbiotic Theory* is discussed elsewhere.

A handful of protozoa were found lacking mitochondria and other organelles. This had suggested they might share ancestry with those primitive eukaryotes that acquired mitochondria by endosymbiosis. However, since such cells contain other organelles such as *hydrogenosomes* and *mitosomes*, it is thought more likely that these species *once had, but then lost mitochondria*. Therefore, descendants of ancient eukaryotic cells missing mitochondria probably no longer exist.

Chloroplasts and *cyanobacteria* contain chlorophyll and use a similar photosynthetic mechanism to make glucose. A typical chloroplast is shown in the micrograph below (left). A chloroplast beginning to store nutrient sugar as starch is at the right.

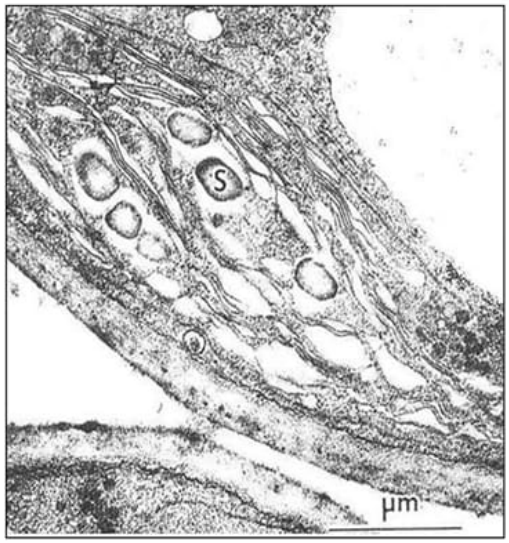


CC-BY-SA 3.0; From: By and3k and caper437 - Own work by uploaders, https://commons.wikimedia.org/w/index.php?curid=7153916



CC-BY; Transmission electron micrograph of chloroplast [T=thylakoids, S = starch] ; from PLOS one; http://redoxbiologycenter.unl.edu/ee00b8d7-d71c-43f8-bab9-5570b2dbd731.pdf

A *leucoplast* is a plastid a chloroplast that has become filled with starch granules. In the micrograph below, you can see that, because of starch accumulation, the grana have become dispersed and indistinct, forming a leucoplast.



From Bergtrom et al., J. Ultrastr. Res. 78:269-282 (S: starch granule). Research by G. Bergtrom

105 Endosymbiosis-Mitochondria & Chloroplasts

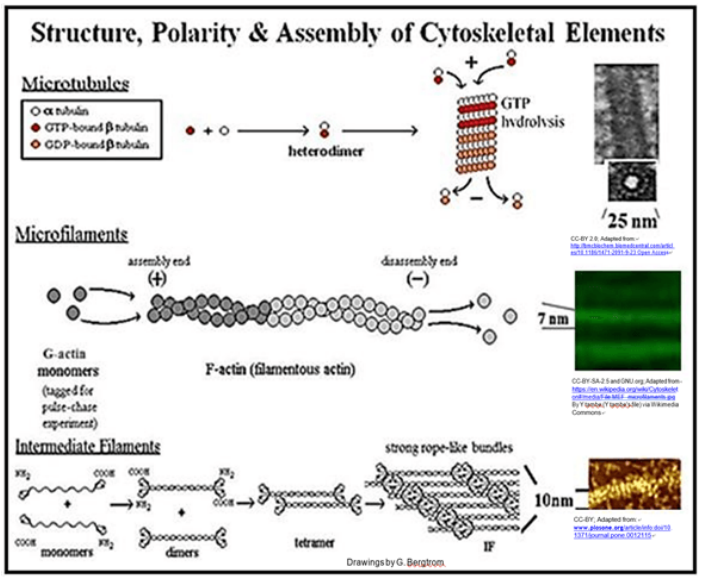
E. Cytoskeletal structures

We have come to understand that the cytoplasm of a eukaryotic cell is highly structured, permeated by rods and tubules. The three main components of this cytoskeleton are ***microfilaments***, ***intermediate filaments*** and ***microtubules.***

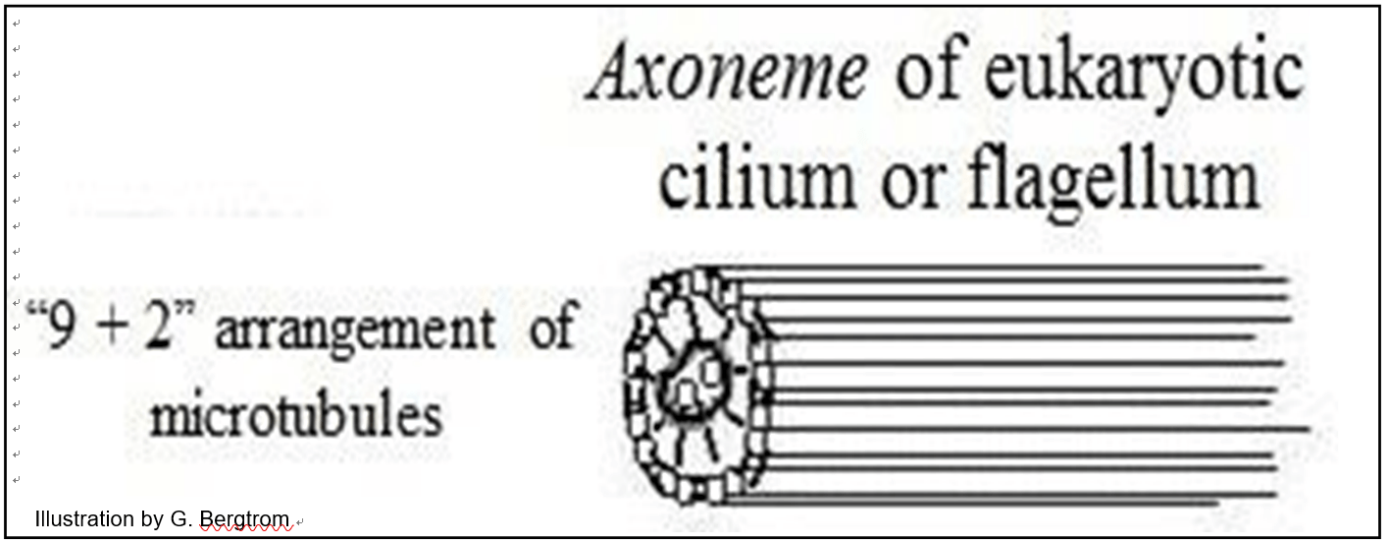
Microtubules are composed of a- and b-*tubulin* protein monomers. Monomeric *actin* proteins make up microfilaments. Intermediate filament proteins are related to *keratin*, a protein found in hair, fingernails, bird feathers, etc. Cytoskeletal rods and tubules not only determine *cell shape*, but also play a role in *cell motility*. This includes the movement of cells from place to place and the movement of structures within cells.

We have already noted that a prokaryotic cytoskeleton is composed in part of proteins homologous to actins and tubulins. As in a eukaryotic cytoskeleton, these bacterial proteins may play a role in maintaining or changing cell shape. On the other hand, flagellum-powered movement in bacteria relies on flagellin, a protein not found in eukaryotic cells. A bacterial flagellum is actually a rigid hook-like structure attached to a molecular motor in the cell membrane that spins to propel the bacterium through a liquid medium.

In contrast, eukaryotic *microtubule*s slide past one another causing a more flexible flagellum to undulate in wave-like motions. Likewise, the motion of a eukaryotic cilium is based on sliding microtubules, in this case causing the cilia to beat rather than undulate. Cilia are involved not only in motility, but also in feeding and sensation. The structures and assembly of the main cytoskeletal components are shown below.



Microtubules in eukaryotic flagella and cilia arise from a *basal body* (similar to *kinetosomes* or *centrioles*). Aligned in a flagellum or cilium, microtubules form an ***axoneme*** surrounded by plasma membrane. In electron micrographs of cross sections, a ciliary or flagellar *axoneme* is typically organized as a ring of nine paired microtubules (called *doublets*) around two *singlet* microtubules (illustrated below).



Centrioles are themselves comprised of a ring of microtubules. In animal cells they participate in spindle fiber formation during mitosis and are the point from which microtubules radiate thorough the cell to help form and maintain its shape. These structures do not involve axonemes. The spindle apparatus in plant cells, which typically lack centrioles, form from an amorphous structure called the *MTOC*, or *MicroTubule Organizing Center*, which serves the same purpose in mitosis and meiosis as centrioles do in animal cells.

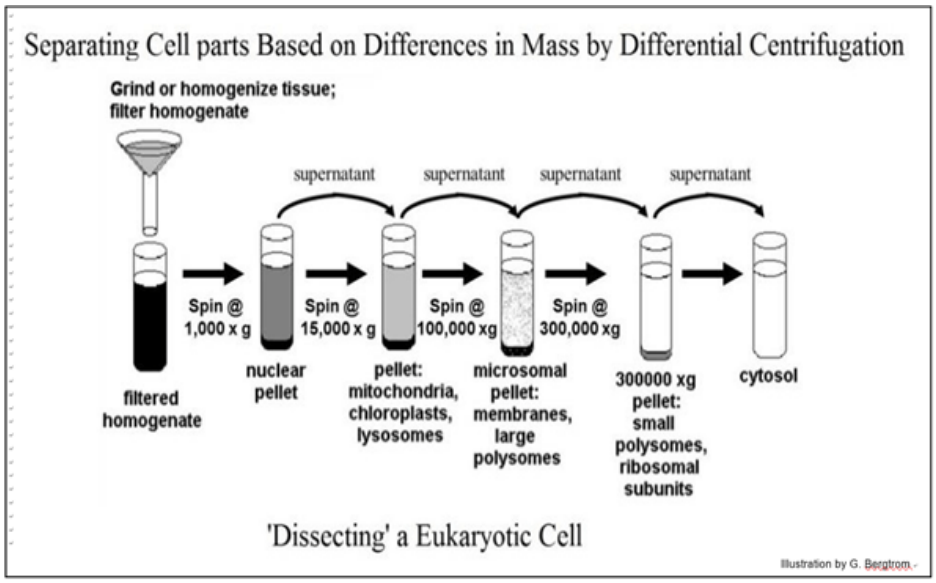
[10.6 Filaments & Tubules of the Cytoskeleton]

Elsewhere, we describe how microfilaments and microtubules interact with motor proteins (*dynein*, *kinesin*, *myosin, etc.*) to generate force that results in the sliding of filaments and tubules to allow cellular movement. You will see that motor proteins can also carry cargo molecules from one place to another in a cell.

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1.5: How We Know the Functions of Cellular Organelles and Structures- Cell Fractionation

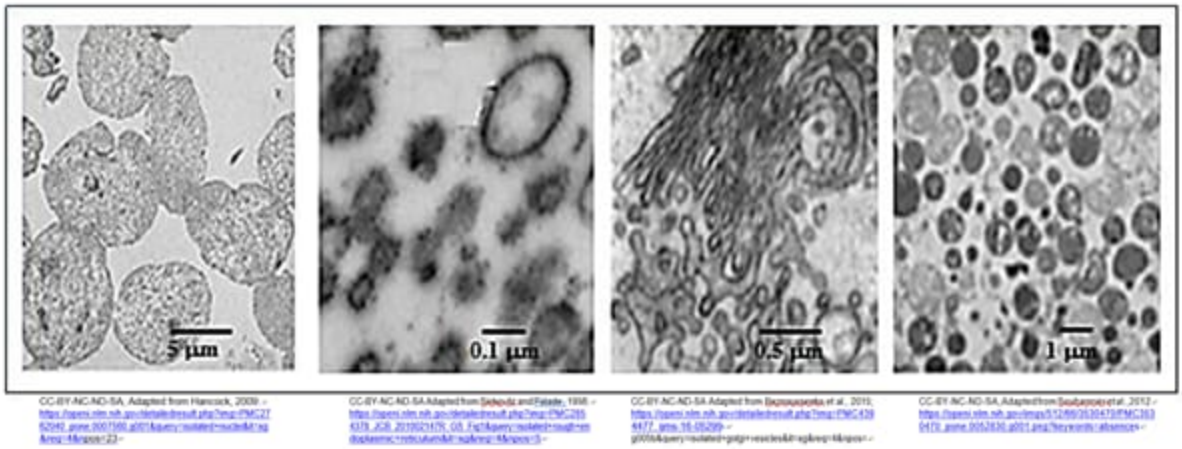
We can see and describe cell parts in the light or electron microscope, but we could not definitively know their function until it became possible to release them from cells and separate them from one another. This became possible with the advent of differential centrifugation. Under centrifugal force generated by a spinning centrifuge, subcellular structures separate by differences in mass. Structures that are more massive reach the bottom of the centrifuge tube before less massive ones. A cell fractionation scheme is illustrated below. Biochemical analysis of the isolated cell fractions can reveal what different organelles and cellular substructures do.



107 Dissecting the Cell; a Cell Fractionation Scheme

Cell fractionation separates cells into their constituent parts. The first step of a cell fractionation is to break open the cells and release their contents. This can be done by physical means such as grinding in a mortar and pestle, tissue grinder or similar device, exposure to ultrasound or high pressure, or exposure to enzymes or other chemicals that can selectively degrade the plasma membrane. The next step is to isolate the subcellular organelles and particles from the cytoplasm (i.e., cytosol) by differential centrifugation. As noted, centrifugation of broken cells at progressively higher centrifugal force separates particulate cell components based on their mass. At the end of this process, a researcher will have isolated mitochondria, chloroplasts, nuclei, ribosomes etc. After re-suspension, each pellet can be re-suspended and prepared for microscopy.

Below are electron micrographs of several isolated subcellular fractions.



These structures can be tentatively identified by microscopy based on their dimensions and appearance. Molecular analyses and biochemical tests on the cell fractions then help to confirm these identities.

108 Isolated Nuclei 109 Isolated RER 110 Isolated Golgi vesicles 111 Lysosomes & Peroxisomes

112 Isolated Mitochondria 113 Isolated Chloroplasts 114 Isolated Plasma Membrane

Can you tell what organelles have been purified in each of these fractions based on the electron micrographs alone? Consider the structures on the left as an example. These were found in a low speed centrifugal pellet, implying that they are large structures. They look a bit like nuclei, which are also the largest structures in a eukaryotic cell. What biochemical or functional tests might you do to confirm that the four structures shown from left to right are isolated nuclei, rough endoplasmic reticulum, Golgi vesicles and mitochondria? Physical separation combined with biochemical-molecular analysis of subcellular structures has revealed their basic functions and continue to reveal previously un-noticed structures and functions in cells.

All of cell and molecular biology is devoted to understanding how prokaryotic and eukaryotic cells (and organisms) use their common structural and biochemical inheritance to meet very different survival strategies. As you progress in your studies, watch for experiments in which cell parts are separated and reassembled, or reconstituted. *Reconstitution* is one of the recurring experimental themes involving the functional analysis of cell components. Look for this theme as you continue your studies. Look also for another theme, namely how evolution can account for the common biochemistry and genetics of life..., *and* its structural diversity!

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1.6: The Origins, Evolution, Speciation, Diversity and Unity of Life

The question of how life began has been with us since the beginnings or recorded history. It is now accepted that there was a time, however brief or long, when the earth was a lifeless (prebiotic) planet. Life's ***origins*** on earth date to some 3.7-4.1 billion years ago under conditions that favored the formation of the first cell, the first entity with all of the properties of life. But couldn't those same conditions have spawned multiple cells independently, each with all of the properties of life? If so, from which of these did life, as we know it today, descend? Whether there were one or more different "first cells", evolution (a property of life) only began with those cells.

115 Properties of Life

The fact that there is no evidence of cells of independent origin may reflect that they never existed. Alternatively, the cell we call our ancestor was evolutionarily successful at the expense of other life forms, which thus became extinct. In any event, whatever this successful ancestor may have looked like, its descendants would have evolved quite different appearances, chemistries and physiologies. These descendant cells would have found different genetic and biochemical solutions to achieving and maintaining life's properties. One of these descendants evolved the solutions we see in force in all cells and organisms alive today, including a common (*universal*) genetic code to store life's information, as well as a common mechanism for retrieving the encoded information. Francis Crick called is commonality the "Central Dogma" of biology. That ancestral cell is called our ***Last Universal Common Ancestor***, or ***LUCA***.

116 The Universal Genetic Code 117 Origins of Life 118 Life Origins vs Evolution

Elsewhere we consider in more detail how we think about the origins of life. For now, our focus is on evolution, the property of life that is the basis of speciation and life's diversity.

Natural selection was Charles Darwin's theory for how evolution led to the *structural* diversity of species. New species arise when beneficial traits are naturally selected from genetically different individuals in a population, with the concomitant culling of less fit individuals from populations over time. If natural selection acts on individuals, evolution results from the persistence and spread of selected, heritable changes through successive generations in a population. Evolution is reflected as *an increase in diversity and complexity* at all levels of biological organization, from species to individual organisms to molecules. For an easy read about the evolution of eyes (whose very existence according to creationists could only have formed by intelligent design by a creator), see the article in National Geographic by E. Yong (Feb., 2016, with beautiful photography by D. Littschwager).

Repeated speciation occurs with the continual divergence of life forms from an ancestral cell through natural selection and evolution. Our shared cellular structures, nucleic acid, protein and metabolic chemistries (the 'unity' of life) supports our common ancestry with all life. These shared features date back to our LUCA! Most living things even share some early *behaviors*. Take our ***biological clock***, an adaptation to our planet's 24 hour daily cycles of light and dark that have been around since the origins of life; all organisms studied so far seem to have one!. The discovery of the genetic and molecular underpinnings of ***circadian rhythms*** (those daily cycles) earned Jeffrey C. Hall, Michael Rosbash and Michael W. Young the 2017 Nobel Prize in Medicine or Physiology (click Molecular Studies of Circadian Rhythms wins Nobel Prize to learn more)!

The molecular relationships common to all living things largely confirm what we have learned from the species represented in the fossil record. Morphological, biochemical and genetic traits that are shared across species are defined as ***homologous***, and can be used to reconstruct evolutionary histories. The biodiversity that scientists (in particular, environmentalists) try to protect is the result of millions of years of speciation and extinction. Biodiversity needs protection from the unwanted acceleration of evolution arising from human activity, including blatant extinctions (think passenger pigeon), and near extinctions (think American bison by the late 1800s). Think also of the consequences the introduction of invasive aquatic and terrestrial species and the effects of climate change.

Let's look at the biochemical and genetic unity among livings things. We've already considered what happens when cells get larger in evolution when we tried to explain how larger cells divided their labors among smaller intracellular structures and organelles. When eukaryotic cells evolved into multicellular organisms, it became necessary for the different cells to communicate with each other and to respond to environmental cues.

Some cells evolved mechanisms to “talk” directly to adjacent cells and others evolved to transmit electrical (neural) signals to other cells and tissues. Still other cells produced hormones to communicate with cells to which they had no physical attachment. As species diversified to live in very different habitats, they also evolved very different nutritional requirements, along with more extensive and elaborate biochemical pathways to digest their nutrients and capture their chemical energy. Nevertheless, despite billions of years of obvious evolution and astonishing diversification, the underlying genetics and biochemistry of living things on this planet is remarkably unchanged. Early in the 20th century, Albert Kluyver first recognized that cells and organisms vary in form appearance in spite of the essential biochemical unity of all organisms (see Albert Kluyver in Wikipedia). This unity amidst the diversity of life is a paradox of life that we will probe further in this course.

A. Genetic Variation, the Basis of Natural Selection

DNA contains the genetic instructions for the structure and function of cells and organisms. When and where a cell or organism’s genetic instructions are used (i.e., to make RNA and proteins) are regulated. Genetic variation results from random mutations. Genetic diversity arising from mutations is in turn, the basis of natural selection during evolution.

119 The Random Basis of Evolution

B. The Genome: An Organism’s Complete Genetic Instructions

We’ve seen that every cell of an organism carries the DNA including gene sequences and other kinds of DNA. The genome of an organism is the *entirety* of its genetic material (DNA, or for some viruses, RNA). The genome of a common experimental strain of *E. coli* was sequenced by 1997 (Blattner FR et al. 1997*The complete genome sequence of Escherichia coli K-12*. Science 277:1452-1474). Sequencing of the human genome was completed by 2001, well ahead of the predicted schedule (Venter JC 2001*The sequence of the human genome*. Science 291:1304-1351). As we have seen in the re-classification of life from five kingdoms into three domains, nucleic acid sequence comparisons can tell us a great deal about evolution. We now know that evolution depends not only on gene sequences, but also, on a much grander scale, on the structure of genomes. Genome sequencing has confirmed not only genetic variation between species, but also considerable variation between individuals of the same species. Genetic variation within species is in fact the raw material of evolution. It is clear from genomic studies that genomes have been shaped and modeled (or remodeled) in evolution. We’ll consider genome remodeling in more detail elsewhere.

C. Genomic ‘Fossils’ Can Confirm Evolutionary relationships.

It had been known for some time that gene and protein sequencing could reveal evolutionary relationships and even familial relationships. Read about an early demonstration of such relationships based on amino acid sequence comparisons across evolutionary time in Zuckerkandl E and Pauling L. (1965) *Molecules as documents of evolutionary theory*. J. Theor. Biol. 8:357-366. It is now possible to extract DNA from fossil bones and teeth, allowing comparisons of extant and extinct species. DNA has been extracted from the fossil remains of humans, other hominids, and many animals. DNA sequencing reveals our relationship to each other, to our hominid ancestors and to animals from bugs to frogs to mice to chimps to Neanderthals to... Unfortunately, DNA from organisms much older than 10,000 years is typically so damaged or simply absent, that relationship building beyond that time is impossible. Now in a clever twist, using what we know from gene sequences of species alive today, investigators recently *‘constructed’* a genetic phylogeny suggesting the sequences of genes of some of our long-gone progenitors, including bacteria (click here to learn more: Deciphering Genomic Fossils). The comparison of these *‘reconstructed’* ancestral DNA sequences suggests when photosynthetic organisms diversified and when our oxygenic planet became a reality.

120 Genomic Fossils- Molecular Evolution

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1.7: Microscopy Reveals Life's Diversity of Structure and Form

Broadly speaking, there are two kinds of microscopy. In *Light Microscopy*, the specimen on the slide is viewed through optical glass lenses. In *Electron Microscopy*, the viewer is looking at an image on a screen created by electrons passing through, or reflected from the specimen. For a sampling of light and electron micrographs, check out this Gallery of Micrographs. Here we compare and contrast different microscopic techniques.

A. Light Microscopy

Historically one form or other of light microscopy has revealed much of what we know of cellular diversity. Check out the Drawings of Mitosis for a reminder of how eukaryotic cells divide and then check out The Optical Microscope for descriptions of different variations of light microscopy (e.g., *bright-field, dark field*, *phase-contrast*, *fluorescence*, etc.). Limits of *magnification* and *resolution* of 1200X and 2 mm, (respectively) are common to all forms of light microscopy. The main variations of light microscopy are briefly described below.

1. *Bright-Field microscopy* is the most common kind of light microscopy, in which the specimen is illuminated from below. Contrast between regions of the specimen comes from the difference between light absorbed by the sample and light passing through it. Live specimens lack contrast in conventional bright-field microscopy because differences in refractive index between components of the specimen (e.g., organelles and cytoplasm in cells) diffuse the resolution of the magnified image. This is why *Bright-Field microscopy* is best suited to fixed and stained specimens.

2. In *Dark-field* illumination, light passing through the center of the specimen is blocked and the light passing through the periphery of the beam is diffracted ("scattered") by the sample. The result is enhanced contrast for certain kinds of specimens, including live, unfixed and unstained ones.

3. In *Polarized light microscopy*, light is polarized before passing through the specimen, allowing the investigator to achieve the highest contrast by rotating the plane of polarized light passing through the sample. Samples can be unfixed, unstained or even live.

4. *Phase-Contrast* or *Interference microscopy* enhances contrast between parts of a specimen with higher refractive indices (e.g., cell organelles) and lower refractive indices (e.g., cytoplasm). *Phase–Contrast* microscopy optics shift the phase of the light entering the specimen from below by a half a wavelength to capture small differences in refractive index and thereby increase contrast. Phase–Contrast microscopy is a most cost-effective tool for examining live, unfixed and unstained specimens.

5. In a *fluorescence microscope*, short wavelength, high-energy (usually UV) light is passed through a specimen that has been treated with a fluorescing chemical covalently attached to other molecules (e.g., antibodies) that fluoresces when struck by the light source. This fluorescent *tag* was chosen to recognize and bind specific molecules or structures in a cell. Thus, in *fluorescence microscopy*, the visible color of fluorescence marks the location of the target molecule/structure in the cell.

6. *Confocal microscopy* is a variant of fluorescence microscopy that enables imaging through thick samples and sections. The result is often 3D-like, with much greater depth of focus than other light microscope methods. Click at Gallery of Confocal Microscopy Images to see a variety of confocal micrographs and related images; look mainly at the specimens.

7. *Lattice Light-Sheet Microscopy* is a 100 year old variant of light microscopy that allows us to follow subcellular structures and macromolecules moving about in living cells. It was recently applied to follow the movement and sub-cellular cellular location of RNA molecules associated with proteins in structures called *RNA granules* (check it out at RNA Organization in a New Light).

B. Electron Microscopy

Unlike light (optical) microscopy, electron microscopy generates an image by passing electrons through, or reflecting electrons from a specimen, and capturing the electron image on a screen. Transmission Electron Microscopy (TEM) can achieve much higher magnification (up to 106X) and resolution (2.0 nm) than any form of optical microscopy! *Scanning Electron Microscopy* (SEM) can magnify up to 105X with a resolution of 3.0-20.0 nm. TEM, together with biochemical and molecular biological studies,

continues to reveal how different cell components work with each other. The higher voltage in *High Voltage Electron microscopy* is an adaptation that allows TEM through thicker sections than regular (low voltage) TEM. The result is micrographs with greater resolution, depth and contrast. SEM allows us to examine the surfaces of tissues, small organisms like insects, and even of cells and organelles. Check this link to Scanning Electron Microscopy for a description of scanning EM, and look at the gallery of SEM images at the end of the entry.

121 Electron Microscopy

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1.8: Key Words and Terms

Actin	Eukaryotes	Nuclear envelope
Archaea	Eukaryotic flagella	Nuclear pores
Bacterial cell walls	Evolution	Nucleoid
Bacterial Flagella	Exocytosis	nucleolus
Binary fission	Extinction	Nucleus
Cell fractionation	Hypothesis	Optical microscopy
Cell theory	Inference	Plant cell walls
Chloroplasts	Intermediate filaments	Plasmid
chromatin	keratin	Progenote
Chromosomes	Kingdoms	Prokaryotes
Cilia	LUCA	Properties of life
Confocal microscopy	Lysosomes	Rough endoplasmic reticulum
Cytoplasm	Meiosis	Scanning electron microscopy
Cytoskeleton	Microbodies	Scientific method
Cytosol	Microfilaments	Secretion vesicles

Deduction	**Microtubules**	**Smooth endoplasmic reticulum**
Differential centrifugation	**Mitochondria**	**Speciation**
Diversity	**Mitosis**	**Theory**
Domains of life	**Motor proteins**	**Tonoplast**
Dynein	**Mutation**	**Transmission electron microscopy**
Endomembrane system	**Natural selection**	**Tubulins**

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2: BASIC CHEMISTRY, ORGANIC CHEMISTRY AND BIOCHEMISTRY

2.1: Introduction

2.2: Atoms and Basic Chemistry

2.3: Chemical bonds

2.4: A Close Look at Water Chemistry

2.5: Some Basic Biochemistry- Carbon, Monomers, Polymers and the Synthesis and Degradation of Macromolecules

2.6: Key Words and Terms

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2.1: Introduction

In this chapter, we start with a review *basic chemistry* from ***atomic structure*** to ***molecular bonds*** to the structure and properties of ***water***, followed by a review of key principles of *organic chemistry* - the chemistry of carbon-based molecules. You may find it useful to have your old general chemistry textbook handy, or check out the excellent introduction to general chemistry by Linus Pauling (1988, *General Chemistry* New York, Springer-Verlag). We'll see how the ***polar covalent bonds*** define the ***structure*** and explain virtually all of ***properties of water***. These range from the energy required to melt a gram of ice to vaporize a gram of water to its surface tension to its ability to hold heat..., not to mention its ability to dissolve a wide variety of ***solutes*** from salts to proteins and other macromolecules. We will distinguish water's ***hydrophilic*** interactions with solutes from its ***hydrophobic*** interactions with fatty molecules. Then, we review some basic biochemistry. Well-known biological molecules include monomers (sugars, amino acids, nucleotides, lipids...) and polymers (polysaccharides, proteins, nucleic acids, fats...).

Biochemical reactions that link *glucose* monomers into polymers on the one hand, and break the polymers down on the other are essential reactions for life on earth. Photosynthetic organisms link glucose monomers into starch, a polysaccharide. Amylose is a simple starch, a large **homopolymer** of repeating glucose monomers. Likewise, polypeptides are **heteropolymers** of 20 different *amino acids*. DNA and RNA nucleic acids are also heteropolymers, made using only four different *nucleotides*.

When you eat, digestive enzymes in your gut catalyze the ***hydrolysis*** of the plant or animal polymers we ate back down to monomers. Hydrolysis adds a water molecule across the bonds linking the monomers in the polymer. Our cells then take up the monomers. Once in our cells, ***condensation*** (***dehydration synthesis***) ***reactions*** remove water molecules from participating monomers to grow new polymers that are more useful to *us*. While they are not, strictly speaking, macromolecules, ***triglycerides*** (fats) and ***phospholipids*** are also broken down by hydrolysis and synthesized in condensation reactions. Triglycerides are energy-rich molecules, while phospholipids (chemical relatives of triglycerides) are the basis of cellular membrane structure.

Relatively weak interactions between macromolecules, for example, ***hydrogen bonds*** (H-bonds), **electrostatic interactions**, ***Van der Waals forces***, etc., hold many cellular structures and molecules together. Individually, these bonds are weak. But millions of them hold can the two complementary DNA strands tightly in a stable double helix. We will see this theme of strength in numbers repeated in other molecular and cellular structures. Monomers also serve other purposes related to energy metabolism, cell signaling etc. Depending on your chemistry background, you may find "Googling" these subjects interesting and useful. The short VOPs in this chapter might help as a guide to understanding the basic chemistry and biochemistry presented here.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. compare and contrast the definitions of *atom*, *element* and *molecule*.

2. List differences between atoms, elements and molecules and between *energy* and

position-based atomic models.

3. describe sub-atomic particle behavior when they absorb and release energy.

4. state the difference between atomic *shells* and *orbitals*.

5. state how *kinetic* and *potential* energy applies to atoms and molecules.

6. explain the behavior of atoms or molecules that fluoresce when excited by high- energy radiation..., and those that do not.

7. distinguish *polar* and *non-polar covalent bonds* and their physical-chemical properties.

8. predict the behavior of electrons in compounds held together by *ionic interactions*.

9. explain how the *properties of water* account for the solubility of salts and macromolecules and the role of H-bonds in support those properties.

10. consider why some salts are not soluble in water in terms of water's properties.

11. describe how molecular linkages form during polymer metabolism and place hydrolytic and dehydration synthetic reactions in a *metabolic context*.

12. distinguish between chemical "bonds" and "linkages" in polymers.

13. categorize different chemical bonds based on their strengths.

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2.2: Atoms and Basic Chemistry

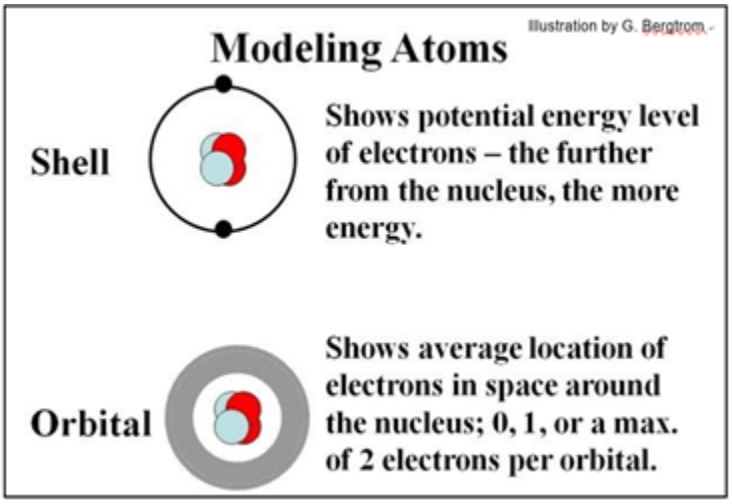
A. Overview of Elements and Atoms

The difference between ***elements*** and ***atoms*** is often confused in casual conversation. Both terms describe ***matter***, substances with ***mass***. Different elements are different kinds of matter distinguished by different physical and chemical properties. In turn, the atom is the fundamental unit of matter..., that is, of an element.

The number of *positively charged* ***protons*** and *neutral* ***neutrons*** in an atomic nucleus account for most of the mass of an atom. Each negatively charged ***electron*** that orbits a nucleus is about 1/2000th of the mass of a proton or neutron. Thus, they do not add much to the mass of an atom. Electrons stay in atomic orbits because of electromagnetic forces, i.e., their attraction to the positively charged nuclei. Nuclear size (mass) and the cloud of electrons around its nucleus define structure of an atom. And that structure dictates the different properties of the elements.

Recall that atoms are chemically most stable when they are electrically uncharged, with an equal number of protons and electrons. ***Isotopes*** of the same element are atoms with the same number of protons and electrons, but a different number of neutrons. Therefore, isotopes are also chemically stable, but they may not be physically stable. For example, the most abundant isotope of hydrogen contains one proton, one electron and *no neutrons*. The nucleus of the ***deuterium*** isotope of hydrogen contains one neutron and that of ***tritium*** contains two neutrons. Both isotopes can be found in water molecules. Deuterium is stable. In contrast, the tritium atom is radioactive, subject to nuclear decay over time. Whether physically stable or not, all isotopes of an element share the same chemical and electromagnetic properties and behave the same way in chemical reactions.

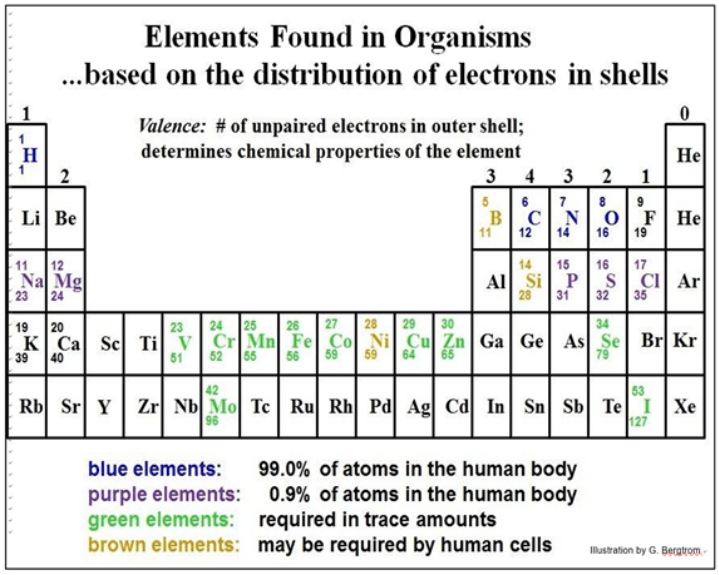
The electromagnetic forces that keep electrons orbiting their nuclei allow the formation of chemical bonds in molecules. We model atoms to illustrate the average physical location of electrons (*the* ***orbital model***) on one hand, and their potential energy levels (the ***Bohr***, or ***shell model***) on the other. Look at the models for helium illustrated below.



Up to two electrons move in a space defined as an ***orbital***. In addition to occupying different areas around the nucleus, electrons exist at different ***energy levels***, moving with different ***kinetic energy***. Electrons can also absorb or lose energy, jumping or falling from one energy level to another.

A unique ***atomic number*** (number of protons) and ***atomic mass*** (usually measured in ***Daltons***, or *Da*) characterize different elements. A unique symbol with a superscripted atomic number and a subscripted atomic mass number defines each element. Take the most common isotope of carbon (C) for example. Its atomic number is 6 (the number of protons in its nucleus) and its mass is 12 Da (6 protons and 6 neutrons at 1 Da each!). Remember that the mass of the electrons in a carbon (C) atom is negligible!

Find the C atom and look at some of the other atoms of elements in the partial periodic table below.

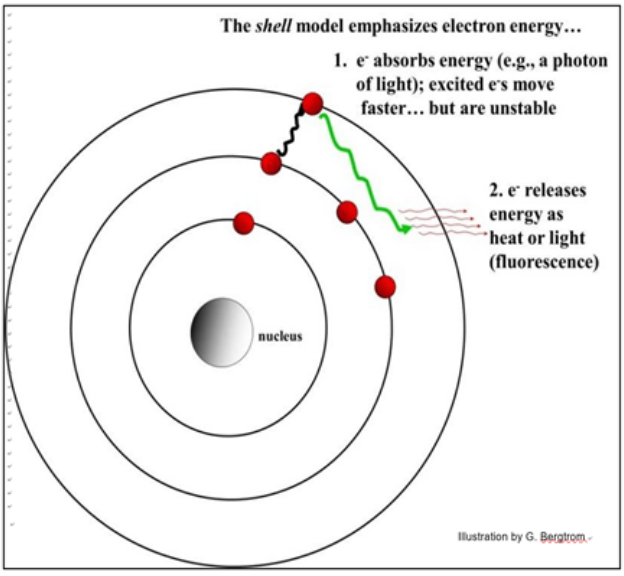


This partial periodic table shows the elements essential for all life in greater or lesser amounts, as well as some that may also be essential in humans.

122 Atoms and Elements

B. Electron Configuration – Shells and Subshells

The *Bohr* model of the atom reveals how electrons can absorb and release energy. The shells indicate the energy levels of electrons. Electrons can absorb different kinds of energy (radiation, light, electrical). For example, beaming UV light at atoms can excite electrons. If an electron absorbs a full ***quantum*** of energy (or a ***photon*** of radiant energy), it will be boosted from the ***ground state*** (the shell it normally occupies) into a higher shell, an ***excited state***. Excited electrons move at greater speed around the nucleus, with more ***kinetic energy*** than it did *at ground*. Excited electrons also have more ***potential energy*** than ground state electrons. This is because they are unstable, releasing some of the energy gained during excitation as they return to *ground*, i.e., their starting energy level (shell), as shown below.



Electrons falling back to ground typically release excitation energy as heat. Atoms whose excited electrons release their energy as light ***fluoresce***; they are ***fluorescent***. A fluorescent light is an example of this phenomenon; electrical energy excites electrons out of atomic orbitals in molecules coating the interior surface of the bulb. As all those excited electrons return to ground state,

they *fluoresce*, releasing light. These atoms can be repeatedly excited by electricity. As we shall see, biologists and chemists have turned fluorescence into tools of biochemistry, molecular biology and microscopy. The ground state is also called the ***resting state***, but electrons at ground are by no means resting! They just move with less kinetic energy excited electrons.

123 Electron Energy & Fluorescence

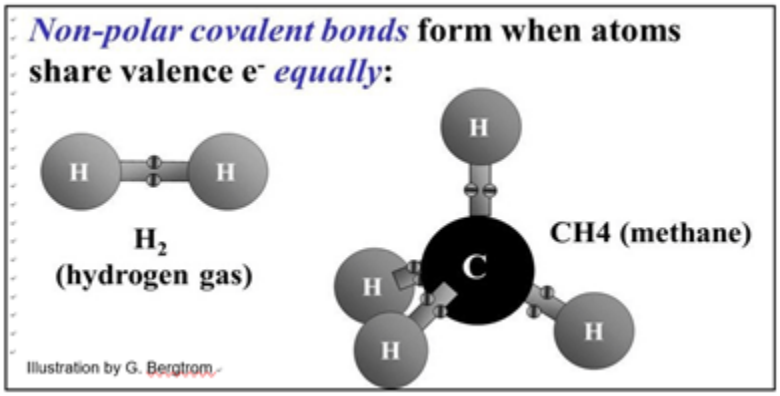
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2.3: Chemical bonds

Atoms form bonds to make molecules. *Covalent bonds* are strong. They can involve unequal *or* equal sharing of a pair of electrons, leading to ***polar covalent bonds*** and ***non-polar covalent bonds*** respectively. ***Ionic bonds*** are weaker than covalent bonds, created by electrostatic interactions between elements that can gain or lose electrons. ***Hydrogen (H-) bonds*** are in a class by themselves! These electrostatic interactions account for the physical and chemical properties of water. They are also involved in interactions between and within other molecules. While atoms can share, gain or lose electrons in chemical reactions, they will neither gain nor lose protons or neutrons. Let's look more closely at chemical bonds and how even the weak bonds are essential to life.

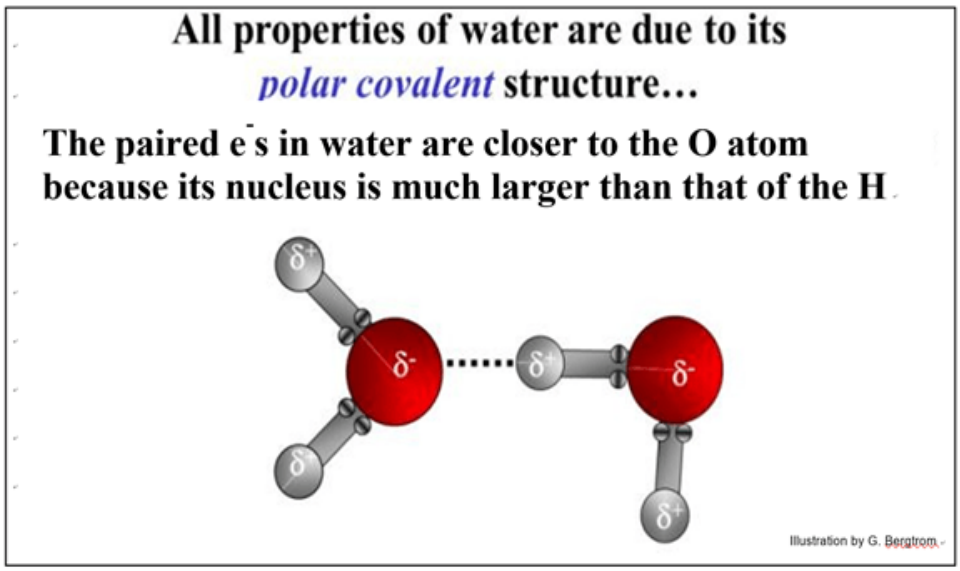
A. Covalent Bonds

Electrons are shared in covalent bonds. Hydrogen gas (H2) is a molecule, not an atom! H atoms in the H2 molecule share their electrons equally. Likewise, the carbon atom in methane (CH4) shares electrons equally with four hydrogen atoms. The equal sharing of electrons in ***non-polar covalent bonds*** in H2 and CH4 is shown below.



A single pair of electrons in H2 forms the covalent bond between two H atoms in the hydrogen molecule. In methane, the carbon (C) atom has four electrons in its outer shell that it can share. Each H atom has a single electron to share. If the C atom shares its four electrons with the four electrons in the four H atoms, there will be four paired electrons (8 electrons in all) moving in filled orbitals around the nucleus of the C atom some of the time, and one pair moving around each of the H atomic nuclei some of the time. Thus, the outer shell of the C atom and each of the H atoms are filled at least some of the time. This stabilizes the molecule. Recall that atoms are most stable when their outer shells are filled and when each electron orbital is filled (i.e., with a pair of electrons).

Polar covalent bonds form when electrons in a molecule are shared unequally. This happens if the atomic nuclei in a molecule are very different in size. This is the case with water, shown below.

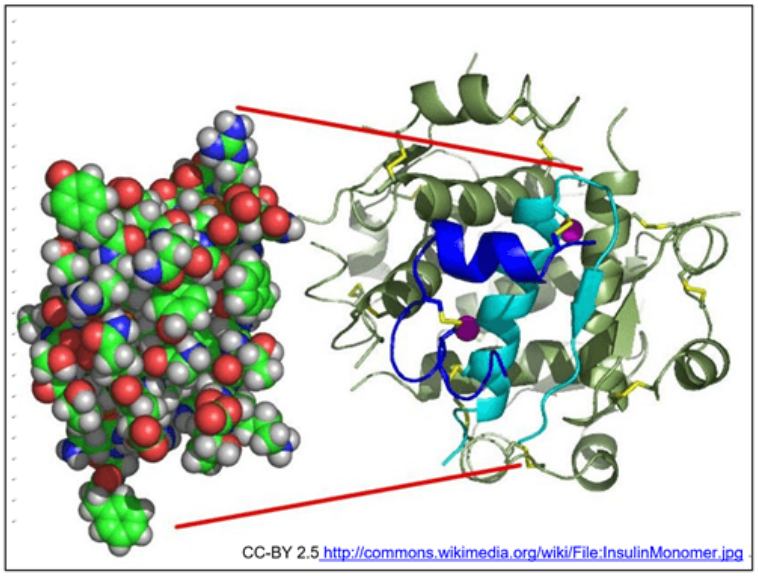


The larger nucleus of the oxygen atom in H2O attracts electrons more strongly than do either of the two H atoms. As a result, the shared electrons spend more of their time orbiting the O atom, such that the O atom carries a *partial negative charge* while each of the H atoms carry a *partial positive charge*. The Greek letter delta (**d**) indicates partial charges in polar covalent bonds. In the two illustrations above, compare the position of the paired electrons in water with those illustrated for hydrogen gas or methane.

Water's polar covalent bonds allow it to attract and interact with other polar covalent molecules, including other water molecules. The polar covalent nature of water also goes a long way to explaining its physical and chemical properties, and why water is essential to life on this planet!

124 Covalent Bonds

Both polar and non-polar covalent bonds play a major role on the structure of macromolecules, like *insulin*, the protein hormone shown below.

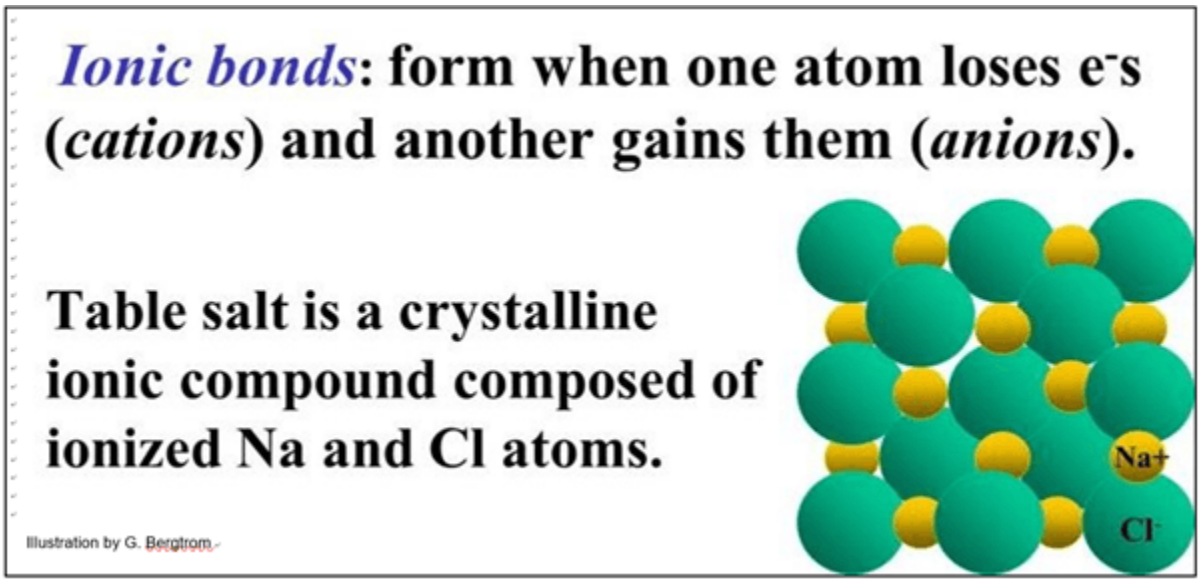


The X-ray image of a space-filling model of the *hexameric* form of stored insulin (above left) emphasizes its tertiary structure in great detail. Regions of internal secondary structure are highlighted in the *ribbon* diagram on the right; as secreted from *Islets of Langerhans* cells of the *pancreas*, active insulin is a dimer of two polypeptides (A and B), shown here in blue and cyan respectively. The subunit structure and the interactions holding the subunits together result from many electrostatic interactions (including H-bonds) and other weak interactions. The disulfide bonds (bridges) seen as yellow 'Vs' in the ribbon diagram stabilize

the associated A and B monomers. We will look at protein structure in more detail in an upcoming chapter.

B. Ionic Bonds

Atoms that gain or lose electrons to achieve a filled outer shell form **ions**, acquiring a negative or a positive charge, respectively. Despite being electrically charged, ions are stable because their outer electron shells are filled. Common table salt is a good example (illustrated below).



Na (sodium) can donate a single electron to Cl (chlorine) atoms, generating Na+ and Cl- ions. The oppositely charged ions then come together forming an ***ionic bond***, an *electrostatic interaction* of opposite charges that holds the Na+ and Cl- ions together in crystal salt. Look up the Bohr models of these two elements and see how ionization of each leaves filled outer shells (energy levels) in the ions.

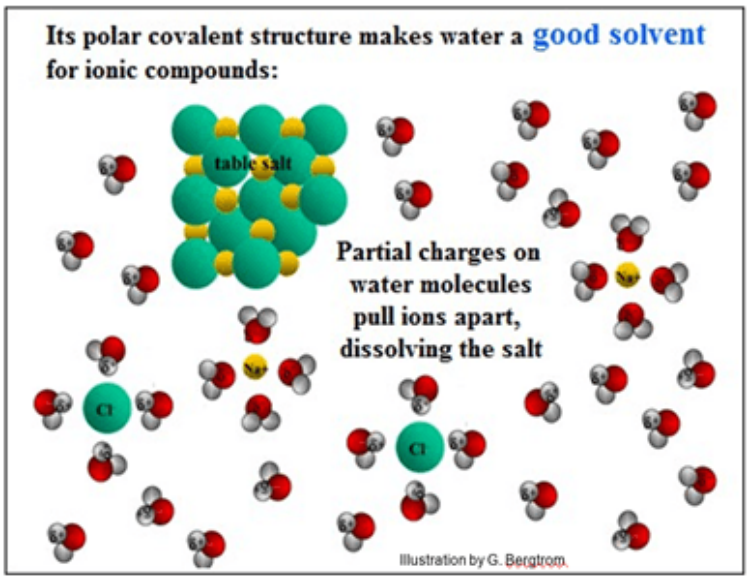
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2.4: A Close Look at Water Chemistry

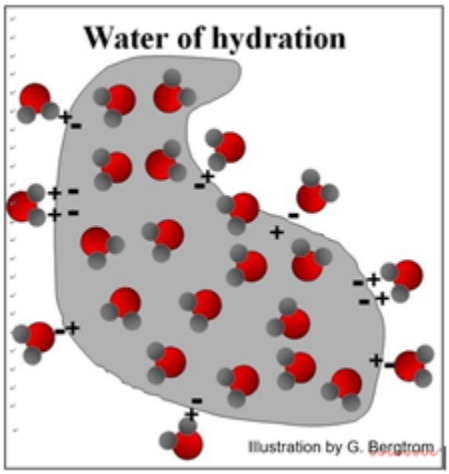
1. Hydrogen Bonds, the Polarity and Properties of Water

Hydrogen bonds are a subcategory of electrostatic interaction (i.e., formed by the attraction of oppositely charges). As noted above, water molecules attract one another (***cohere***) because of strong electrostatic interactions that form H-bonds. Because of water's polar covalent nature, it is able to attract positively and negatively charged groups of solutes, making it a good solvent. Solutes (water-soluble molecules) or molecular surfaces attracted to water are ***hydrophilic***. Lipids like fats and oils are not polar molecules and therefore do not dissolve in water; they are ***hydrophobic*** (from *hydro, water; phobic, fearing)*.

Soluble salts like NaCl dissolve because the Cl- and Na+ ions more strongly attract the partial positive and negative charges (respectively) of water molecules. The result is that the ions separate. We call this separation of salt *ionization*. The ionization of NaCl dissolving in water is shown below.



Water is also a good solvent for macromolecules (proteins, nucleic acids) with exposed polar chemical groups on their surfaces that attract water molecules, as shown below.



125 Water, Hydrogen & ionic Bonds

In addition to being a good solvent, we recognize the following properties of water, all of which result from its polar nature and H-bonding abilities:

1. ***Cohesion***: the ability of water molecules to stick together via H-bonds.
2. ***High Surface tension***: water's high cohesion means that it can be hard to break the surface; think the water strider, an insect that 'walks' on water.
3. ***Adhesion***: this results from water's electrostatic interactions with ions and the partial charges on polar covalent molecules or functional groups. Adhesion explains water's solvent properties and (at least in part) capillary action where water molecules 'crawl' along hydrophilic surfaces, often against the force of gravity.
4. ***High specific heat***: The cohesion of water molecules is so strong that it takes a lot of energy to separate the molecules and make them move faster, i.e., to heat water; specifically it takes 1 *Kcal*, (1 *Calorie*, with a capital C) to heat a gram of water 1oC. Incidentally, high specific heat also explains why water "holds its heat" (i.e., stays hotter longer that the pot that it's in!).
5. ***High heat of vaporization***: It takes even more energy per gram of water to turn it into water vapor!

2. Water Ionization and pH

One last property of water: it ionizes weakly to form H+ and OH- ions, - or more correctly, pairs of water molecules form H3O+ and OH- ions. You can think of this as happening in the following two reactions:

1. $H_2O + H_2O \rightleftharpoons 2H^+ + 2OH^-$
2. $2H^+ + 2OH^- \rightleftharpoons H_3O^+ + OH^-$

Acid molecules added to water dissociate and release protons. This drives reaction #2, forming more H3O+ ions in the solution, in turn driving reaction #1 forward. A pH meter measures the relative acidity or concentration of protons in a solution. Acidic solutions have a pH below 7.0 (neutrality).

Bases ionizing in water release OH- (hydroxyl) ions. The increase in OH- ions removes protons from the solution, driving both reaction in reverse and raising the pH of the solution.

To summarize acid-base chemistry:

When dissolved in water

1. Acids release H+
2. Bases accept H+

Since the pH of a solution is the negative logarithm of the hydrogen ion concentration,

PH and solution

1. at pH 7.0, a solution is neutral
2. below a pH of 7.0, a solution is acidic
3. above a pH of 7.0, a solution is basic

Check a basic chemistry book to remind yourself of the relationship between pH and the [H+] in a solution!

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2.5: Some Basic Biochemistry- Carbon, Monomers, Polymers and the Synthesis and Degradation of Macromolecules

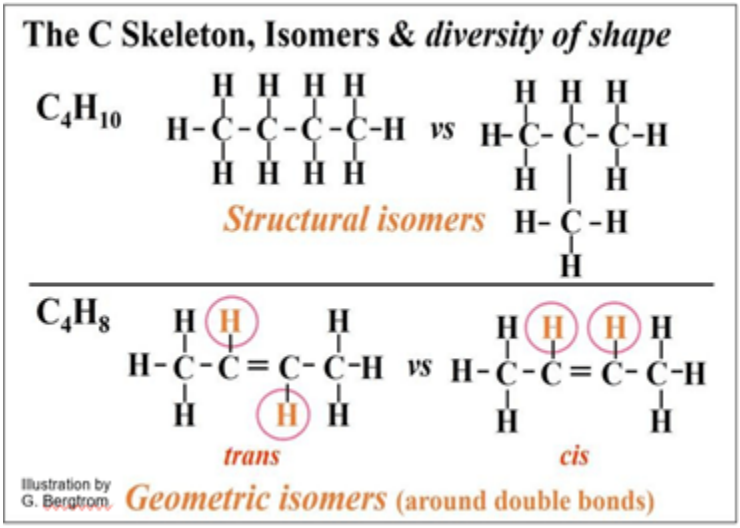
Like evolution, the origin of life involved some prebiotic 'natural selection' of chemicals in the environment. As with evolution, this *chemical selection* would favor expanding possibility and diversity. In simple terms, atoms that could interact with a maximal number of other atoms to form the largest number of stable molecules would have been most likely to accumulate in the environment. The tetravalent C atom met these criteria for chemical selection, proving perfect for building an organic chemistry set.

At the same time, water turned out to be the perfect place to launch prebiotic *chemical selection* experiments. Water persists as the life's universal solvent, which by way, is why evidence of water in places beyond our earth gets us all excited!

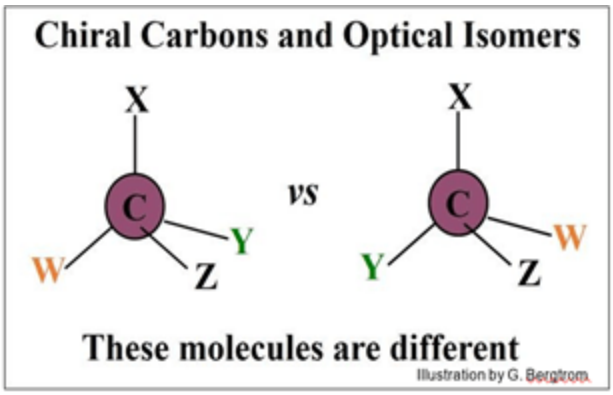
A. Isomerism in Organic Molecules and the Diversity of Shape

The ***carbon skeleton*** is a perfect platform of organic molecule diversity. The differences in arrangement of atoms and functional chemical groups around C atom result in ***isomerism***. Isomers of an organic molecule have the same chemical formula but different shapes (and so, potentially different chemical properties and biochemical function). The larger the carbon skeleton of an organic molecule, the greater the diversity of molecular shapes available for chemical selection.

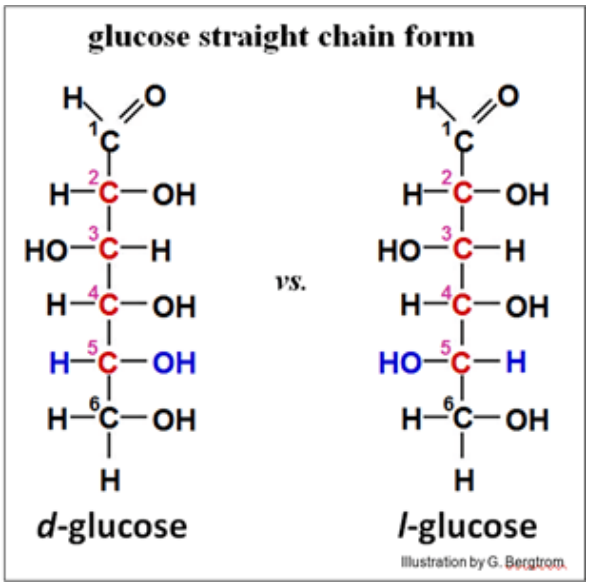
Look at the examples of ***structural isomers*** and ***geometric isomers*** below.



It is easy to see that the structural isomers of C4H10 (above left and right) have different shapes. You cannot convert one structural isomer to the other without breaking covalent bonds. In the geometric isomers of C4H8 in the lower panel, the H atoms on the double-bonded C atoms can be on the same (cis) or opposite (trans) side of the *planar* double bond. Geometric isomers too, cannot be interconverted without breaking chemical bonds. ***Optical isomers*** are yet a third kind of isomer. They exist around ***optically active*** (***asymmetric***, or ***chiral***) carbons. A chiral C is one that is covalently linked to four different atoms and/or molecular groups. The principle of chirality is illustrated below.



Optical isomers (also called ***enantiomers***) also differ in *shape*, and just like structural and geometric isomers, they can't be converted from one to the other without breaking and re-making covalent bonds. We say that optical isomers are *optically active* because they bend, or rotate light in opposite directions in a *polarimeter*. Light passing through a solution of one optical isomer is rotated in one direction while light passing through the other isomer is rotated in the opposite direction. These directions are referred to as ***l,*** or *levo* (meaning left) and ***d*** or *dextro* (meaning right). If a molecule has more than one chiral C (glucose for example has four chiral carbons), its behavior in a polarimeter will be based on the sum of optical activities of all the chiral carbons. The common isomer of glucose in our diet is *d-glucose*. The d- and l- isomers of glucose are illustrated below (showing chiral carbons in red).

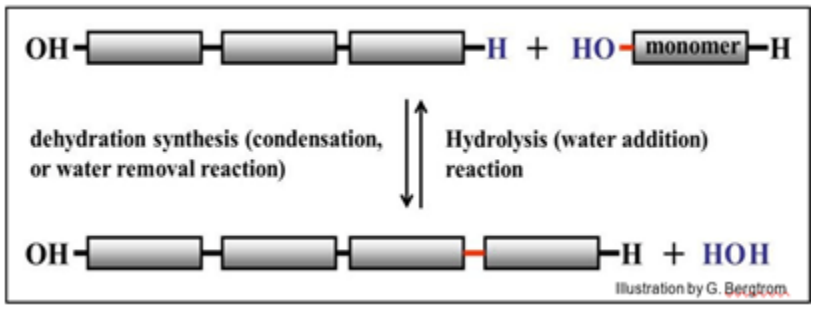


Glucose enantiomers are also referred to as ***D*** and ***L*** respectively. This is a convention based on the configuration of the four different atoms or groups around the *last optically active carbon* in a molecule (**5C** in glucose). For glucose, *d* and *l* in fact correspond to *D* and *L* respectively. As we will see for some molecules, the upper case designation of a chiral molecule does not always indicate how it bends light in a polarimeter, while the lower case *d* and *l* always do!

Remember that the shape and chemical properties of any molecule dictates its function. Isomerism in organic (carbon-based) molecules increased the diversity of molecular shapes available for chemical selection. An early selection of isomers (and specific optical isomers in particular) during chemical evolution contributed greatly to chemical functions and reactions we recognize in cells... even before there was life on earth. That all life uses the same isomers of glucose in energy reactions and the same isomers of amino acids to build proteins confirms the prebiotic selection of those isomers!

B. Monomers to Polymers and Back: Dehydration Synthesis and Hydrolysis

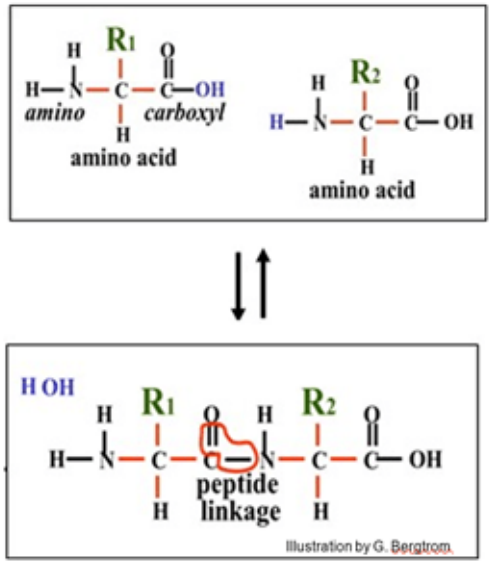
All living things build and break down polymers (macromolecules) by ***dehydration synthesis*** (***condensation*** reactions) and ***hydrolysis***, respectively. *Dehydration synthesis* and *hydrolysis* reactions are essentially the reverse of each other, as illustrated below:



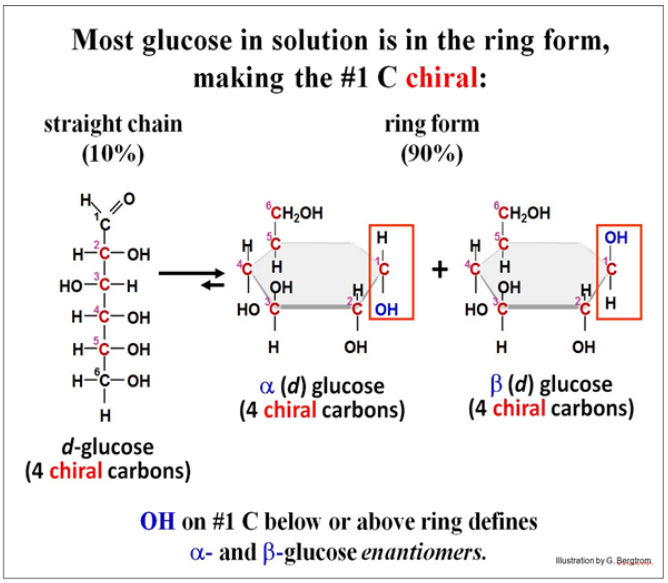
Condensation reactions build macromolecules by removing a water molecule from interacting monomers. The 'bond' that forms in a condensation reaction *is not a single bond*, but a ***linkage*** involving several bonds! The linkages form by removing an OH from one monomer and an H group from the other to form a water molecule.

126 Organic Molecules Monomers & Polymers

Repeated condensation reactions such as the one between two amino acids shown below form the peptide linkages that build polypeptides during translation.

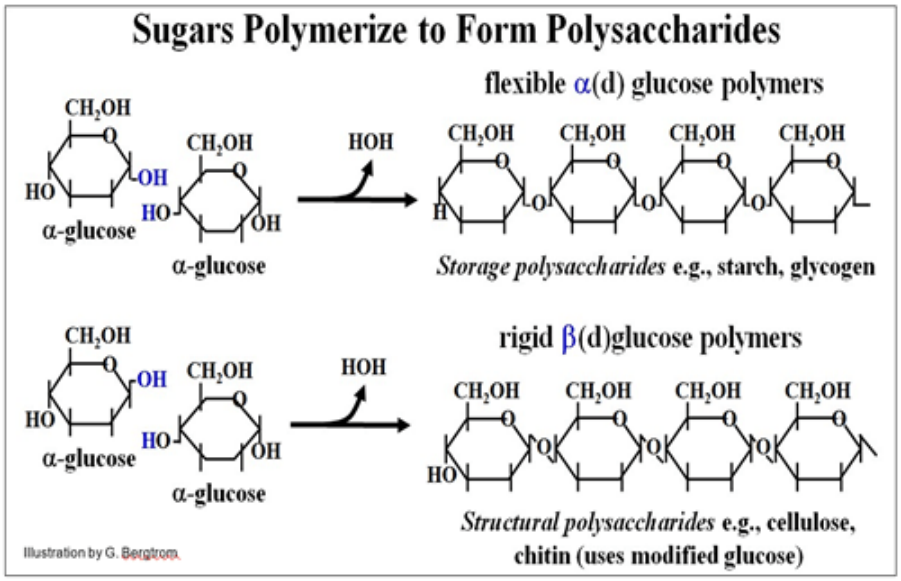


Cells perform repeated condensation reactions to build other polymers, including polysaccharides and polynucleotides (the RNA and DNA nucleic acids). Consider the polymerization of glucose monomers into storage or structural polysaccharides for example. Cells use only ***(d)glucose*** is used by cells to make polysaccharides. Straight-chain (***d)glucose*** with four chiral carbons becomes cyclic when dissolved in water, where the cyclic molecule acquires a fifth chiral carbon, shown below.



The fifth pair of enantiomers in solution are called **a** and **b**, or more correctly, ***a(d)glucose*** and ***b(d)glucose***. Having selected *d-glucose* for most cellular energy metabolism, life then exploited the additional chiral carbon in cyclic *d-glucose* to make the different polysaccharide polymers we now find in plants and animals.

The condensation reactions shown below link glucose monomers, forming storage and structural polysaccharides.



The -OH (hydroxyl) groups on the #1 C of ***a(d)glucose*** are *below* the glucose rings. The condensation reaction removes a water molecule, linking the sugars by an ***a1,4 glycoside linkage*** in the dimer, connecting them by their # 1 and # 4 carbons. Other *linkages* are possible; diverse *a-glycoside* linkages characterize *branched* ***storage polysaccharides*** like *glycogen* in animals and the *starches* in plants. When ***b(d)glucose*** enantiomers polymerize, they form rigid ***structural polysaccharides*** such as those of cellulose in plant cell walls. A modified b-glucose called *N-acetyl glucosamine* (not shown) polymerizes to form *chitin*, the principal component of fungal cell walls and of the tough exoskeleton of arthropods (insects, crustacea). In another chapter, we'll revisit the linkage of amino acids the in the process of *translation* to build a polypeptide, using *only* ***L*** amino acids to make their proteins! We'll also look at the details of *replication* and *transcription* that cells use to catalyze condensation reactions to synthesizing DNA and RNA from nucleotide monomers.

To summarize:

1. Linkages in these biopolymers are broken and formed daily in our lives! After a protein- and carb-containing meal, digestion, the hydrolysis of glycoside and peptide linkages, begins in your mouth and continues in your stomach and small intestines. Then our cells use condensation reactions to complete the job of turning carrot- and cow-derived monomers into you or me!
2. Prebiotic ***chemical evolution*** has selected only one of the optical isomers (enantiomers) of glucose, amino acid and other monomers with which to build polymers. This is so even though some of the different isomers are available and even used for different purposes. The flexible ***a(d)glucose*** polymer was selected to be the storage polysaccharides that we use for energy, a selection probably made by cells themselves. Storage polysaccharides include the plant *starches* and animal *glycogen*. Likewise, the rigid inflexibility of ***b(d)glucose*** polymers would have been selected to reinforce cell structure and stability. Since all organisms store carbohydrate energy in ***a(d)glucose*** polymers and since ***b(d)glucose*** polymers are almost universally used to strengthen cell structure, these selections must have occurred early in the history of life.

127 Carbohydrates: Sugars & Polysaccharides

128 Lipids: Triglycerides & Phospholipids

129Proteins: Amino Acids & Polypeptides

130 DNA & RNA: Nucleotides & Nucleic Acids

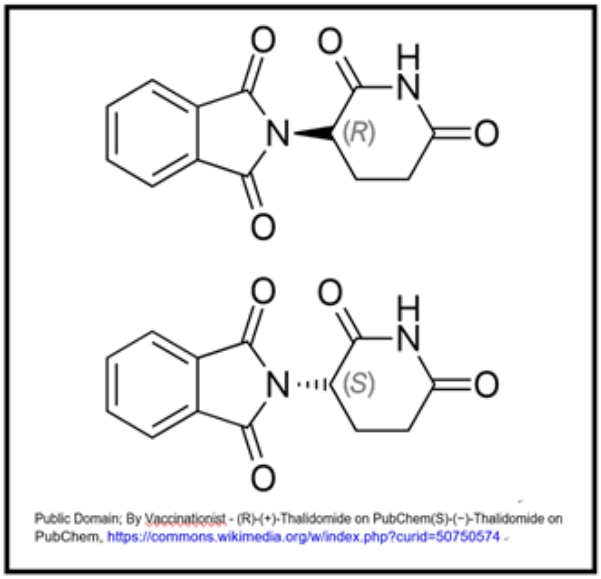
To conclude this chapter and to emphasize the significance of chirality to life, here is what can happen when the wrong isomer ends up in the wrong place at the wrong time...

C. A Tale of Chirality Gone Awry

Consider the story of *Thalidomide*, a tragic example of what happens if we are unaware of enantiomeric possibilities. Introduced in 1957, Thalidomide sold as an over-the-counter anti-nausea drug for cancer treatments and as a very effective morning sickness remedy for pregnant women. However, by the early 1960s, the birth of about 10,000 infants with severely deformed limbs was connected to the drug. The half of the infants affected that survived did so with other defects as well. Of course, the response was to pull Thalidomide off the market.

Thalidomide is a *teratogen*. Teratogens are substances or conditions (drugs, chemicals, radiation, illness during pregnancy, etc.) that cause deformities during embryogenesis and fetal development. The chemical basis of Thalidomide's effects are based on its *enantiomeric* (*chiral*) structure in which an amine-containing ring can exist in front of, or behind the rest of the molecule.

The structure of Thalidomide is shown below.



Public Domain; By Vaccinationist - (R)-(+)-Thalidomide on PubChem(S)-(−)-Thalidomide on PubChem, https://commons.wikimedia.org/w/index.php?curid=50750574

The two isomers are referred to as 'S' and 'R'. Of these, the S isomer is the teratogen. While synthesis of pure R is possible, when used in treatment, R and S easily interconvert, creating a ***racemic mixture***. In the mother, S is transferred to the embryo or fetus, with its terrible consequences. Remarkably, there were relatively few cases of Thalidomide-induced birth deformities in the United States because our FDA (*Food and Drug Administration*) had not yet approved the drug for clinical use. Of course, we already knew that cells synthesized polymers from specific optical isomers of their precursor monomers. So the sad Thalidomide story resulted from the untested effects of an unexpected optical isomer. Many countries quickly tightened their pre-approval drug testing regulations because of this tragedy.

In a more hopeful twist of the tale, Thalidomide has turned out to be effective in treating cancer, leprosy, rheumatoid arthritis and other autoimmune diseases. Such therapeutic benefits may be due to its *anti-inflammatory effects*. Its effects on tumor growth seems to be due to its inhibition of *angiogenesis* (development of blood vessels) in the tumors. Ironically, blockade of angiogenesis might also have contributed to the failure of proper limb growth during pregnancy.

To conclude, when all is normal, the shapes of molecules, particularly macromolecules, are essential for the specificity of reactions essential to life.

131 Shape & the Specificity of Molecular Interactions

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2.6: Key Words and Terms

acids and bases	geometric isomers	polar covalent bonds
adhesion	glycogen	polymers
a-glucose	glycoside linkage	polynucleotides
amino acids	heat of vaporization	polypeptides
angiogenesis	hydrogen bonds	polysaccharides
atom	hydrolysis	potential energy
atomic mass	hydrophilic	properties of water
b-glucose	hydrophobic	protons
Bohr model	ionic bonds	quantum
carbohydrates	ionization	racemic mixture
cellulose	isomers	RNA
chirality	isotopes	salts
chiral carbon	kinetic energy	scanning tunneling microscope
chitin	lipids	sharing electrons
cohesion	macromolecules	solutes
condensation reaction	molecule	specific heat

dehydration synthesis	**monomers**	**starches**
digestion	**neutrons**	**structural isomers**
DNA	**nucleotides**	**surface tension**
electron shell	**optical isomers**	**teratogen(ic)**
electrons	**orbitals**	**Thalidomide**
electrostatic interaction	**partial charge**	**triglycerides**
element	**peptide linkage**	**valence**
enantiomers	**pH**	**Van der Waals forces**
ester linkage	**phosphate ester linkage**	**water ions**
excitation	**phosphodiester linkage**	**water of hydration**
fats	**phospholipids**	
fluorescence	**photon**	

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3: DETAILS OF PROTEIN STRUCTURE

3.1: Introduction

3.2: Levels (Orders) of Protein Structure

3.3: Changes in Protein Shape Can Cause Disease

3.4: Quaternary Structure

3.5: Some Proteins are Chemically Modified and Others Require Prosthetic Groups to be Biologically Active

3.6: Protein Domains, Motifs, and Folds in Protein Structure

3.7: Proteins, Genes and Evolution- How Many Proteins are We?

3.8: View 3D Animated Images of Proteins in the NCBI Database

3.9: Key Words and Terms

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3.1: Introduction

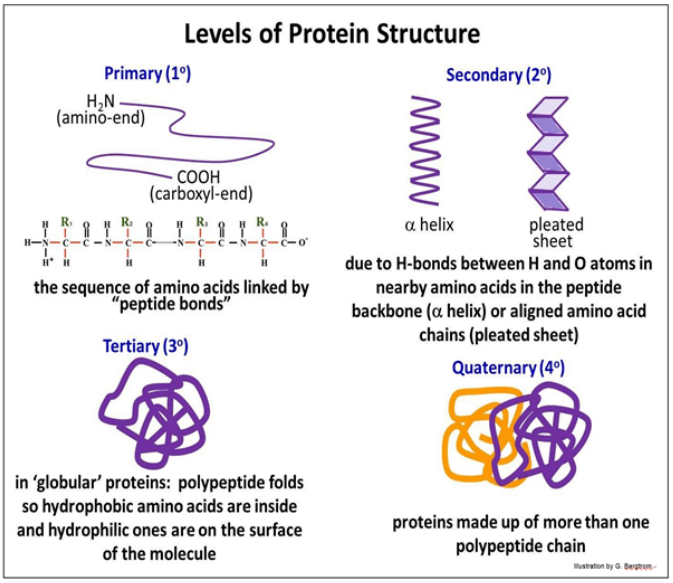
Proteins are the workhorses of cells, responsible for just about all aspects of life (look at oxytocin in the cartoon)! Comprised of one or more *polypeptides*, they:

1. are the ***catalysts*** that make biochemical reactions possible.
2. are ***membrane components*** that selectively let substances in and out of the cell.
3. allow ***cell-cell communication*** and cell's ***response to environmental change***.
4. form the internal structure of cells (***cytoskeleton***) and nuclei (***nucleoskeleton***).
5. enable the ***motility*** of cells and things inside cells.
6. are in fact responsible for other cell functions too numerous to summarize here!

We owe much of what we know about biomolecular structure to the development of X-ray crystallography. In fact an early determination of the structure of insulin (as well as penicillin and vitamin B12) using X-ray crystallography earned Dorothy Hodgkins the 1964 Nobel Prize in Chemistry. In this chapter, we look at the different levels of protein structure..., in fact what it takes to be a functional protein.

The ***primary structure*** (***1o structure***) of a polypeptide is its amino acid sequence. Interactions between amino acids near each other in the sequence cause the polypeptide to fold into ***secondary (2o) structures,*** including a helix and b-, or pleated sheet conformations. ***Tertiary (3o) structures*** form when non-covalent interactions between amino acid side-chains at some distance from one another in the primary sequence cause the polypeptide further folds into more a complex 3-dimensional structure. Other proteins (called *chaperones*!) facilitate the accurate folding of a polypeptide into correct, bioactive, 3-dimensional conformations. **Quaternary (4o) structure** refers to proteins made up of two or more polypeptides. Refer to the four levels of structure on the next page as we explore how each level affects the shape and biological/biochemical function of the protein.

Covalent bonds between specific amino acids (e.g., cysteines) that end up near each other after folding may stabilize tertiary and quaternary structures. Many proteins also bind metal ions (e.g., Mg++, Mn++) or small organic molecules (e.g., heme) before they become functionally active. Finally, we look beyond these orders of structure at their ***domains*** and ***motifs*** that have evolved to perform one or another specific protein functions.



Clearly, in trying to understand molecular (especially macromolecular) function, a recurring theme emerges: the function of a protein depends on its ***conformation***. *In turn,* protein conformation is based on the location and physical and chemical properties of critical ***functional groups***, usually amino acid side chains. Watch for this theme as we look at enzyme catalysis, the movement of molecules in and out of cells, the response of cells their environment, the ability of cells and organelles to move, how DNA replicates, how gene transcription and protein synthesis are regulated..., just about everything a cell does! We will conclude this chapter with a look at some techniques for studying protein structure.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. define and distinguish between the orders of protein structure.

2. differentiate between beta sheet, alpha helix and random coil structure based on the atomic interactions involved on each.

3. trace the path to the formation of a polypeptide; define its primary structure and how it is determined by ‘protein sequencing’.

4. describe how globular proteins arise from the hydrophobic and hydrophilic interactions that drive protein folding and how changes in protein shape can cause disease.

5. formulate an hypothesis (or look one up) to explain why the amino acid glycine is a disruptor of alpha helical polypeptide structure.

6. compare and contrast motif and domain structure of proteins and polypeptides, and their contribution to protein function.

describe different techniques for studying proteins and the physical/chemical differences between proteins that make each technique possible.

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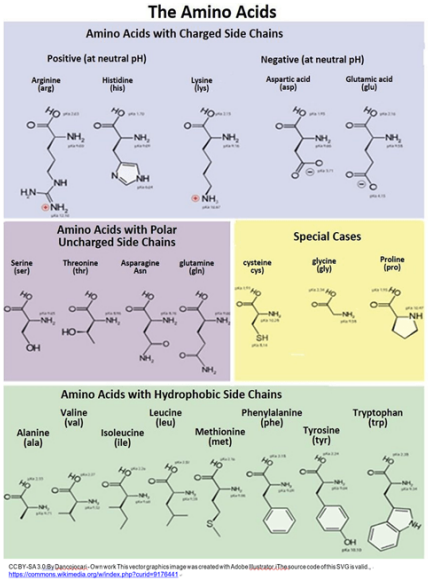
3.2: Levels (Orders) of Protein Structure

A. Primary Structure

1. L amino acids and the C-N-C-N-... polypeptide backbone

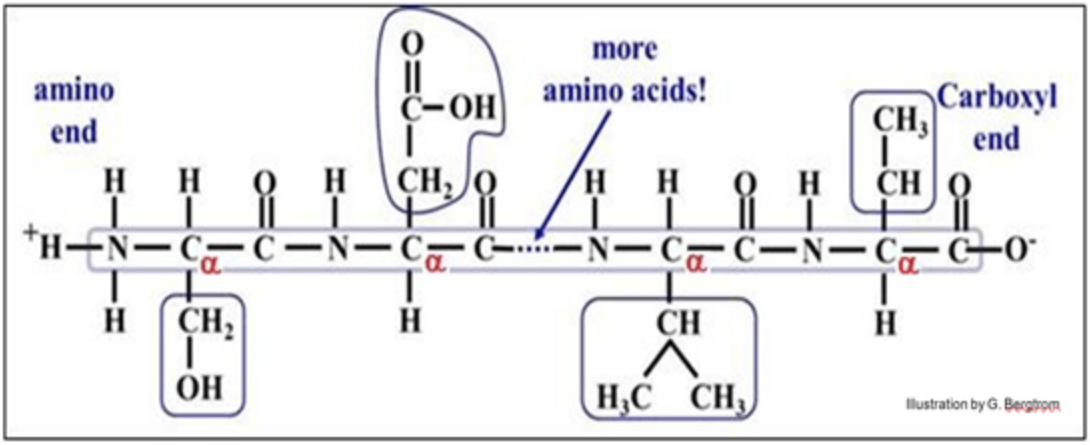
The ***primary structure*** of a protein refers to the amino acid sequence of its polypeptide chain(s). Cells use only 20 amino acids to make polypeptides and proteins, although they do use a few additional amino acids for other purposes. ***Peptide linkages*** between amino acids in polypeptides form in *condensation reactions* in cells during protein synthesis (i.e., ***translation***). The linkages involve multiple covalent bonds. They break and rearrange between the *carboxyl* and *amino* groups of amino acids during linkage formation.

The 20 amino acids found in proteins are shown below.



Except for glycine, the ***a-carbon*** in the 19 other amino acids is bound to four different groups, making them *chiral* or *optically active*.

Recall that chiral carbons allow for mirror image ***D*** and ***L*** or ***d*** and ***l*** optical isomers. Recall also that *only* the lower case ***d*** or ***l*** defines the optical properties of isomers. Just to make life interesting, ***L*** amino acids are actually dextrorotary in a polarimeter, making them ***d-amino acids***! While both enantiomers exist in cells, only dextrorotary ***d*** (i.e., ***L***) amino acids (along with glycine) are used by cells to build polypeptides and proteins. A partial polypeptide is illustrated below.



The result of translation in a cell is a polypeptide chain with a ***carboxyl end*** and an **amino end**. Amino acid side chains (circled above) end up alternating on opposite sides of a ***C-N-C-N-... polypeptide backbone*** because of the covalent bond angles along the backbone. You could prove this to yourself by assembling a short polypeptide with a molecular modeling kit, the kind you might have used in a chemistry class! The C-N-C-N-...backbone is the underlying basis of higher orders, or levels of protein structure (see below).

132 Amino Acid Sequence & Protein Primary Structure

2. Determining Protein Primary Structure - Polypeptide Sequencing

Frederick Sanger was the first to demonstrate a practical method for sequencing proteins when he reported the amino acid sequence of the two polypeptides of *Bovine* (cow) *insulin*. Briefly, the technique involves stepwise hydrolysis of polypeptide fragments, called an *Edman Degradation*. Each hydrolysis leaves behind a polypeptide fragment shortened by one amino acid that can be identified. Sanger received a Nobel Prize in 1958 for this feat. By convention, the display and counting of amino acids always starts at the amino-, or N-terminal end (the end with a free NH2-group). Primary structure is dictated directly by the gene encoding the protein. After transcription of a gene), a ribosome *translates* the resulting mRNA into a polypeptide.

For some time now, the sequencing of DNA has replaced most direct protein sequencing. The method of DNA sequencing, colloquially referred to as the *Sanger dideoxy* method, quickly became widespread and was eventually automated, enabling rapid gene (and even whole genome) sequencing. Now, instead of directly sequencing polypeptides, we can infer amino acid sequences from gene sequences isolated by cloning or revealed after complete genome sequencing projects. This is the same Sanger who first sequenced proteins, and yes..., he won a second Nobel Prize for the DNA sequencing work in 1980!

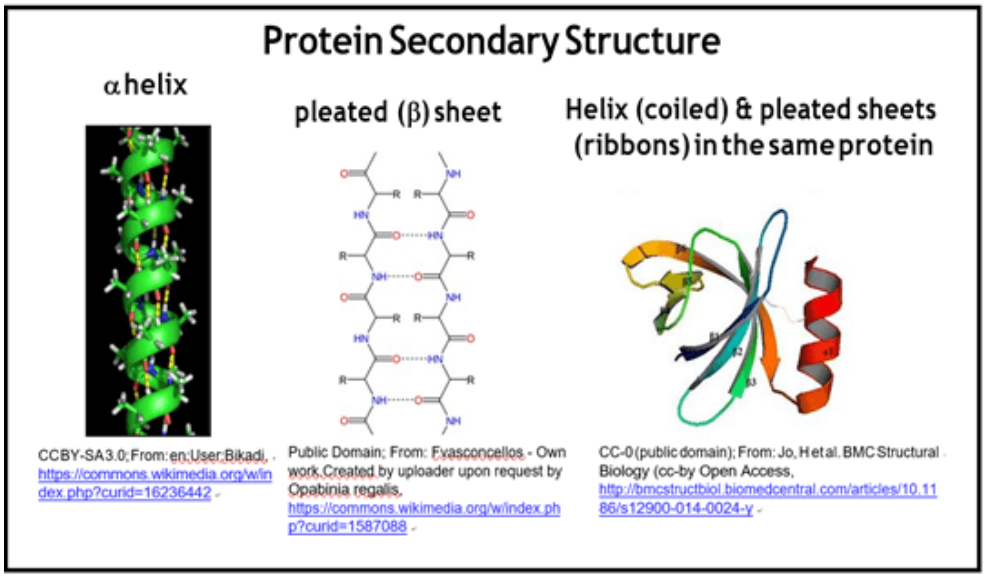
The different physical and chemical properties of amino acids themselves result from the side chains on their *a-carbons*. The unique physical and chemical properties of polypeptides and proteins are determined by their unique combination of amino acid side chains and their interactions within and between polypeptides. In this way, primary structure reflects the genetic underpinnings of polypeptide and protein function. The higher order structures that account for the functional motifs and domains of a mature protein derive from its primary structure. Christian Anfinsen won a half-share of the 1972 Nobel Prize in Chemistry for demonstrating that this was the case for the ribonuclease enzyme (Stanford Moore and William H. Stein earned their share of the prize for relating the structure of the active site of the enzyme to its catalytic function). See 1972 Nobel Prize in Chemistry for more.

B. Secondary Structure

Secondary structure refers to highly regular local structures within a polypeptide (e.g., ***a helix***) and either within or between polypeptides (***b-pleated sheets***). Linus Pauling and coworkers suggested these two types of secondary structure in 1951. A little Linus Pauling history is would be relevant here! By 1932, Pauling had developed his *Electronegativity Scale* of the elements that could predict the strength of atomic bonds in molecules. He contributed much to our understanding of atomic orbitals and later to the structure of biological molecules. He earned the 1954 Nobel Prize in Chemistry for this work. He and his colleagues later discovered that sickle cell anemia was due to an abnormal hemoglobin, and went on to predict the alpha helical and pleated sheet secondary structure of proteins. While he did not earn a second Nobel for these novel molecular genetics studies, he did

win the 1962 Nobel Peace prize for convincing almost 10,000 scientists to petition the United Nations to vote to ban atmospheric nuclear bomb tests. A more detailed review of his extraordinary life (e.g., at Linus Pauling-Short Biography) is worth a read!

Secondary structure conformations occur due to the spontaneous formation of hydrogen bonds between amino groups and oxygens along the polypeptide backbone, as shown in the two left panels in the drawing below. Note that amino acid side chains play no significant role in secondary structure.

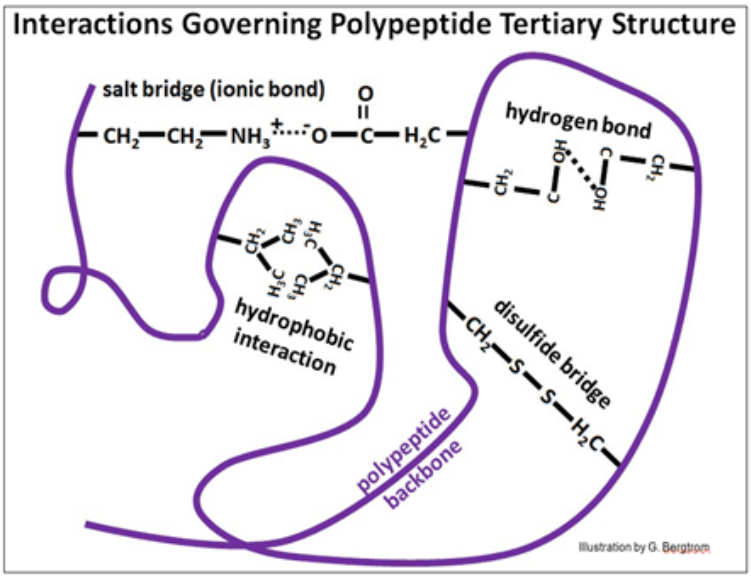


133 Protein secondary structure

The a helix or b sheets are a most stable arrangement of H-bonds in the chain(s). These regions of ordered secondary structure in a polypeptide can be separated by varying lengths of less structured peptide called ***random coils***. All three of these elements of secondary structure can occur in a single polypeptide or protein that has folded into its tertiary structure, as shown at the right in the illustration above. The pleated sheets are shown as ribbons with arrowheads representing *N-to-C* or *C-to-N* polarity of the sheets. As you can see, a pair of peptide regions forming a pleated sheet may do so either in the parallel or antiparallel directions (look at the arrowheads of the ribbons), which will depend on other influences dictating protein folding to form tertiary structure. Some polypeptides never go beyond their secondary structure, remaining fibrous and insoluble. Keratin is perhaps the best-known example of a ***fibrous protein***, making up hair, fingernails, bird feathers, and even filaments of the cytoskeleton. Most polypeptides and proteins however, do fold and assume tertiary structure, becoming soluble ***globular proteins***.

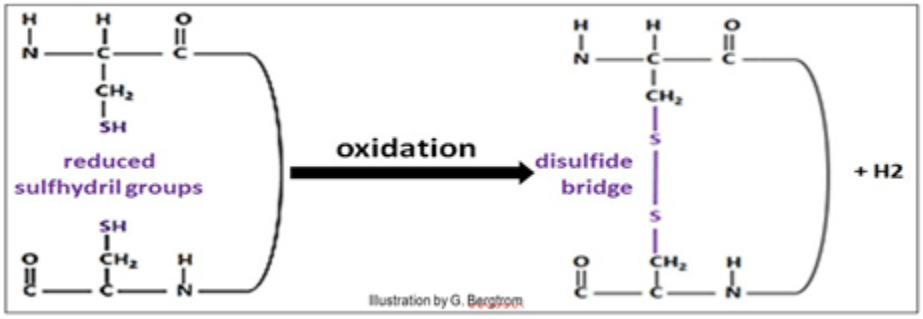
C. Tertiary Structure

Polypeptides acquire their *tertiary structure* when *hydrophobic* and non-polar side chains spontaneously come together to exclude water, aided by the formation of *salt bridges* and H-bonds between polar side chains that find themselves inside the globular polypeptide. In this way, a helices or b sheets are folded and incorporated into globular shapes. The forces that cooperate to form and stabilize 3-dimensional polypeptide and protein structures are illustrated below.



Polar (*hydrophilic*) side chains that can find no other side-chain partners are typically found on the outer surface of the ''globule', where they interact with water and thus dissolve the protein (recall *water of hydration*). Based on non-covalent bonds, tertiary structures are nonetheless strong simply because of the large numbers of otherwise weak interactions that form them. Nonetheless, covalent disulfide bonds between cysteine amino acids in the polypeptide (shown above) can further stabilize tertiary structure. Disulfide bonds (bridges) form when cysteines far apart in the primary structure of the molecule end up near each other in a folded polypeptide. Then the **–SH** (***sulfhydryl***) groups in the cysteine side chains are oxidized, forming the disulfide **(–S-S-**) bonds.

The sulfhydryl oxidation reaction is shown below.



134 Protein Tertiary (3⁰) Structure

135 Disulfide Bridges Stabilize 3⁰ Structure

To better understand how disulfide bridges can support the 3-dimensional structure of a protein, just imagine its physical and chemical environment. Changing the temperature or salt concentration surrounding a protein might disrupt non-covalent bonds involved in the 3D shape of the active protein. Unaffected by these changes, disulfide bridges limit the disruption and enable the protein to re-fold correctly and quickly when conditions return to normal (think *homeostasis*!).

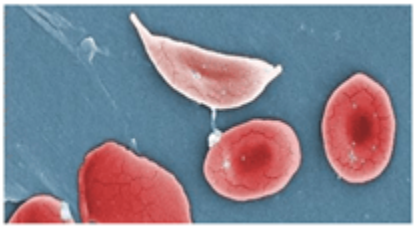
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3.3: Changes in Protein Shape Can Cause Disease

While the conformation of a protein determines its biological function, an allosteric change (change in shape) can moderate or disrupt its function. Under normal circumstances, cells use changes in protein shape to regulate metabolism. Such *allosteric regulation* is well documented in familiar biochemical pathways such as glycolysis and is discussed in more detail elsewhere. Less well understood is how (or why) is why conformational change in some proteins cells has devastating effects.

A. Sickle Cell Anemia

Mutant genes for globins can result in hemoglobin disorders characterized by inefficient oxygen delivery by blood. In the 1940s, the British biochemist J.B.S. Haldane made a correlation between southern African regions with high incidences hemoglobin disorders and malaria, suggesting that heterozygous individuals (i.e., those that had only one copy of a mutant hemoglobin gene), were somehow protected from malaria. Another well-known example of a hemoglobin disorder is *sickle cell anemia*, caused by a single base change in the gene for human b-hemoglobin, one of the polypeptides in hemoglobin. Since red blood cells are rich in hemoglobin, sickling hemoglobins can cause the cells themselves to become sickle-shape. Sickled cells disrupt capillary flow and oxygen delivery, resulting in the symptoms of anemia. Sickle cell anemia originated in Africa and spread to the United States during the slave trade. Once out of Africa and regions where malaria was epidemic, the mutation was of no value, and was just a source of disease. Individuals heterozygous for the sickle cell mutation have *sickle cell trait* and are generally unaffected because at least some of their hemoglobin is normal. Homozygous individuals make only the sickle cell variant of b-hemoglobin; they will suffer more frequent and severe suffer episodes of the disease. Stressors that can trigger sickling include infection or dehydration. Compare normal red blood cells to a sickle cell below.



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The sickle cell gene affects perhaps more than 100 million people worldwide, including 8-10% of African Americans. For more demographic information, see Sickle Cell Trait Demographics Article and Sickle Cell Data from the CDC. In Africa, heterozygotes with sickle cell trait are protected from malaria, confirming Haldane's hypothesis. But patients homozygous for the b-hemoglobin mutation derive little benefit from its anti- malarial effects.

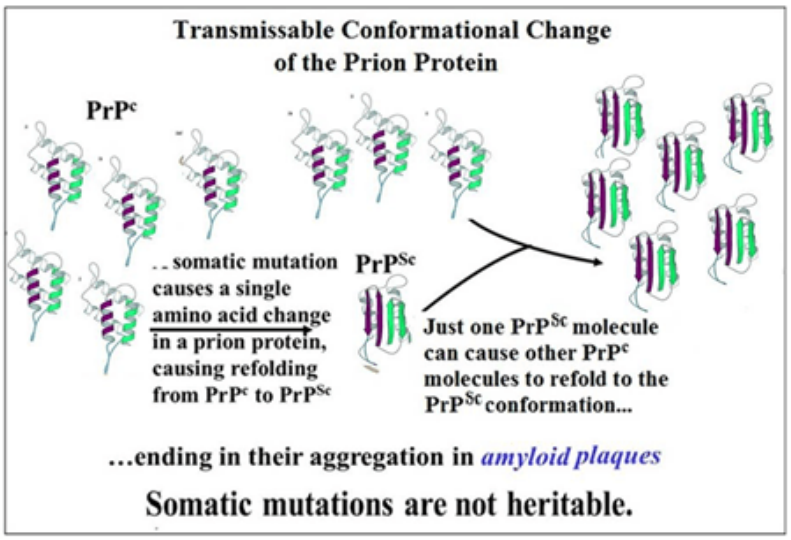
In the meantime, despite a 33% reduction in cases of malaria, the disease (caused by a mosquito-borne parasite) still threatens half of the people on the planet, causing over a half-million deaths per year. There are treatments other than mosquito nets and killing mosquitos, but at this time, there is still no preventive vaccine.

B. The Mis-Folding of Prions and Alzheimer's Disease

1. The Prion Protein

When first discovered, prion proteins seemed to behave as infectious agents that could reproduce without DNA or other nucleic acid. As you can imagine, this highly unorthodox and novel hereditary mechanism generated its share of controversy. Read about research on the cellular *PrPc* prion protein at en.Wikipedia.org/wiki/Prion.

Of course, prions turned out *not* to be reproductive agents of infection after all. Recent studies of prions have revealed several normal prion protein functions such as roles in memory formation in mice and in sporulation in yeast (Check out *Prion Proteins May Play a Role in Memory Formation*). A mutant version of the prion protein (*PrPSc*) is able to mis-fold, assuming an abnormal shape. The deformed *PrPSc* can then induce abnormal folding even normal PrPc! These events, illustrated below, result in the formation of so-called *amyloid plaques*.



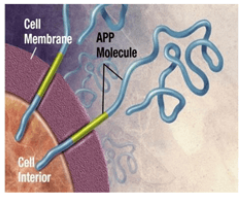
Flickr, Creative Commons License. Adapted from Author: AJC1, 18 April 2007; https://www.learner.org/courses/physics/visual/visual.html?shortname=protein_folding .

In their abnormally folded state, prions have been associated Alzheimer's Disease (which affects about 5.5 million Americans, as well as with *Mad Cow disease* and *Creutzfeldt-Jakob-Disease* (mad cow disease in humans), as well as *Scrapie* in sheep, among others. We are beginning to understand that the role of prion proteins in ***Alzheimer's Disease*** is less causal and somewhat indirect.

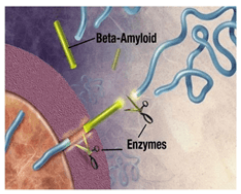
2. The *amyloid beta* (*Ab*) peptide

The post-mortem brains of patients that suffered Alzheimer's disease exhibit characteristic *extracellular* ***amyloid plaques*** composed largely of the ***amyloid beta*** (***Ab***) peptide. Enzymatic cleavage of the **APP** protein (***amyloid precursor protein***) generates extracellular 39-43 amino acid ***Ab*** peptides. Under normal conditions, excess ***Ab*** peptides are themselves digested.

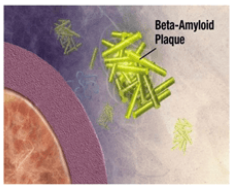
Unregulated ***Ab*** peptide formation however, leads to the formation of *beta amyloid plaques seen in Alzheimer's disease.as* illustrated below.



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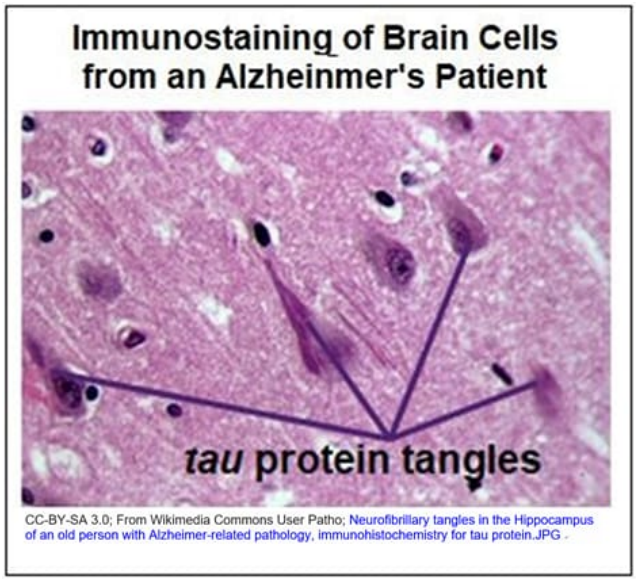
Public Domain,; By National institute on aging - http://www.nia.nih.gov/Alzheimers/Publications/UnravelingTheMystery/Part1/Hallmarks.htm , https://commons.wikimedia.org/w/index.php?curid=3481649 .

The scissors in the illustration represent two enzymes that digest the APP. Prion proteins are not a proximal cause of Alzheimer's Disease, but may have a role in initiating events that lead to it. Normal prion protein (***PrPc***), itself a membrane receptor, is thought to bind ***Ab*** peptides, effectively preventing their aggregation into plaques. An experimental reduction of *PrPc* was shown to increases extracellular ***Ab*** peptides. Presumably prion protein aggregation induced by mutant PrP (***PrPsc***) prevents prion proteins

from binding to ***Ab*** peptide, leading to its accumulation and ultimately to amyloid plaque formation and neurodegeneration.

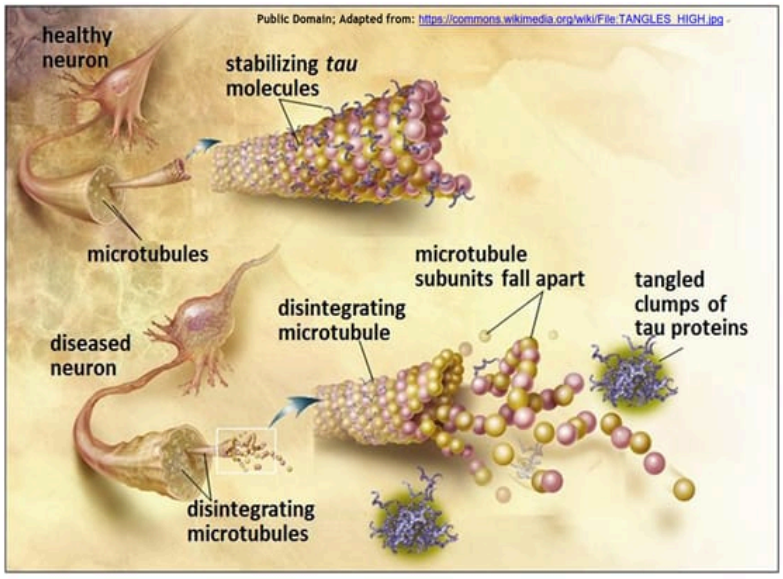
3. The Tau protein

A protein called ***tau*** is also associated with Alzheimer's Disease. Misshapen tau accumulating in ***neurofibrillary tangles*** in hippocampus brain neurons may be a more immediate cause of the neuronal disfunction associated with the disease. In normal neurons, a **Microtubule-Associated Protein Tau** (***MAP-T***) is phosphory- lated and then binds to, and stabilizes microtubules. But when neuronal *tau* becomes *hyper-phosphorylated,* its conformation changes. No longer stabilized, the microtubules disassemble and the deformed tau proteins form ***neurofibrillary tangles.*** Immunostaining of *hippocampal* neurons with antibodies to tau protein localizes the ***neurofibrillary tangles*** as seen in the micrograph below.



CC-BY-SA 3.0; From Wikimedia Commons User Patho; Neurofibrillary tangles in the Hippocampus of an old person with Alzheimer-related pathology, immunohistochemistry for tau protein.JPG

The formation of neurofibrillary tau protein tangles in a diseased neuron is compared to normal neurons in the illustration below.



The" tangles clumps of tau proteins" in this illustration are what stain deep purple in the micrograph of immunostained neurons in the light micrograph.

There is no cure for Alzheimer's disease, although treatments with *cholinesterase inhibitors* seem to slow its advancement. For example, the drug *Aricept* inhibits acetylcholine breakdown by *acetylcholinesterase*, thereby enhancing cholinergic

neurotransmission, which may in turn prolong brain neural function. Unfortunately, there is as yet no treatment to restore lost memories and the significant cognitive decline associated with Alzheimer's disease. Perhaps more promising in this respect, the recent development of a blood test may detect people at risk for Alzheimer's disease. As it happens, ***Aβ*** molecules escape into the blood stream as much as 8 years before Alzheimer's symptoms appear. The prospect of early *Aβ* detection has raised hopes that new therapies might be on the horizon. For a brief review, see Early Detection of Alzheimer's Disease.

C. Some Relatives of Alzheimer's Disease

Some of the same protein abnormalities that are seen in Alzheimer's disease also characterize other neurodegenerative diseases as well as traumatic brain damage, as discussed below.

1. Chronic Traumatic Encephalopathy

An abnormal accumulation of tau protein is diagnostic of ***CTE*** (*Chronic Traumatic Encephalopathy*). In the early 20th century, disoriented boxers staggering about after a fight were called 'punch drunk', suffering from *dementia pugilistica*. We now know they suffered from *CTE*, as do other athletes exposed to repetitive mild-to- severe brain trauma, such as football players. Immunostaining of whole brains and brain tissue from autopsied *CTE* patients with antibodies to tau protein show accumulations of abnormal tau proteins and tau neurofibrillary tangles very much like those found in of Alzheimer's patients. Many National Football League and other football players have been diagnosed post-mortem with *CTE*, and many still living show signs of degenerative cognition and behavior consistent with *CTE* (see a List of NFL players with chronic traumatic encephalopathy to see how many!

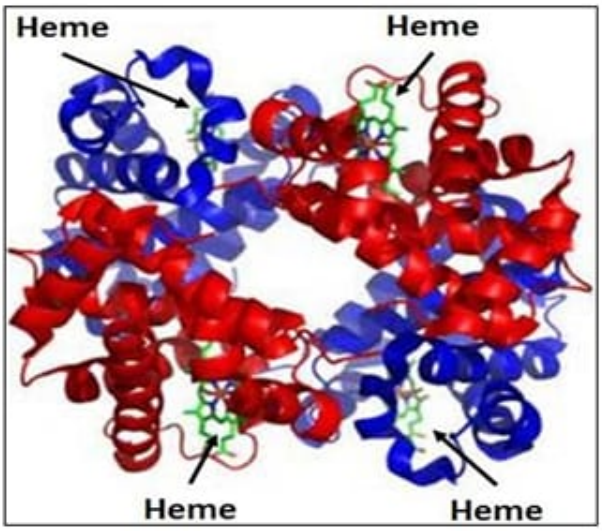
2. Parkinson's Disease

This is yet another example of a neurodegenerative disease that results when a single protein changes shape in brain cells. Though not characterized as plaques, aggregates can form in brain cells when the protein ***alpha-synuclein*** undergoes anomalous conformational change. The change results in ***MSA*** *(Multiple System Atrophy)* or Parkinson's Disease (click Synuclein Allostery and Aggregation in Parkinson's Disease to read more details about this recent research). Much of the high-resolution electron microscopy that reveals protein structure and that can capture conformational changes we now recognize, comes from the work of Jacques Dubochet, Joachim Frank and Richard Henderson who received the 2017 Chemistry Nobel Prize for Chemistry for developing and refining cryo-electron microscopy for biomolecular imaging (see 2017 Nobel Prize for Chemistry for more).

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3.4: Quaternary Structure

Quaternary structure describes a protein composed of two or more polypeptides. Like tertiary structure, multimeric polypeptide are formed by the same kinds of non-covalent interactions and may be stabilized disulfide bonds. Specifically, a *dimer* contains two, a *trimer* three, a *tetramer* four polypeptides... and so on. Multimers made up of identical subunits are referred to with a prefix of "homo-" (e.g. a homotetramer). Those made up of different subunits are *heteromers*. The vertebrate hemoglobin molecule, consisting of two a- and two b- globins (shown below) is a *heterotetramer*.



GNU Free Documentation License; Adapted from: https://en.wikipedia.org/wiki/Hemoglobin .

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3.5: Some Proteins are Chemically Modified and Others Require Prosthetic Groups to be Biologically Active

Many polypeptides are modified after translation, for instance by *phosphorylation* or *glycosylation* (addition of one or more phosphates or sugars respectively to specific amino acids in the chain). These modifications account for and enhance the molecular and functional diversity of proteins within and across species.

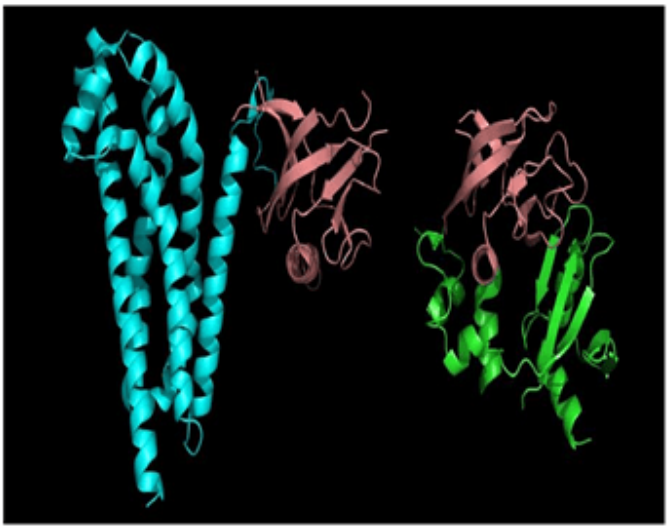
Hemoglobins exemplify another feature of the structure of many proteins. To be biologically active, globin polypeptides must associate with a *prosthetic group*, in this case a cyclic organic molecule called *heme*. At the center of each heme is the iron that reversibly binds oxygen. All kinds of organisms, from bacteria to plants and animals and even in some anaerobic organisms contain some kind of hemoglobin. Other proteins must be bound to different metal ions (magnesium, manganese, cobalt...) to be biologically active.

136 Protein Quaternary Structure & Prosthetic Groups

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3.6: Protein Domains, Motifs, and Folds in Protein Structure

The structures of two different proteins shown below share a common *PH* (Pleckstrin Homology) *domain* (maroon).



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These two and many other proteins have this domain, allowing them to bind a molecule of *phosphatidyl-inositol triphosphate* that is generated as part of a common cell-signaling pathway. The implication of this common *domain* is that a cell can have signaling pathways that allowing it to respond to different signals that lead to the same response, albeit under different conditions and probably at different times. Proteins are typically described as consisting of several distinct sub-structures, discussed below.

A. Domains

A *structural domain* is an element of the protein's overall structure that is stable and often *folds* independently of the rest of the protein chain. Like the PH domain above, many domains are not unique to the protein products of one gene, but instead appear in a variety of proteins. Proteins sharing *more than a few* common domains are encoded by members of evolutionarily related genes comprising ***gene families***. Genes for proteins that share only one or a few domains may belong to ***gene superfamilies***. Superfamily members can have one function in common, but their sequences are otherwise unrelated. Domain names often derive from their prominent biological function in the protein they belong to (e.g., *the calcium-binding domain* of *calmodulin*), or from their discoverers (the PH domain!). The domain swapping that gives rise to gene families and superfamilies are natural genetic events. Because protein domains can also be "swapped" by genetic engineering to make *chimeric proteins* with novel functions.

137 Protein Domain Structure & Function

B. Motifs

Protein *motifs* are small regions of protein three-dimensional structure or amino acid sequence shared among different proteins. They are recognizable regions of protein structure that may (or may not) be defined by a unique chemical or biological function.

C. Supersecondary Structure

Supersecondary structure refers to a combination of secondary structure elements, such as *beta-alpha-beta* units or the *helix-turn-helix motif*. They may be also referred to as structural motifs. “Google” *Supersecondary structure* for examples.

D. Protein Folds

A *protein fold* refers to a general aspect of protein architecture, like *helix bundle*, *beta- barrel*, *Rossman fold* or other "folds" provided in the Structural Classification of Proteins database. Click Protein Folds to read more about these structures.

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3.7: Proteins, Genes and Evolution- How Many Proteins are We?

If evolution did not have to select totally new proteins for each new cellular function, then how many genes does it take to make an organism? The number of genes in an organism that encode proteins may be far fewer than the number of proteins they actually make. Current estimates suggest that it takes just 25,000 genes make and operate a human and all its proteins (check out Pertea and Salzberg at Estimating the number of genes in the human genome). However, our cells (and those of eukaryotes generally) may express as many as 100,000 different proteins. How is this possible? Are there more efficient ways to evolve new and useful cellular tasks than evolving a new genes?

As we already noted, the use of the same 20 amino acids to make proteins in all living things speaks to their early (even pre-biotic) selection and to the common ancestry of all living things. Complex conserved domain structures shared among otherwise different proteins imply that evolution of protein function has occurred as much by recombinatorial exchange of DNA segments encoding these substructures, as by an accumulation of base substitutions in otherwise redundant genes. Likewise, motifs and folds might also be shared in this way. Protein number can exceed gene number in eukaryotes, in part because cells can produce different RNA variants from the same genes by “alternative splicing”, which can create mRNAs that code different combinations of substructures from same gene! Alternate splicing is discussed in detail in a later chapter). The conservation of amino acid sequences across species (e.g., histones, globins, etc.) is testimony to the common ancestry of eukaryotes. Along with the synthesis of alternate versions of an RNA, an ongoing repurposing of useful regions of protein structure may prove a strategy for producing new proteins without adding new genes to a genome.

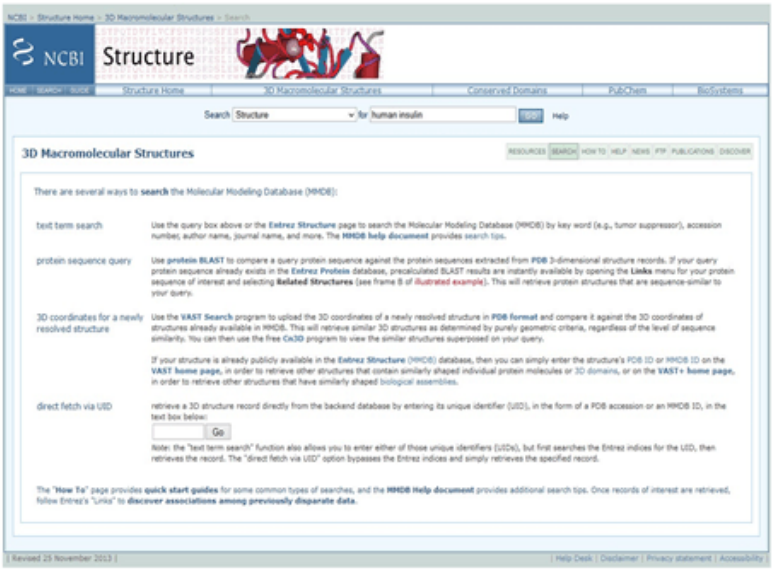
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3.8: View 3D Animated Images of Proteins in the NCBI Database

We can't see them with our own eyes, but viewed by X-Ray diffraction, proteins exhibit exquisite diversity. You can get an X-Ray eye's view of protein structures at *National Center for Biological Information's* Cn3D database. Here's how to access three-dimensional animated images of proteins from the database:

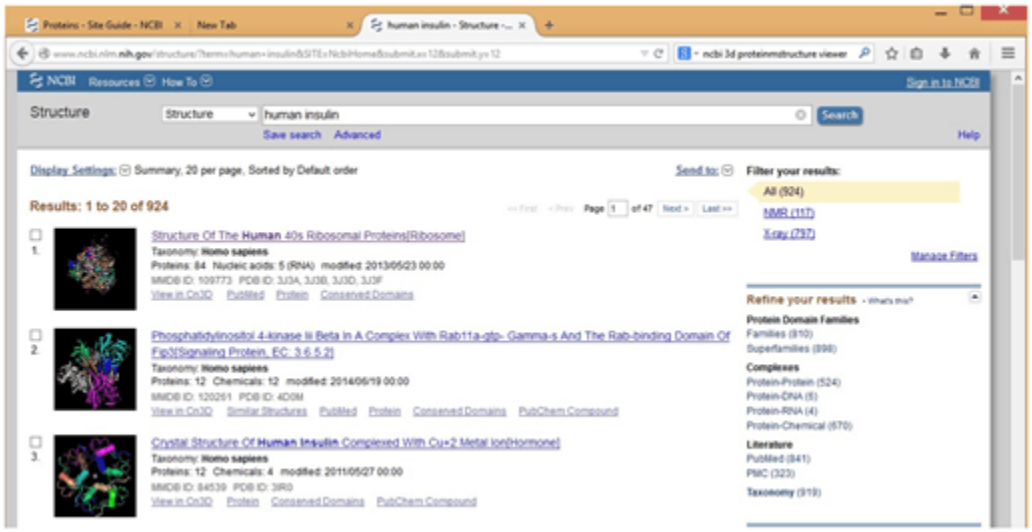
Click http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dinstall.shtml to download the Cn3D-4.3.1_setup file (for Windows or Mac). The software will reside on your computer and will activate when you go to a macromolecule database search site.

Click http://www.ncbi.nlm.nih.gov/Structure/MMDB/docs/mmdb_search.html to enter the protein structure database (below):

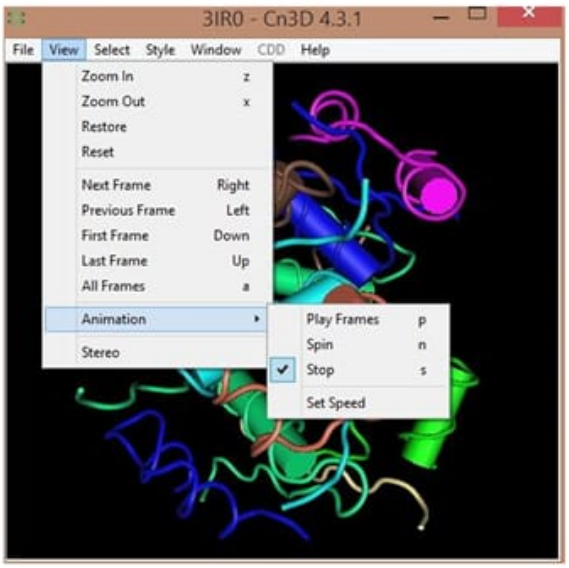


The search example shown above for human insulin takes you to this link: http://www.ncbi.nlm.nih.gov/structure/?term=human+insulin&SITE=NcbiHome&submit

.x=12&submit.y=12 The website is shown below:



Click *View in Cn3D* for the desired protein. For human insulin see this:



To rotate the molecule, click *View* then *Animation,* then *Spin*... and enjoy!

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3.9: Key Words and Terms

Ab	functional groups	primary structure
a-carbon	gene family	prion
alpha helix	gene superfamily	protein folding
allosteric regulation	glycosylation	PrP
Alzheimer's disease	helix-turn-helix motif	quaternary structure
amino acid residues	hemoglobin	random coil
amino end	hydrophobic interactions	recombinatorial exchange
amyloid beta protein	levels of protein structure	salt bridges
amyloid plaques	Mad Cow disease	salt bridges
beta barrel	multimer	secondary structure
beta sheet	NCBI Cn3D database	sequence motifs
carboxyl end	neurofibrillary tangles	sickle cell anemia
catalysts	nucleoskeleton	side chains
chaperones	orders of protein structure	structural domain
configuration	Parkinson's disease	structural motif
Creutzfeldt-Jakob disease	peptide bonds	sulfhydryl groups

cytoskeleton	**peptide linkages**	**tau protein**
disulfide bonds	**phosphorylation**	**tertiary structure**
Edman degradation	**pleated sheet**	
enzymes	**polypeptide backbone**	

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4: BIOENERGETICS

4.1: Introduction
4.2: Kinds of Energy
4.3: Deriving Simple Energy Relationships
4.4: Key Words and Terms

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4.1: Introduction

Three ***Laws of Thermodynamics*** describe the flow and transfer of energy in the universe.

1. **Energy can neither be created nor destroyed.**

2. ***Universal entropy (disorder) is always increasing***.

3. ***Entropy declines with temperature*** -as temperatures approach absolute zero, so goes entropy

In living systems, we do not have to worry about the third law because equations for energy exchange in living systems already reflect the temperature dependence of entropy changes during reactions. Here we look at how we came to understand basic thermodynamic principles and how they apply to living systems. First, we will look at different kinds of energy and at how redox reactions govern the flow of energy through living things. Next, we will try to understand some simple arithmetic statements of the *Laws of Thermodynamics* for *closed systems* and then at how they apply to chemical reactions conducted under ***standard conditions***. Finally, since there really is no such thing as a ***closed system***, we look at the energetics of reactions occurring in open systems. For an excellent discussion of how basic thermodynamic principles apply to living things, see Lehninger A. (1971) *Bioenergetics: The Molecular Basis of Biological Energy Transformation*. Benjamin Cummings, San Francisco.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain the difference between *energy transfer* and *energy transduction*.

2. compare and contrast *potential* vs. *kinetic* as well as other categories of energy (e.g.,

mass, heat, light..., etc.).

3. explain the reciprocal changes in universal free energy and entropy.

4. derive the algebraic relationship between *free energy*, *enthalpy* and *entropy*.

5. state the difference between *exothermic*, *endothermic*, *exergonic*, and *endergonic*

reactions

6. predict changes in free energy based on changes in the concentrations of reactants and products in closed systems and open systems.

7. explain how the same reaction can be endergonic but will (under appropriate conditions) release free energy.

8. predict whether a biochemical reaction will release free energy if it is exothermic, and if so, under what conditions (you should be able to do this after working some sample problems of closed system energetics).

9. distinguish between the equilibrium and steady-state of reactions and explain how an endergonic reaction could also be spontaneous (i.e., could release free energy).

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4.2: Kinds of Energy

We can easily recognize different kinds of energy around us like ***heat***, ***light***, ***electrical***, ***chemical***, ***nuclear***, ***sound***, etc., and you probably know that energy is measurable (calories, joules, volts, decibels, quanta, photons...). Even mass is a form of energy, as you may recall from Albert Einstein's famous e=mc2 equation (the *law of relativity*).

The problem in thinking about thermodynamics is that the universe is big and there are too many kinds of energy to contemplate at once! To simplify, imagine only two kinds of energy in the universe: ***potential energy*** and ***kinetic energy***. A helpful example is a dam. The water above the dam has *potential energy*. As the water flows over (or through) the dam, its potential energy is released as *kinetic energy*. In the old days the kinetic energy of flowing water could be used to power (i.e., turn) a millstone to grind wheat or other grains into flour. These days, water is more likely to flow through a hydroelectric dam where kinetic energy is converted (*transduced*) to electricity. In this simple view, heat (molecular motion), electricity (a current of electrons), sound (waves), and light (waves OR moving 'particles') are different forms of kinetic energy. The energy of mass or its position in the universe is potential energy. Thus chemical energy, for example the energy in a mole of ATP, is potential energy. Physicists talk a lot about potential energy and about kinetic energy flow and conversion.

An equally simple way to conceptualize energy is as ***useful*** *vs.* ***useless***. This concept led directly to the arithmetic formulation of the thermodynamic laws. In this utilitarian way of thinking about energy, useless energy is ***entropy***, while useful energy can be any of the other forms of energy (potential or kinetic).

A key to understanding bioenergetics is recognizing the difference between closed and open systems in the universe. Systems such as biochemical reactions in a test tube, reach ***equilibrium***. Such systems are considered *closed****. Closed systems*** are artificial, possible only in the laboratory, where one can restrict and measure the amount of energy and mass getting into or escaping the system. Cells on the other hand (in fact every reaction or event in the rest of the universe) are ***open systems***. Open systems readily exchange energy and mass with their surroundings.

YouTube Video

This interactive element is not available in this format. Visit the link or scan the QR code to access it online.

View this YouTube Video online

Video 4.2.1: Different Kinds of Energy; Chemical Equilibrium. (Gerry Bergtrom)

With this brief introduction, we can imagine ourselves to be early scientists trying to understand energy flow in the universe, asking how the ***Laws of Thermodynamics*** apply to living systems (*bioenergetics*). We'll see that the *Laws* can be demonstrated because all kinds of energy can be measured (as heat in calories or joules, electricity in volts, light in quanta, matter in units of mass, etc.).

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4.3: Deriving Simple Energy Relationships

A. Energy in the Universe: the Universe is a *Closed System*

Consider an event, any even. I think we can agree that when stuff happens, participants in the happening go from an unstable state to a relatively more stable state. For example, you carrying a bag of marbles and you accidentally drop the bag. The marbles would fall to the floor, roll and spread out, eventually coming to a stop. At that point, the marbles are in a more *stable state* than they were when you were holding the bag.

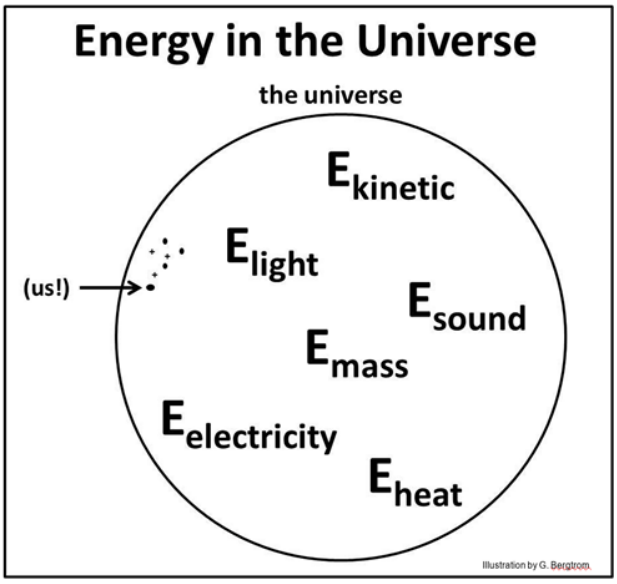
If asked, you would say that gravity made the marbles fall from the bag. That is certainly true. Could we then say that the drive to greater stability is what made the marbles fall? In fact, regardless of the force or impetus for the event, we can say that the drive to achieve greater stability that makes things/events happen! This is the essence of the ***Second Law of Thermodynamics***: *all universal energy transfer events occur with an increase in stability..., that is, an increase in entropy*. We'll consider the second law and entropy in detail shortly.

The tendency of things go from unstable to more stable is a natural, rational state of affairs..., like those marbles on the floor, or a messy bedroom with clothes strewn about. Intuitively, messy and disordered is more stable than ordered. Of course, marbles dropping or clothing going from folded and hung to wrinkled on the floor releases energy (potential energy) as they fall (kinetic energy). If you don't believe that this release of energy is real, just think of how much energy you will need to pick up the marbles or re-fold your clothes (after laundering them of course!).

We can model the flow of energy in the universe in a way that is consistent with thermodynamic laws. Since the ***First law of Thermodynamics*** says that *energy can be neither created nor destroyed*, a simple statement of the First Law could be:

$$E_{universal} = E_{light} + E_{heat} + E_{electrical} + E_{mass}....$$

The equation sums up the different kinds of energy in the universe. Look at it this way:



Energy cannot get in or out of the universe. Energy can only be transferred from one place to another, or converted from one form to another. It follows then that **Euniversal** is the sum of all kinds of energy in the universe, and that this *sum* is a constant. The equation below is consistent with this idea.

$$E_{light} + E_{heat} + E_{electrical} + E_{mass} + \ldots = \text{a constant}$$

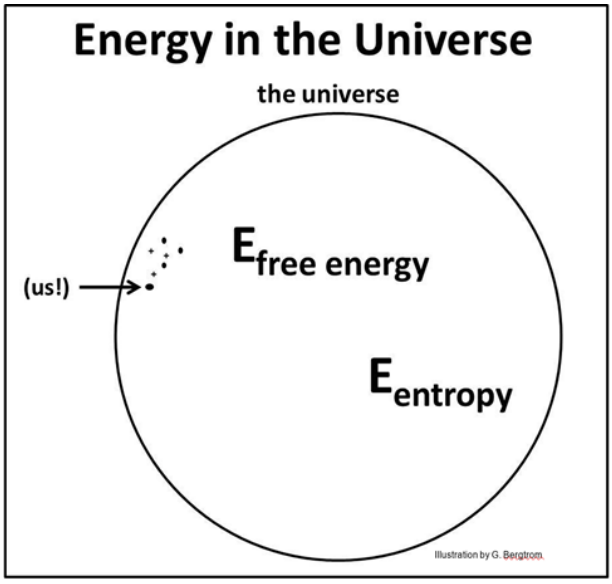
This is a statement of the *First Law of Thermodynamics*.

139 First Law of Thermodynamics

If we go with the simpler binary notion or ***useful*** and ***useless*** energy, our equation shortens to the sum of just two kinds of energy in the universe:

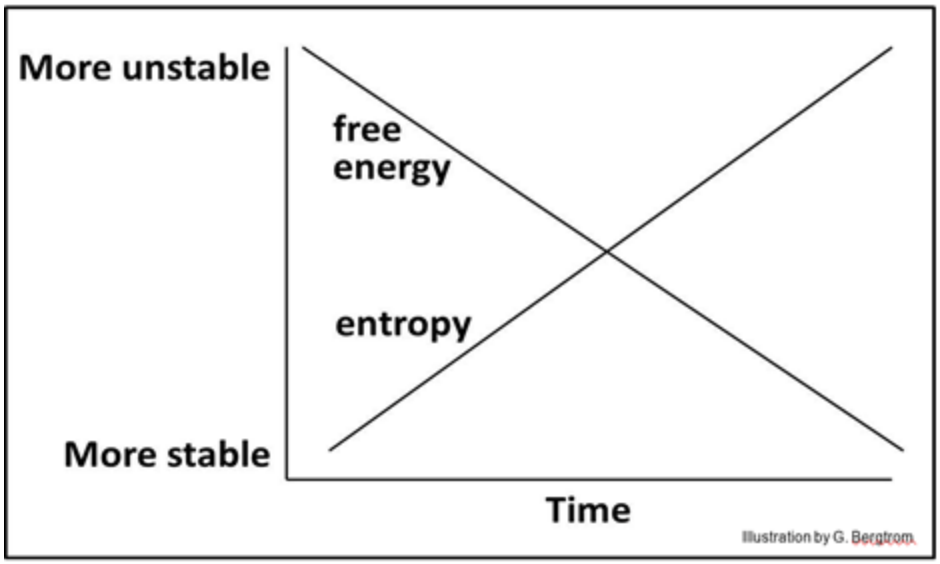
$$E_{universal} = G_{universal} + TS_{universal}$$

In this equation, G is useful energy ("Gibbs" free energy), S is useless energy (entropy), and T is absolute temperature (included because of the third law). This is also a statement of the *First Law*. Here is our revised circle diagram:



By the way, segregating things and concepts into circles is a way of logically viewing relationships between them. John Venn first formalized this approach in the late 19th century. The *Venn diagrams* used here to describe the universe are very simple. For examples of more complex overlapping components of the universe that share some but not all attributes, "Google" Venn Diagrams.

In this binary energy model, it follows that as universal entropy increases, free energy in the universe must decrease:



Free or 'potentially useful' energy is higher in more ordered, complex and therefore relatively unstable systems. Such unstable, ordered systems will release free energy *spontaneously*.

140 Second Law Thermodynamics

While the arithmetic statements about changes in energy are useful concepts, the parameters are of course, not measurable! But if we isolate a bit of the universe, we *can* measure energies and watch energy flow.

B. Energy is Exchanged Between Systems in the Universe

If we can measure the amount of energy put into or removed from ***a system within the universe***, we can write a more useful equation to follow the transfer of energy between a system and its surroundings:

$$\Delta H = \Delta G + T\Delta S \tag{4.3.1}$$

In this formula, ΔH is the change (D) in enthalpy, i.e., as energy entering/leaving the system in units of heat energy); ΔG is change in free energy and ΔS is change in entropy; **T** = absolute temperature (oK).

Heat given off in a reaction (or other event) is often confused with entropy. It is true that much of the increase in entropy that occurs in living things is indeed in the form of random molecular motion, or heat. But remember that heat can have its uses; not all heat is entropic! Hence, it is more interesting (and accurate!) to think of energy in terms of changes in enthalpy, free energy and entropy during energy transfers.

According to the equation $\Delta H = \Delta G + T\Delta S$, interacting systems in our universe would seem to be *closed systems*. Accordingly, energy put into or removed from the system (ΔH) will be exactly balanced by increases and/or decreases in the other two terms in the equation ($\Delta G + T\Delta S$**)**. Recall that we refer to these systems as closed systems *not* because they are really closed, but because we can isolate them well enough to account for energy flow into and out of the system. The value of this (or any) algebraic equation with three variables is that if you know two of the values, you can calculate the third! Here is a simple situation to illustrate the point: If I put a liter of water on a burner and light the flame, the water gets hot. If the temperature of the liter of water rises by 1oC, we know that it has absorbed 1000 calories (one Kcal, or one *food* Calorie) of the heat from the burner.

Since energy interactions depend on different physical conditions, such as temperature and air pressure, we need to standardize those conditions when conducting experiments that measure energy changes in experimentally isolated systems. For more on how standardizing these physical parameters enables measuring energy change in chemical reactions (in fact, any energy exchange), click the link below.

141 Deriving Closed System Thermodynamics

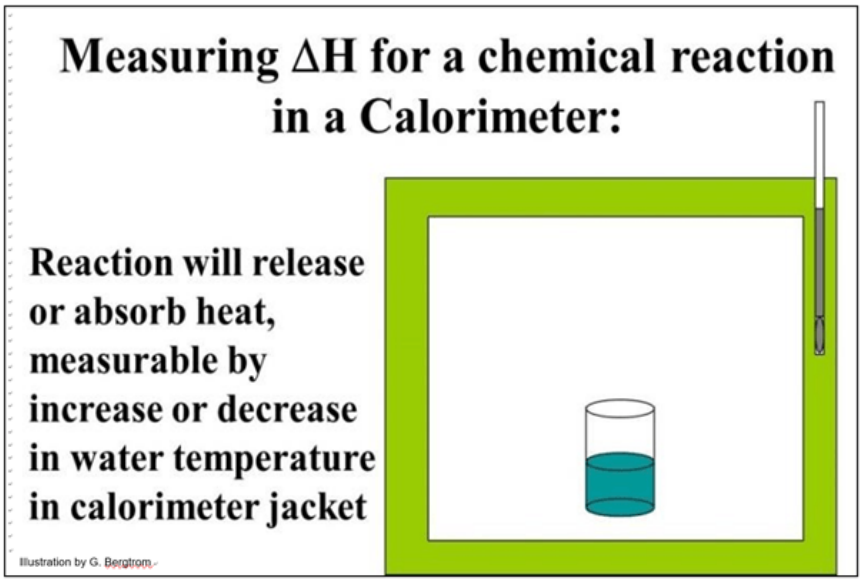
Turning to ***bioenergetics***, let's apply the equation $\Delta H = \Delta G + T\Delta S$ to chemical reactions in cells. Because most life on earth

lives at sea level where the air pressure is 1 atmosphere and the temperature is in the 20's (Celsius), typical determinations of ΔH, ΔG, and ΔS are made under ***standard conditions*** where T=298oK (25oC), an atmospheric pressure of pressures of 1 atm, and a constant pH of 7.0. In addition, measured values are adjusted to calculate unimolar quantities of reactants (see below). Our equation for reactions under these standard conditions becomes: $\Delta H = \Delta Go + T\Delta S$**,** where ΔGo is the ***standard free energy change*** for the reaction conducted in a *closed system* under standard conditions, while ΔH and ΔS are still the enthalpy and entropy changes, but determined under standard conditions.

What are *unimolar conditions* in practice? It means that if you burn 180 mg of glucose in a calorimeter, multiply the calories released (ΔH) by 1000. This gives you the calories released by burning 180 gm (i.e., a whole mole) of the stuff. Now we are ready to consider examples of how we determine the energetics of reactions.

c. How is Enthalpy Change (ΔH) Determined?

ΔH for a chemical reaction can easily be determined by conducting the reaction under standard conditions in a ***bomb calorimeter*** (illustrated below).



Food manufacturers determine the ***Calorie*** content of food using a bomb calorimeter. As a reaction takes place in the beaker in the illustration, it will either release or absorb heat, either heating or cooling the water in the calorimeter jacket, as measured by the thermometer. A reaction that releases heat as it reaches equilibrium is defined as ***exothermic***, and the ΔH for an exothermic reaction will be negative. For example, a package says that a chocolate bar has 90 Calories. This means that burning the bar will generate 90 kilocalories (Kcal) of heat as measured in the calorimeter. Recall that one Calorie (with a capital C) = 1000 calories, or one Kcal. One calorie (lower case) is the energy needed to raise a gram of water by 1oC).

You are probably most familiar with reactions that release heat, but some chemical reactions actually absorb heat. Take the common hospital cold pack for example. Squeeze it to get it going and toss it in the calorimeter. You can watch the temperature in the calorimeter drop as the pack absorbs heat from the surroundings! Such reactions are defined as ***endothermic***, with a positive ΔH. OK, so we can determine the value of one of the energy parameters... we need to know at least one other, either ΔGo or ΔS before the equation $\Delta H = \Delta Go + T\Delta S$ becomes useful.

D. How is Standard Free Energy Change (ΔGo) Determined?

As it turns out, standard free energy change, ΔGo , is *directly proportional* to the concentrations of reactants and products of a reaction conducted to completion (i.e., ***equilibrium***) under standard conditions. Therefore, to determine ΔGo, we need to be able to measure the concentration of reactants and reaction products before and after a chemical reaction (i.e., when the reaction reaches *equilibrium*). Take the following generic chemical reaction:

$$2A + B <===> 2C + D \tag{4.3.2}$$

The following equation relates ΔGo the equilibrium concentrations of A, B, C and D:

$$\Delta Go = -RTlnKeq = -RTln((C^2 + D)/(A^2 + B)) \tag{4.3.3}$$

R= the gas constant (1.8006 cal/mole-deg), **T** = 298oK and **Keq** is the ***equilibrium constant***. This is the ***Boltzman equation***. As you can see, the Keq for the reaction is the ratio of the product of the concentrations of the products (raised to their stoichiometric powers) to the product of the concentrations of the reactants (raised to *their* stoichiometric powers).

If you can determine equilibrium concentrations of reactants and products in a chemical reaction, you can use this equation to calculate ΔGo (*standard free energy change*) for a reaction. Consider the following generic chemical reaction:

$$A + B \Leftrightarrow C + D \tag{4.3.4}$$

If the ΔGo is a negative number, the reaction is defined as ***exergonic***. We say that exergonic reactions release free energy. If the ΔGo is a positive number, the reaction absorbs free energy and is defined as ***endergonic***.

142 Determining DH & DG in Closed Systems

E. Working an Example Using These Equations for Closed Systems

Consider the following reaction:

$$X \Leftrightarrow Y \tag{4.3.5}$$

If you are given [X] and [Y], you can also do the math. At equilibrium, the concentrations of the reactants and products for this reaction measured (assayed), with the following results:

[X] = 2.5 Kcal/Mole; [Y] = 500 cal/Mole

Use the Boltzmann equation (above) to calculate the standard free energy for this reaction. What is the **Keq** for this reaction? What is the ΔGo for the reaction? If you did not come up with a Keq of 0.2 and an absolute value for the standard free energy for**|** ΔGo**|** of 866.2 Kcal/mole, re-calculate or collaborate with a classmate. Hint: make sure that you convert the units in your equation so that they are all the same!). Based on the calculated value of ΔGo, is this reaction endergonic or exergonic?

If you conduct the reaction in a bomb calorimeter, it proceeds to equilibrium with a ΔH = -750 Kcal/Mole. What kind of reaction is this? Together with the *enthalpy change*, it is now possible to calculate an absolute value for the entropy change to be **|**ΔS**|** = 116.2 cal/mol-deg for this reaction. At equilibrium, did the reaction proceed with an increase or decrease in entropy under standard conditions? Again, if you did not get the correct answer, re-calculate or collaborate with a classmate.

143 Determining DS in Closed System

F. Summary: The Properties of Closed Systems

First, let's reiterate that there is no such thing as a closed system, unless of course the universe is one! What we call a closed system is simply one in which we can measure the energy going into or coming out of the system, and within which we can measure energy transfers and transductions (changes from one kind of energy to another.

Features of systems can be defined by their properties:

Properties of Closed Systems

1. **Closed systems are experimentally *defined by an investigator*.**
2. ***Standard conditions* apply.**
3. **Energy entering or leaving the system is *measurable*.**
4. **Reactions reach *equilibrium* regardless of reaction rate.**
5. **Product and reactant concentrations at equilibrium are constant.**
6. **Measured energy transfer/transduction values are *constant*.**

G. Actual Free Energy Change in Open Systems

Later we will be discussing the flow of energy through living things, from sunlight to chemical energy in nutrient molecules into ATP, from chemical energy as ATP into the performance of all manner of cellular work. Cells are ***open systems*** that constantly exchange mass and energy with their environment and ***never reach equilibrium***. In addition, diverse organisms live under very different atmospheric conditions and maintain different body temperatures (e.g., your cat has a higher body temperature than you do!). Clearly, the conditions under which cells conduct their biochemical reactions are decidedly *non-standard*. However, while *open systems* do not reach equilibrium, they *do* achieve a ***steady state*** in which the rate of input of energy and matter is equal to the rate of output of energy and matter. Think of a biochemical pathway like glycolysis. If a cell's energy needs are constant, the pathway will reach a *steady state*. Of course, a cell's need for energy (as ATP) can change as energy needs change. If it does, then the steady state of ATP production will change to meet the needs of the cell.

Since reaction rates can change (and are in fact regulated in cells), implying that the steady state of a reaction or biochemical pathway can change. We characterize open systems by their properties, as listed below.

Properties of Open Systems

1. **Open systems *exchange energy and mass with their surroundings*.**
2. **Open systems *never reach equilibrium***
3. **They *achieve steady state* where the energy input rate = output rate.**
4. **The *steady state can change*.**
5. **In open systems, *endergonic reactions can be energetically favorable* (spontaneous).**

Fortunately, there is an equation to determine free energy changes in open systems. For our chemical reaction $2A + B <===> 2C + D$, this equation would be:

$$\Delta G' = \Delta Go + RT \ln(((Css)^2 + Dss)/((Ass)^2 + Bss)) \tag{4.3.6}$$

Here, $\Delta G'$ is the ***actual free energy change*** for a reaction in an open system. ΔGo is the standard free energy change for the same reaction under standard conditions. In a closed system. **R** is again the gas constant (1.806 cal/mole-deg) and **T** is the absolute temperature in which the reaction is actually occurring. The subscript '**ss**' designates reactant and product concentrations measured under *steady state* conditions. To determine the actual free energy of a biochemical reaction in a cell (in fact in any living tissue), all you need to know are the ΔGo for the reaction, the steady state concentrations of reaction components in the cells/tissues, and the absolute T under which the reactions are occurring.

144 The Energetics of Open Systems

Elsewhere, we will use the reactions of the glycolytic pathway to exemplify the energetics of open and closed systems. At that time, pay careful attention to the application of the terminology of energetics in describing energy flow in *closed* vs. *open systems*.

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4.4: Key Words and Terms

actual free energy	endothermic	Law of Conservation
ATP	energy	Laws of Thermodynamics
bioenergetics	energy transduction	light
Boltzman equation	energy transfer	mass
calories	enthalpy	open system properties
calorimeter	entropy	open systems
chemical energy	equilibrium constant	order vs. entropy
chemical equilibrium	exergonic	standard conditions
closed systems	exothermic	standard free energy

decibels	free energy	steady state
e=mc2	gas constant	useful energy
electricity	Gibbs free energy	useless energy
endergonic	Keq	volts

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5: ENZYME CATALYSIS AND KINETICS

5.1: Introduction
5.2: Enzymes
5.3: Key Words and Terms

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5.1: Introduction

In this chapter, we look at the properties and mechanism of action of enzymes. These include allosteric change (***induced fit***, ***enzyme regulation***), energetic events (changes in ***activation energy***), and how enzymes work in open and closed (experimental) systems. Any catalyst, by definition, accelerates a chemical reaction. But enzymes and inorganic catalysts differ in important ways (blue and red in the table below).

Enzymes *vs* Inorganic Catalysts

Inorganic Catalysts	Enzymes
e.g., Ni, Pl, Ag, etc.	e.g., pepsin, trypsin, ATP synthase, ribonuclease, etc.
increase rxn rate	increase rxn rate
unchanged at end of rxn	unchanged at end of rxn
non-specific	highly specific
rigid, inflexible	flexible - can undergo *allosteric* change…
cannot be regulated	can be regulated

Enzymes are long polymers that can fold into intricate shapes. As a result, they can be more specific than inorganic catalysts in which substrates they recognize and bind to. Finally, enzymes are flexible and can be regulated in ways that rigid, inflexible, metallic inorganic catalysis cannot. The specificity of an enzyme lies in the structure and flexibility of its ***active site***. We will see that the active site of enzymes undergo conformational change during catalysis. The flexibility of enzymes also explains the effects of enzymes to cellular metabolites that indicate the biochemical status of the cell. When such metabolites bind to an enzyme, they force a conformational change in the enzyme that change the catalytic rate of the reaction, a phenomenon called ***allosteric regulation***. As you might imagine, changing the rate of a biochemical reaction can change the rate of an entire biochemical pathway…, and ultimately the steady state concentrations of products and reactants in the pathway.

To understand the importance of allosteric regulation, we'll look at how we measure the speed of enzyme catalysis. As we consider the classic early 20th century enzyme kinetic studies of Leonor Michaelis and Maud Menten, we'll focus on the significance of the ***Km*** and ***Vmax*** values that they derived from their data. But, before we begin our discussion here, remember that chemical reactions are by definition, reversible. The action of catalysts, either inorganic or organic, depends on this concept of reversibility.

Finally, let's give a nod to recent human ingenuity that enabled enzyme action to turn an *extracellular profit*! You can now find enzymes in household cleaning products like detergents, where they digest and remove stains caused by fats and pigmented proteins. Enzymes added to meat tenderizers also digest (hydrolyze) animal proteins down to smaller peptides. Enzymes can even clean a clogged drain!

Learning objectives

When you have mastered the information in this chapter, you should be able to:

1. describe how the molecular flexibility of protein and RNA molecules make them ideal biological catalysts.

2. compare and contrast the properties of *inorganic* and *organic* catalysts.

3. explain why *catalysts do not change equilibrium concentrations* of a reaction conducted in a closed system.

4. compare the activation energies of catalyzed and un-catalyzed reactions and explain the roles of *allosteric effectors* in enzymatic reactions.

5. discuss how allosteric sites interact with an enzyme's active site and explain the concept of the rate limiting reaction in a biochemical pathway.

6. write simple rate equations for chemical reactions.

7. write the possible rate equations for equations for catalyzed reactions.

8. distinguish between *Vmax* and *Km* in the Michaelis-Menten kinetics equation.

9. state what Vmax and Km say about the progress of an enzyme catalyzed reaction.

10. interpret enzyme kinetic data and the *progress of an enzyme-catalyzed reaction* from this data.

11. more accurately identify Leonor Michaelis and Maud Menten!

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5.2: Enzymes

By 1941, their studies correlating mutations with enzyme deficiencies in *Neurospora crassa* (bread mold) and *Drosophila melanogaster* led George Beadle and Edward Tatum to propose the ***one-gene/one-enzyme hypothesis*** in 1941. In 1958, they shared Nobel Prize in Physiology and Medicine for this work. By the time of the award, their hypothesis had morphed twice: first into the ***one-gene/one-protein***, and then the ***one-gene/one- polypeptide*** hypothesis. This rightfully revered history helped launch the age of molecular biology, making the discovery of RNA catalysts quite a surprise! The discovery of RNA catalysts, dubbed ***ribozymes***, earned Sidney Altman and Thomas Cech the Nobel Prize in Chemistry in 1989. Ribozymes are now known that catalyze RNA splicing (the removal of unwanted regions of a precursor RNA. ***Ribozymes*** are also associated with a region of ribosomal RNA, where they participate in the catalysis of protein synthesis. Here, the focus here is based on the long history of *protein enzyme catalysis*, but you should recognize the mechanisms of enzyme catalytic mechanisms to be discussed involve essential features in all biocatalysts.

Enzymes are generally soluble in or outside cells while a few are part of membranes or other cellular structures. In all cases, they bind to soluble ***substrates*** (the reactants in enzyme-catalyzed reactions). The large size and exquisite diversity of protein structures make enzymes highly specific catalysts. The specificity of an enzyme results from the shape of the ***active*** site of the enzyme, which is dependent on the three-dimensional arrangement of amino acids in and around the region. The *substrates* of a catalyzed biochemical reaction are bound to, and held in place on the enzyme while rapid bond rearrangements take place. Because of their flexibility, enzymes undergo change in shape at the active site during catalysis itself. In addition, this flexibility enables small metabolites in cells to interact with and change the shapes of many enzymes. The latter phenomenon enables ***allosteric regulation***, allowing cells to control the rates and even the direction of biochemical reactions and pathways. As we will see, enzymes may also be bound to *prosthetic groups* or ions that contribute to the shape and activity of the enzyme.

Almost no chemical reaction occurs that is not directly the result of enzyme catalysis, from the digestion of nutrients in your mouth, stomach and small intestines to pretty much every chemical reaction inside your cells [check out Kornberg A (1989) *Never a Dull Enzyme*. Ann. Rev. Biochem. 58:1-30].

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A. The Mechanisms of Enzyme Catalysis

We describe the action of biological catalysis in two ways. One way takes into account structural features of the enzyme (active site shape, overall conformation, the *affinities* of the enzyme for its substrates). The other way involves the energetic of enzyme action. We'll see that enzymes lower the ***activation energy*** of a chemical reaction. Activation energy is an inherent energy barrier to the reaction. Of course, structural and energy considerations of enzyme catalysis are related.

1. Structural Considerations of Catalysis

From a chemistry course, you may recall that the rate of an uncatalyzed reaction is dependent on the concentration of the reactants in solution. This is the *Law of Mass Action*, recognized in the 19th century. Look at this simple reaction:

$$\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{C} + \mathrm{D} \tag{5.2.1}$$

The *Law of Mass Action* makes two key assumptions:

a. At any given time following the start of the reaction, the rate of product formation is proportional to the concentrations of the

reactants and products ([A], [B], [C] and [D] in this case).

b. Chemical reactions in the laboratory eventually reach equilibrium, at which point the net rate of formation of reaction products is zero (i.e., the forward and reverse reactions occur at the same rate).

At the start of the reaction written above, since there are no products yet, the reaction rate should be directly proportional only to the concentration of the reactants. Therefore, the *Law of Mass Action* predicts that a chemical reaction will occur faster at higher concentrations of A & B. This is because there are more reactant molecules in solution and a greater likelihood that they will collide in an orientation that allows the bond rearrangements for the reaction to occur.

Of course, reactant concentrations decline as products accumulate over time. Then the rate of formation of C & D should slows down, now affected by product as well as reactant concentrations; remember, all chemical reactions are inherently reversible! You may recognize the chemical rate equations from a chemistry course; these enable quantitation of reaction rates for our sample reaction. Here is the rate of formation of the products, C and D:

Misplaced '&'

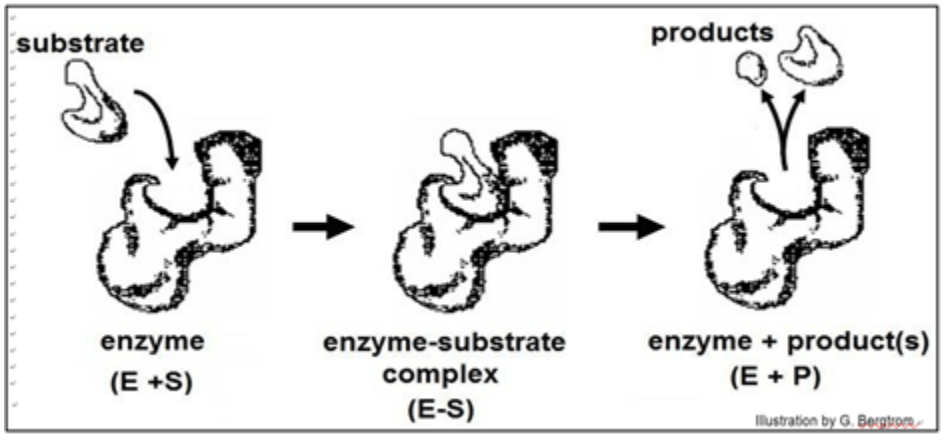
This equation recognizes that the reaction is reversible. Thus, the net reaction rate is equal to the *rate of the forward reaction* $\mathrm{k_1[A][B]}$ minus the *rate of the back reaction* $\mathrm{k_{-1}[C][D]}$. The equation is valid (applicable) at any time during the reaction. $\mathrm{k_1}$ and $\mathrm{k_{-1}}$ are ***rate constants*** for the forward and reverse reactions, respectively.

So how do catalysts work? Catalysts increase chemical reaction rates by bringing reactants together more rapidly than they would encounter each other based just on random molecular motion in solution. This is possible because catalysts have an ***affinity*** for their substrates.

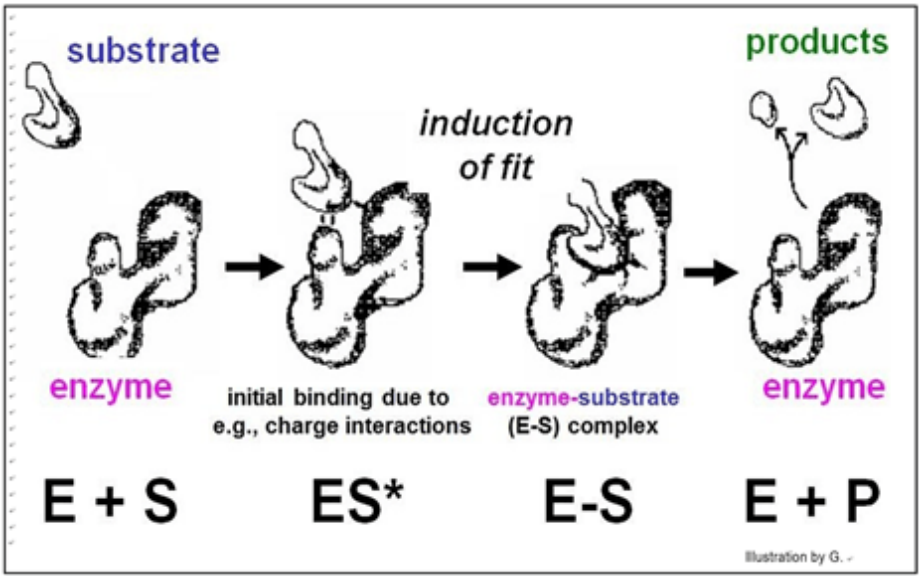
In the case of inorganic catalysts, relatively weak, generic forces account for the affinity of reactants and inorganic catalysts. Thus, a metallic catalyst (e.g., silver, platinum) attracts molecules with the appropriate (e.g., charge) configuration. If the attraction (*affinity*) is sufficient, the metal will hold reactants in place long enough to catalyze the bond rearrangements of a chemical reaction.

Unlike inorganic catalysts, enzymes have evolved highly specific shapes with physical-chemical properties. As a result, enzymes typically attract only the substrates necessary for a particular biochemical reaction. The active site of an enzyme has the exquisitely selective affinity for its substrate(s). This affinity is many times greater than those of inorganic catalysts for generic reactants. The result is that enzymes are more efficient, faster catalysts.

Early ideas of how substrate-enzyme interaction could be so specific suggested a ***Lock and Key*** mechanism, illustrated below.



According to this model, the affinity of enzyme for substrate brings them together, after which the substrate uniquely fits into the active site like a key into a lock. Once in the active site, the substrate(s) would undergo the bond rearrangements specific for the catalyzed reaction to generate products and regenerate an unchanged enzyme. But X-ray crystallography of enzyme-substrate interaction revealed that the active site of the enzyme changes shape during catalysis. This *allosteric change* suggested the revised, ***Induced Fit*** mechanism of enzyme action modeled below.



In this model, enzyme-substrate affinity causes the substrate to bind to the enzyme surface. Once bound, the enzyme undergoes an allosteric change, drawing the substrate(s) more tightly into the active site and catalyzing the reaction. Of course, after the reaction products come off, the enzyme returns to its original shape.

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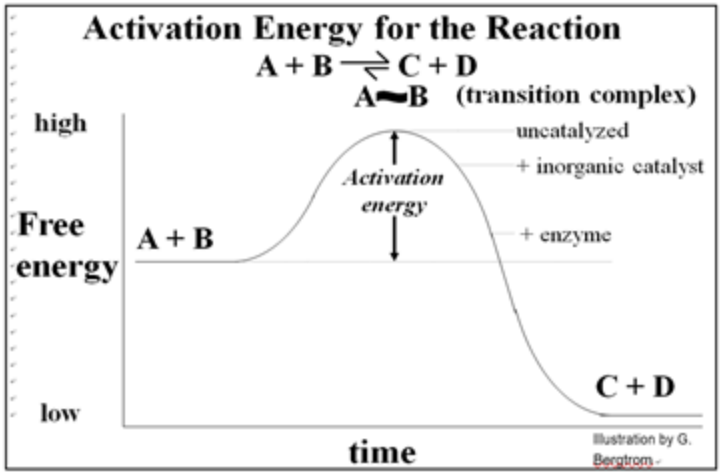
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2. *Energetic Considerations of Catalysis*

Catalysts work by lowering the ***activation energy*** (E_a) for a reaction, thereby dramatically increasing the rate of the reaction. Activation energy is essentially a barrier to getting interacting substrates together to actually undergo a biochemical reaction. Compare the random motion of substrates in solution that occasionally encounter one another. They even more rarely bump into one another in just the right way to cause a reaction. This explains why adding more reactants or increasing the temperature of a reaction can speed it up..., by increasing the number of random as well as productive molecular collisions. Unlike molecules and reactions in a test tube, living organisms do not have these options for conducting fast biochemical reactions, or controlling reaction rates.

Inorganic catalytic surfaces attract reactants where catalysis can occur. The attractions are weak compared to those of enzymes and their substrates. An enzyme's active site attracts otherwise randomly distributed substrates very strongly, making enzyme catalysis faster than inorganic catalysis. Again, cells cannot use inorganic catalysts, most of which are insoluble and would attract reactants indiscriminately... not a good way for cells to control metabolism! The advent of enzymes with their specificity and high rate of catalysis was a key event in *chemical evolution* required for the origins of life. As we saw, allosteric change during the 'induction of fit' enables specific catalysis. In fact, a catalyzed reaction will be faster than the same reaction catalyzed by a piece of metal, and of course much faster (millions of times faster!) than the uncatalyzed reaction. The energetic of catalysis helps to explain why. Take a look at the energetic of a simple reaction in which A & B are converted to C & D, shown below.



Conducted in a closed system, enzyme-catalyzed reactions reach their equilibrium more rapidly. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium concentrations of reactants and products of these reactions. The roughly 4000 biochemical reactions known to be catalyzed in cells are undoubtedly an underestimate! But remember too, that we estimate that the human genome has only 20,000 to 25,000 different genes!

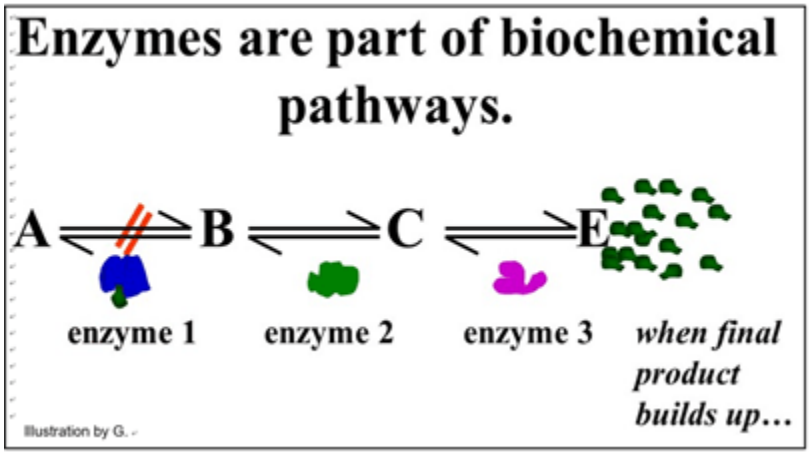
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B. Enzyme Regulation

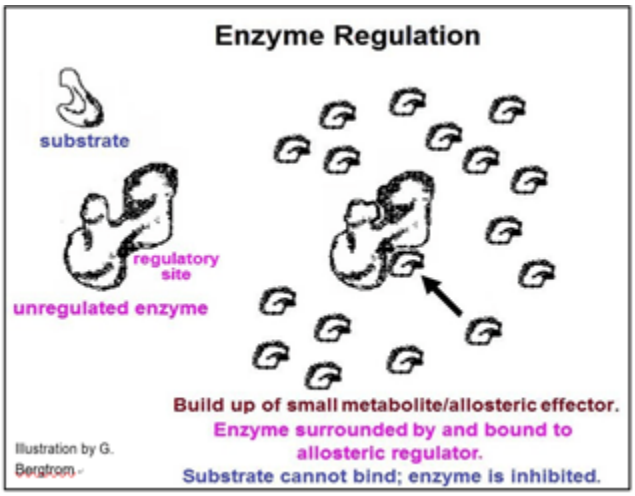
We noted that some enzymes are regulated, which just means that factors in the cell can slow down or speed up their rate of catalysis. In this way, the cell can respond quickly to metabolic needs reflected by the intracellular levels of these factors. Factors that slow down catalysis are called ***inhibitors***. Those that speed up catalysis are called ***activators***. In addition to responding to intracellular molecular indicators of the metabolic status of the cell, enzymes may be inhibited by drugs, poisons or changes in the chemical milieu (e.g. pH). Since cellular reactions occur as part of biochemical pathways, regulating a single enzyme can affect an entire pathway. For example, look at the generic pathway illustrated below.



This pathway exists to produce substance **E**. Under normal conditions, another series of metabolic reactions would consume **E**. However, if the cell is meeting its metabolic needs and no longer needs so much **E**, it will accumulate in the cell. If there is an excess of **E** in the cell, it might bind to one of the enzymes. In the pathway shown, **E** binds to *enzyme 1*. This binding causes an allosteric change inhibiting catalysis and slowing down the entire pathway. In this example, of ***allosteric regulation***, we can assume that inhibitory regulation of **enzyme 1** evolved to control the rate of production of substance **E**. This is a common mode of enzyme allosteric regulation, called ***feedback inhibition***.

Enzymes can be regulated precisely because they can be *bent out of shape* (or into shape for that matter!). Some small metabolites become chemical information when they accumulate in cells, becoming the indicators of cellular metabolic status. The result is a decrease or increase enzyme activities to achieve an appropriate cellular response.

Whether an activator or an inhibitor of enzyme catalysis, regulatory molecules typically bind to enzymes at ***allosteric regulatory*** sites, causing local allosteric changes that is transmitted to the active site. *Enzyme inhibition* will occur if a change in shape reduces the affinity of enzyme for substrate or the rate of the bond rearrangements after the substrate has entered the active site. *Enzyme activation* would occur if the allosteric effect were to increase this affinity and/or catalytic rate. The mechanism of allosteric regulation of enzyme activity is illustrated below.



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We can understand the effects changing rates of enzyme catalysis by determining *enzyme kinetics*. By comparing kinetic data for each enzyme in a biochemical pathway, one can determine a standard *rate-limiting reaction* under a given set of conditions. For example, if clinical tests reveal that a patient is producing too much of a biochemical metabolite, then the catalytic rate of the normally rate-limiting enzyme in its pathway of synthesis may have increased. What then, if the patient is producing too little of the metabolite? Either the catalytic rate of the rate-limiting enzyme has decreased or the catalytic rate of another enzyme in the biochemical pathway has become rate limiting. Reasons why a cellular biochemical would deviate from 'normal' levels include:

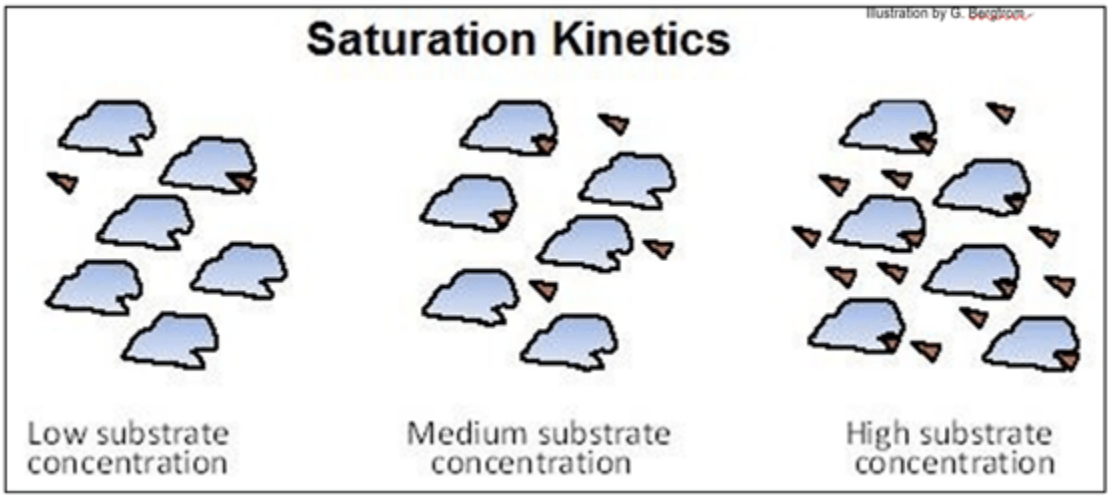
1. ***Viral & bacterial infection or environmental poisons:*** these can interfere with a specific reaction in a metabolic pathway; remedies depend on this information!
2. ***Chronic illness resulting from mutational enzyme deficiencies:*** treatments might include medications designed to enhance or inhibit (as appropriate) enzyme activity.
3. ***Genetic illness tied to metabolic deficiency:*** if a specific enzyme is the culprit, investigation of a pre- and/or post-natal course of treatment might be possible.

4. ***Life-style changes and choices:*** these might include eating habits, usually remediated by a change in diet.
5. ***Life-Style changes brought on by circumstance rather than choice:*** these are changes due to aging, such as the possibility of onset of Type 2 Diabetes; this can be delayed by switching to a low carb diet favoring hormonal changes that improve proper sugar metabolism.

Knowing the rate-limiting reaction(s) in biochemical pathways can identify regulated enzymes and lead to a remedy to correct a metabolic imbalance. As noted, ribozymes are RNA molecules that catalyze biochemical reactions; their kinetics can also be analyzed and classified. Next, we look at an overview of enzyme kinetics (for clear, detailed explanations of enzyme catalytic mechanisms, check out Jencks WP [1987, *Catalysis in Chemistry and Enzymology*. Mineola, NY, Courier Dover Publications]). We will consider how enzymes are regulated later, when we discuss glycolysis, a biochemical pathway that most living things use to extract energy from nutrients.

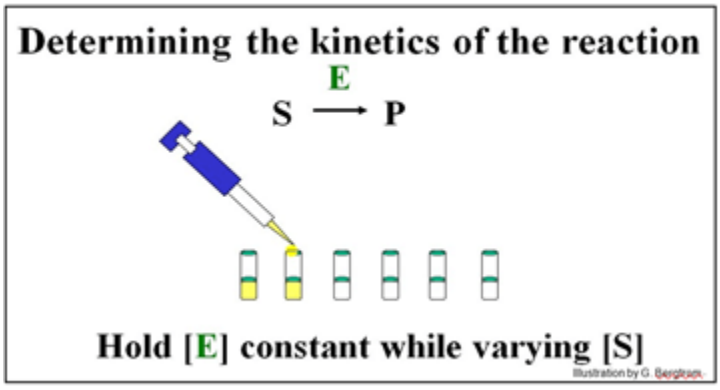
C. Enzyme Kinetics

All catalyzed chemical reactions display *saturation kinetics*, as shown below.

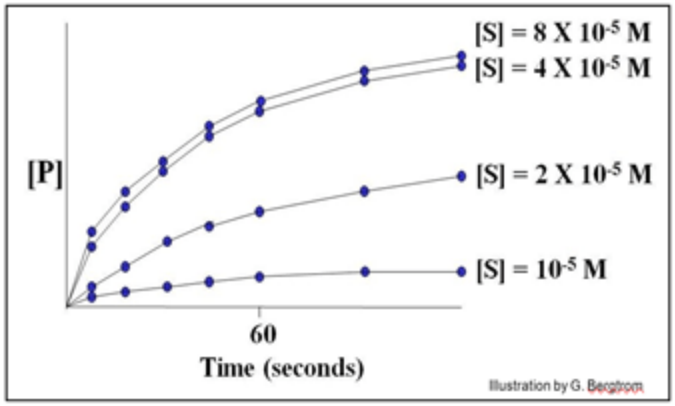


Note how at high substrate concentration, the active sites on all the enzyme molecules are bound to substrate molecules.

The experiment described below will determine the kinetics of the conversion of S to P by enzyme E.



A series of reaction tubes are set up, each containing the same concentration of enzyme ([E]) but different concentrations of substrate ([S]). The concentration of P ([P]) produced at different times just after the start of the reaction in each tube is plotted to determine the *initial rate* of P formation for each concentration of substrate tested (see below).



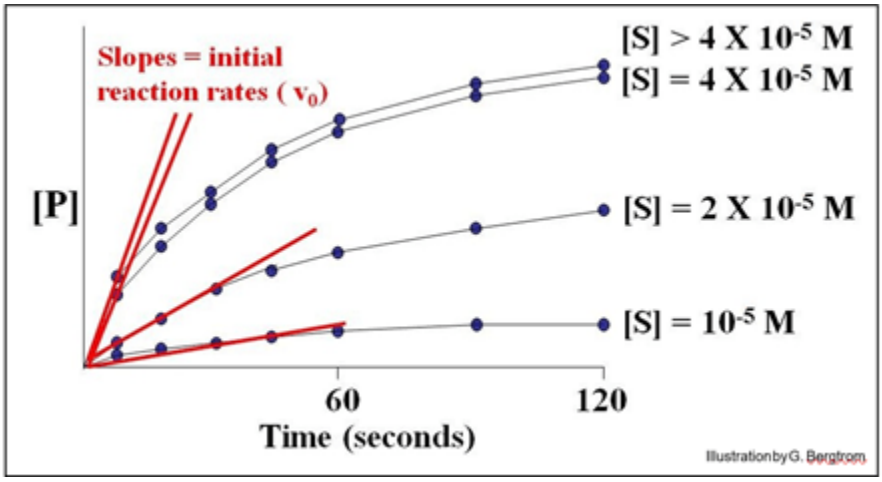
In this hypothetical example, the rates of the reactions (amount of P made over time) do not increase at substrate concentrations higher than 4 X10^{-5} M. The upper curves therefore represent the maximal rate of the reaction at the experimental concentration of enzyme. We say that the maximal reaction rate occurs at ***saturation***.

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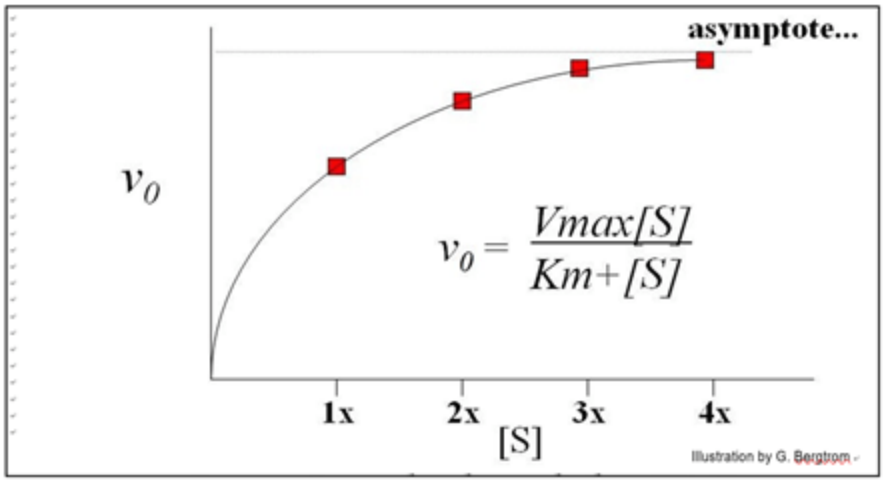
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Next, we can estimate the initial reaction rate (v_o) at each substrate concentration by plotting the slope of the first few time points through the origin of each curve in the graph. Consider the graph below, of the initial reaction rates estimated in this way.



Each straight line is the v_o for the reaction at a different [S] near the very beginning of the reaction, when [S] is high and [P] is vanishingly low. Next, we plot these rates (slopes, or v_o values) against the different concentrations of S in the experiment to get the curve of the reaction kinetics below.



This is an example of Michaelis-Menten kinetics common to many enzymes, named after the two biochemists who realized that the curve described ***rectangular hyperbola***.

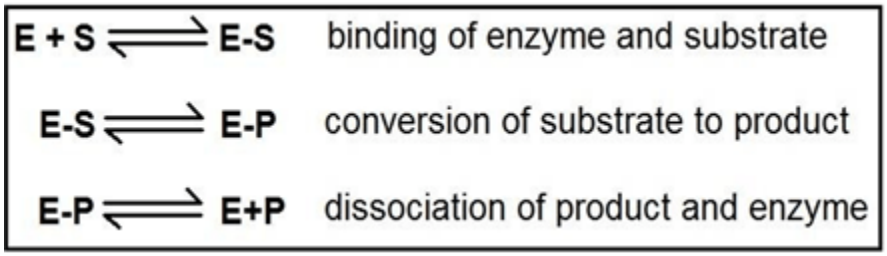
Put another way, the equation mathematically describes the mechanism of catalysis of the enzyme. The equation below mathematically describes a rectangular hyperbola:

$$\mathrm{y} = \frac{\mathrm{xa}}{\mathrm{x} + \mathrm{b}} \tag{5.2.2}$$

You might be asked to understand the derivation of (or even derive!) the Michaelis- Menten equation in a Biochemistry course. Suffice it to say here that Michaelis and Menten started with some simple assumptions about how an enzyme-catalyzed reaction would proceed and wrote reasonable chemical and rate equations for those reactions. Here is one way to write the chemical equation for a simple reaction in which an enzyme (E) catalyzes the conversion of substrate (S) to product (P):

$$\mathrm{S} \underset{\mathrm{E}}{\rightleftharpoons} \mathrm{P} \tag{5.2.3}$$

Michaelis and Menten rationalized that this reaction might actually proceed in three steps. In each step, **E**, the enzyme is treated as a reactant in the conversion of S to P. The resulting chemical equations are shown below.



Reasoning that the middle reaction (the conversion of E-S to E-P) would be the fastest one, and therefore would not be the *rate-limiting reaction of catalysis*, they only considered the first and third reactions to be relevant in determining the overall kinetics of product formation. Then they wrote the following rate equations for just these two chemical reactions (as one would in an introductory chemistry course):

$$V_{\text{E-S formation}} = \mathrm{k}_1[\mathrm{E}][\mathrm{S}] - \mathrm{k}_{-1}[\text{E-S}] \tag{5.2.4}$$

$$V_{\text{P formation}} = \mathrm{k}_2[\text{E-S}] - \mathrm{k}_{-2}[\mathrm{E}][\mathrm{P}] \tag{5.2.5}$$

Both of these equations describe a straight line, which does not describe the observed hyperbolic reaction kinetics. Solving one for e.g., E-S and substituting the solution for E-S in the other equation left a single equation that also described a straight line. Again, not the expected rectangular hyperbola. To arrive at a chemical rate equation consistent with a rectangular hyperbola, Michaelis and Menten made several assumptions, including those made by G. E. Briggs and J. B. S. Haldane about how E, S and P would behave in a catalyzed reaction.

It was those assumptions allowed them to re-write each equation, combine and rewrite them into a single mathematical equation that did indeed describe a rectangular hyperbola. Here are Briggs and Haldane's assumptions:

1. [S] >> [E] at start and during the 'steady state'
2. [P] << [S] at start of reaction
3. All E is bound to S at start of reaction
4. $[E]_{total} = [E]_{free} + [E\text{-}S]$ at all times

We have already seen the equation that Michaelis and Menten derived and now known as the Michaelis-Menten equation:

$$v_0 = \frac{\mathrm{Vmax}[S]}{\mathrm{Km} + [S]} \tag{5.2.6}$$

The take-home message here is that the assumptions about an enzyme-catalyzed reaction are a good approximation of how the reaction proceeds over time.

Michaelis and Menten defined Vmax and Km as key kinetic factors in enzymatic reactions. In the generic example of substrate conversion to product, we saw that increasing [S] results in a higher rate of product formation because a higher rate of encounters of enzyme and substrate molecules. At some point however, increasing [S] does not increase the initial reaction rate any further. Instead, v_0 ***asymptotically*** approaches a theoretical maximum for the reaction, defined as ***Vmax***, the *maximum initial rate*. As we have already seen, *Vmax* occurs when all available enzyme active sites are saturated (occupied by substrate). At this point, the intrinsic catalytic rate determines the *turnover rate* of the enzyme. The substrate concentration at which the reaction rate has reached ½*Vmax* is defined as $\boldsymbol{K_M}$ (the *Michaelis-Menten constant*). The Km is a ratio of rate constants remaining after rewriting the rate equations for the catalyzed reaction.

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To recapitulate, the two most important kinetic properties of an enzyme are:

1. how quickly the enzyme becomes saturated with a particular substrate, which is related to the *Km* for the reaction, and
2. the maximum rate of the catalyzed reaction, described by the *Vmax* for the reaction.

Knowing these properties suggests how an enzyme might behave under cellular conditions, and can show how the enzyme should respond to allosteric regulation by natural inhibitory or activating factors..., and to poisons or other anomalous chemicals. You can find more details of how kinetic equations are derived (a necessary step in understanding how the enzyme works) in any good biochemistry textbook, or check out the *Michaelis-Menten Kinetics* entry in in the Enzymes Wikipedia link.

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5.3: Key Words and Terms

activation energy	enzyme	Michaelis-Menten constant
active site	enzyme activation	Michaelis-Menten kinetics
allosteric change	enzyme inhibition	rate-limiting reaction
allosteric regulation	enzyme kinetics	ribozyme
allosteric site	enzyme regulation	saturation kinetics
biochemical pathway	induced fit	substrate specificity
catalytic RNAs	inorganic catalyst	substrates
conformation	Km	Vmax

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6: GLYCOLYSIS, THE KREBS CYCLE AND THE ATKINS DIET

6.1: Introduction

6.2: Glycolysis (from the Greek glyco (sugar) lysis (Separation), or Sugar Breakdown

6.3: Some Details of Glycolysis

6.4: Gluconeogenesis

6.5: The Atkins Diet and Gluconeogenesis

6.6: The Krebs/TCA/Citric acid cycle

6.7: Key Words and Terms

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6.1: Introduction

We used to get metabolic pathways charts like the one you see here free from vendors of biochemical reagents. This one is a high-resolution image; if you zoom in, you can actually read the content..., but don't feel you must! The big picture is correct in macro- detail, but the chart is likely out of date in small new details. In this chapter, we 'zoom in' on the small region in the middle of the chart, encompassing glycolysis and the Krebs cycle.

We have looked at the principles governing *thermodynamics* (the flow of energy in the universe) and *bioenergetics* (energy flow in living systems). We saw evidence that energy can be exchanged between components in the universe, but that it can be *neither created nor destroyed*. That makes the universe a *closed system*, a conclusion codified as the *first law of thermodynamics*. Personally, I find it troubling that there is no escape from the universe..., that is, until I remind myself that the universe is a pretty big place, and I am but a small part of a small system. You can define systems for yourself: the solar system, planet earth, the country you pledge allegiance to, your city or village, your school, a farm or homestead...! Then you may derive comfort from the realization that you can move from one system to another and even exchange goods and services between them. This is a metaphor for energy flow between systems in the universe. We also said that the first law applies to *closed systems within the universe*..., and that there are no closed systems in the universe! Any system in the universe is open, always exchanging energy and mass with neighboring systems. What we mean by the term 'closed system' is that we can define and *isolate* some small part of the universe, and then *measure* any energy that this isolated system gives up to its environment, or takes in from it. The simplest demonstration of the first law in action was the *bomb calorimeter* that measures heat released or absorbed during a chemical reaction.

The second concept said that energy flows from one place to another only *when it can*. In the vernacular, we say that ***energy flows downhill***. Anything that happens in the universe (a galaxy moving through space, a planet rotating, you getting out of bed, coffee perking, sugar burning in your cells, your DNA replicating) does so because of a downhill flow of energy. We saw that by definition, any happening or event in the universe, however large or small, is ***spontaneous***, occurring with a release of *free energy*. Remember, spontaneous means "by itself" and not necessarily instantaneous or fast! Finally, we noted that that when enzymes catalyze biochemical reactions in a closed system, the reactions still reach equilibrium, despite the higher rate of the catalyzed reaction. What does this tell you about the energetics of catalyzed reactions in closed systems?

With this brief reminder about energy flow and what enzymes do, we'll turn to the question of how our cells capture ***nutrient free energy***. This will include examples of the energetics of closed systems that reach equilibrium, and open systems that don't! First we'll tackle ***glycolysis***, an anaerobic pathway for generating chemical energy from glucose, as well as the first of several pathways of *respiration*. Then we'll look at *Gluconeogenesis*, a regulated reversal of glycolysis. We ask when, where and why we would want to make, rather than burn glucose. Finally, we begin a discussion of respiration with a look at the ***Krebs Cycle***.

The complete respiratory pathway can be summarized by the following equation:

$$C6H12O6 + 6O2 \Leftrightarrow 6CO2 + 6H2O \tag{6.1.1}$$

The *standard free energy change* for this reaction *(ΔGo)* is about -687Kcal/mole. That is the maximum amount of nutrient free energy that is (at least in theory) available from the complete respiration of a mole of glucose. Given the cost of about 7.3 Kcal to make each mole of ATP (*adenosine triphosphate*), how many moles of ATP might a cell produce after burning a mole of glucose? We'll figure this out here.

learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. explain the difference between fermentation and respiratory glycolysis and the role of redox reactions in both processes.

2. calculate and then compare and contrast *DGo and DG'* for the same reaction, and explain any differences in free energy in open and closed systems.

3. describe and explain the major events of the first stage of glycolysis and trace the free energy changes through the formation of G-3-P.

4. describe and explain the major events of the second stage of glycolysis and trace the free energy changes through the formation of pyruvate and lactic acid.

5. state the role of *redox reactions* in glycolysis and fermentation.

6. compare and contrast glucose (i.e., carbohydrates in general), ATP, NADH and FADH2 as *high-energy* molecules. [Just for fun, click Power in the Primordial Soup to read some *far out* speculations on prebiotic high-energy molecules that might have been around when ATP was being hired for the job!].

7. explain why only a few cell types in the human body conduct *gluconeogenesis*.

8. explain why gluconeogenesis, an energetically unfavorable pathway, occurs at all.

9. explain why the *Atkins Diet* works and speculate on its downside (and that of the related South Beach Diet).

10. explain the concept of a *super-catalyst*.

11. explain why a *super-catalyst* like the *Krebs Cycle* would have evolved.

12. explain the role of high energy linkages and electron carriers in the Krebs cycle.

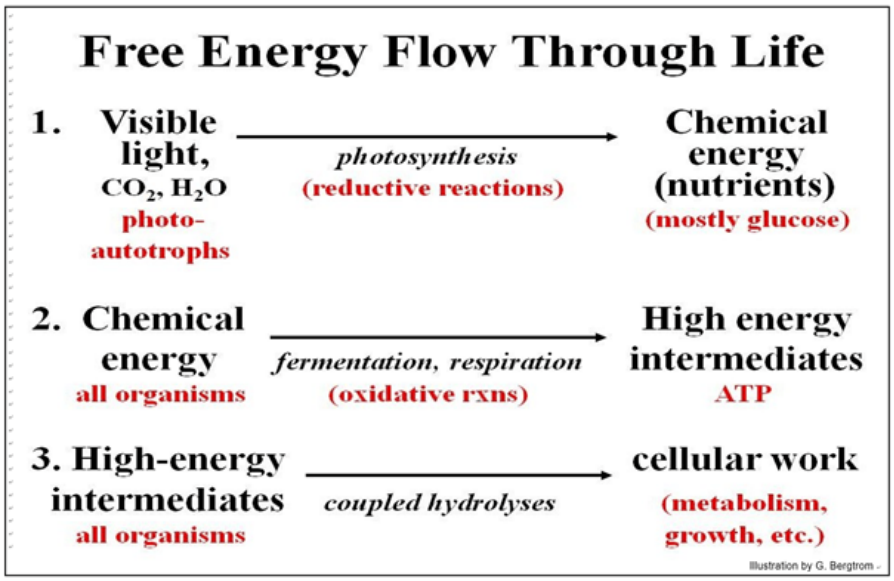
13. compare *phosphate ester linkages* in ATP and GTP, and *thioester linkage* in *acetyl-S- CoA* and *succinyl-S-CoA* in terms of energetics and their biochemical reactions.

speculate on why the *Krebs Cycle* in *E. coli* generates GTP molecules and why it generates ATP molecules eukaryotes.

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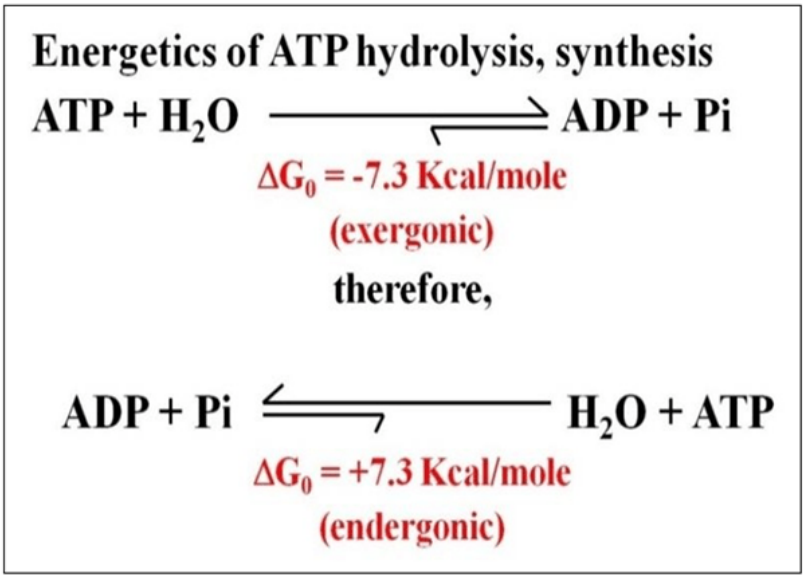
6.2: Glycolysis (from the Greek glyco (sugar) lysis (Separation), or Sugar Breakdown

One of the properties of life is that living things require energy. The pathways of energy flow through life are shown below.

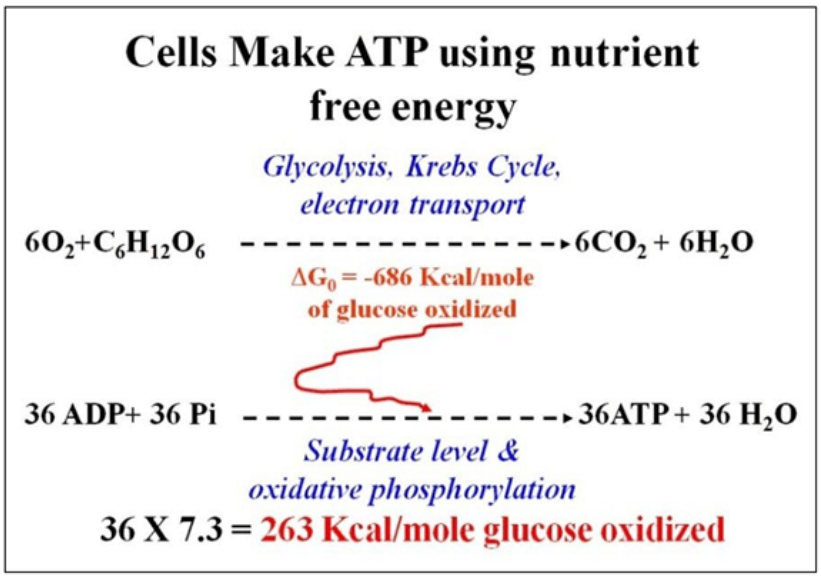


To begin with, the most common intracellular *energy currency* with which livings things "pay" for cellular work is **ATP**. The energy to make ATP on planet earth ultimately comes from the sun via *photosynthesis*. Recall that light energy fuels the formation of glucose and O2 from CO2 and water in green plants, algae, cyanobacteria and a few other bacteria. Photosynthesis even produces some ATP directly, but not enough to fuel all cellular and organismic growth and metabolism. So all cells, even plant cells, use ***fermentation*** or ***respiration*** (***anaerobic*** or ***aerobic*** processes respectively) to capture nutrient free energy (mostly) as ATP.

ATP is called a ***high-energy intermediate*** because its hydrolysis releases a large amount to free energy. In the condensation reactions that make ATP, it takes about 7.3 Kcal of free energy to link a phosphate to ADP in a *phosphate ester* linkage. Having captured nutrient free energy in a form that cells can use, ATP hydrolysis releases that free energy to fuel cellular work, including bending cilia, whipping flagella, contracting muscles, transmitting neural information, building polymers from monomers, and more. The energetics of ATP hydrolysis and synthesis are summarized below.

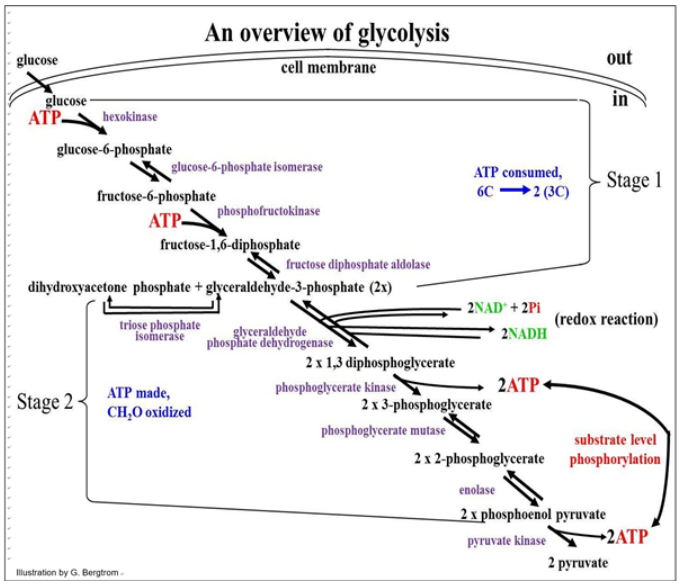


The free energy needed to make ATP in animal cells comes exclusively from nutrients (sugars, fats, proteins). As noted, plants get free energy directly from sunlight, but they mobilize nutrient free energy they make in much the same way as the rest of us get it from what we eat! Glucose oxidation releases a considerable amount of free energy, enough to synthesize many molecules of ATP, as shown below.



Cellular respiration, the oxidation of glucose, starts with ***glycolysis***. Otto Myerhoff and Archibald V. Hill shared a Nobel Prize in Physiology or Medicine with in 1923 for isolating enzymes of glucose metabolism from muscle cells. Thanks to the efforts of others (e.g., Gustav Embden, Otto Meyerhof, Otto Warburg, Gerty Cori, Carl Cori), all the enzymes and reactions of the *glycolyitic pathway* were known by 1940, and the pathway became known as the *Embden-Myerhoff Pathway*. As we will see, glycolysis is an evolutionarily conserved biochemical pathway used by all organisms to capture a small amount of nutrient free energy. For more detail, check out Fothergill-Gilmore LA [(1986) *The evolution of the glycolytic pathway*. Trends Biochem. Sci. 11:47-51]. The glycolytic pathway occurs in the cytosol of cells where it breaks down each molecule of glucose (C6H12O6) into two molecules of ***pyruvic acid*** (***pyruvate***; CH3COCOOH). This occurs in two stages, capturing nutrient free energy in two ATP molecules per glucose molecule that enters the pathway.

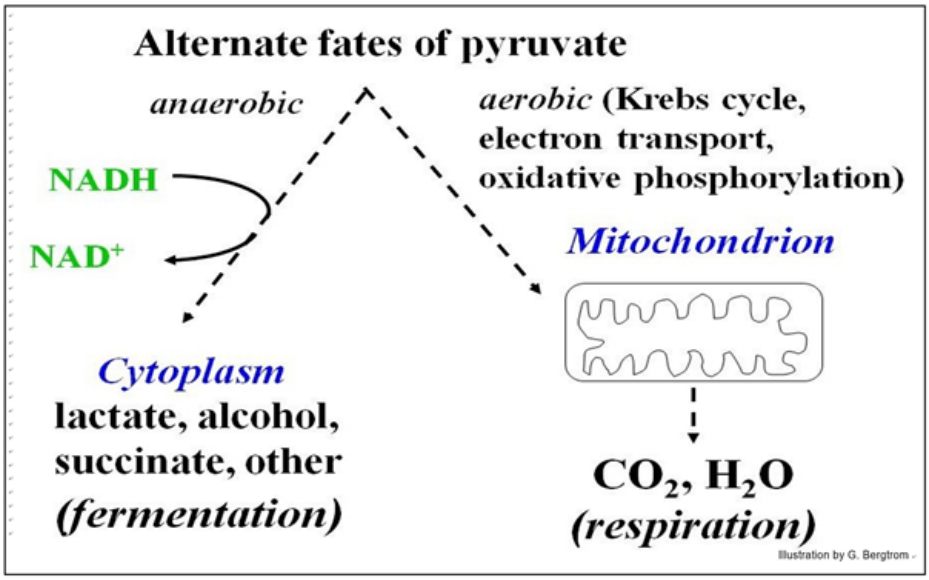
Glycolytic reactions are summarized below, highlighting the two stages of the pathway.



Stage 1 of glycolysis actually consumes ATP. Phosphates are transferred from ATP first to glucose and then to fructose-6-phosphate, reactions catalyzed by ***hexokinase*** and ***phosphofructokinase*** respectively. So, these *Stage 1* phosphorylations *consume* free energy. Later, in ***Stage 2*** of glycolysis, nutrient free energy is captured in ATP and ***NADH*** (reduced *nicotinamide adenine dinucleotide*). NADH forms in ***redox reactions*** in which **NAD+** is reduced as some metabolite is oxidized. In *Stage 2*, it is *glyceraldehyde- 3-phosphate* that is oxidized..., but more later!

In fact, by the end of glycolysis, four molecules of ATP and two of NADH have been formed and a single starting glucose molecule has been split into two molecules of ***pyruvate***. Pyruvate will be metabolized either anaerobically or aerobically.

The alternate fates of pyruvate are summarized below.



151 Overview of Glycolysis

Anaerobic (complete) glycolysis is a fermentation pathway. In *anaerobic glycolysis* the electrons in NADH produced in *Stage 2* of glycolysis are used to reduce pyruvate, so that in the end, there is no consumption of O2 and no net oxidation of nutrient (i.e., glucose). A familiar anaerobic glycolytic pathway is the production of alcohol by yeast in the absence of oxygen. Another one is the *muscle fatigue* you might have experienced after especially vigorous and prolonged exercise. This results from a fermentation that produces an anaerobic build-up of *lactic acid* in skeletal muscle cells. In *anaerobic glycolysis*, the reduction of pyruvate can lead to one of several other *fermentation end products*, along with a net yield of two ATPs per glucose fermented.

Aerobic* (*incomplete*) *glycolysis also produced two ATPs, and is the first step in the complete oxidation of glucose, the ***respiration*** pathway oxidizing glucose to CO2 and H2O, leaving no carbohydrates behind. Pyruvate is completely oxidized in mitochondria. As we look at the reactions of glycolysis and the Krebs cycle, watch for redox reactions in both pathways.

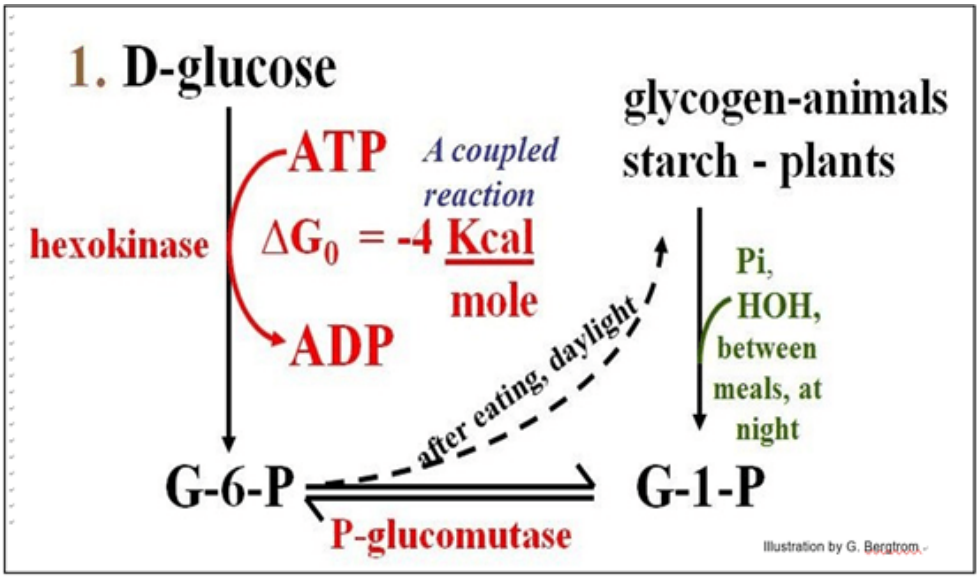
Along the way, we'll also consider ***Gluconeogenesis***, a pathway that essentially reverses the glycolysis and results in glucose synthesis. Gluconeogenesis occurs both under normal conditions, during in high-protein/low carb diets, and during fasting or starvation. In another chapter, we'll look at electron transport and oxidative phosphorylation, the pathways that complete the oxidation of glucose. Here, we begin with a closer look at glycolysis, focusing on the enzyme-catalyzed reactions and free energy transfers between pathway components. We will consider the energetics and enzymatic features of each reaction.

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6.3: Some Details of Glycolysis

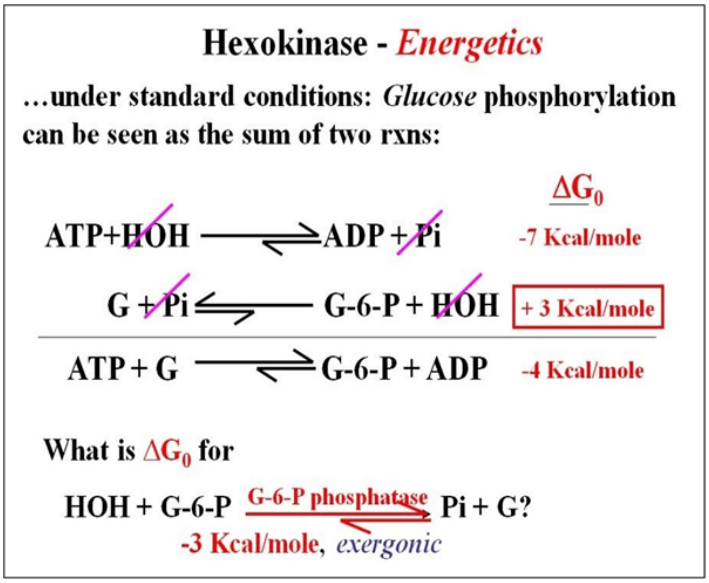
A. Glycolysis, Stage 1

Reaction 1: In the first reaction of glycolysis, the enzyme ***hexokinase*** rapidly phosphorylates glucose entering the cell, forming *glucose-6-phosphate* (**G-6-P**). As shown below, the overall reaction is ***exergonic***; the *free energy change* for the reaction is -4 Kcal per mole of G-6-P synthesized.



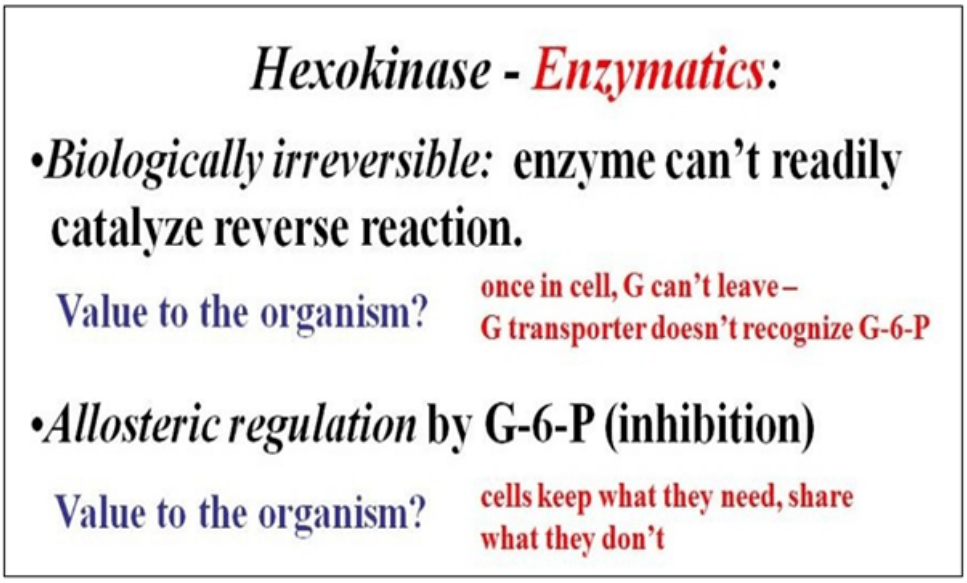
This is a ***coupled reaction***, in which *phosphorylation* of glucose is coupled to ATP hydrolysis. The free energy of ATP hydrolysis (an energetically favorable reaction) fuels the glucose phosphorylation (an energetically *un*favorable reaction). The reaction is also ***biologically irreversible***, as shown by the single vertical arrow. Excess dietary glucose can be stored in most cells (especially liver and kidney cells) as a highly branched polymer of glucose monomers called ***glycogen***. In green algae and plants, glucose made by photosynthesis is stored as polymers of starch. When glucose is necessary for energy, glycogen and starch hydrolysis forms glucose-1- phosphate (**G-1-P**) which is then converted to **G-6-P**.

Let's look at the energetics (free energy flow) of the hexokinase-catalyzed reaction. This reaction can be seen as the sum of two reactions shown below.



Recall that ATP hydrolysis is an *exergonic reaction*, releasing ~7 Kcal/mole (rounding down!) in a closed system under standard conditions. The condensation reaction of glucose phosphorylation occurs with a DGo of +3 Kcal/mole. This is an *endergonic* reaction under standard conditions. Summing up the free energy changes of the two reactions, we can calculate the overall DGo of -4 Kcal/mole for the coupled reaction under standard conditions in a closed system.

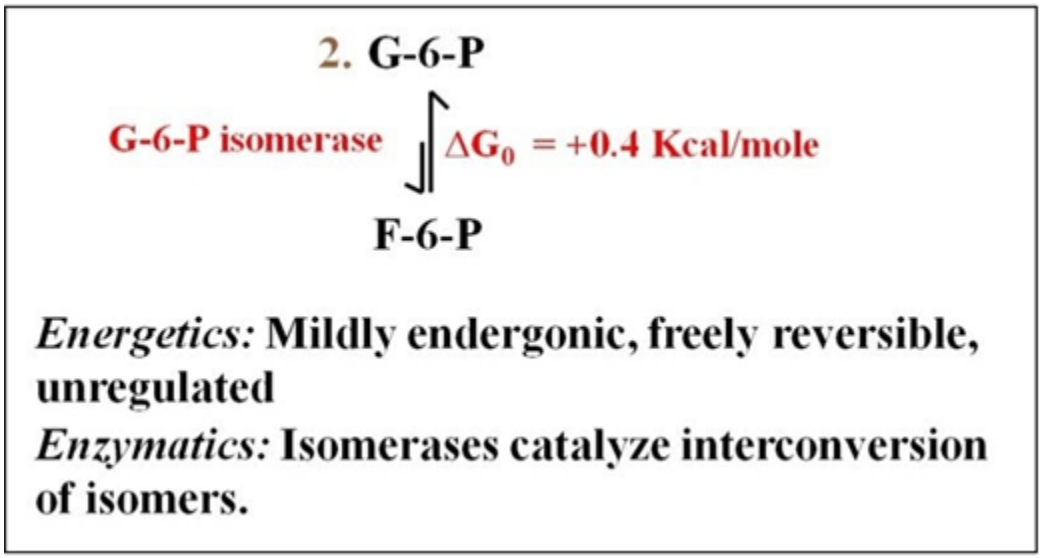
The reactions above are written as if they are reversible. However, we said that the overall coupled reaction is *biologically irreversible*. Where's the contradiction? To explain, we say that an enzyme-catalyzed reaction is biologically irreversible when the products have a relatively low affinity for the enzyme active site, making catalysis (acceleration) of the reverse reaction very inefficient. Enzymes catalyzing biologically irreversible reactions don't allow going back to reactants, but they are often allosterically regulated. This is the case for hexokinase. Imagine a cell that slows its consumption of G-6-P because its energy needs are being met. What happens when G-6-P levels rise in cells? You might expect the hexokinase reaction to slow down so that the cell doesn't unnecessarily consume a precious nutrient energy resource. The *allosteric regulation* of hexokinase is illustrated below.



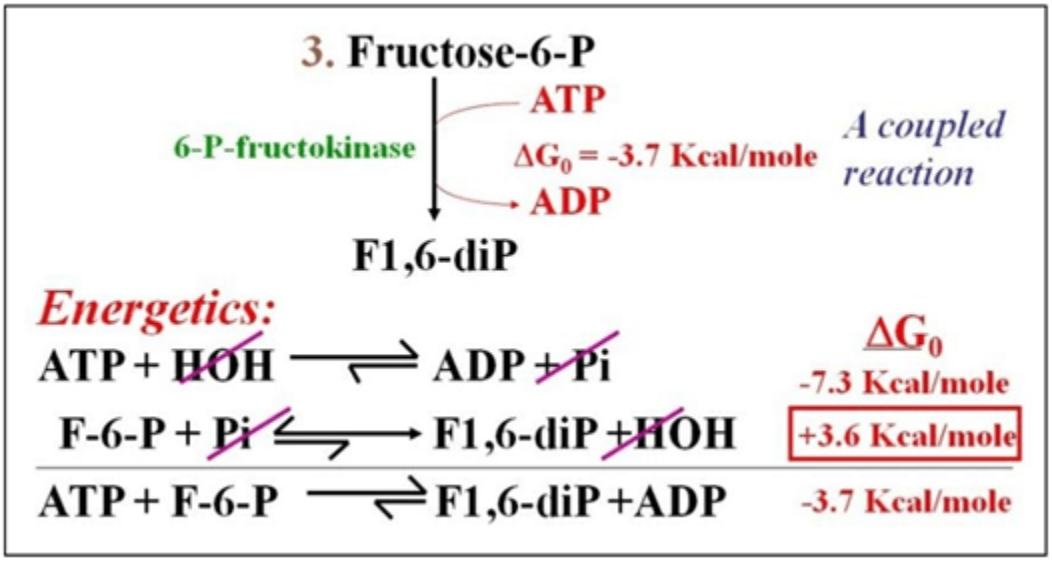
As G-6-P concentrations rise in the cell, *excess* G-6-P binds to an allosteric site on hexokinase. The conformational change in the enzyme is then transferred to the active site, inhibiting the reaction.

152 Glycolysis Stage 1, Reaction 1

Reaction 2: In this slightly endergonic and reversible reaction, *isomerase* catalyzes the isomerization of **G-6-P** to *fructose-6-P* (**F-6-P**), as shown below.

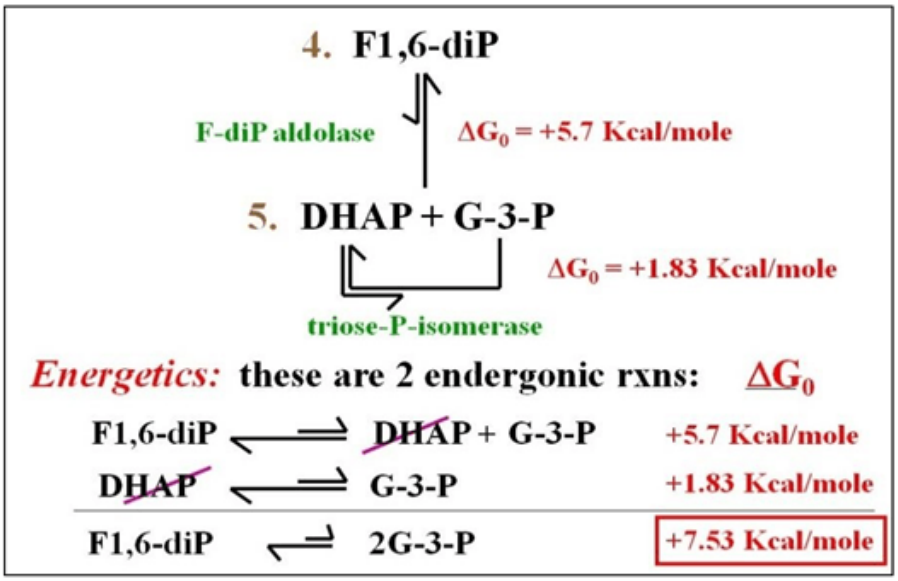


Reaction 3: In this biologically irreversible reaction, *6-phosphofructokinase* (6-P- fructokinase) catalyzes the phosphorylation of F-6-P to make *fructose 1,6 di- phosphate* (**F1,6 diP**). This is also a *coupled reaction*, in which ATP provides the second phosphate. The overall reaction is written as the sum of two reactions, as shown below.



Like the hexokinase reaction, the *6-P-fructokinase* reaction is a coupled, exergonic and allosterically regulated reaction. Multiple ***allosteric effectors***, including ATP, ADP and AMP and long-chain fatty acids regulate this enzyme.

Reactions 4 and 5: These are the last reactions of the first stage of glycolysis. In *reaction 4*, F1,6 diP (a 6-C sugar) is reversibly split into *dihydroxyacetone phosphate* (**DHAP**) and *glyceraldehyde-3-phosphate* (**G-3-P**). In *reaction 5* (also reversible), DHAP is converted into another G-3-P. Here are the reactions:



The net result is the formation of two molecules of G-3-P in the last reactions of *Stage 1* of glycolysis. The enzymes *F-diP aldolase* and *triose-P-isomerase* both catalyze freely reversible reactions. Also, both reactions proceed with a positive free energy change and are therefore *endergonic*. The sum of the free energy changes for the splitting of F1,6 diP into two G-3-Ps is a whopping +7.5 Kcal per mole, a very energetically unfavorable process.

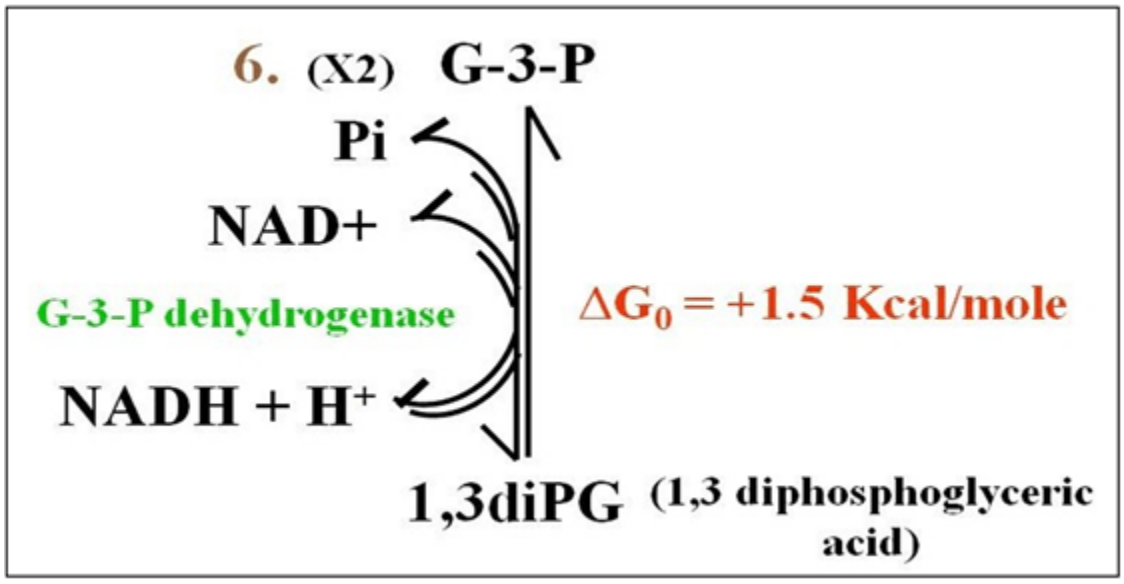
Summing up, by the end of *Stage 1* of glycolysis, we have consumed two ATP molecules, and split one 6C carbohydrate into two 3-C carbohydrates. We have also seen two biologically irreversible and allosterically regulated enzymes.

153 Glycolysis Stage 1; Reactions 2-5

B. Glycolysis, Stage 2

We will follow just one of the two molecules of G-3-P generated by the end of *Stage 1* of glycolysis, but remember that both are proceeding through *Stage 2* of glycolysis**.**

Reaction 6: This is a redox reaction. G-3-P is oxidized to *1,3, diphosphoglyceric acid* (**1,3, diPG**) and NAD+ is reduced to NADH. The reaction catalyzed by g*lyceraldehyde-3-phopsphate dehydrogenase* is shown below.

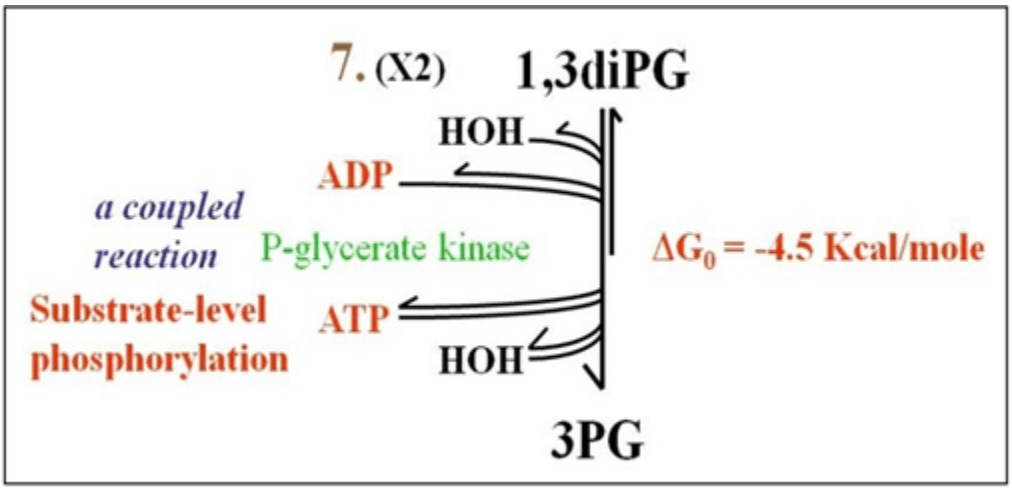


In this *freely reversible endergonic* reaction, a hydrogen molecule (**H2**) is removed from G-3-P, leaving behind phosphoglyceric acid. This short-lived oxidation intermediate is phosphorylated to make *1,3 diphosphoglyceric acid* (**1,3diPG**). At the same time, the hydrogen molecule is split into a hydride ion (**H-**) and a proton (**H+**). The H- ions reduce **NAD+** to NADH, leaving the protons behind in solution. Remember that all of this is happening in the active site of the same enzyme!

Even though it catalyzes a reversible reaction, *G-3-P dehydrogenase* is allosterically regulated. However, in contrast to the regulation of hexokinase, that of G-3-P dehydrogenase is more complicated! The regulator is NAD+ and the mechanism of allosteric regulation of *G-3-P dehydrogenase* by NAD+ is called ***negative cooperativity***. It turns out that the higher the [NAD+] in the cell, the lower the affinity of the enzyme for more NAD+ and the faster the reaction in the cell! The mechanism is discussed at the link below.

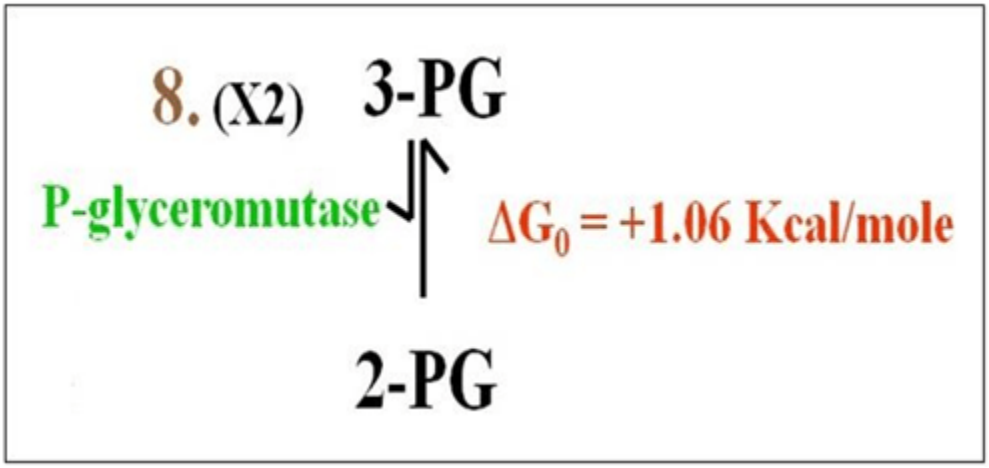
154 Glycolysis Stage 2; Reaction 6

Reaction 7: The reaction shown below is catalyzed by *phosphoglycerate kinase*. It is freely reversible and *exergonic*, yielding ATP and *3-phosphoglyceric acid* (**3PG**).



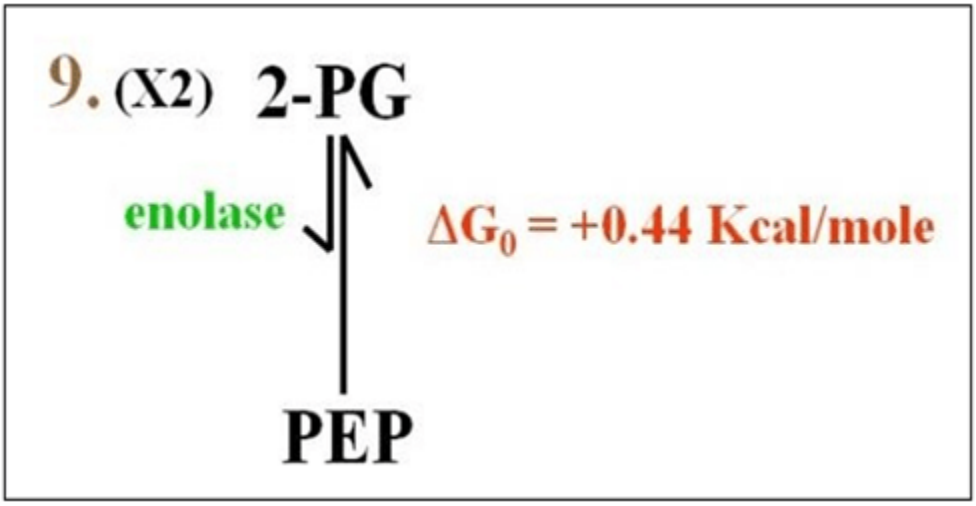
Catalysis of phosphate group transfer between molecules by kinases is called ***substrate-level phosphorylation***, often the phosphorylation of ADP to make ATP. In this *coupled reaction* the free energy released by hydrolyzing a phosphate from 1,3 diPG is used to make ATP. Remember that this reaction occurs twice per starting glucose. Two ATPs have been synthesized to this point in glycolysis. We call 1,3 diPG a ***very high-energy phosphate compound***.

Reaction 8: This freely reversible endergonic reaction moves the phosphate from the number 3 carbon of 3PG to the number 2 carbon as shown below.

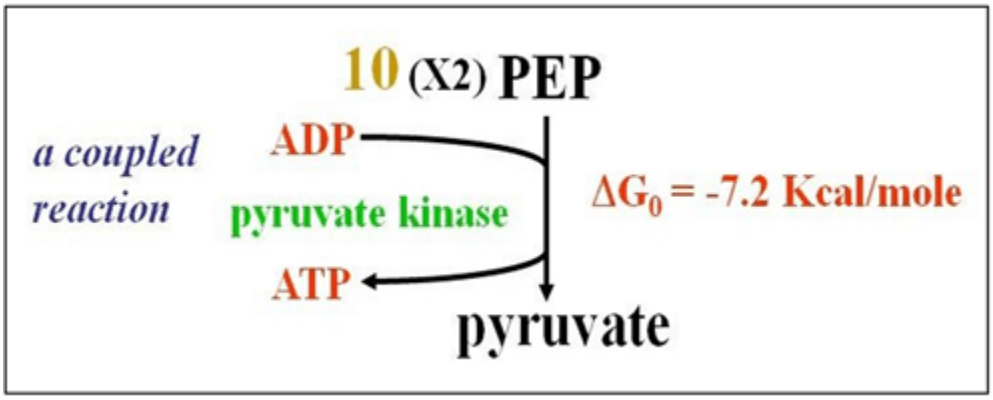


Mutases like *phoshoglycerate mutase* catalyze transfer of functional groups within a molecule.

Reaction 9: In this reaction (shown below), *enolase* catalyzes the conversion of 2PG to *phosphoenol pyruvate* (**PEP**).



Reaction 10: This reaction results in the formation of *pyruvic acid* (***pyruvate***), as shown below. Remember again, two pyruvates are produced per starting glucose molecule.



The enzyme *pyruvate kinase* couples the *biologically irreversible,* exergonic hydrolysis of a phosphate from PEP and transfer of the phosphate to ADP in a *coupled reaction*. The reaction product, PEP, is another *very high-energy* phosphate compound.

155 Glycolysis Stage 2: Reactions 7-10

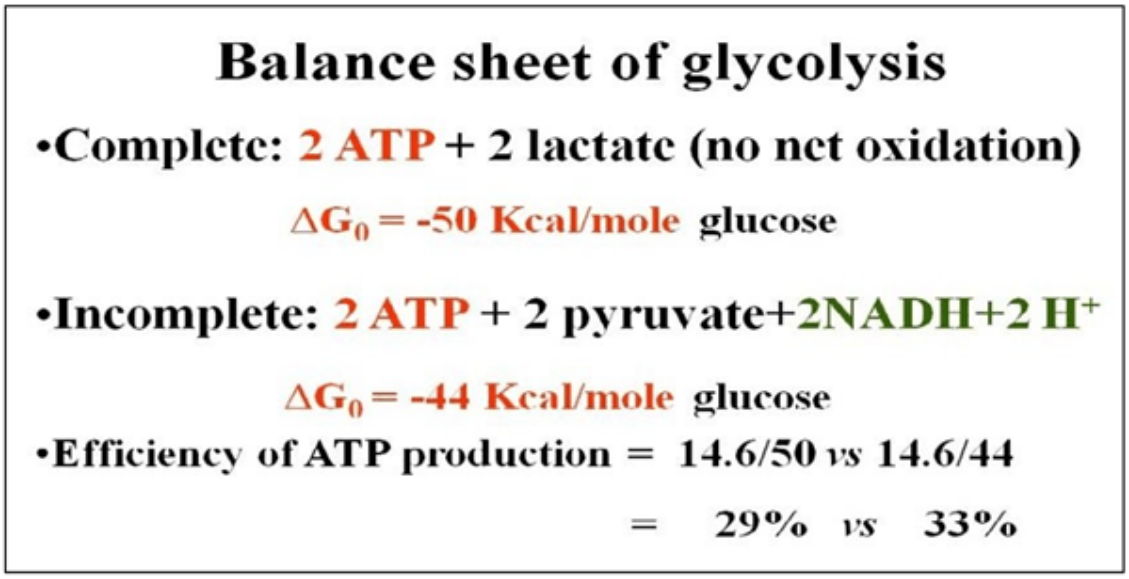
Pyruvate kinase is allosterically regulated by ATP, citric acid, long-chain fatty acids, F1,6 diP, and one of its own substrates, PEP.

In *incomplete (aerobic) glycolysis*, pyruvate is oxidized in mitochondria during respiration (see the *Alternate Fates of Pyruvate* above). ***Fermentations*** are called *complete glycolysis* because pyruvate is reduced to one or another end product. Recall that muscle fatigue results when skeletal muscle uses anaerobic fermentation to get energy during vigorous exercise. When pyruvate is reduced to ***lactic acid*** (***lactate***), lactic acid accumulation causes muscle fatigue. The enzyme *Lactate Dehydrogenase* (**LDH**) that catalyzes this reaction is regulated, but not allosterically. Instead different muscle tissues regulate LDH by making different versions of the enzyme! Click the Link below for an explanation.

156 Fermentation: Regulation of Pyruvate Reduction is NOT Allosteric!

C. A Chemical and Energy Balance Sheet for Glycolysis

Compare the balance sheets for complete glycolysis (fermentation) to lactic acid and *incomplete* (aerobic) glycolysis, showing chemical products and energy transfers.



There are two reactions in *Stage 2* of glycolysis that each yield a molecule of ATP. Since each of these reactions occurs twice per starting glucose molecule, Stage 2 of glycolysis produces four ATP molecules. Since *Stage 1* consumed two ATPs, the net yield of chemical energy as ATP by the end of glycolysis is two ATPs, whether complete to lactate or incomplete to pyruvate! Because they can't make use of oxygen, anaerobes have to settle for the paltry 15 Kcal worth of ATP that they get from a fermentation. Since there are 687 Kcal potentially available from the complete combustion of a mole of glucose, there is a lot more free energy left to be captured during the rest of respiration.

157 Balance Sheet of Glycolysis

Remember also that the only redox reaction in aerobic glycolysis is in Stage 2. This is the oxidation of G-3-P, a 3C glycolytic intermediate. Now check out the redox reaction a fermentation pathway. Since pyruvate, also a 3C intermediate, was reduced, there has been *no net oxidation of glucose* (i.e., glycolytic intermediates) in complete glycolysis.

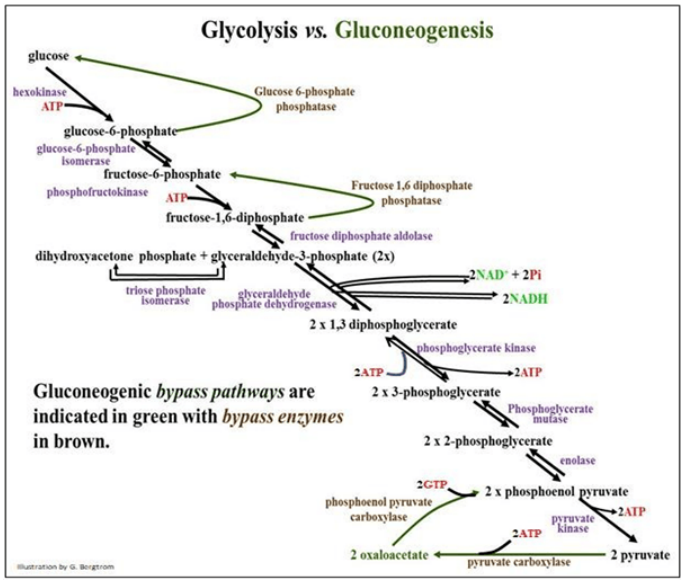
By this time, you will have realized that glycolysis is a net energetically favorable (*downhill*, *spontaneous*) reaction pathway in a closed system, with an overall negative ΔGo. Glycolysis is also normally spontaneous in most of our cells, driven by a constant need for energy to do cellular work. Thus the actual free energy of glycolysis, or ΔG', is also negative. In fact, glycolysis in actively respiring cells proceeds with release of more free energy than it would in a closed system. In other words, the ΔG' for glycolysis in active cells is more negative than the ΔGo of glycolysis!

Now, for a moment, let's look at gluconeogenesis, the Atkins Diet and some not-so- normal circumstances when glycolysis essentially goes in reverse, at least in a few cell types. Under these conditions, glycolysis is energetically unfavorable, and those reverse reactions are the ones proceeding with a negative ΔG'!

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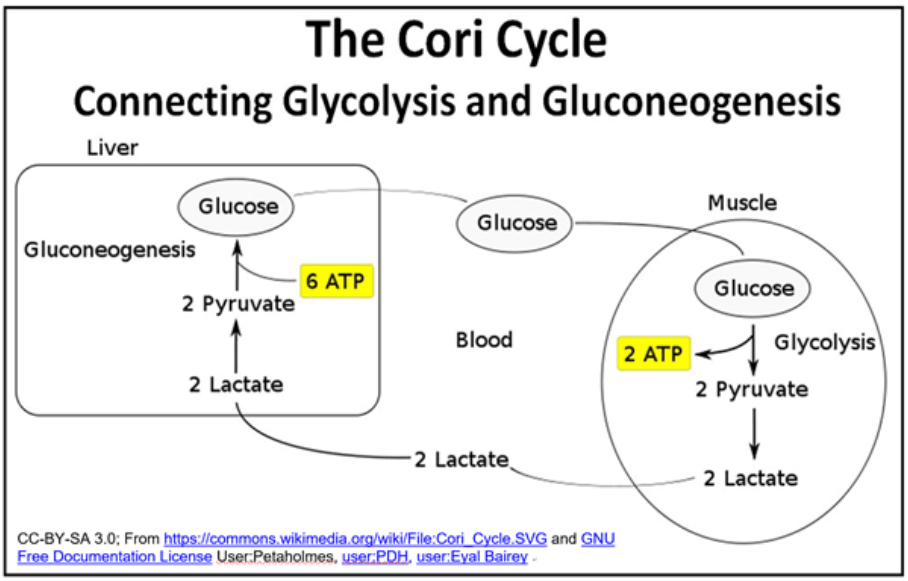
6.4: Gluconeogenesis

In a well-fed animal, most cells can store a small amount of glucose as glycogen. All cells break glycogen down as needed to retrieve nutrient energy as G-6-P. Glycogen hydrolysis, or ***glycogenolysis***, produces G-1-P that is converted to G-6-P, as we saw at the top of *Stage* 1 of glycolysis. But, glycogen in most cells is quickly used up between meals. Therefore, most cells depend on a different, external source of glucose other than diet. Those sources are liver and to a lesser extent, kidney cells, that can store large amounts of glycogen after meals. In continual feeders (for examples cows and other ruminants), glycogenolysis is ongoing. In *intermittent feeders* (like us), liver glycogenolysis can supply glucose to the blood for 6-8 hours between meals, to be distributed as needed to all cells of the body. Thus, you can expect to use up liver and kidney glycogen reserves after a good night's sleep, a period of intense exercise, or any prolonged period of low carbohydrate intake (fasting or starvation). Under these circumstances, animals use **gluconeogenesis** (literally, *new glucose synthesis*) in liver and kidney cells to provide systemic glucose to nourish other cells. As always in otherwise healthy individuals, the hormones insulin and glucagon regulate blood *glucose homeostasis*, protecting against *hypoglycemia* (low blood sugar) and *hyperglycemia* (high blood sugar) respectively. The gluconeogenic pathway produces glucose from carbohydrate and non-carbohydrate precursor substrates. These precursors include pyruvate, lactate, glycerol and *gluconeogenic amino acids*. The latter are amino acids that can be converted to alanine. Look at the side-by-side reactions of glycolysis and gluconeogenesis on the next page. Look for the two *bypass* reactions, catalyzed by two *carboxylases* and two *phosphatases* (brown in the illustration) and the glycolytic reactions that function in reverse during gluconeogenesis.



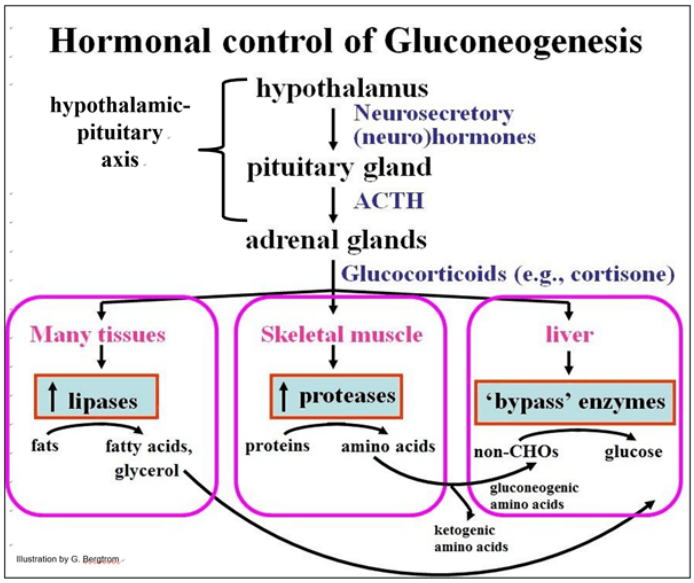
If glycolysis is an exergonic pathway, then gluconeogenesis must be an endergonic one. In fact, while glycolysis through two pyruvates generates a net of two ATPs, gluconeogenesis from two pyruvate to glucose costs 4 ATPs and two GTPs! Likewise, gluconeogenesis is only possible if the bypass enzymes are present. These are necessary to get around the three biologically irreversible reactions of glycolysis. Except for the *bypass reactions*, gluconeogenesis is essentially a reversal of glycolysis.

As drawn in the pathways above, glycolysis and gluconeogenesis would seem to be cyclic. In fact this apparent cycle was recognized by Carl and Gerti Cori, who shared the 1947 Nobel Prize for Medicine or Physiology with Bernardo Houssay for discovering how glycogen is broken down to pyruvate in muscle (in fact most) cells, which can then be used to resynthesize glucose in liver cells. Named after the Coris, The Cori Cycle, shown below, recognizes the interdependence of liver and muscle in glucose breakdown and resynthesis.



In spite of this free energy requirement, gluconeogenesis is energetically favorable in liver and kidney cells! This is because the cells are open systems. The accumulation of pyruvate in liver cells and a rapid release of new glucose into the blood drives the energetically favorable reactions of gluconeogenesis forward. Thus, under gluconeogenic conditions, glucose synthesis occurs with a negative DG', a decline in actual free energy. Of course, glycolysis and gluconeogenesis are not simultaneous! Which pathways operate in which cells is tightly controlled.

Glycolysis is the norm in all cell types, even in liver and kidney. However, the cessation of glycolysis in favor of gluconeogenesis in the latter cells is under hormonal control, as illustrated below.



Key in turning on liver gluconeogenesis is the role of glucocorticoid hormones. What causes the secretion of glucocorticoids? A long night's sleep, fasting and more extremely, starvation are forms of *stress*. Stress responses starts in the *hypothalamic-pituitary axis*. Different stressors cause the *hypothalamus* to secrete a *neurohormone* that in turn, stimulates the release of *ACTH* (*adrenocorticotropic hormone*) from the *pituitary gland*. ACTH then stimulates the release of cortisone and other glucocorticoids from the cortex (outer layer) of the adrenal glands. As the name glucocorticoid suggests, these hormones participate in the regulation of glucose metabolism. Here is what happens at times of low blood sugar (e.g., when carbohydrate intake is low):

1. Glucocorticoids stimulate the synthesis of gluconeogenic bypass enzymes in liver cells.

2. Glucocorticoids stimulate *protease* synthesis in skeletal muscle, causing hydrolysis of the peptide bonds between amino acids.

Gluconeogenic amino acids circulate to the liver where they are converted to pyruvate, a major precursor of gluconeogenesis. Some amino acids are ketogenic; they are converted to Acetyl-S-CoA, a precursor to *ketone bodies*.

(1). Glucocorticoids stimulate increased levels of enzymes including *lipases* that catalyze hydrolysis of the ester linkages in triglycerides (fat) in adipose and other cells. This generates *fatty acids* and *glycerol*.

(2). Glycerol circulates to liver cells that take it up convert it to G-3-P, augmenting gluconeogenesis. Fatty acids circulate to liver cells where they are oxidized to Acetyl-S-CoA that is then converted to ketone bodies. and released to the cisculation.

(3). Most cells use fatty acids as an alternate energy nutrient when glucose is limiting. , and while heart and brain cells depend on glucose for energy, brain cells can use ket9one bodies as an alternate energy course.

essential roles of glucocorticoids

1. It's a pity that we humans cannot use fatty acids as gluconeogenic substrates! Plants and some lower animals have a *glyoxalate cycle* pathway that can convert the products of fatty acid oxidation (acetate) directly into carbohydrates that can enter the gluconeogenic pathway. Lacking this pathway, we (and higher animals in general) cannot convert fats to carbohydrates, in spite of the fact that we can all too easily convert the latter to the former!

   The dark side of bad eating habits is prolonged starvation that can overwhelm the gluconeogenic response. You see this in reports from third world regions suffering starvation due to drought or other natural disaster, or war. The spindly arms and legs of starving children result from muscle wasting as the body tries to provide the glucose necessary for survival. When the gluconeogenic response is inadequate to the task, the body can resort to ketogenic fat metabolism. Think of this as a *last* resort, leading to the production of ketone bodies and the "acetone breath" in starving people.

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6.5: The Atkins Diet and Gluconeogenesis

You may know that the *Atkins Diet* is an ultra-low carb diet. It is one of several low-carb *ketogenic diets*. The glucocorticoid hormones released on a low carb diet trick the body into a constant gluconeogenic state. While the liver can produce enough glucose for brain and heart cells, the rest of the cells in our bodies switch to burning fats, hence the weight loss. Discredited some years ago, the Atkins Diet (and similar ones e.g., South Beach) is now back in favor. Some folks on these diets restrict their intake of carbohydrates so much that they can develop “acetone breath”! Nevertheless, low carb diets are important in the control of diabetes. In older folks, type 2 (adult-onset) diabetics can control their disease with a low carb diet and a drug called *metformin*, which blocks gluconeogenesis and therefore prevents glucose synthesis from gluconeogenic substrates, at the same time stimulating cellular receptors to take up available glucose. For more details on the mechanism of *metformin* action, check out Hundal RS et al. [(2000) *Mechanism by Which Metformin Reduces Glucose Production in Type 2 Diabetes.* Diabetes 49 (12): 2063–9]. Given the prevalence of obesity and type 2 diabetes in the U.S., it’s likely that someone you know is taking *metformin* or other similar medication!

158 Gluconeogenesis & the Atkins Diet

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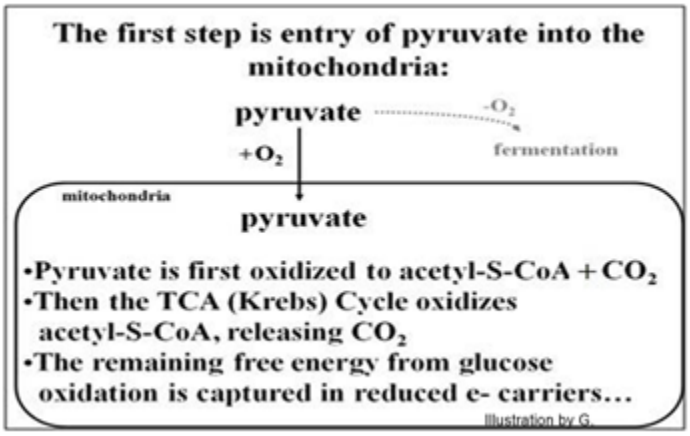
6.6: The Krebs/TCA/Citric acid cycle

Glycolysis through fermentative reactions produces ATP anaerobically. The evolution of respiration (the aerobic use of oxygen to efficiently burn nutrient fuels) had to wait until photosynthesis created the oxygenic atmosphere we live in now. Read more about the source of our oxygenic atmosphere in Dismukes GC et al. [(2001) *The origin of atmospheric oxygen on earth: the innovation of oxygenic photosynthesis.* Proc. Nat. Acad. Sci. USA 98:2170-2175].

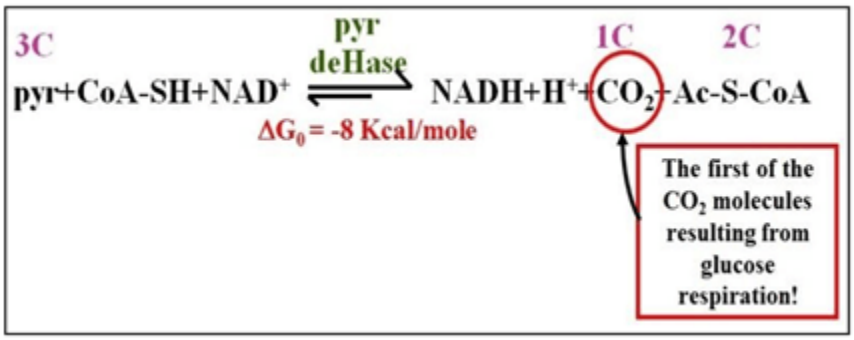
The ***Krebs cycle*** is the first pathway of oxygenic respiration. Evolution of this respiration and the chemical bridge from glycolysis to the Krebs cycle, no doubt occurred a few reactions at a time, perhaps at first as a means of protecting anaerobic cells from the 'poisonous' effects of oxygen. Later, natural selection fleshed out the aerobic Krebs cycle, electron transport and oxidative phosphorylation pathways we see today.

Whatever its initial utility, these reactions were an adaptive response to the increase in oxygen in the earth's atmosphere. As a pathway for getting energy out of nutrients, respiration is much more efficient than glycolysis. Animals rely on it, but even plants and photosynthetic algae use the respiratory pathway when sunlight is not available! Here we focus on oxidative reactions in mitochondria, beginning with pyruvate oxidation and continuing to the redox reactions of the Krebs cycle.

After entering the mitochondria, *pyruvate dehydrogenase* catalyzes pyruvate oxidation to ***Acetyl-S-Coenzyme A*** (***Ac-S-CoA***). Then the Krebs cycle completely oxidizes the *Ac-S- CoA*. These mitochondrial redox reactions generate CO2 and lot of reduced electron carriers (NADH, FADH2). The free energy released in these redox reactions is coupled to the synthesis of only one ATP per pyruvate oxidized (i.e., two per the glucose we started with!). It is the NADH and FADH2 molecules have captured most of the free energy in the original glucose molecules. These entry of pyruvate into the mitochondrion and its oxidation are summarized below.

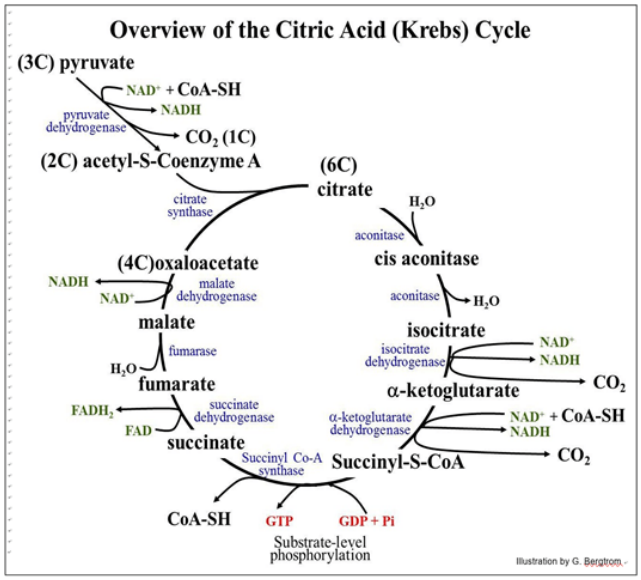


Pyruvate oxidation converts a 3C carbohydrate into acetate, a 2C molecule, releasing a molecule of CO2. In this highly exergonic reaction, CoA-SH forms a *high-energy thioester linkage* with the acetate in Ac-S-CoA. The oxidation of pyruvic acid results in the reduction of NAD+, production of ***Ac-S-CoA*** and a molecule of CO2, as shown below.



The **Krebs cycle** functions during respiration to oxidize Ac-S-CoA and to reduce NAD+ and FAD to NADH and FADH2 (respectively). Intermediates of the Krebs cycle also function in amino acid metabolism and interconversions. All aerobic organisms alive today share the Krebs cycle we see in humans. This is consistent with its spread early in the evolution of our oxygen environment. Because of the central role of Krebs cycle intermediates in other biochemical pathways, parts of the pathway may even have pre- dated the complete respiratory pathway. The Krebs cycle takes place in mitochondria of eukaryotic cells.

After the oxidation of pyruvate, the Ac-S-CoA enters the Krebs cycle, condensing with *oxaloacetate* in the cycle to form *citrate*. There are four redox reactions in the Krebs cycle. As we discuss the Krebs cycle, look for the accumulation of reduced electron carriers (FADH2, NADH) and a small amount of ATP synthesis by substrate-level phosphorylation. Also, follow the carbons in pyruvate into CO2. The Krebs Cycle as it occurs in animals is summarized below.



To help you understand the events of the cycle,

1. find the two molecules of CO2 produced in the Krebs cycle itself.

2. find GTP (which quickly transfers its phosphate to ADP to make ATP). Note that in bacteria, ATP is made directly at this step.

3. count all of the reduced electron carriers (NADH, FADH2). Both of these electron carriers carry a pair of electrons. If you include the electrons on each of the NADH molecules made in glycolysis, how many electrons have been removed from glucose during its complete oxidation?

Remember that glycolysis produces two pyruvates per glucose, and thus two molecules of Ac-S-CoA. Thus, the Krebs cycle turns twice for each glucose entering the glycolytic pathway. The *high-energy thioester bonds* formed in the Krebs cycle fuel ATP synthesis as well as the condensation of oxaloacetate and acetate to form citrate in the first reaction. Each NADH carries about 50 Kcal of the 687 Kcal of free energy originally available in a mole of glucose; each FADH2 carries about 45 Kcal of this free energy. This energy will fuel ATP production during electron transport and oxidative phosphorylation.

159 Highlights of the Krebs Cycle

Finally, the story of the discovery of the Krebs cycle is as interesting as the cycle itself! Albert Szent-Györgyi won a Nobel Prize in 1937 for discovering some organic acid oxidation reactions initially thought to be part of a linear pathway. Hans Krebs did the elegant experiments showing that the reactions were part of a cyclic pathway. He proposed (correctly!) that the cycle would be a *supercatalyst* that would catalyze the oxidation of yet another organic acid. Some of the experiments are described by Krebs and his coworkers in their classic paper: Krebs HA, et al. [(1938) *The formation of citric and α-ketoglutaric acids in the mammalian body.* Biochem. J. 32: 113–117]. Hans Krebs and Fritz Lipmann shared the 1953 Nobel Prize in Physiology or Medicine. Krebs was recognized for his elucidation of the TCA cycle, which now more commonly carries his name. Lipmann was recognized for proposing ATP as the mediator between food (nutrient) energy and intracellular work energy, and for discovering the reactions that oxidize pyruvate and synthesize Ac-S-CoA, bridging the Krebs Cycle and oxidative phosphorylation (to be considered iin the next chapter).

16.0 Discovery of the Krebs Cycle

You can read Krebs' review of his own research in Krebs HA [(1970) *The history of the tricarboxylic acid cycle*. Perspect. Biol. Med. 14:154-170]. For a classic read on how Krebs described his supercatalyst suggestion, click Hans Krebs Autobiographical Comments. For more about the life of Lipmann, check out the brief Nobel note on the Fritz Lipmann Biography.

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6.7: Key Words and Terms

Acetyl-S-coenzyme A (Ac-S-CoA)	free energy capture	phosphatase enzymes
ADP, ATP, GDP, GTP	fructose	phosphate-ester linkage
aerobic	G, G6P, F6P, F1,6-diP	redox reactions
anaerobic	gluconeogenesis	reducing agent
Atkins Diet	gluconeogenic amino acids	respiration
biochemical pathways	glycolysis	SDH (succinate dehydrogenase)
bioenergetics	glyoxalate cycle	spontaneous reaction
bypass reactions, enzymes	high energy bond (linkage)	stage 1
C6H12O6 (glucose)	high energy molecules	stage 2
cells as open systems	isomerase enzymes	standard conditions
Cori Cycle	kinase enzymes	steady state
dehydrogenase enzymes	Krebs (TCA, citric acid) cycle	stoichiometry of glycolysis
DHAP, G3P,1,3-diPG, 3PG, 2PG, PEP, Pyr	metabolic effects of low carb diet	substrate level phosphorylation
diabetes	metformin	Succinyl-S-CoA
energetics of glycolysis	mitochondria	super-catalyst

energy flow in cells	**mutase enzymes**	**synthase enzymes**
equilibrium	**NAD+ (oxidized nicotinamide adenine di- Phosphate)**	**thioester linkage**
FAD (oxidized nicotinamide adenine di- Phosphate)	**NADH (reduced nicotinamide adenine di- Phosphate)**	**DG' (actual free energy change)**
FADH2 (reduced flavin adenine di-Phosphate)	**nutrients**	**DGo (standard free energy change)**
fermentation	**oxidation, reduction**	
free energy	**oxidizing agent**	

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7: ELECTRON TRANSPORT, OXIDATIVE PHOSPHORYLATION, AND PHOTOSYNTHESIS

7.1: Introduction

7.2: The Electron Transport Chain (ETC)

7.3: Oxidative Phosphorylation

7.4: Photosynthesis

7.5: More Thoughts on the Mechanisms and Evolution of Respiration and Photosynthesis

7.6: Key Words and Terms

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7.1: Introduction

We have seen that glycolysis generates two pyruvate molecules per glucose molecule, and that the subsequent oxidation of each pyruvate generates two Ac-S-CoA molecules. After the further oxidation of each Ac-S-CoA by the *Krebs cycle*, aerobic cells have captured about 30 Kcal out of the 687 Kcal potentially available from a mole of glucose in two molecules of ATP. Not much for all that biochemical effort! However, a total of 24 H+ (protons) pulled from glucose in *redox reactions* have also been captured, in the form or the *reduced electron carriers* NADH and FADH2. We begin here with a look at ***electron transport*** and ***oxidative phosphorylation***, the linked ("coupled") mechanism that transfers much of nutrient free energy into ATP. We will see that the free energy released by the transport of electrons from the reduced electron carriers is captured in a ***proton (H+) gradient***. Then we'll see how dissipation of this gradient releases free energy to fuel ATP synthesis by *oxidative phosphorylation*. Next, we will contrast mitochondrial oxidative phosphorylation with the *substrate-level phosphorylation* we saw in glycolysis and the Krebs cycle. After presenting an energy balance sheet for respiration, we look at how cells capture of free energy from alternate nutrients. Then we discuss ***photosynthesis*** (overall, the opposite of respiration) and conclude by comparing photosynthesis and respiration.

learning objectives

When you have mastered the information in this chapter, you should be able to:

1. explain the *centrality* of the Krebs Cycle to aerobic metabolism.

2. identify *sources of electrons in redox reactions* leading to and within the Krebs cycle.

3. illustrate the *path of electrons* from the Krebs cycle to and through the electron transport chain.

4. trace the *evolution of the electron transport chain* from its location on an aerobic bacterial membrane to its location in eukaryotic cells.

5. list the expected properties of a *proton gate* and a *proton pump*.

6. *interpret experiments* involving redox reactions, ATP synthesis and ATP hydrolysis conducted with intact mitochondria and separated mitochondrial membranes.

7. distinguish between the *pH, H+ and electrical gradients* established by electron transport.

8. explain the *chemiosmotic mechanism of ATP synthesis* and contrast it with *substrate- level phosphorylation*.

9. compare and contrast the role of electron transport in respiration and photosynthesis and discuss the evolution of each.

10. trace and explain the different paths that electrons can take in photosynthesis.

11. explain the presence of similar (or even identical) biochemical intermediates in respiration and photosynthesis.

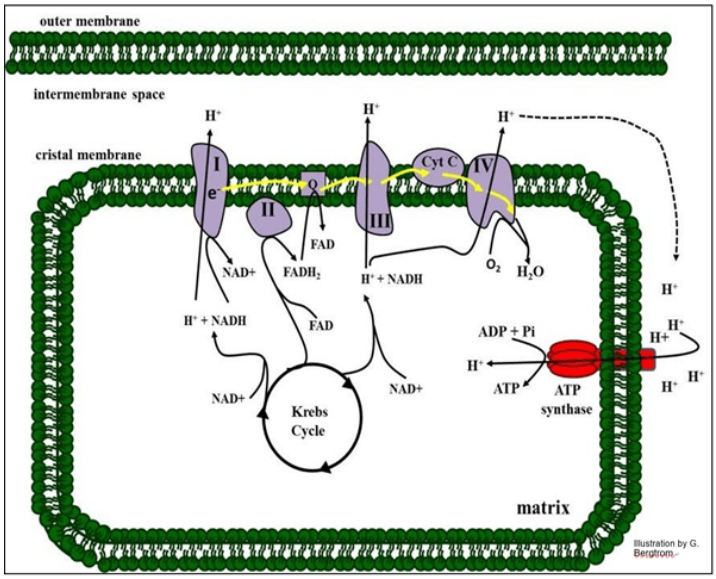
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7.2: The Electron Transport Chain (ETC)

All cells use an electron transport chain (ETC) to oxidize substrates in *exergonic* reactions. The electron flow from reduced substrates through an ETC is like the movement of electrons between the poles of a battery. In the case of the battery, the electron flow releases free energy to power a motor, light, cell phone, etc. In the mitochondrial ETC, electrons flow when the reduced electron (NADH, FADH2) are oxidized. In plants and other photosynthetic organisms, an ETC serves to oxidize NADPH (a phosphorylated version of the electron carrier NADH). In both cases, free energy released when the redox reactions of an ETC are coupled to the active transport of protons (H+ ions) across a membrane. The result is a chemical gradient of H+ ions as well as a pH gradient. Since protons are charged, the proton gradient is also an electrical gradient. In a kind of shorthand, we say that the free energy once in reduced substrates is now in an ***electrochemical gradient***. That gradient free energy is captured in ATP synthesis reactions coupled to the flow (diffusion) of protons back across the membrane in the process called *oxidative phosphorylation*. In aerobic respiration, electrons are ultimately transferred from components at the end of the ETC to a final electron acceptor molecular oxygen, O2, making water. In photosynthesis, electron transfer reduces CO2 to sugars.

The ***Chemiosmotic Mechanism*** explained how the creation of an electrochemical gradient and how gradient free energy ends up in ATP. For this insight, Peter Mitchell won the Nobel Prize in Chemistry in 1978. You can read Mitchell's original proposal of the *chemiosmosis model* of mitochondrial ATP synthesis in Mitchell P (1961) *Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism*. Nature 191:144-148. Here we focus on the details of respiration as it occurs in the mitochondria of eukaryotic cells. The end products of electron transport are NAD+, FAD, water and protons. The protons end up outside the mitochondrial matrix because they are pumped across the cristal membrane using the free energy of electron transport.

Electron transport and ***oxidative phosphorylation*** are summarized in the illustration below.



161 Electron Transport Oxidizes Reduced Electron Carriers

162 Finding the Free Energy of Electron Transport

163 Separating Electron Transport from Oxidative Phosphorylation

Roman numbered protein complexes along with Coenzyme Q (just "Q" in the drawing) and cytochrome C (Cyt c) constitute the ETC), the sequence of reactions that oxidize NADH or FADH2 to NAD+ and FAD (respectively). The electrons from these reduced electron carriers are transferred from one ETC complex to the next. At the end of chain, electrons, protons and oxygen unite in complex IV to make water. As you might expect, under standard conditions in a closed system, electron transport is downhill, with an overall release of free energy (negative DGo) at equilibrium.

In the illustration above, we can see three sites in the respiratory ETC that function as H+ pumps. At these sites, the negative change in free energy of electron transfer is large and coupled to the action of a pump. The result is that protons accumulate outside the matrix of the mitochondrion. Because the outer mitochondrial membrane is freely permeable to protons, the electrochemical gradient is in effect between the cytoplasm and the mitochondrial matrix. Proton flow back into the mitochondrial matrix through lollipop- shaped ATP synthase complexes releases the gradient free energy that is harnessed as chemical energy.

164 Proton Pumps Store Free Energy of the ETC in Proton Gradients

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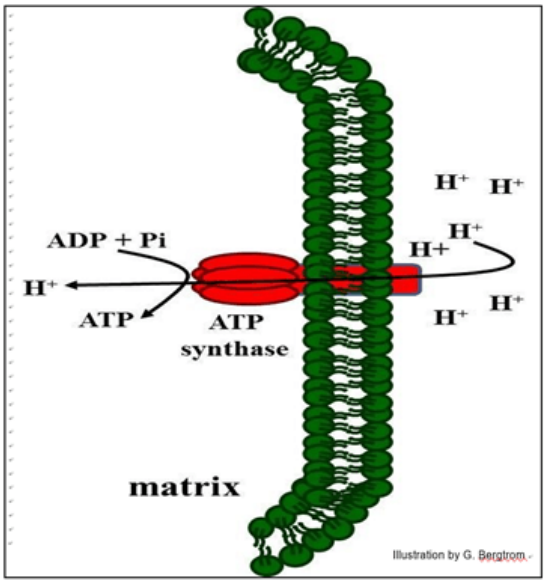
7.3: Oxidative Phosphorylation

Oxidative phosphorylation is the mechanism that by which ATP captures the free energy in the mitochondrial proton gradient. Most of the ATP made in aerobic organisms is made by oxidative phosphorylation, rather than by substrate phosphorylation (the mechanism of ATP synthesis in glycolysis or the Krebs cycle). Some aerobic chemistry may have evolved in response to the toxic effects of rising environmental oxygen levels. Later elaboration of respiratory metabolism were undoubtedly selected because it turns out to be more efficient at making ATP than anaerobic fermentations such as 'complete' glycolysis. In other words, oxidative phosphorylation fueled by electron transport is more efficient that substrate-level phosphorylation. Oxidative phosphorylation couples controlled diffusion of protons through the cristal membrane **ATP synthase** to ATP production.

To summarize here, the movement of electrons down the electron transport chain fuels three proton pumps that establish a proton gradient across the *cristal* membrane that stores free energy. We say that the proton gradient has a ***proton-motive force***, resulting from the difference in proton concentration (an H+ or pH gradient) and a difference in electric potential. The use of this force to make ATP is regulated.

Conditions in the cell control when the energy stored in this gradient will be released to make ATP. The switch that allows protons to flow across the cristal membrane to relieve the proton gradient is an *ATP synthase*, a tiny, complex enzymatic protein motor. For a clear discussion of this complex enzyme, see P. D. Boyer (1997) *The ATP synthase – a splendid molecular machine*. Ann. Rev. Biochem. 66:717-749.

The capture of free energy of protons flowing through the complex is summarized below.



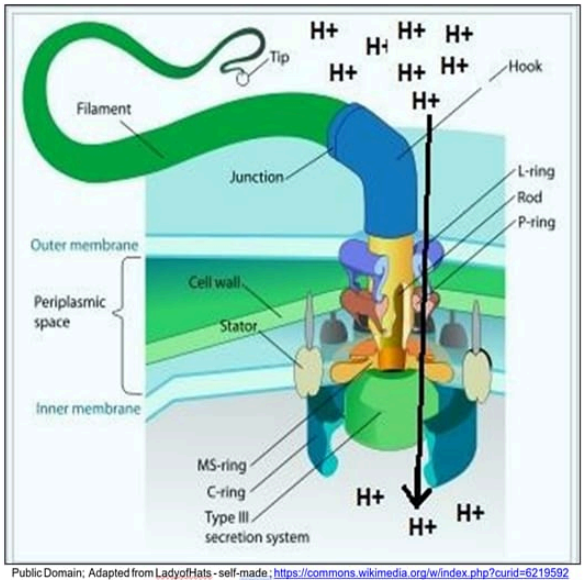
In mitochondria, the protons **pumped** out of the mitochondrial matrix (using the free energy released by electron transport) can then flow back into the matrix through the *ATP synthase*. If the three ETC sites in the cristal membrane that actively transport protons are **proton pumps**, then the cristal membrane ATP synthase complexes function as regulated **proton gates** that catalyzes ATP synthesis when protons are allowed to flow through them. For their discovery of the details of ATP synthase function, P. D. Boyer and J. E. Walker shared the Nobel Prize in Chemistry in 1997.

165 Proton Gates Capture Proton Gradient Free Energy as ATP

The ratio of ATP to ADP concentrations regulates proton flow through the ATP synthase gates is regulated. A high *ATP/ADP* ratio in the mitochondrial matrix indicates that the cell does not need more ATP, closing the proton gate remains closed so that the proton gradient cannot be relieved. On the other hand, a low *ATP/ADP* ratio in the matrix means that the cell is hydrolyzing a lot of ATP..., and that the cell needs more. Then the proton gate opens, and protons flow through cristal membrane ATP synthases back into the matrix along a concentration gradient. As they flow, they release the free energy that powers a protein motor in the enzyme that in turn, activates ATP synthesis. Recall that according to the *endosymbiotic theory*, aerobic bacteria are the

evolutionary ancestor to mitochondria; in fact, the cell membrane of aerobic bacteria house an ETC and *chemiosmotic mechanism* of ATP generation much like that in mitochondria.

Proton gradients do not only power ATP synthesis, but can also power cellular work quite directly. The well-known example is the bacterial flagellum driven directly by proton flow through cell membrane proton gate/molecular motor complex (below).



Public Domain; Adapted from LadyofHats - self-made ; https://commons.wikimedia.org/w/index.php?curid=6219592

Electron transport in the cell membrane creates the gradient, and relief of the gradient directly powers the flagellum.

Just as we did for glycolysis, we can count the ATPs and see how much free energy we get from aerobic respiration, i.e., the complete oxidation of glucose. You can see this in the link below.

166 A Balance Sheet for Respiration

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7.4: Photosynthesis

Chemically, photosynthesis is the reverse reaction of respiration. Compare the two reactions:

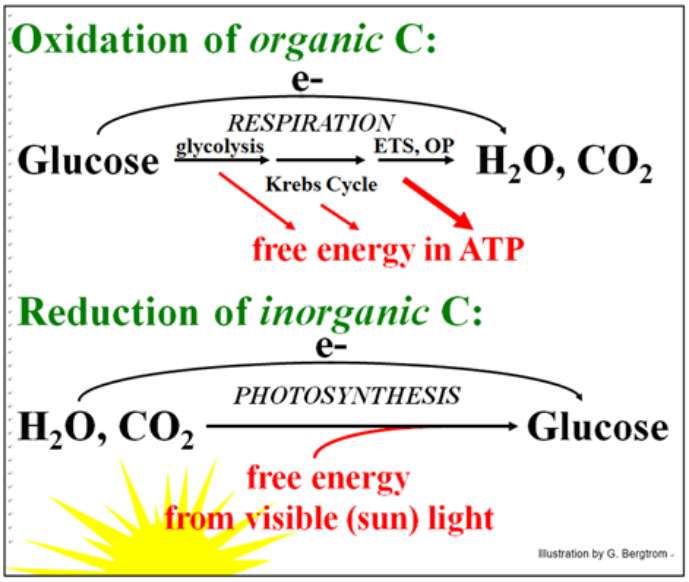
$$C6H12O6 + 6O2 \Leftrightarrow 6CO2 + 6H2O \tag{7.4.1}$$

(DGo = -687Kcal/mole)

$$6CO2 + 6H2O \Leftrightarrow C6H12O6 + 6O2 \tag{7.4.2}$$

(DGo = +687Kcal/mole)

If respiration (reaction 1) is the complete oxidation of glucose to H2O and CO2, then photosynthesis (reaction 2) is the reduction of CO2 using electrons from H2O. Photosynthesis is thus an endergonic reaction. During photosynthesis, sunlight (specifically visible light), fuels the reduction of CO2 (summarized below).

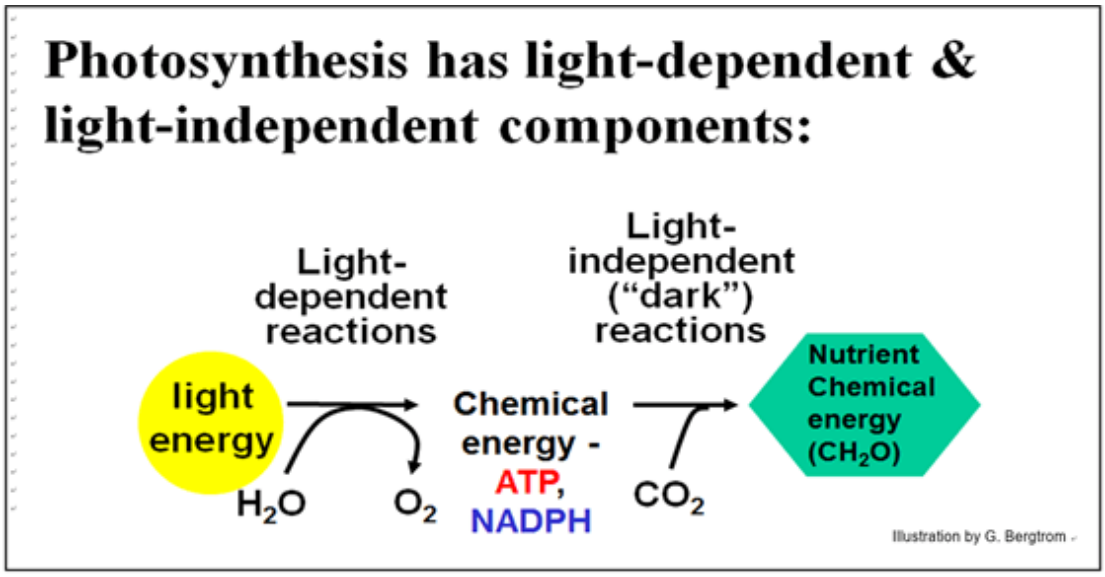


Photosynthesis began in the absence of oxygen; it came before oxygenic respiration on earth. Increasing oxygen in the atmosphere led to selection of oxygenic respiratory pathways (the Krebs cycle, electron transport and oxidative phosphorylation). When we look at photosynthesis in some detail, we will see that photosynthesis and respiration have electron transport-ATP synthesizing systems with similar features. This suggests that they share a common evolutionary ancestry. Elsewhere, we will consider what a common ancestral system might have looked like. Two biochemical pathways make up photosynthesis:

· ***Light-dependent reactions*** that use visible light energy to remove electrons from water, reduce electron carriers, pump protons and make ATP;

· ***Light-independent reactions*** that use ATP to transfer electrons from the reduced electron carriers to CO2 to synthesize glucose.

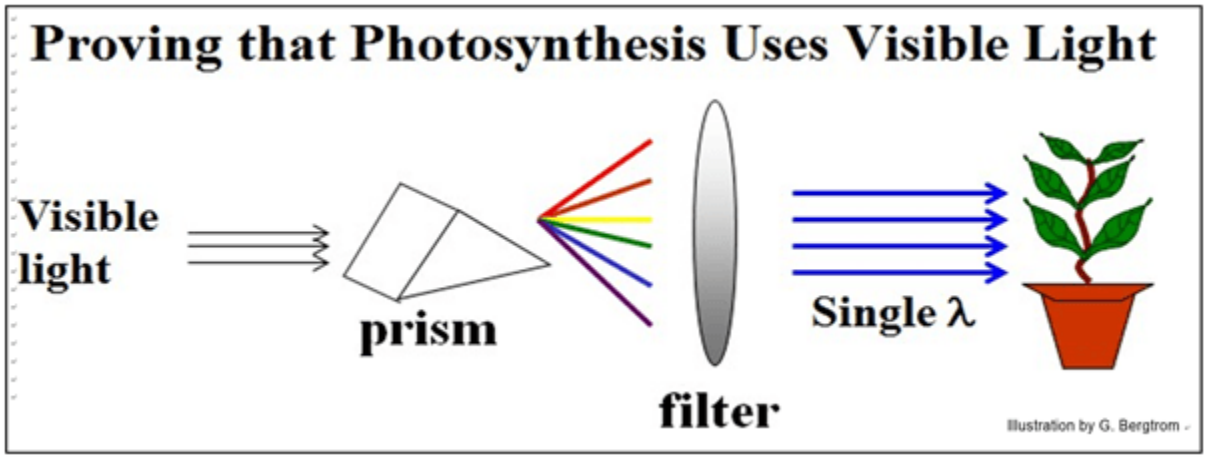
The two pathways are summarized below.



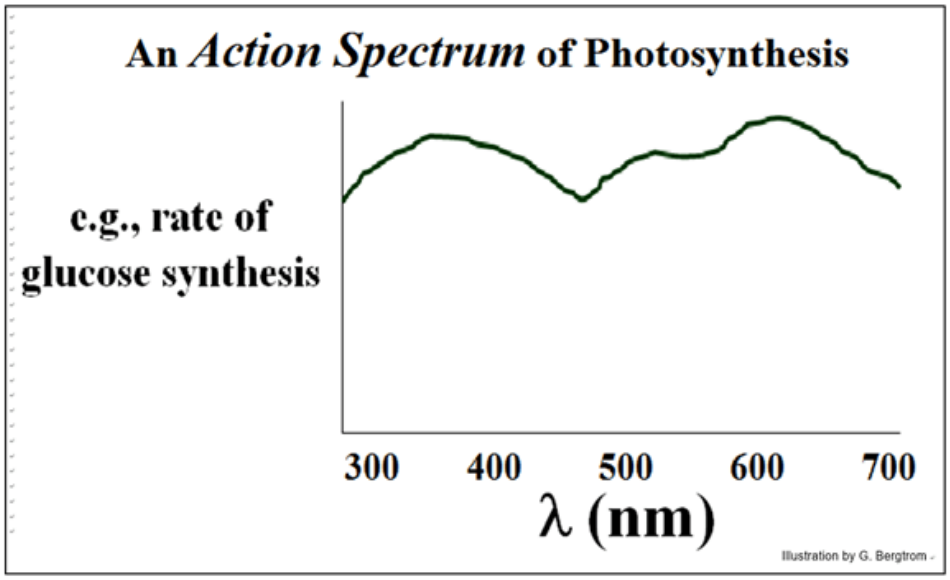
A. The Light Dependent Reactions

Colored substances contain **pigments** that reflect the colors that we see and at the same time, absorb all the other colors of visible light. Early studies asked which plant pigments absorbed the light that allowed (we say supported) photosynthesis. Chlorophyll, the abundant pigment we see in plant tissues is actually two separate green pigments, ***chlorophyll a*** and ***chlorophyll b***. One might therefore predict that light absorbed by chlorophyll will support photosynthesis, but light absorbed by other pigments in plant cells would not.

The experiment to test this hypothesis is illustrated below.

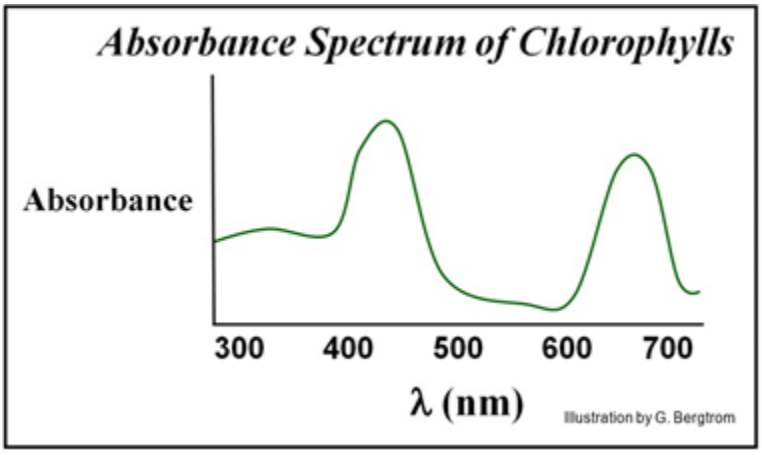


The ***action spectrum*** of photosynthesis below plots the results of this experiment.

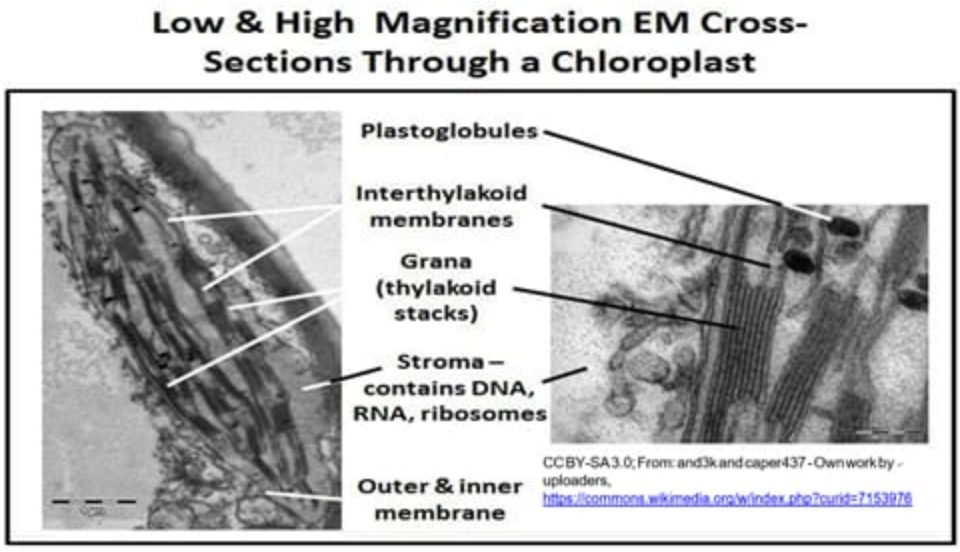


The spectrum shows that all wavelengths of visible light energy support photosynthesis. In addition, other experiments revealed that radiation other than visible light (e.g., ultraviolet and infrared light) do not support photosynthesis. One can conclude that chlorophylls alone are likely not the only pigments to support photosynthesis.

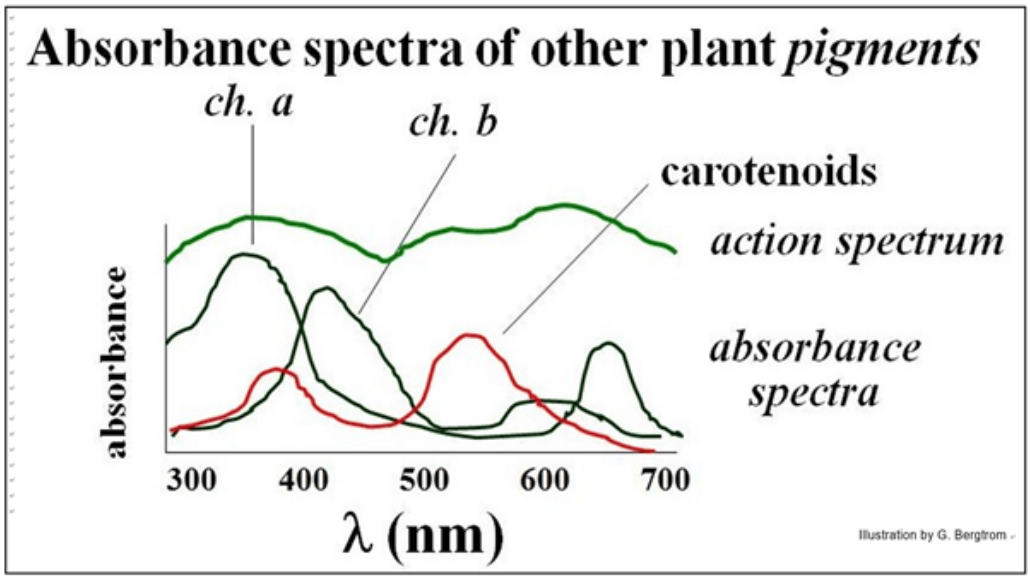
Chlorophylls are easily purified from leaves. The graph below shows an average **absorbance spectrum for** of chlorophylls. The absorbance of *chlorophyll a* and *chlorophyll b* are slightly different, but center at wavelengths at 450 nm and 675 nm.



We can conclude from the *absorbance spectra* that chlorophylls do support photosynthesis, but that, indeed, they are not alone in doing so. Chlorophylls alone do not account for *the action spectrum* of photosynthesis! Clearly, other pigments absorbing elsewhere in the visible spectrum also support photosynthesis. Of course, we knew that leaves and other photosynthetic plant tissues contained a variety of different pigments, many of which we see as fall colors. All of these pigments (including chlorophylls) are found in the **chloroplasts**, the organelles that conduct photosynthesis in plants. Examine the structure of chloroplasts in the electron micrographs below.



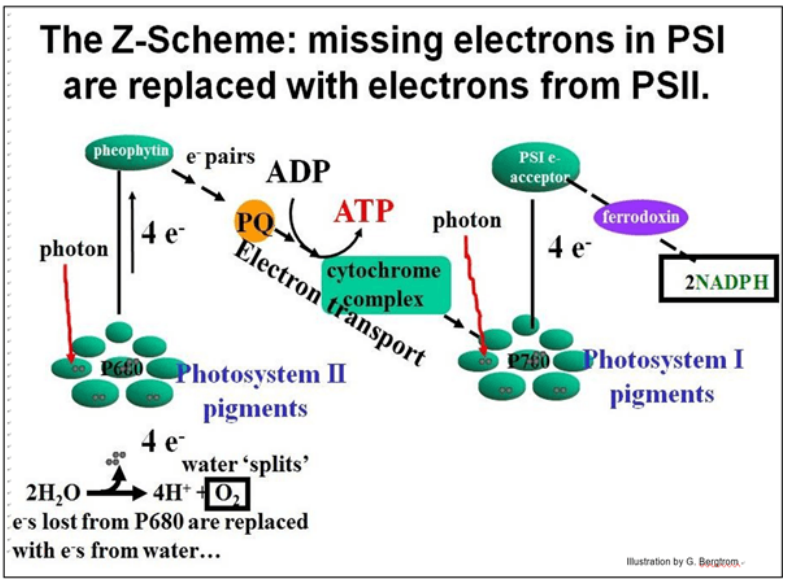
The visible light *absorbance spectra* of three different kinds of plant pigments shown below do coincide with the *action spectrum* of photosynthesis. This implies that absorption of light by those pigments is responsible for photosynthesis.



Carotenoids, **chlorophyll b** and other ***accessory pigments*** participate in capturing light energy for photosynthesis. Two clusters of pigments capture light energy. These ***reaction centers*** are part of ***photosystems 1*** and ***photosystem 2*** on **thylakoid membranes** of chloroplasts. Johann Deisenhofer, Robert Huber and Hartmut Michel first determined the 3D structure of a bacterial reaction center. Then they and unraveled the relationship between the structure of the proteins in the center and the membrane in which they were embedded. For this, they shared the 1988 Nobel Prize in Chemistry.

The activities of Photosystem I are animated at Photosystem 1 Action. You should see light (a photon) excite electron (e-) pairs excited from Photosystem I pigments that then transfer their energy from pigment to pigment, ultimately to chlorophyll a *P700*. The impact of the electron pair then excites a pair of electrons from chlorophyll a *P700*. This e- pair is captured by a *photosystem I* (***PSI***) e- acceptor. Next, the reduced PSI acceptor is oxidized as electrons move down a short ETC, eventually reducing NADP+ to NADPH. Electrons on NADPH will eventually be used to reduce

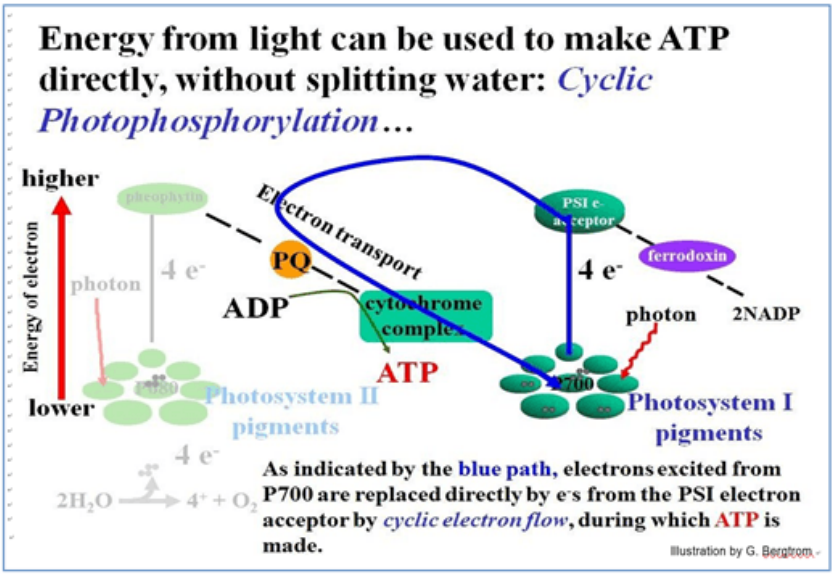
CO2 to a carbohydrate. So far, so good! But that leaves an electron deficit in Photosystem I. The Z-Scheme illustrated below follows electrons *taken from water* (absorbed through roots) into *photosystem II* (***PSII***), which will replace those missing from PSI.



Let's summarize the flow of electrons from water through the Z-scheme. Light excites an e- pair from the ***P680*** form of *chlorophyll a* in PSII. A PSII electron acceptor in the thylakoid membrane, identified as ***pheophytin***, captures these electrons. An electron transport chain oxidizes the pheophytin, transferring e- pairs down to PSI. Some of the free energy released pumps protons from the *stroma* into the space surrounded by the thylakoid membranes. The gradient free energy fuels ATP synthesis as protons flow back into the stroma through a chloroplast ***ATP synthase***. The link at Action in the Z-Scheme animates the entire Z-Scheme, showing first how PSI electrons reduce NADP+ and then how PSII electrons replace missing PSI electrons, making ATP along the way. The oxygen released by splitting water ends up in the atmosphere.

B. Cyclic Photophosphorylation

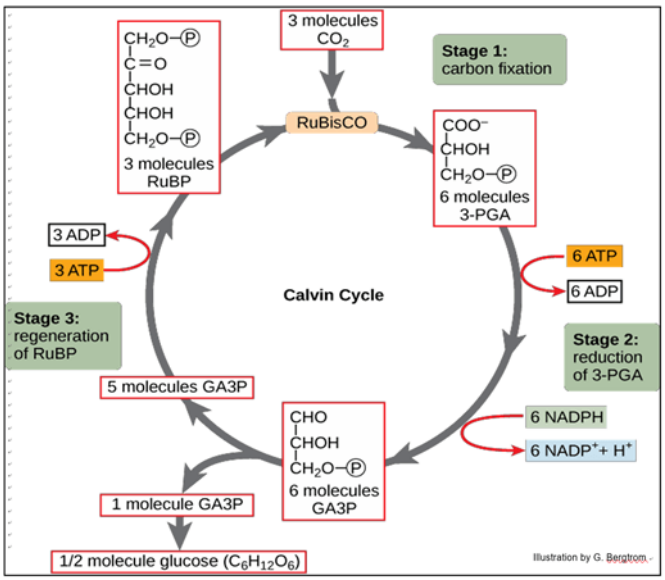
The Z-Scheme does not in fact make enough ATP to power the Calvin Cycle. But when the need for ATP exceeds the capacity of the tissues to make sugar, the photosynthetic apparatus can *take a time-out,* resorting to **Cyclic Photo- phosphorylation** for a while. Cyclic Photophosphorylation simply takes electrons excited to the PSI electron acceptor, and instead of sending them to NADP+, deposits them on PC (*plastocyanin*) in the electron transport chain between PSII and PSI. These electrons then flow down this 'long line' of the Z, right back to PSI, releasing their free energy to make ATP. In light, the electrons just go up and around, hence the name *Cyclic Photophosphorylation*. The path of electrons is shown below and animated at Action in Cyclic Photophosphorylation.



C. The Light-Independent ("Dark") Reactions

1. The Dark Reactions of C3 Photosynthesis

As we have seen, the light-dependent reactions of photosynthesis require light energy and water and generate O2, ATP and NADPH. In the ***light-independent*** (or ***'dark'***) reactions, the ATP and NADPH will provide free energy and electrons (respectively) for carbon fixation (the reduction of CO2 to make carbohydrates). CO2 enters photosynthetic tissues through ***stomata***. Stomata are pores in leaves that can be open or closed, depending on light, temperature conditions and water availability. In addition to allowing CO2 into photosynthetic tissues, stomata also function in ***transpiration***, which allows excess water in cells to leave the plants by transpiration (sometimes called *evapotranspiration*). C3 photosynthesis is the mechanism of *carbon fixation* in most plants, so called because its first carbohydrate product is a 3-C molecule, 3-phosphoglyceric acid (3-PG). You should recognize 3-PG; it is also a glycolytic intermediate. The Calvin Cycle is the most common dark reaction pathway. For its discoverer, M. Calvin received the Nobel Prize in Chemistry in 1961. The Calvin Cycle is shown below.



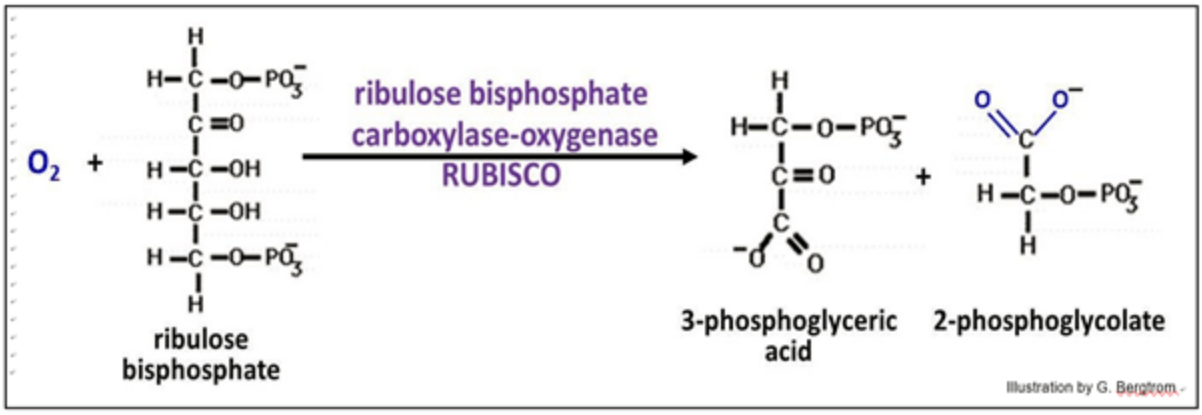
Check the animation at Action in the Calvin Cycle.

Each carbon dioxide entering the Calvin cycle is "fixed" to a 5-carbon *ribulose bisphosphate* molecule (RuBP), catalyzed by the enzyme ***RuBP carboxylase- oxygenase***, or ***Rubisco*** for short. The expected 6-C molecule must be quickly split into two 3-C molecules since it has not been detected as an intermediate to date! The first detectable products are two molecules of 3-PG. Each 3-PG is in turn reduced to glyceraldehyde-3-phosphate (G-3-P, another familiar molecule).

The cycle regenerates the RuBP ***and*** produces glucose. Perhaps the easiest way to see this is to imagine the cycle going around 12 times, fixing 12 molecules of carbon dioxide, as shown in the link above. Two of the G-3-P molecules are linked together to make a single 6-C molecule of glucose (which is polymerized into starch for storage in daylight). That leaves 10 molecules of G-3-P, for a total of 30 carbons. The latter part of the cycle will regenerate 5 molecules of new RuBP, accounting for our 30 carbons!

2. Photorespiration

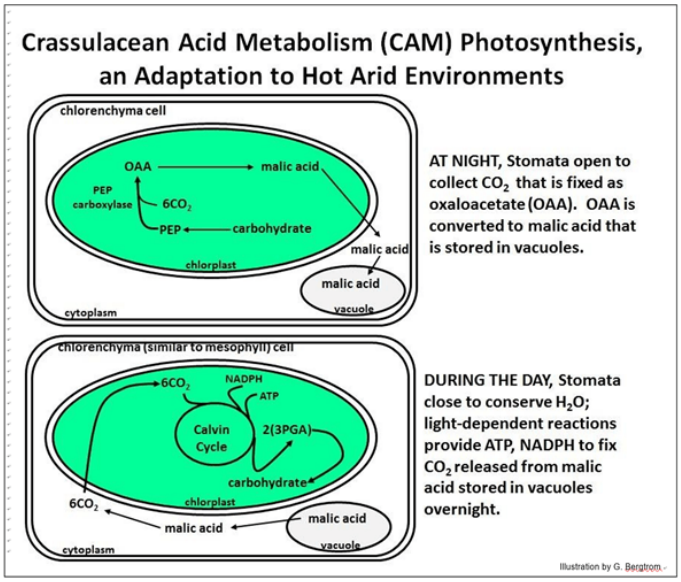
There are times that even plants in temperate environments suffer prolonged hot, dry spells. Perhaps you have seen a lawn grow more slowly and turn brown after a dry heat wave in summer, only to grow and re-green after the rains resume. C3 plants resort to ***photorespiration*** during drought and dry weather, closing their stomata to conserve water. Under these conditions, CO2 can't get into the leaves... and O2 can't get out! As CO2 levels drop and O2 rise in photosynthetic cells, the Calvin Cycle slows down. Instead of fixing CO2, the enzyme ***Rubisco*** now catalyzes "*O2 fixation*" using its *oxygenase* activity. The combination of RuBP with O2 splits RuBP into a 3-carbon and a 2-carbon molecule: **3-phosphoglyceric acid** (3-PG) and **phosphoglycolate** respectively. The reaction is shown below.



Not only does photorespiration result in only one 3-carbon carbohydrate (compared to two in the Calvin Cycle), but the phosphoglycolate produced is *cytotoxic* (not healthy for cells!). Removing the phosphate and metabolizing the remaining *glycolic acid* costs energy. Therefore, photorespiration can only be sustained for a short time. On the other hand, plants that have adapted to live in hot arid environments all the time have evolved one of two alternate pathways. One is the ***CAM*** (***Crassulacean Acid Metabolism***); the other is the ***C4*** pathway. Each is an alternative to C3 carbon fixation.

3. The CAM Photosynthetic Pathway

Crassulacean acid metabolism (**CAM**) was discovered in the *Crassulaceae*. These are succulents like sedum (a common ground cover), cactuses and jade plants, and some orchids. The CAM pathway was selected in evolution to allow plants to conserve water, especially during the high daytime temperatures. *Stomata* in chlorenchymal (***mesophyll***) leaf cells close during the day to minimize water loss by ***transpiration***. The stomata open at night, allowing plant tissues to take up CO2. CAM plants fix CO2 by combining it with ***PEP*** (*phosphoenol pyruvate*). This eventually produces ***malic acid*** that is stored in plant cell vacuoles. By day, stored malic acid retrieved from the vacuoles splits into pyruvate and CO2. The CO2 then enters chloroplasts and joins the Calvin Cycle to make glucose and the starches. The CAM pathway is shown below.



In sum, CAM plant *mesophyll* cells

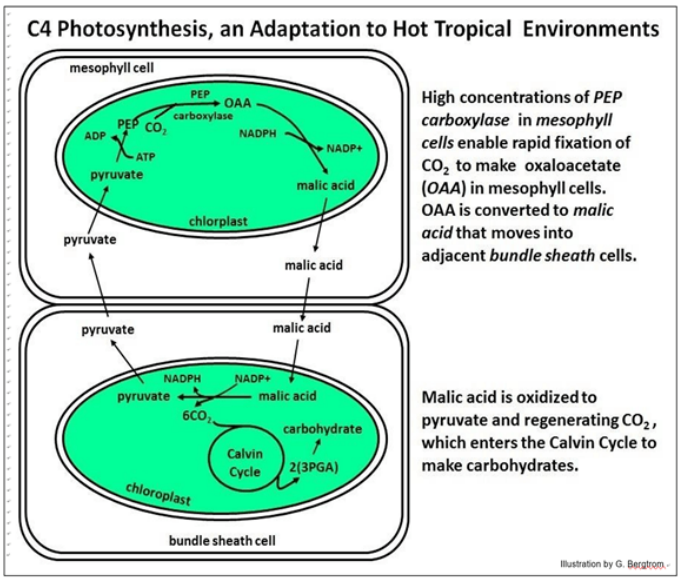
1. open stomata to collect, fix and store CO2 as an organic acid at night.

2. close stomata to conserve water in the daytime.

3. re-fix the stored CO2 as carbohydrate using the NADPH and ATP from the light reaction the next the day.

4. The C4 Photosynthetic Pathway

C4 refers to malic acid, the 4-carbon end product of CO2 fixation. In this regard, the C4 pathway is the same as in CAM metabolism! In both pathways, PEP carboxylase is the catalyst of carbon fixation, converting phosphoenol pyruvate (PEP) to oxaloacetate (OAA). The OAA is then reduced to malic acid, as shown below.



C4 metabolism diverges from CAM pathway after malic acid formation. PEP carboxylase catalysis is rapid in C4 plants, in part because malic acid does not accumulate in the *mesophyll* cells. Instead, it is rapidly transferred from mesophyll to adjacent ***bundle sheath*** cells, where it enters chloroplasts. The result is that C4 plants can keep stomata open for CO2 capture (unlike CAM plants), but closed at least part of the day to conserve water. The 4-carbon malic acid is oxidized to pyruvate (three carbons) in the bundle sheath cell chloroplasts. The CO2 released enters the Calvin cycle to be rapidly fixed by Rubisco. Of course, this system allows more efficient water use and faster carbon fixation under high heat, dry conditions than does C3 photosynthesis. Corn is perhaps the best-known C4 plant!

By the way, can you recognize several more intermediates common to respiration and the light-independent photosynthetic reactions?

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7.5: More Thoughts on the Mechanisms and Evolution of Respiration and Photosynthesis

We can assume that the abundance of chemical energy on our cooling planet favored the formation of cells that could capture free energy from these nutrients in the absence of any oxygen. For a time, we thought that the first cells would have extracted nutrient free energy by non-oxidative, fermentation pathways. And they would have been voracious feeders, quickly depleting their environmental nutrient resources. In this scenario, the evolution of autotrophic life forms saved life from an early extinction! That is because autotrophs could create organic molecules extracting free energy from inorganic molecules or from light.

An alternative scenario that is gaining traction, suggests that the first cells may have started with oxidative reactions that used something other than oxygen as a final electron acceptor. In this scenario (to be considered in more detail elsewhere), non-oxygenic 'oxidative' chemistries came first, followed by the evolution of anoxic fermentative chemistries, then followed by photosynthesis, and finally respiratory pathways. In either scenario, we can safely assume that photosynthesis existed before oxygenic respiration.

We also assume that oxygenic photoautotrophs that capture free energy from light would become the most abundant autotrophs, if for no other reason than sunlight is always available (at least during the day), and oxygen is abundant in the air! The early photoautotrophs were likely the ancestors of today's cyanobacteria. In fact, a phylogenetic study of many genes including "plastid-encoded proteins, nucleus-encoded proteins of plastid origin..., as well as wide-ranging genome data from cyanobacteria" suggests a common ancestry of freshwater cyanobacteria and eukaryotic chloroplasts (Ponce-Toledo, R.I. et al., 2017, *An Early-Branching Freshwater Cyanobacterium at the Origin of Plastids*. Current Biology 27:386-391).

But what about the origins of respiratory metabolism and the endosymbiotic origins of mitochondria? Let's start by asking how respiration co-opted photosynthetic electron transport reactions that captured the electrons from H2O needed to reduce CO2, turning those reactions to the task of burning sugars back to H2O and CO2. As photosynthetic organisms emerged and atmospheric oxygen increased, elevated oxygen levels would have been toxic to most living things. Still, some autotrophic cells must have had a pre- existing genetic potential to conduct detoxifying respiratory chemistry. These would have been facultative aerobes with the ability to switch from photosynthesis to respiration when oxygen levels rose. Today's purple non-sulfur bacteria such as *Rhodobacter sphaeroides* are just such facultative aerobes! Perhaps we aerobes descend from the ancestors of such cells that survived and spread from localized environments where small amounts of oxygen threatened their otherwise strictly anaerobic neighbors. Is it possible that the endosymbiotic critter that became the first mitochondrion in a eukaryotic cell was not just any aerobic bacterium, but a purple photosynthetic bacterium?

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7.6: Key Words and Terms

active transport of protons	energy efficiency of glucose metabolism	PEP carboxylase
ATP synthase	energy flow in glycolysis	pH gradient
bacterial flagellum	energy flow in the Krebs Cycle	pheophytin
C4 photosynthesis	F1 ATPase	photosynthesis
Calvin Cycle	FAD	Photosystems
CAM photosynthesis	FADH2	proton gate
carotene	Light-dependent reactions	proton gradient
chemiosmotic mechanism	Light-independent reactions	proton pump
Chlorophyll a	Malic acid	PSI electron acceptor
Chlorophyll b	mitochondria	PSII electron acceptor
	molecular motor	redox reactions
complex I, II, III, IV	NAD+	reduced electron carriers

Crassulaceae	NADH	RUBISCO
cristal membrane	NADP+	RuBP
Cyclic photophosphorylation	NADPH	Splitting water

cytochromes	**outer membrane**	**stoichiometry of glycolysis**
Dark Reactions	**oxidative phosphorylation**	**stoichiometry of the Krebs Cycle**
electrochemical gradient	**oxidative phosphorylation**	**substrate-level phosphorylation**
electron transport system (chain)	**P680**	**Z-scheme**
endosymbiotic theory	**P700**	

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8: DNA, CHROMOSOMES AND CHROMATIN

8.1: Introduction
8.2: The Stuff of Genes
8.3: DNA Structure
8.4: Genes and Chromatin in Eukaryotes
8.5: Structure and Organization of DNA in Bacteria
8.6: Key Words and Terms

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8.1: Introduction

Here we look at classic experiments that led to our understanding that genes are composed of ***DNA***. We already knew that genes were on chromosomes (*chromo* – colored; *soma*-body). Early 20th century ***gene mapping*** even showed the relative location (***locus***) of genes on chromosomes. Compared to eukaryotes, bacteria contain a very small amount of DNA per cell. Subsequent bacterial *gene mapping* and electron microscopy revealed that the *E. coli* "chromosome is little more than a small closed, circular DNA double helix. In contrast, linear eukaryotic chromosomes are highly *condensed* structures composed of DNA and protein, visible only during ***mitosis*** or ***meiosis***. During the much longer ***interphase*** portion of the eukaryotic ***cell cycle***, chromosomes de-condense to ***chromatin***, a less organized form of protein-associated DNA in the nucleus. Chromatin is the gatekeeper of *gene activity* in eukaryotic cells, a situation quite different from bacterial cells. Since we know that all cells of an organism contain the same DNA, and all cells must alter patterns of *gene expression* over time, understanding the structure and organization of DNA in cells is essential to an understanding of how and when cells turn genes on and off.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. summarize the evidence that led to acceptance that genes are made of DNA.

2. discuss how Chargaff''s DNA *base ratios* support DNA as the "stuff of genes".

3. interpret the results of Griffith, Avery et al. and Hershey & Chase, in historical context.

4. outline and explain how Watson and Crick built their model of a DNA double helix.

5. distinguish between conservative, semiconservative and dispersive replication.

6. describe and/or draw the progress of a *viral infection*.

7. trace the fate of *35SO4 (sulfate)* into proteins synthesized in cultured bacteria.

8. distinguish between the organization of DNA in chromatin and chromosomes and speculate on how this organization impacts replication.

9. list some different uses of karyotypes.

10. compare and contrast euchromatin and heterochromatin structure and function.

11. outline an experiment to purify *histone H1* from chromatin.

12. formulate an *hypothesis* to explain why chromatin is found only in eukaryotes.

13. describe the roles of different histones in nucleosome structure.

14. explain the role of Hfr strains in mapping genes in *E. coli*.

15. explain the chemical rationale of using different salt concentrations to extract 10 nm nucleosome fibers *vs*. 30nm solenoid structures from chromatin.

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8.2: The Stuff of Genes

That all eukaryotic cells contain a nucleus was understood by the late 19th century. By then, histological studies had shown that nuclei contained largely proteins and DNA. At around the same time, the notion that the nucleus contains genetic information was gaining traction. In 1910, Albrecht Kossel received the 1910 Nobel Prize in Physiology or Medicine for his discovery of the adenine, thymine, cytosine and guanine (the four DNA bases), as well as of uracil in RNA. Mendel's Laws of Inheritance, presented in 1865, were not widely understood, probably because they relied on a strong dose of arithmetic and statistics, when the utility of quantitative biology was not much appreciated. But, following the re-discovery three decades later, the number of known inherited traits in any given organism increased rapidly. At that time, DNA was known as a small, simple molecule, made up of only the four nucleotides (see *DNA Structure* below for additional historical perspective). So, the question was how could such a small, simple account for the inheritance of so many different physical traits? The recognition that enzyme activities were inherited in the same way as morphological characteristics led to the ***one- gene-one enzyme*** hypothesis that earned G. W. Beadle, E. L. Tatum and J. Lederberg the 1958 Nobel Prize for Physiology and Medicine. When enzymes were later shown to be proteins, the hypothesis became *one-gene-one protein*. When proteins were shown to be composed of one or more polypeptides, the final hypothesis became *one-gene-one- polypeptide*. However, this relationship between genes and proteins failed to shed any light on how DNA might be the genetic material. In fact, quite the contrary! As chains of up to 20 different amino acids, polypeptides and proteins had the potential for enough structural diversity to account for the growing number of heritable traits in a given organism. Thus, proteins seemed more likely candidates for the molecules of inheritance.

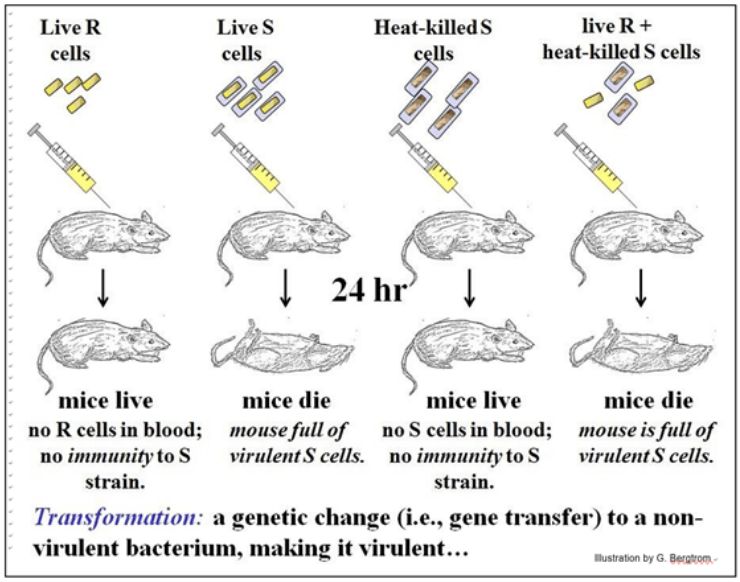
The experiments you will read about here began around the start of World War I and lasted until just after World War 2. During this time, we learned that DNA was no mere tetramer, but was in fact a long polymer. This led to some very clever experiments that eventually forced the scientific community to the conclusion that DNA, not protein, was the genetic molecule, despite being composed of just four monomeric units. Finally, we look at the classic work of *Watson, Crick, Franklin* and *Wilkins* that revealed the structure of the genetic molecule.

A. Griffith's Experiment

Fred Neufeld, a German bacteriologist studying pneumococcal bacteria in the early 1900s discovered three immunologically different strains of *Streptococcus pneumonia (*Types I, II and III*)*. The virulent strain (Type III) was responsible for much of the mortality during the **Spanish Flu** (influenza) *pandemic* of 1918-1920. This pandemic killed between 20 and 100 million people, many because the influenza viral infection weakened the immune system of infected individuals, making them susceptible to bacterial infection by *Streptococcus pneumonia*.

In the 1920s, *Frederick Griffith* was working with virulent ***wild type*** (*Type III*) and ***benign*** (*Type II*) strains of *S. pneumonia*. The two strains were easy to tell apart petri dishes because the virulent strain grew in morphologically *smooth colonies*, while the benign strain formed *rough colonies*. For this reason, the two bacterial strains were called ***S*** and ***R***, respectively. We now know that *S* cells are coated with a polysaccharide (mucus) capsule, making colonies appear smooth. In contrast, *R* cell colonies look rough (don't glisten) because they lack the polysaccharide coating.

Griffith knew that injecting mice with *S* cells killed them within about a day! Injecting the non-lethal *R* cells on the other hand, caused no harm. Then, he surmised that, perhaps, the exposure of mice to the R strain of *S. pneumonia* first would immunize them against lethal infection by S cells. His experimental protocol and results, published in 1928, are summarized below.



To test his hypothesis, Griffith injected mice with R cells. Sometime later, he injected them with S cells. However, the attempt to immunize the mice against S. *pneumonia* was unsuccessful! The control mice injected with S strain cells *and* the experimental mice that received the R strain cells first and then S cells, all died in short order! As expected, mice injected with R cells only survived.

Griffith also checked the blood of his mice for the presence of bacterial cells:

· Mice injected with benign **R (**rough) strain cells survived and after plating blood from the mice on nutrient medium, no bacterial cells grew.

· Many colonies of S cells grew from the blood of dead mice injected with S cells.

Griffith performed two other experiments, shown in the illustration:

1. He injected mice with heat-killed S cells; as expected, these mice survived. Blood from these mice contained no bacterial cells. This was “expected” since heating the S cells should have the same effect as pasteurization has on bacteria in milk!

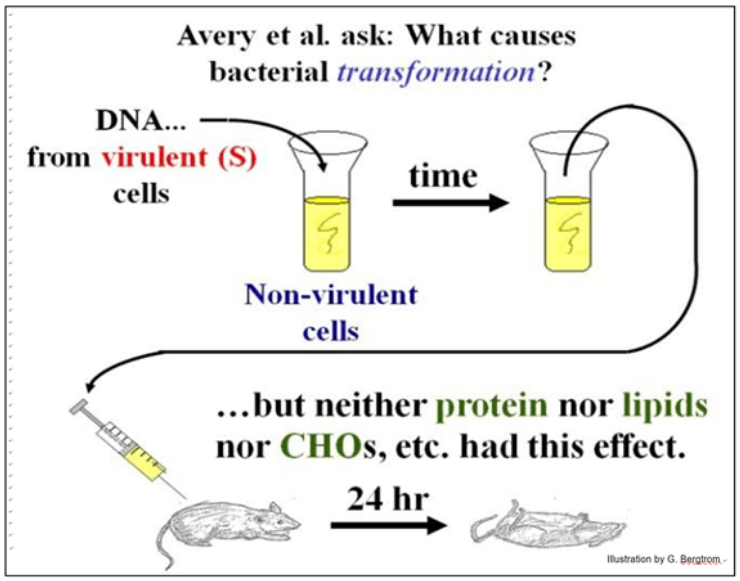
2. Griffith also injected mice with a *mixture of live R cells and heat-killed S cells*, in the hope that the combination might induce immunity in the mouse where injecting the R cells alone had failed. You can imagine his surprise when, far from becoming immunized, the injected mice died and abundant S cells had accumulated in their blood.

Griffith realized that something important had happened in his experiments. In the mixture of live R cells and heat-killed S cells, something released from the dead S cells had transformed some R cells. Griffith named this “something” the **transforming principle**, a molecule present in the debris of dead S cells and sometimes acquired by a few live R cells, turning them into virulent S cells. We now know that R cells lack polysaccharide coat, and that the host cell’s immune system can attack and clear R cells before a serious infection can take hold.

B. The Avery-MacLeod-McCarty Experiment

Griffith didn’t know the chemical identity of the transforming principle. However, his experiments led to studies that proved DNA was the “stuff of genes”. With improved molecular purification techniques developed in the 1930s, O. Avery, C. MacLeod, and M. McCarty transformed R cells *in vitro* (that is, without the help of a mouse!). They purified heat-killed S-cell components (DNA, proteins, carbohydrates, lipids…) and separately tested the transforming ability of each molecular component on R cells in a test tube.

The experiments of Avery et al. are summarized below.



Since only the ***DNA fraction*** of the dead S cells could cause transformation, Avery et al. concluded that DNA must be the ***Transforming Principle***. In spite of these results, DNA was not readily accepted as the stuff of genes. The sticking point was that DNA was composed of only four nucleotides. Even though scientists knew that DNA was a large polymer, they still thought of DNA as that simple molecule, for example a polymer made up of repeating sequences of the four nucleotides:

...AGCTAGCTAGCTAGCTAGCT...

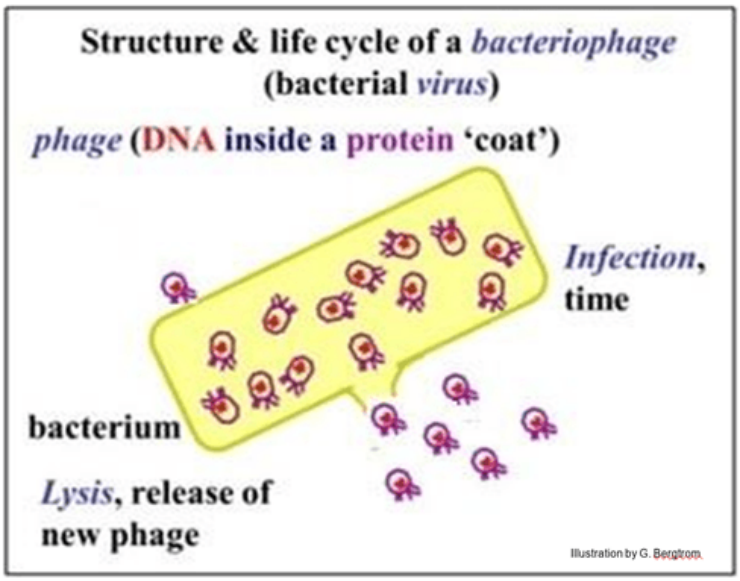
Only the seemingly endless combinations of 20 amino acids in proteins promised the biological specificity necessary to account for an organism's many genetic traits. Lacking structural diversity, DNA was explained as a mere scaffold for protein genes. To adapt Marshal McLuhan's famous statement that *the medium is the message* (i.e., airwaves do not merely *convey*, but are the message), many still believed that proteins are the medium of genetic information *as well as* the functional message itself.

The reluctance of influential scientists of the day to accept a DNA *transforming principle* deprived its discoverers of the Nobel Prize stature it deserved. After new evidence made further resistance to that acceptance untenable, even the Nobel Committee admitted that failure to award a Nobel Prize for the discoveries of Avery et al. was an error. The key experiments of Alfred Hershey and Martha Chase finally put to rest any notion that proteins were genes.

167 Transformation In & Out of Mice; Griffith, McCarthy et al.

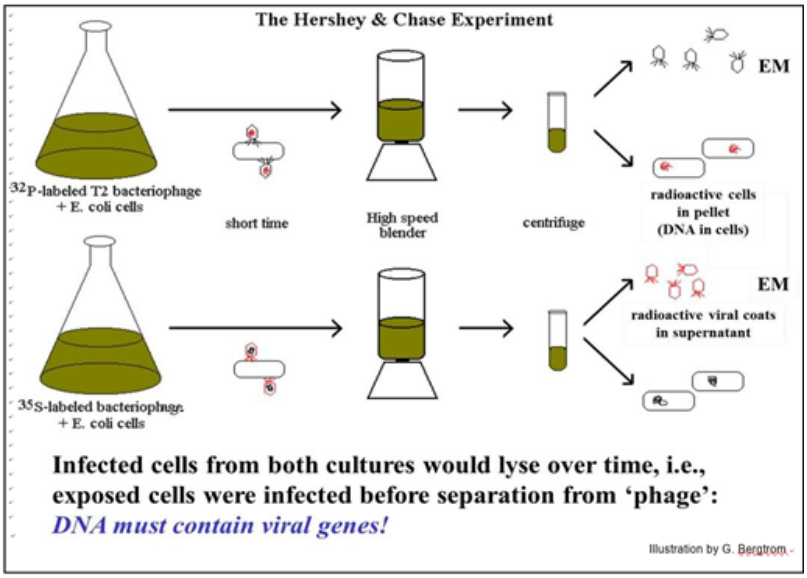
C. The Hershey-Chase Experiment

Biochemically, bacterial viruses were known consist of DNA enclosed in a protein capsule. The life cycle of bacterial viruses (bacteriophage, or phage for short) begins with infection of a bacterium, as illustrated below.



Phages are inert particles until they bind to and infect bacterial cells. Phage particles added to a bacterial culture could be seen attach to bacterial surfaces in an electron microscope. Investigators found that they could detach phage particles from bacteria by agitation in a blender (similar to one you might have in your kitchen). Centrifugation then separated the bacterial cells in a pellet at the bottom of the centrifuge tube, leaving the detached phage particles in the supernatant. By adding phage to bacteria and then detaching the phage from the bacteria at different times, it was possible to determine how long it the phage had to remain attached before the bacteria become infected. It turned out that pelleted cells that had been attached to phage for short times would survive and reproduce when re-suspended in growth medium. But pelleted cells left attached to phage for longer times had become infected; centrifugally separated from the detached phage and resuspended in fresh medium, these cells would go on and lyse, producing new phage. Therefore, the transfer of genetic information for virulence from virus to phage took some time. The viral genetic material responsible for infection and virulence was apparently no longer associated with the phage capsule, which could be recovered from the centrifugal supernatant.

Alfred Hershey and Martha Chase designed an experiment to determine whether the *DNA* enclosed by the viral *protein capsule* or the capsule protein itself caused phage to infect the bacterium. In the experiment, they separately grew *E. coli* cells infected with ***T2 bacteriophage*** in the presence of either ^{32}**P** or ^{35}**S** (*radioactive* isotopes of phosphorous and sulfur, respectively). The result was to produce phage that contained either radioactive DNA or radioactive proteins, but not both (recall that only DNA contains phosphorous and only proteins contain sulfur). They then separately infected fresh *E. coli* cells with either ^{32}P- or ^{35}S-labeled, radioactive phage. Their experiment is described below.



Phage and cells were incubated with either 32P or 35S just long enough to allow infection. Some of each culture was allowed to go on and lyse to prove that the cells were infected. The remainder of each mixture was sent to the blender. After centrifugation of each blend, the pellets and supernatants were examined to see where the radioactive proteins or DNA had gone. From the results, the 32P always ended up in the pellet of bacterial cells while the 35S was found in the phage remnant in the supernatant. Hershey and Chase concluded that the genetic material of bacterial viruses was DNA and not protein, just as Avery et al. had suggested that DNA was the bacterial transforming principle.

Given the earlier resistance to “simple” DNA being the genetic material, Hershey and Chase used cautious language in framing their conclusions. They need not have; all subsequent experiments confirmed that DNA was the genetic material. Concurrent with these confirmations were experiments demonstrating that DNA might not be (indeed, was not) such a simple, uncomplicated molecule! For their final contributions to pinning down DNA as the “stuff of genes”, Alfred D. Hershey shared the 1969 Nobel Prize in Physiology or Medicine with Max Delbruck and Salvador E. Luria.

168 Hershey and Chase: Viral Genes are in Viral DNA

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8.3: DNA Structure

A. Early Clues and Ongoing Misconceptions

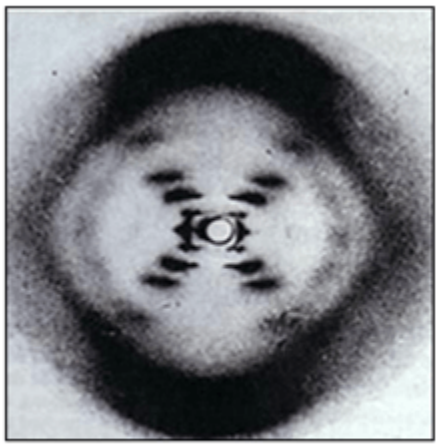
By 1878, a substance in the pus of wounded soldiers derived from cell nuclei (called *nuclein*) was shown to be composed of 5 bases (the familiar ones of DNA and RNA). The four bases known to make up DNA (as part of nucleotides) were thought to be connected through the phosphate groups in short repeating chains of four nucleotides. By the 1940s, we knew that DNA was a long polymer. Nevertheless, it was still considered too simple to account for genes (see above). After the Hershey and Chase experiments, only a few holdouts would not accept DNA as the genetic material. So, the question remaining was how such a "simple" molecule could account for all the genes, even in so simple an organism as a bacterium. The answer to this question was to lie at least in part in an understanding of the physical structure of DNA, made possible by the advent of ***X-Ray Crystallography***.

If a substance can be crystallized, the crystal will diffract X-rays at angles revealing regular (repeating) structures of the crystal. William Astbury demonstrated that high molecular weight DNA had just such a regular structure. His ***crystallographs*** suggested DNA to be a linear polymer of stacked bases (nucleotides), each nucleotide separated from the next by 0.34 nm. Astbury is also remembered for coining the term "*molecular biology*" to describe his studies. The term now covers as all aspects of biomolecular structure, as well as molecular functions (e.g. replication, transcription, translation, gene regulation...).

In an irony of history, the Russian biologist Nikolai Koltsov had already intuited in 1927 that the basis of genetic transfer of traits would be a "giant hereditary molecule" made up of "two mirror strands that would replicate in a semi-conservative fashion using each strand as a template". A pretty fantastic inference if you think about it since it was proposed long before Watson and Crick and their colleagues worked out the structure of the DNA double-helix!

B. Wilkins, Franklin, Watson & Crick

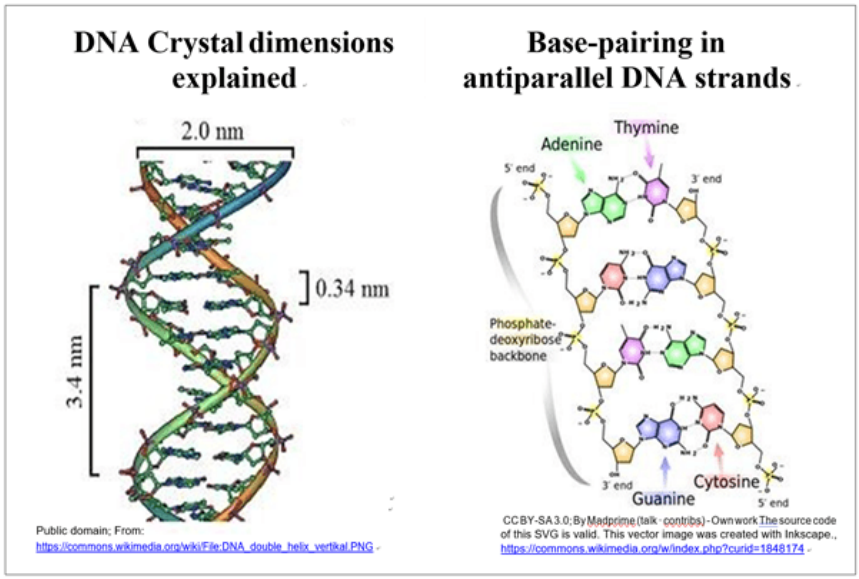
Maurice Wilkins, an English biochemist, was the first to isolate highly pure, high molecular weight DNA. Working in Wilkins laboratory, Rosalind Franklin was able to crystalize this DNA and produce very high-resolution X-Ray diffraction images of the DNA crystals. Franklin's most famous (and definitive) crystallography was "Photo 51", shown below.



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This image confirmed Astbury's **0.34 nm** repeat dimension and revealed two more numbers, **3.4 nm** and **2 nm**, reflecting additional repeat structures in the DNA crystal. When James Watson and Francis Crick got hold of these numbers, they used them along with other data to build DNA models out of nuts, bolts and plumbing. Their models eventually revealed DNA to be a pair of **antiparallel complementary** of nucleic acid polymers..., shades of Koltsov's mirror-image macromolecules! Each strand is a string of nucleotides linked by *phosphodiester bonds*, the two strands held together in a *double helix* by complementary H-bond interactions.

Let's look at the evidence for these conclusions and as we do, refer to the two illustrations of the double helix below.



Recalling that Astbury's 0.34 nm dimension was the ***distance between successive nucleotides*** in a DNA strand, Watson and Crick surmised that the 3.4 nm repeat was a structurally meaningful 10-fold multiple of Astbury's number. When they began building their DNA models, they realized from the bond angles connecting the nucleotides that the strand was forming a helix, from which they concluded that the 3.4 nm repeat was the ***pitch*** of the helix, i.e., the distance of one complete turn of the helix. This meant that there were 10 bases per turn of the helix. They further reasoned that the 2.0 nm number might reflect the diameter of helix. When their scale model of a single stranded DNA helix predicted a helical diameter much less than 2.0 nm, they were able to model a ***double helix*** that more nearly met the 2.0 nm diameter requirement. In building their double helix, Watson and Crick realized that bases in opposing strands would come together to form H-bonds, holding the helix together. However, for their double helix to have a constant diameter of 2.0 nm, they also realized that the smaller **pyrimidine** bases, ***Thymine*** (**T**) and ***Cytosine*** (**C**), would have to H-bond to the larger **purine** bases, ***Adenine*** (**A**) and ***Guanosine*** (**G**).

Now to the question of how a "simple" DNA molecule could have the structural diversity needed to encode thousands of different polypeptides and proteins. In early studies, purified *E. coli* DNA was chemically hydrolyzed down to nucleotide monomers. The hydrolysis products contained nearly equal amounts of each base, reinforcing the notion that DNA was that simple molecule that could not encode genes. But Watson and Crick had private access to revealing data from Erwin Chargaff. Chargaff had determined the base composition of DNA isolated from different species, including *E. coli*. He found that the base composition of DNA from different species was not always *equimolar*, meaning that for some species, the DNA was not composed of equal amounts of each of the four bases (see some of this data below).

Base Compositions of DNA from Different Organisms

base		*human*	*yeast*	*fly*
purine	A	27%	21%	15%
pyrimidine	C	23%	32%	35%
purine	G	23%	32%	35%
pyrimidine	T	27%	21%	15%

The mere fact that DNA from some species could have base compositions that deviated from equimolarity put to rest the argument that DNA had to be a very simple sequence. Finally, it was safe to accept that to accept the obvious, namely that DNA

was indeed the “stuff of genes”.

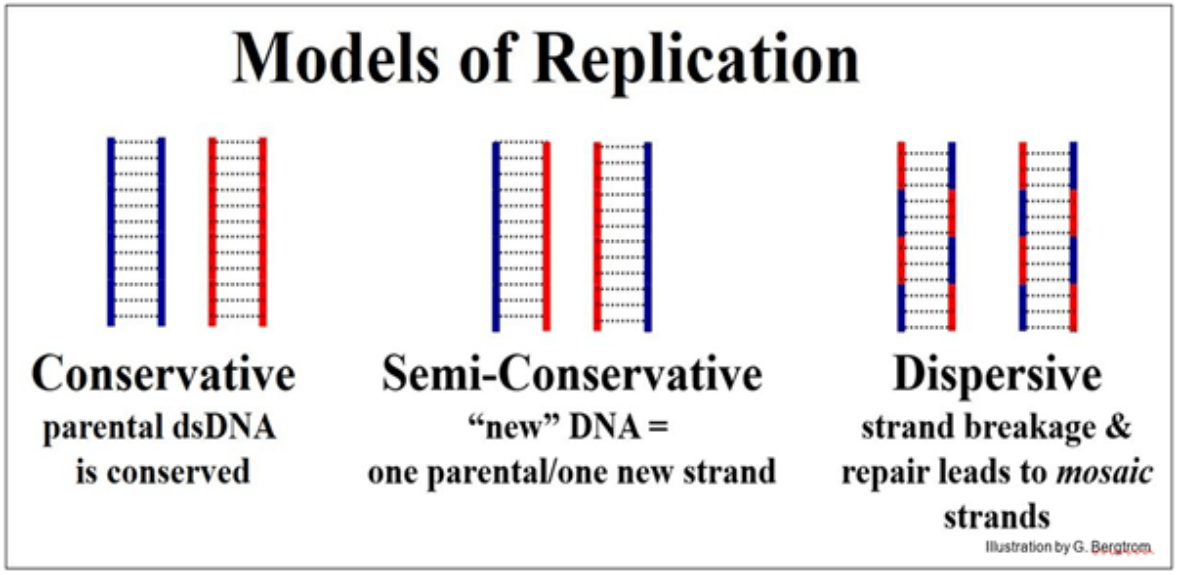
Chargaff’s data also showed a unique pattern of base ratios. Although base compositions could vary between species, the **A/T** and **G/C** *ratio* was always one, for every species. Likewise the ratio of **(A+C)/(G+T)** and **(A+G)/(C+T)**. From this information, Watson and Crick inferred that **A** (a purine) would H-bond with **T (a pyrimidine)**, and **G** (a purine) would H-bond with **C** (a pyrimidine) in the double helix. When building their model with this new information, they also found H-bonding between the complementary bases would be maximal only if the two DNA strands were **antiparallel**, leading to the most stable structure of the double helix.

Watson and Crick published their conclusions about the structure of DNA in 1953 (Click here to read their seminal article: Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Their article is also famous for predicting a semi-conservative mechanism of replication, something that had been predicted by Koltsov 26 years earlier, albeit based on intuition... and much less evidence!

Watson, Crick and Wilkins shared a Nobel Prize in 1962 for their work on DNA structure. Unfortunately, Franklin died in 1958 and Nobel prizes were not awarded posthumously. There is still controversy about why Franklin did not get appropriate credit for her role in the work. But she has been getting well-deserved, long-delayed recognition, including a university in Chicago named in her honor!

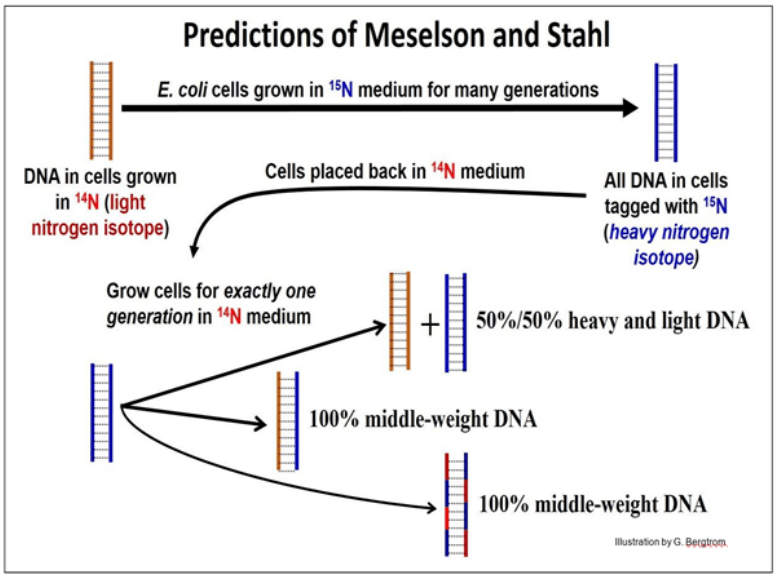
169 Unraveling the Structure of DNA

Confirmation of Watson & Crick’s suggestion of semiconservative replication came from Meselson and Stahl’s very elegant experiment, which tested the three possible models of replication shown below.



In their experiment, *E. coli* cells were grown for in medium containing 15N, a ‘heavy’ nitrogen isotope. After many generations, all of the DNA in the cells had become labeled with the heavy isotope. At that point, the 15N-tagged cells were placed back in medium containing the more common, ‘light’ 14N isotope and allowed to grow for exactly one generation.

Meselson and Stahl’s predictions and their experimental design are shown below.



Meselson and Stahl knew that 14N-labeled and 15N-labeled DNA would form separate bands after centrifugation on *CsCl chloride density gradients*. They tested their predictions by purifying and centrifuging the DNA from the 15N-labeled cells grown in 14N medium for one generation. They found that this DNA formed a single band with a density between that of 15N-labeled DNA and 14N-labeled DNA. This result *eliminated* a conservative model of DNA replication (as Watson and Crick also predicted. That left two possibilities: replication was either semiconservative or dispersive. The dispersive model was *eliminated* when DNA isolated from cells grown for a 2nd generation on 14N were shown to contain two bands of DNA on the CsCl density gradients.

17.0 Replication is Semiconservative

Chromosomes

We understood from the start of the 20th century that chromosomes contained genes. Therefore, it becomes necessary to understand the relationship between chromosomes, chromatin, DNA and genes. As noted earlier, chromosomes are a specialized, *condensed* version of chromatin, with key structural features shown below.

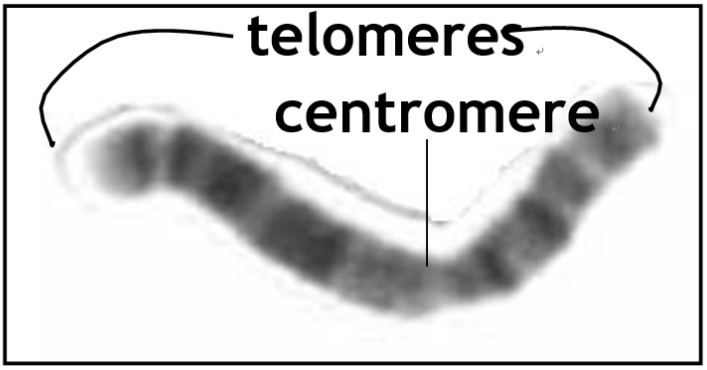
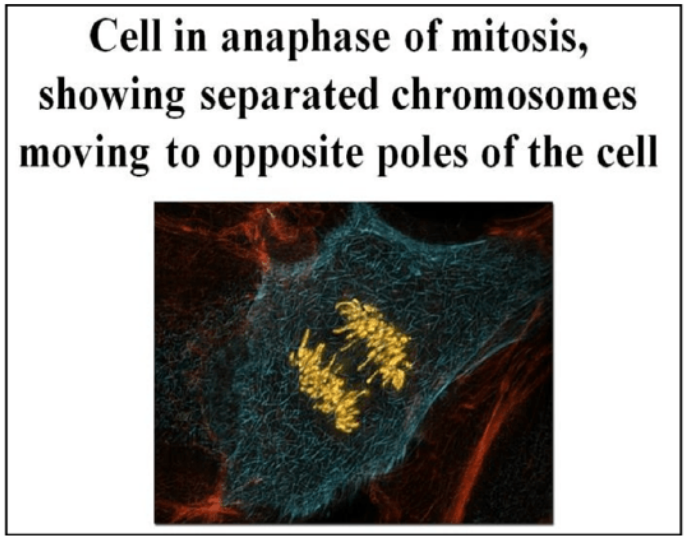


Illustration by G. Bergtrom

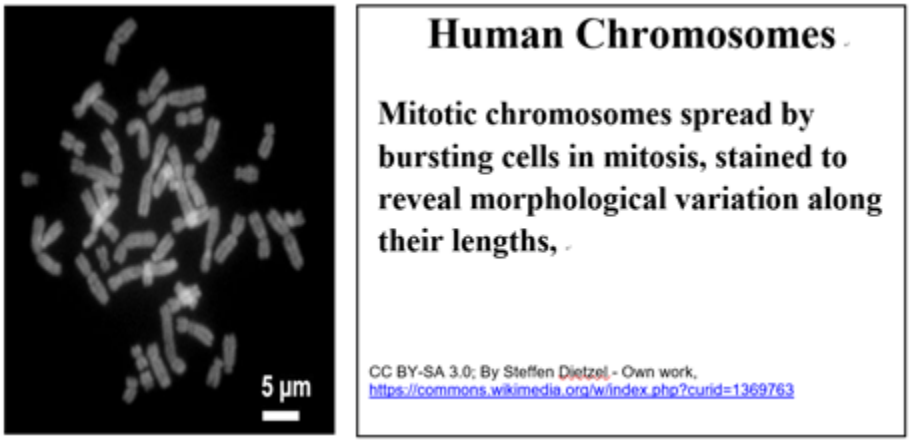
Public Domain; Adapted from National Human Genome Research Institute - Cropped from File:Human male karyotpe high resolution.jpg, https://commons.wikimedia.org/w/index.php?curid=40960106

We now know that the compact structure of a chromosome prevents damage to the DNA during cell division. This damage can occur when forces on centromeres generated by mitotic or meiotic spindle fibers pull chromatids apart. As the nucleus breaks down during mitosis or meiosis, late 19th century microscopists saw chromosomes condense from the dispersed cytoplasmic background. These chromosomes remained visible as they separated, moving to opposite poles of the cell during cell division. Such observations of chromosome behavior during cell division pointed to their role in heredity. A computer- colorized cell in mitosis is shown below.

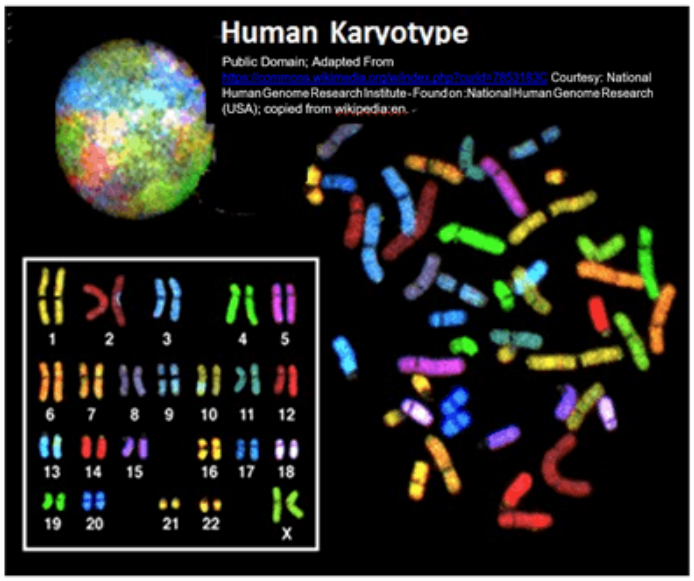


CC-BY-SA-NC; From: https://fascience.wikispaces.com/10+Cell+Growth+and+Division

It is possible to distinguish one chromosome from another by ***karyotyping***. When cells in metaphase of mitosis are placed under pressure, they burst and the chromosomes spread apart. Such a chromosome spread is shown below.



By the early 1900s, the number, sizes and shapes of chromosomes were shown to be species-specific. What's more, a close look at chromosome spreads revealed that chromosomes came in morphologically matched pairs. This was so reminiscent of Gregor Mendel's paired hereditary factors that chromosomes were then widely accepted as the structural seat of genetic inheritance. Cutting apart micrographs like the one above and pairing the chromosomes by their morphology generates a ***karyotype***. Paired human homologs are easily identified in the colorized micrograph below.



Captured in mitosis, all dividing human cells contain 23 pairs of homologous chromosomes. The ***karyotype*** is from a female; note the pair of homologous sex ("X") chromosomes (lower right of the inset). X and Y chromosomes in males are not truly homologous. Chromosomes in the original spread and in the aligned karyotype stained with fluorescent antibodies to chromosome-specific DNA sequences, 'light up' the different chromosomes.

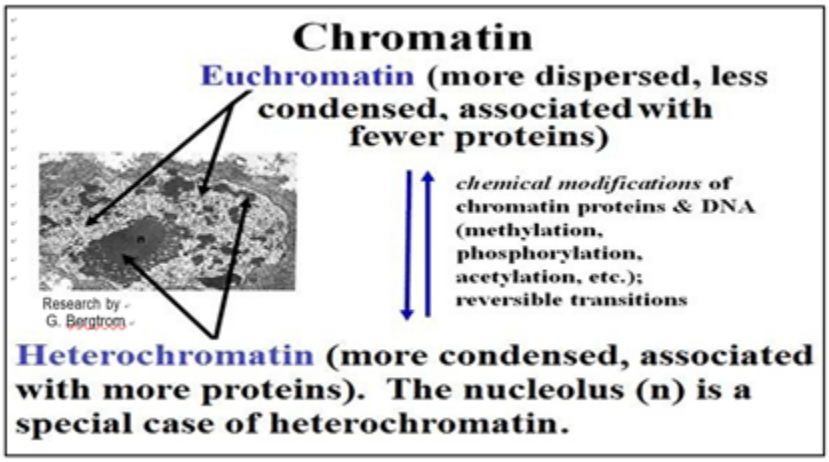
171 DNA, Chromosomes, Karyotypes & Gene Maps

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8.4: Genes and Chromatin in Eukaryotes

Chromosomes and chromatin are a uniquely eukaryotic association of DNA with more or less protein. Bacterial DNA (and prokaryotic DNA generally) is relatively 'naked' – not visibly associated with protein.

The electron micrograph of an *interphase* cell (below) reveals that the chromatin can itself exist in various states of condensation.



Chromatin is maximally condensed during mitosis, forming chromosomes. During interphase, chromatin exists in more or less condensed forms, called ***Heterochromatin*** and ***euchromatin*** respectively. Transition between these chromatin forms involve changes in the amounts and types of proteins bound to the chromatin, and can that can occur during gene regulation, i.e., when genes are turned on or off. Active genes tend to be in the more dispersed euchromatin so that enzymes of replication and transcription have easier access to the DNA. Genes that are inactive in transcription are heterochromatic, obscured by additional chromatin proteins present in heterochromatin. We'll be looking at some experiments that demonstrate this in a later chapter.

We can define three levels of chromatin organization in general terms:

1. DNA wrapped around histone proteins form *nucleosomes* in a "beads on a string" structure.

2. Multiple nucleosomes coil (condense), forming 30 nm fiber (solenoid) structures.

3. Higher-order packing of the 30 nm fiber leads to formation of metaphase chromosomes seen in mitosis & meiosis.

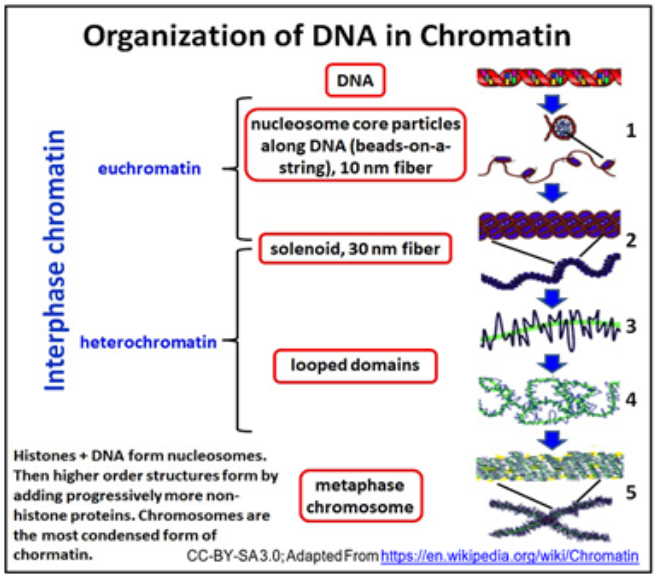
The levels of chromatin structure were determined in part by selective isolation and extraction of interphase cell chromatin, followed by selective chemical extraction of chromatin components. The steps are:

· Nuclei are first isolated from the cells.

· The nuclear envelope gently ruptured so as not to physically disrupt chromatin structure.

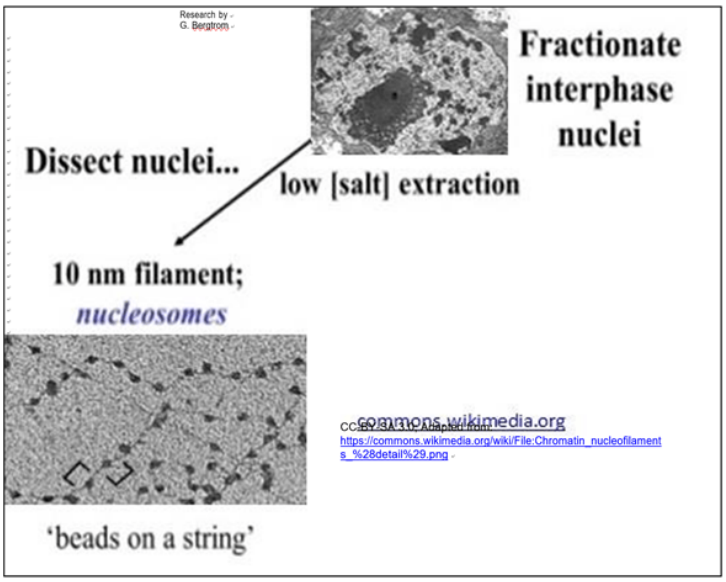
· the chromatin can be gently extracted by one of several different chemical treatments (high salt, low salt, acid...).

The levels of chromatin structure are illustrated below.

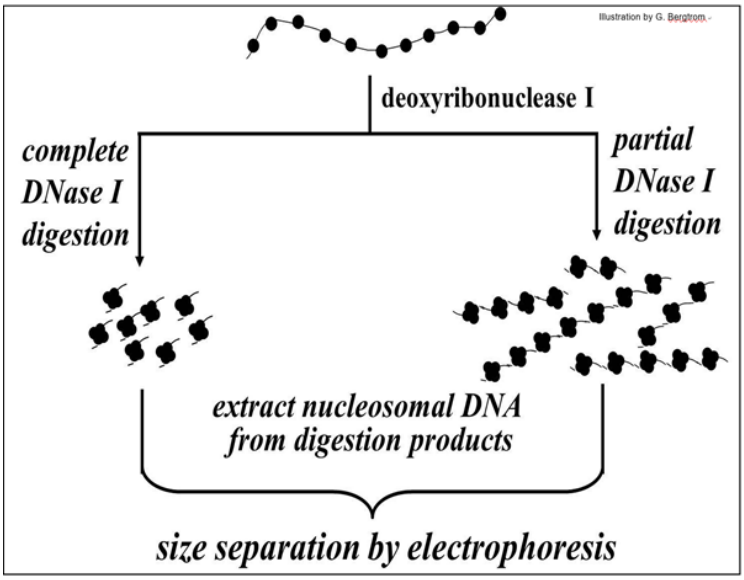


Salt extraction dissociates most of the proteins from the chromatin. When a low salt extract is centrifuged and the pellet resuspended, the remaining chromatin looks like *beads on a string*. DNA-wrapped *nucleosomes* are the beads, which are in turn linked by uniform lengths of metaphorical DNA "string' (**# 1** in the illustration above). A high salt chromatin extract appears as a coil of nucleosomes, or *30 nm solenoid fiber* **(# 2** above). Other extraction protocols revealed other aspects of chromatin structure shown in #s **3** and **4** above. Chromosomes seen in metaphase of mitosis are the 'highest order', most condensed form of chromatin.

The 10 nm filament of nucleosome 'beads-on-a-string' remaining after a low salt extraction can be seen in an electron microscope as shown below.



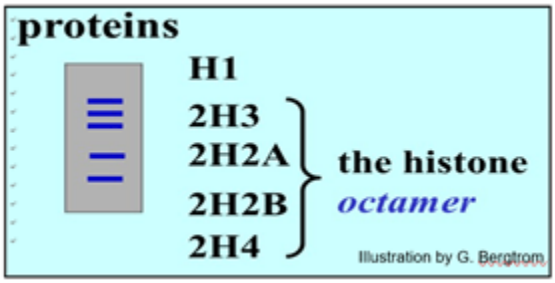
When these nucleosome necklaces were digested with the enzyme *deoxyribonuclease* (**DNAse**), the DNA between the 'beads' was degraded, leaving behind shortened 10nm filaments after a short digest period, or just single beads the beads after a longer digestion (below).



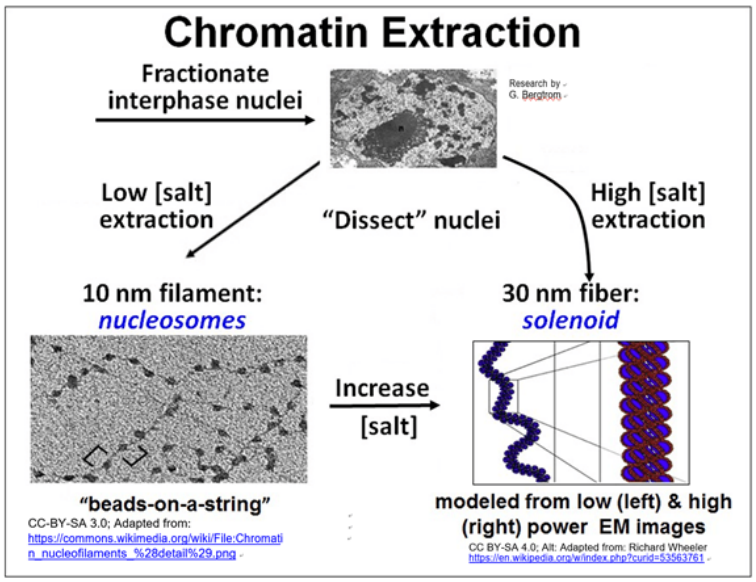
Roger Kornberg (son of Nobel Laureate Arthur Kornberg who discovered the first DNA polymerase enzyme of replication) participated in the discovery and characterization of nucleosomes while he was still a post-doc! Electrophoresis of DNA extracted from these digests revealed *nucleosomes* separated by a "linker" DNA stretch of about 80 base pairs. DNA extracted from the nucleosomes was about 147 base pairs long. This is the DNA that had been wrapped around the proteins of the nucleosome.

172 Nucleosomes-DNA & Protein

After separating all of the proteins from nucleosomal DNA, five proteins were identified (illustrated below).



Histones are basic proteins containing many *lysine* and *arginine* amino acids. Their positively charged side chains enable these amino acids bind the acidic, negatively charged *phosphodiester backbone* of double helical DNA. The DNA wraps around an octamer of histones (2 each of 4 of the histone proteins) to form the *nucleosome*. About a gram of histones is associated with each gram of DNA. After a high salt chromatin extraction, the structure visible in the electron microscope is the 30nm solenoid, the coil of nucleosomes modeled in the figure below.

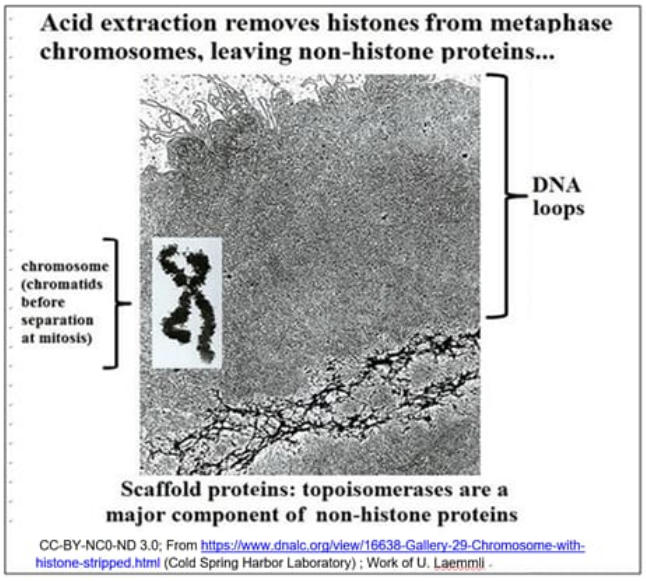


As shown above, simply increasing the salt concentration of an already extracted nucleosome preparation will cause the 'necklace' to fold into the 30nm solenoid structure.

173 Chromatin Structure: Dissecting Chromatin

As you might guess, an acidic extraction of chromatin should selectively remove the basic histone proteins, leaving behind an association of DNA with non-histone proteins. This proved to be the case. An electron micrograph of the chromatin remnant after an acid extraction of metaphase chromosomes is shown on the next page.

DNA freed of the regularly spaced histone-based nucleosomes, loops out, away from the long axis of the chromatin. Dark material along this axis is a protein scaffolding that makes up what's left after histone extraction. Much of this protein is *topoisomerase*, an enzyme that prevents DNA from breaking apart under the strain of replication.



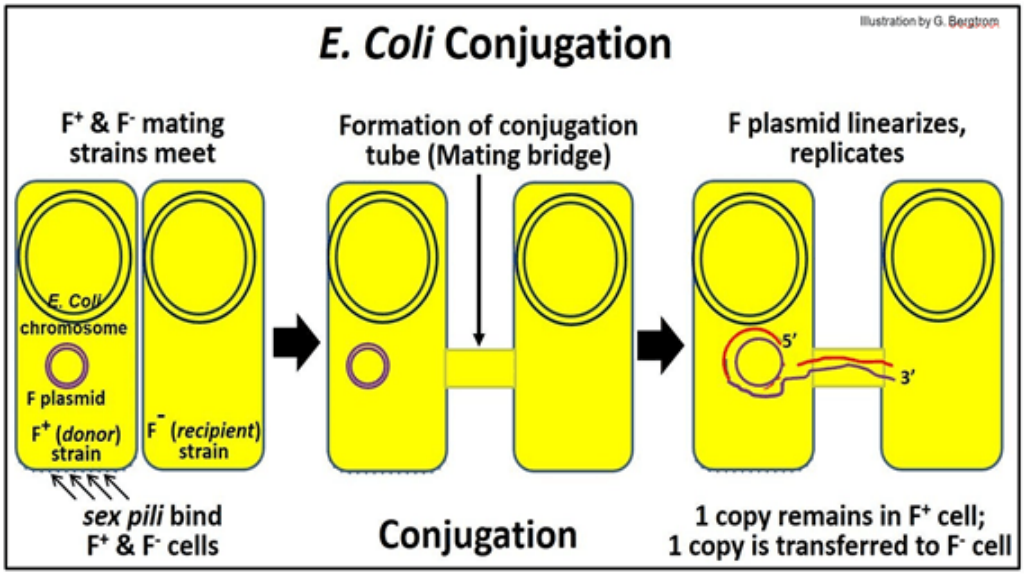
174 Histones and Non-Histone Proteins

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8.5: Structure and Organization of DNA in Bacteria

Sexual reproduction allows compatible genders (think *male* and *female*) to share genes, a strategy that increases species diversity. It turns out that bacteria and other single celled organisms can also share genes... and spread diversity. We will close this chapter with a look at sex (*E. coli* style!), and gene-mapping experiments showing linearly arranged genes on a circular bacterial DNA molecule (the bacterial 'chromosome').

E. coli sex begins when F+ and F- cells meet. These cells are "opposite" mating types that can share DNA during ***conjugation***. F+ cells contain the ***F plasmid***, a small circular DNA molecule that is separate from the *E. coli* chromosome. The F (fertility) plasmid has genes that encode ***sex pili*** on F+, as well as factors needed to form a mating bridge, or conjugation tube. The behavior of the F plasmid during conjugation is shown below.



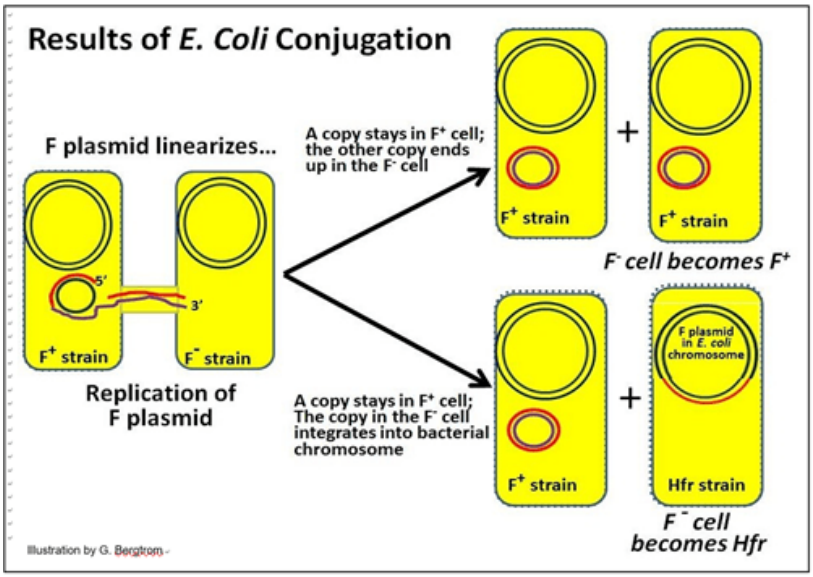
When an F+ (*donor*) cell encounters an F- (*recipient*) cell, sex pili on the donor cell initiate recognition. Next, a conjugation tube forms, linking the cytoplasms of the two cells. After nicking one strand of the F plasmid DNA, the nicked begins to roll into the conjugation tube and into the recipient (F-) cell. The DNA strand entering the recipient cell replicates, as does the intact circle remaining in the donor cell (replicating DNA is shown in red in the illustration).

E. coli conjugation can have different outcomes:

1. One outcome is that one of two semi-conservatively replicated F plasmids remains in the donor cell and another is now in the recipient cell. In this case, the recipient cell becomes a new F+ *donor* cell!

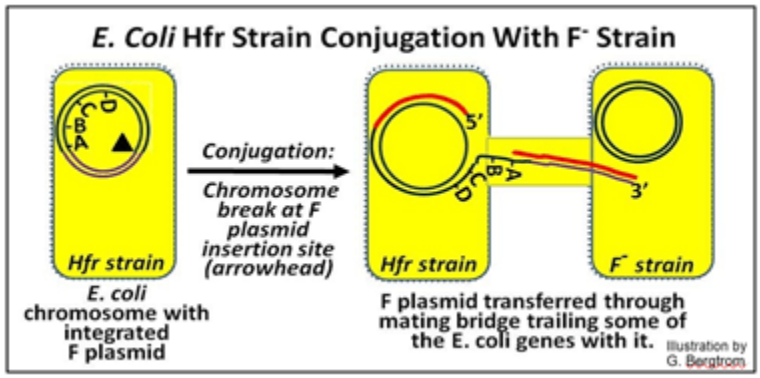
2. The other outcome is integration of the F plasmid into recipient cell chromosomal DNA. Insertion is typically at specific sites in the DNA where there is sufficient sequence similarity between the plasmid and chromosomal DNA to allow insertion by *recombination*. The result is that the recipient cell becomes an ***Hfr*** (*High-frequency recombination*) cell. This cell will produce ***Hfr strain*** progeny cells.

These two possible results of conjugation in *E. coli* are illustrated below.



Hfr cells readily express their integrated F plasmid genes, and like F+ cells, develop sex pili and form a conjugation tube with an F- cell. One strand of the bacterial chromosomal DNA will be nicked at the original insertion site of the F plasmid.

The next events parallel the replicative transfer of an F plasmid during F+/F- conjugation, except that only part of the Hfr donor chromosomal DNA is transferred, as shown below.



In this illustration, the F plasmid has inserted '*in front of*' an A gene so that when it enters the conjugation tube, it brings along several *E. coli* chromosomal genes Because of the size of the bacterial chromosome, only a few bacterial genes enter the recipient gene before transfer is aborted. But in the brief time of DNA transfer, at least some genes did get in to the recipient F- strain where they can be expressed. Here is an outline of an experiment that allowed mapping bacterial genes on a circular DNA chromosome:

1. Hfr cells containing functional **A, B, C, D** genes were mated with recipient cells containing mutants of either the A, the B, the C or the D gene.

2. Conjugation was mechanically disrupted at different times after the formation of a conjugation tube.

3. Recipient cells from each of the disrupted conjugations were then grown in culture and analyzed for specific gene function.

In this hypothetical example, the results were that a recipient cell with a *mutant A* gene acquired a wild type A gene (and therefore A-gene function) after a short time before conjugation disruption. Progressively longer times of conjugation (measured in separate experiments) were required to transfer gens B, C and D (respectively) to the recipient cell. Thus the order of these genes on the bacterial chromosome was

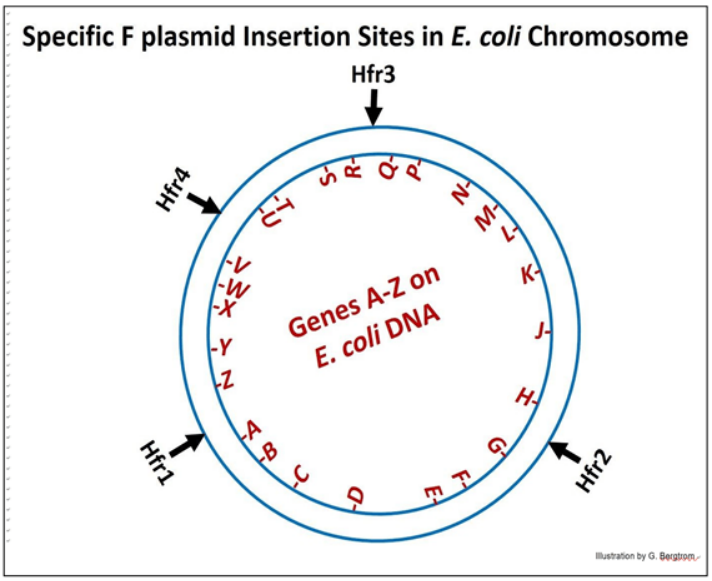
-A-B-C-D-

The timing of conjugation that led to F- mutants acquiring a functional gene from the Hfr strain was so refined that not only could the gene locus be determined, but even the size) length) of the genes! Thus, the time to transfer a complete gene to an F- cell reflects the size (length) of the gene. The other important conclusion is that genes are arranged linearly on bacterial DNA.

Recall that genes already mapped along the length of eukaryotic chromosomes implied a linear order of the genes. However, little was known about eukaryotic chromosome structure at the time, and the role of DNA as the 'stuff of genes' was not appreciated. These bacterial mating experiments demonstrated for the first time that genes are linearly arranged not just along a chromosome, but also along the DNA molecule.

Over time, many bacterial genes were mapped all along the *E. coli* chromosome by isolating many different Hfr strains in which an F plasmid had inserted into different sites around the DNA circle. These Hfr strains were mated to F- bacteria, each with mutations in one or another known bacterial gene. As in the original 'ABCD' experiment, the order of many genes was determined, and even shown to be linked at a greater or lesser distance to those ABCD genes and each other.

The map that results from such a study is diagrammed below.



Using the different Hfr strains (numbered in the diagram) in conjugation experiments, it was shown that in fact, the different Hfr cells transferred different genes into the recipient cells in the order implied by the diagram. What's more, when the experiment was done with *Hfr4* (in this generic diagram); the order of genes transferred after longer times of conjugation was found to be:

V-W-X-Y-Z-A-B....

The obvious conclusion from experiments like these was that the *E. coli* DNA molecule (its 'chromosome') was a closed circle! We will see visual evidence of circular *E. coli* chromosomes in the next chapter, with some discussion of how this evidence informed our understanding of DNA replication.

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8.6: Key Words and Terms

10 nm fiber	double helix	mutations
30 nm fiber	euchromatin	non-histone proteins
5' -to-3' replication	F and Hfr plasmids	nuclear proteins
antiparallel DNA strands	F- strain	nucleosomes
bacterial conjugation	F+ strain	recipient cell
base ratios	fertility plasmid	replication
beads-on-a-string	heterochromatin	S. *pneumonia* type III-S
chromatin	Hfr strain	S. *pneumonia* type II-R
chromosomes	histone octamer	semi-conservative replication
conjugation tube	histone proteins	sex pili
conservative replication	influenza	solenoid fiber
deoxyribonuclease	karyotype	spindle fibers
discontinuous replication	levels of chromatin packing	transforming principle
dispersive replication	mating bridge	X & Y chromosomes
DNA	metaphase chromatin	X-ray crystallography
donor cell	mitosis & meiosis	X-ray diffraction

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9: DETAILS OF DNA REPLICATION AND REPAIR

9.1: Introduction
9.2: DNA Replication
9.3: DNA Repair
9.4: Key Words and Terms

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9.1: Introduction

Replication begins at one or more **origins of replication** along DNA, where **helicase enzymes** catalyze unwinding of the double helix. DNA unwinding creates replicating bubbles, or **replicons**, with **replication forks** at either end. Making a new DNA strand starts with making an RNA **primer** with RNA nucleotides and **primase** enzymes. DNA nucleotides are then added to the 3'-ends of primers by one a **DNA polymerase**. Later, other DNA polymerases catalyze removal of the RNA primers and replacement of the hydrolyzed ribonucleotides with new deoxyribonucleotides. Finally, **DNA ligases** stitch together the fragments of new DNA synthesized at the replication forks. This complex mechanism is common to the replication of 'naked' prokaryotic DNA and of chromatinencased eukaryotic DNA, and must therefore have arisen early in the evolution of replication biochemistry. In this chapter, we look at the details of replication and the differences in detail between prokaryotic and eukaryotic replication that arise because of differences in DNA packing. As with any complex process with many moving parts, replication is error-prone. Therefore, we will also look at how the overall **fidelity** of replication relies mechanisms of **DNA repair** that target specific kinds of replication mistakes, or **mutations**. At the same time, lest we think that uncorrected errors in replication are always a bad thing, they usually *do not* have bad outcomes. Instead, they leave behind the very mutations that allow *natural selection* and the *evolution of diversity*.

Learning Objectives

1. Explain how Cairns interpreted his theta (Θ) images.
2. Compare and contrast the activities of enzymes required for replication.
3. Describe the order of events at an origin of replication and at each *replication fork*.
4. Compare *unidirectional* and *bidirectional* DNA synthesis from an origin of replication.
5. Outline the basic synthesis and proofreading functions of *DNA polymerase*.
6. Identify the major players and their roles in the initiation of replication.
7. Explain how Okazaki's experimental results were not entirely consistent with how both strands of DNA replicate
8. List the major molecular players (enzymes, etc.) that elongate a growing DNA strand.
9. List the non-enzymatic players in replication and describe their functions.
10. Describe how the structure of *telomerase* enables proper replication.
11. Compare the activities of topoisomerases 1 and 2.
12. Explain the reasoning behind the hypothesis of *processive replication*.
13. Compare and contrast the impacts of germline and somatic mutations.
14. Describe common forms of DNA damage.
15. List enzymes of replication that were adapted to tasks of DNA repair.
16. Explain why a DNA glycosylase is useful in DNA repair.
17. Explain why the connection between 'breast cancer genes' and DNA repair.

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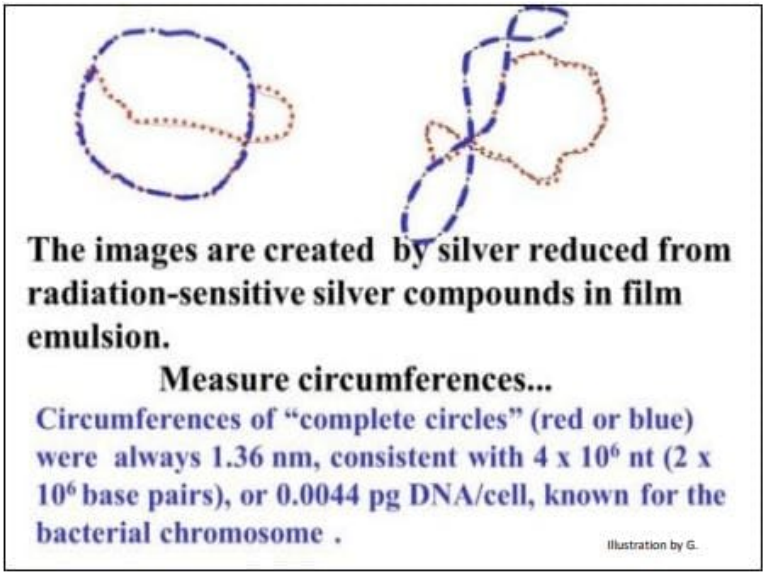
9.2: DNA Replication

As we've seen, DNA strands have directionality, with a 5' nucleotide-phosphate and a 3' deoxyribose hydroxyl end. This is even true for circular bacterial chromosomes..., if the circle is broken! Because the strands of the double helix are *antiparallel*, the 5' end of one strand aligns with the 3'end of the other at both ends of the double helix. The complementary pairing of bases in DNA means that the base sequence of one strand can be used as a template to make a new complementary strand. As we'll see, this structure of DNA created some interesting dilemmas for understanding the biochemistry of replication. The puzzlement surrounding how replication proceeds begins with experiments that visualize replicating DNA.

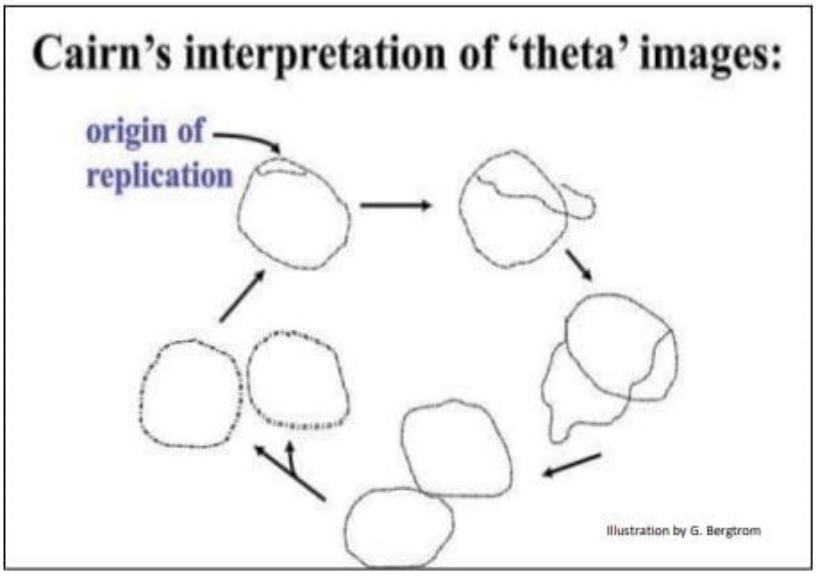
A. Visualizing Replication and Replication Forks

Recall the phenomenon of bacterial conjugation allowed a demonstration bacterial chromosomes were circular. In 1963, John Cairns confirmed this fact by direct visualization of bacterial DNA. He cultured *E. coli* cells for long periods on 3Hthymidine (3H-T) to make all of their cellular DNA radioactive. He then disrupted the cells gently to minimize damage to the DNA. The DNA released was allowed to settle and adhere to membranes. A sensitive film was placed over the membrane and time was allowed for the radiation to expose the film. After Cairns developed the autoradiographs, he examined the results in the electron microscope. He saw tracks of silver grains in the autoradiographs (the same kind of silver grains that create an image on film in old-fashioned photography). Look at the two drawings of his **autoradiographs** on the next page.

Cairns measured the length of the "silver" tracks, which usually consisted of three possible closed loops, or circles. The circumferences of two of these circles were always equal, their length closely predicted by the DNA content of a single, nondividing cell. Cairns therefore interpreted these images to be bacterial DNA in the process of replication. Cairns' autoradiographs and the measurements that led him to conclude that he was looking at images of bacterial circular chromosomes are illustrated below.



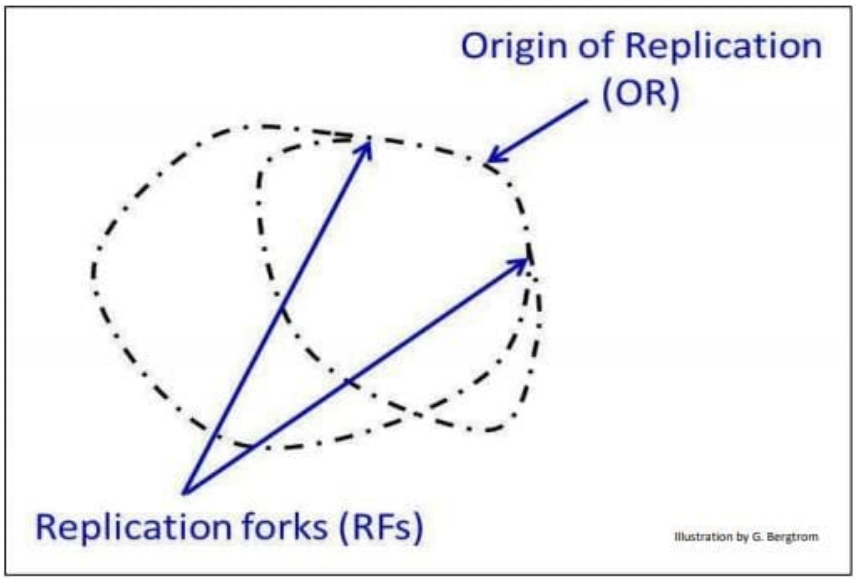
He arranged his autoradiograph images in a sequence (below) to make his point.



Because the replicating chromosomes looked (vaguely!) like the Greek letter **\(\theta \)**, Cairns called them **theta images**. He inferred that replication starts at a single **origin of replication** on the bacterial chromosome, proceeding around the circle to completion.

175 Seeing *E.Coli* Chromosomes

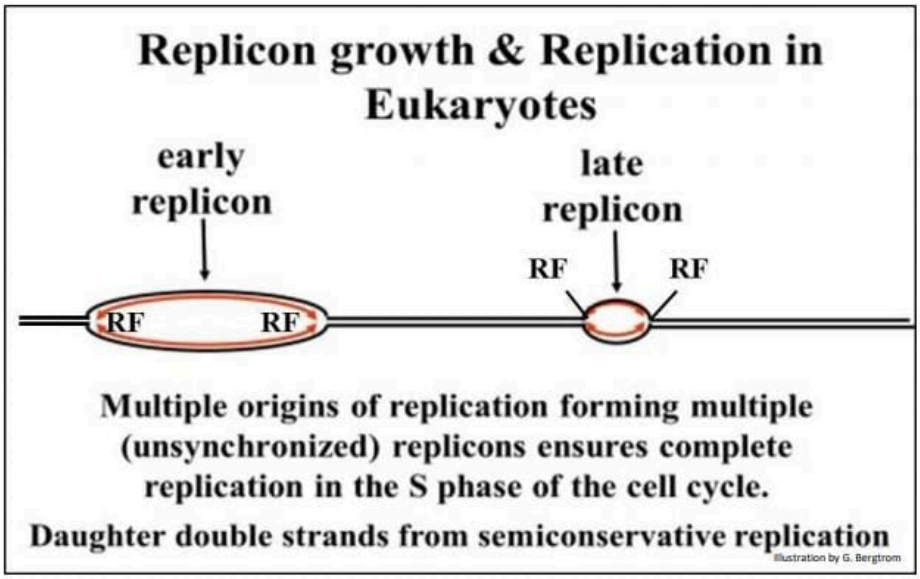
Subsequent experiments by David Prescott demonstrated **bidirectional replication**..., that replication did indeed begin at an origin of replication, after which the double helix was unwound and replicated in both directions, away from the origins, forming **two replication forks** (illustrated below).



176 Semiconservative Bidirectional Replication From Two RFs

Bacterial cells can divide every hour (or even less); the rate of bacterial DNA synthesis is about 2 X 10⁶ base pairs per hour. A typical eukaryotic cell nucleus contains thousands of times as much DNA as a bacterium, and typical eukaryotic cells double every 15-20 hours. Even a small chromosome can contain hundreds or thousands of times as much DNA as a bacterium. It appeared that eukaryotic cells could not afford to double their DNA at a bacterial rate of replication! Eukaryotes solved this problem not by evolving a faster biochemistry of replication, but by using multiple origins of replication from which DNA synthesis proceeds in both directions. This results in the creation of multiple **replicons**.

Each replicon enlarges, eventually meeting other growing replicons on either side to replicate most of each linear chromosome, suggested in the illustration below.

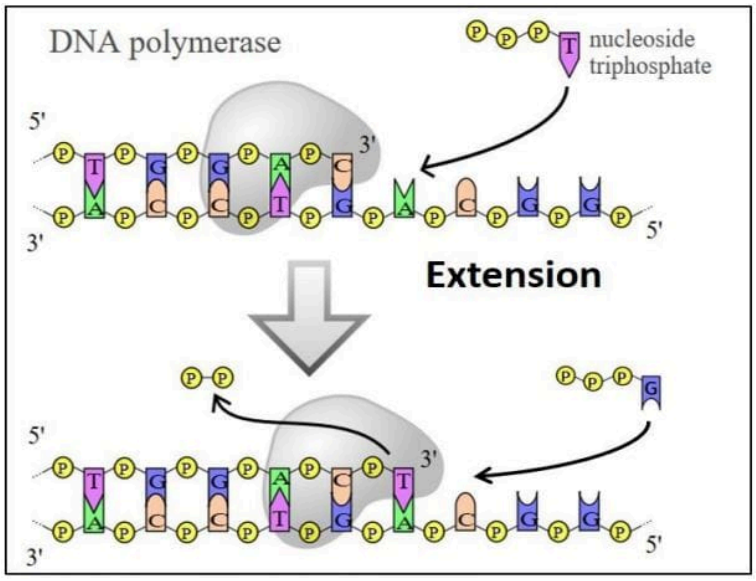


Before we consider the biochemical events at replication forks in detail, let's look at the role of DNA polymerase enzymes in the process.

177 Multiple Replicons in Eukaryotes

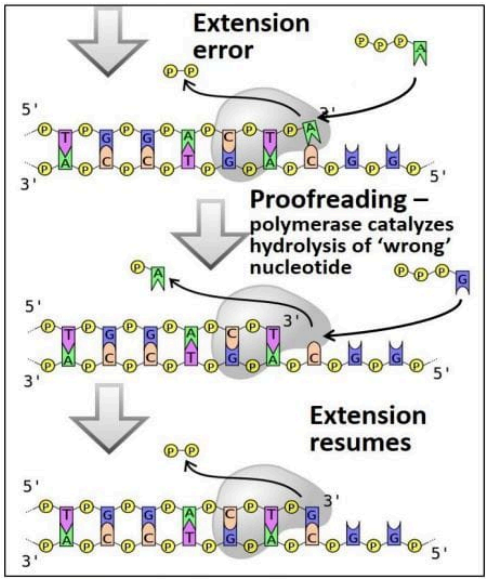
B. DNA Polymerases Catalyze Replication

The first of these enzymes was discovered in *E. coli* by Arthur Kornberg, for which he received the 1959 Nobel Prize in Chemistry. Thomas Kornberg, one of Arthur's sons later found two more of DNA polymerases! All DNA polymerases require a template strand against which to synthesize a new complementary strand. They all grow new DNA by adding to the 3' end of the growing DNA chain in successive condensation reactions. And finally, all DNA polymerases also have the odd property that they can only add to a pre-existing strand of nucleic acid, raising the question of where the 'preexisting' strand comes from! DNA polymerases catalyze the formation of a phosphodiester linkage between the end of a growing strand and the incoming nucleotide complementary to the template strand. The energy for the formation of the phosphodiester linkage comes in part from the hydrolysis of two phosphates (pyrophosphate) from the incoming nucleotide during the reaction. While replication requires the participation of many nuclear proteins in both prokaryotes and eukaryotes, DNA polymerases perform the basic steps of replication, as shown in the illustration below.



178 DNA Polymerases & Their Activities

Although DNA polymerases replicate DNA with high fidelity with as few as one error per 10⁷ nucleotides, mistakes do occur. The proofreading ability of some DNA polymerases corrects many of these mistakes. The polymerase can sense a mismatched base pair, slow down and then catalyze repeated hydrolyses of nucleotides until it reaches the mismatched base pair. This basic proofreading by DNA polymerase is shown below.



After mismatch repair, DNA polymerase resumes forward movement. Of course, not all mistakes are caught by this or other repair mechanisms (see *DNA Repair*, below). Mutations in the eukaryotic germ line cells that elude correction can cause genetic diseases. However, most are the mutations that fuel evolution. Without mutations in germ line cells (egg and sperm), there would be no mutations and no evolution, and without evolution, life itself would have reached a quick dead end! Other replication mistakes can generate mutations somatic cells. If these somatic mutations escape correction, they can have serious consequences, including the generation of tumors and cancers.

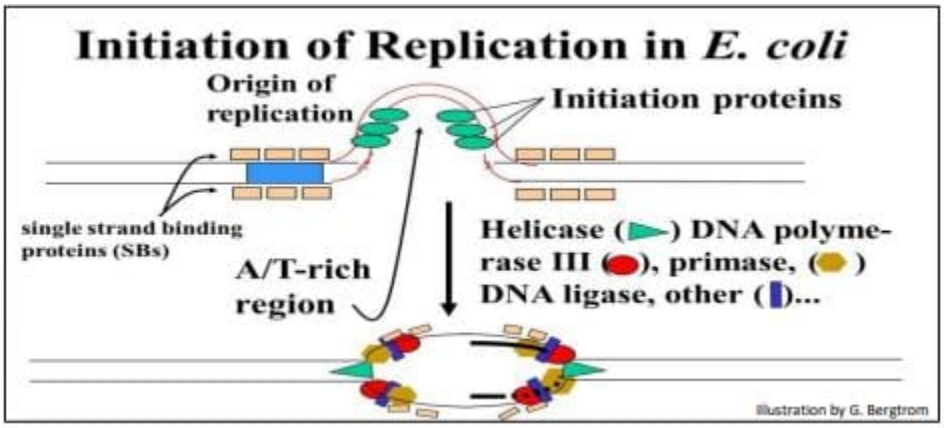
C. The Process of Replication

DNA replication is a sequence of repeated condensation (dehydration synthesis) reactions linking nucleotide monomers into a DNA polymer. Like all biological polymerizations, replication proceeds in three enzymatically catalyzed and coordinated steps:

initiation, **elongation** and **termination**.

1. Initiation

As we have seen, DNA synthesis starts at one or more origins or replication. These are DNA sequences targeted by *initiator proteins* in *E. coli* (below).



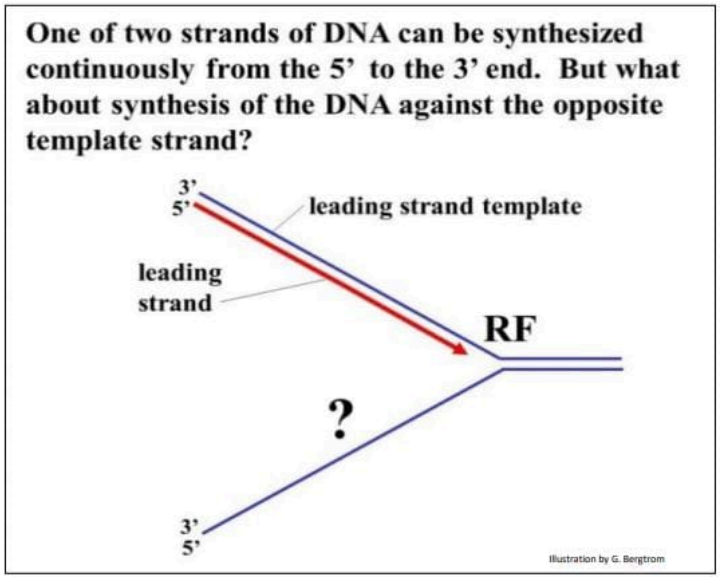
After breaking hydrogen bonds at the origin of replication, the DNA double helix is progressively unzipped in both directions (i.e., by *bidirectional replication*). The separated DNA strands serve as templates for new DNA synthesis. Sequences at replication origins that bind to initiation proteins tend to be rich in adenine and thymine bases. This is because A-T base pairs have two hydrogen (H-) bonds that require less energy to break than the three H-bonds holding G-C pairs together. Once initiation proteins loosen H-bonds at a replication origin, **DNA helicase** uses the energy of ATP hydrolysis to unwind the double helix. DNA polymerase III is the main enzyme that then elongates new DNA. Once initiated, a replication bubble (replicon) forms as repeated cycles of elongation proceed at opposite replication forks.

179 Replication Initiation in *E. coli*

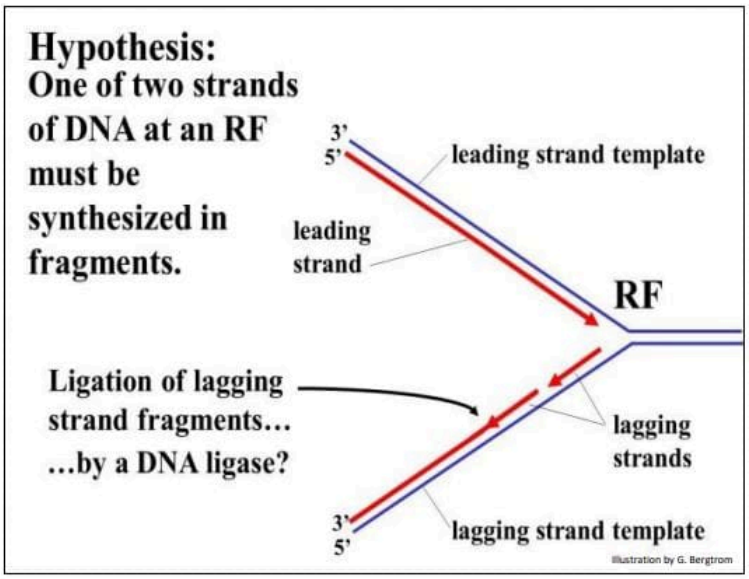
Recalling that new nucleotides can only be added to the free 3' hydroxyl group of a pre-existing nucleic acid strand. Since no known DNA polymerase can start synthesizing new DNA strands from scratch, this is a problem! The action of DNA polymerases therefore requires a **primer**, a nucleic acid strand to which to add nucleotides. The questions were..., what is the primer and where does it come from? Since *RNA polymerases* (enzymes that catalyze RNA synthesis) are the only nucleotide polymerase that can grow a new nucleic acid strand against a DNA template from scratch (i.e., from the first base), it was suggested that RNA might be the primer, After synthesis of a short RNA primer, new *deoxynucleotides* would be added to its 3' end by DNA polymerase. The discovery of short stretches of RNA nucleotides at the 5' end of Okazaki fragments confirmed the notion of RNA primers. We now know that cells use **primase**, a special RNA polymerase active during replication, to make those RNA primers against DNA templates before a DNA polymerase can grow the DNA strands at replication forks. As we will see now, the requirement for RNA primers is nowhere more in evidence in events at a replication fork.

2. Elongation

Looking at elongation at one replication fork (below), we see another problem:

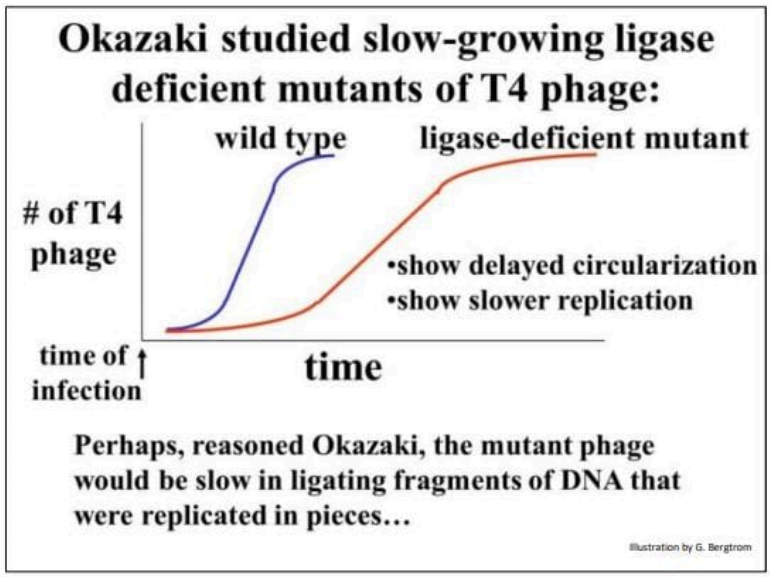


One of the two new DNA strands can grow continuously towards the replication fork as the double helix unwinds. But what about the other strand? Either this other strand must grow in pieces in the opposite direction, or it must wait to begin synthesis until the double helix is fully unwound. If one strand of DNA must be replicated in fragments, then those fragments would have to be stitched (i.e., ligated) together. The problem is illustrated below.



According to this hypothesis, a new **leading strand** of DNA is lengthened continuously by sequential addition of nucleotides to its 3' end against its **leading strand template**. The other strand however, would be made in pieces that would be joined in phosphodiester linkages in a subsequent reaction. Because of the extra step and presumably extra time it takes to make and join these new DNA fragments, this new DNA is called the **lagging strand**, making its template the **lagging strand template**.

Reiji Okazaki and his colleagues were studying mutants of T4 phage that grew slowly in their *E. coli* host cells. They graphed the growth rates of wild-type and mutant T4 phage and demonstrated that slow growth was due to a deficient **DNA ligase** enzyme, already known to catalyze the circularization of linear phage DNA molecules being replicated in infected host cells. The graph below summarizes their results.



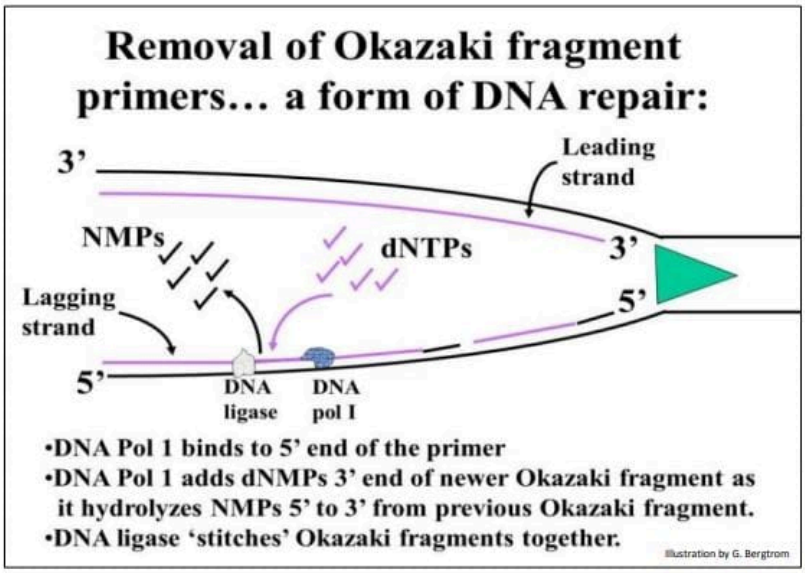
Okazaki's hypothesis was that the deficient DNA ligase in the mutant phage not only slowed down circularization of replicating T4 phage DNA, but would also be slow at joining phage DNA fragments replicated against at least one of the two template DNA strands. When the hypothesis was tested, the Okazakis found that short DNA fragments did indeed accumulate in *E. coli* cells infected with ligasedeficient mutants, but not in cells infected with wild type phage. The lagging strand fragments are now called **Okazaki fragments**.

180 Okazaki Experiments & Fragments - Solving a Problem at an RF

181 Okazaki Fragments are Made Beginning with RNA Primers

You can check out Okazaki's original research at this link.

Each Okazaki fragment would have to begin with a 5' RNA primer, creating yet another dilemma! The RNA primer must be replaced with deoxynucleotides before stitching the fragments together. This in fact happens, and the process illustrated below

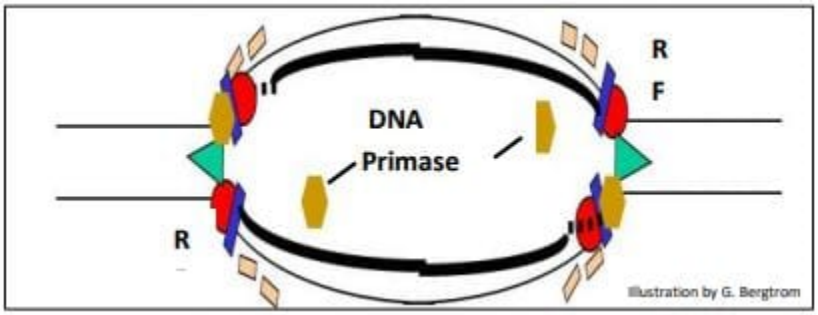


Removal of RNA primer nucleotides from Okazali fragments requires the action of **DNA polymerase I,** an enzyme that can also catalyze hydrolysis of the phosphodiester bonds between the RNA (or DNA) nucleotides from the 5'-end of a nucleic acid strand. *Flap Endonuclease 1* (**FEN 1**) also plays a role in removing 'flaps' of nucleic acid from the 5' ends of the fragments often displaced by polymerase as it replaces the replication primer. At the same time as the RNA nucleotides are removed, DNA polymerase

I catalyzes their replacement by the appropriate deoxynucleotides. Finally, when a fragment is entirely DNA, **DNA ligase** links it to the rest of the already assembled lagging strand DNA. Because of its 5' exonuclease activity (not found in other DNA polymerases), DNA polymerase 1 also plays unique roles in DNA repair (discussed further below). As Cairn's suggested and others demonstrated, replication proceeds in two directions from the origin to form a replicon with its two replication forks (RFs). Each RF has a primase associated with replication of Okazaki fragments along lagging strand templates.

As Cairn's suggested and others demonstrated, replication proceeds in two directions from the origin to form a replicon with its two replication forks (RFs). Each RF has a primase associated with replication of Okazaki fragments along lagging strand templates.

The requirement for primases at replication forks is shown below.

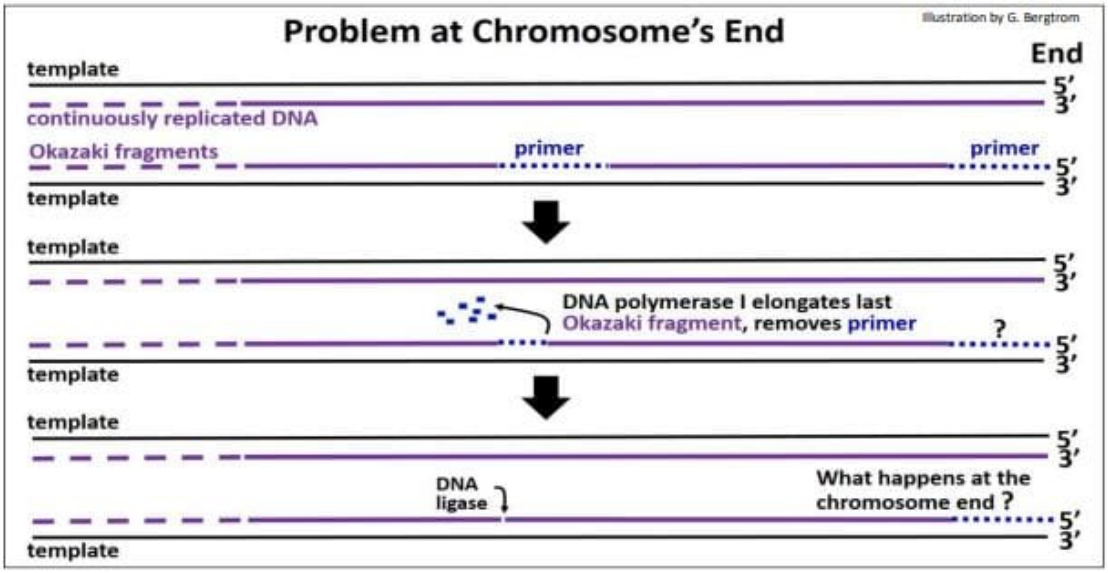


Now we can ask what happens when replicons reach the ends of linear chromosomes in eukaryotes.

182 Replication Elongation in *E.coli*

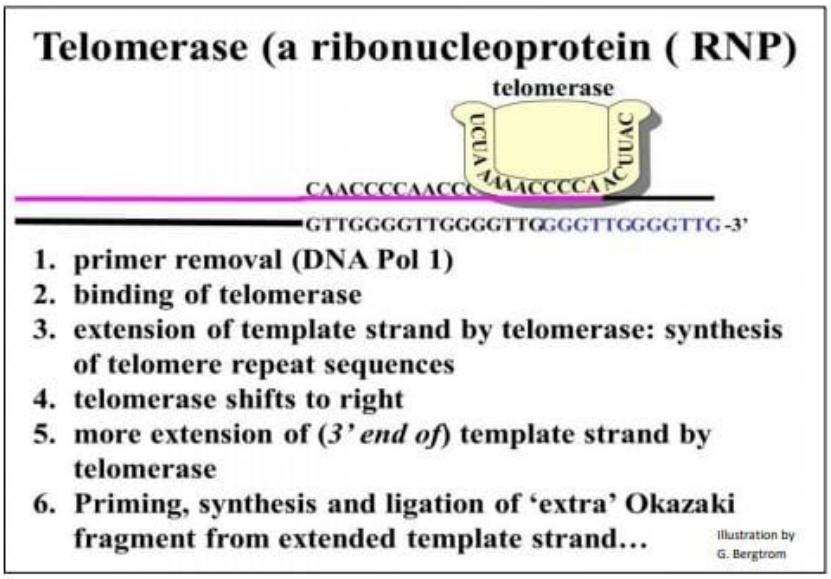
3. Termination

In prokaryotes, replication is complete when two replication forks meet after replicating their portion of the circular DNA molecule. In eukaryotes, many replicons fuse to become larger replicons, eventually reaching the ends of the chromosomes..., where there is yet another problem (below)!



When a replicon nears the end of a chromosome (i.e. a double-stranded DNA molecule), the strand synthesized continuously stops when it reaches the 5' end of its template DNA. In theory, synthesis of a last Okazaki fragment can be primed from the 3' end of the lagging template strand. The illustration above implies removal of a primer from the penultimate Okazaki fragment and DNA polymerase catalyzed replacement with DNA nucleotides. But what about the last Okazaki fragment? Would its primer be hydrolyzed? Moreover, without a free 3' end to add to, how are those RNA nucleotides replaced with DNA nucleotides? The

problem here is that every time a cell replicates, one strand of new DNA (likely both) would get shorter and shorter. Of course, this would not do..., and does not happen! Eukaryotic replication undergoes a *termination* process involving extending the length of one of the two strands by the enzyme **telomerase**. The action of telomerase is summarized in the illustration below



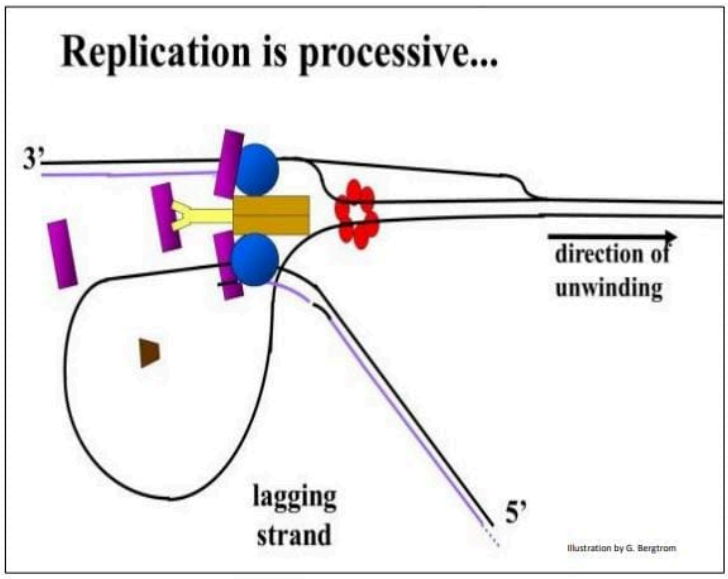
Telomerase consists of several proteins and an RNA. From the drawing,, the RNA component serves as a template for 5'-> 3' extension of the problematic DNA strand. The protein with the requisite reverse transcriptase activity is called ***Te****lomerase* ***R****everse* ***T****ranscriptase*, or **TERT**. The ***Te****lomerase* ***R****NA* ***C****omponent* is called **TERC**. Carol Greider, Jack Szostak and Elizabeth Blackburn shared the 2009 Nobel Prize in Physiology or Medicine for discovering telomerase.

183 Telomerase Replication Prevents Chromosome Shortening https://youtu.be/M4dmfrxGKKU

One of the more interesting recent observations was that differentiated, nondividing cells no longer produce the telomerase enzyme. On the other hand, the telomerase genes are active in normal dividing cells (e.g., stem cells) and cancer cells, which contain abundant telomerase.

4. Is Replication Processive?

Drawings of replicons and replication forks suggest separate events on each DNA strand. Yet events at replication forks seem to be coordinated. Replication may be **processive**, meaning both new DNA strands are replicated in the same direction at the same time, smoothing out the process. How might this be possible? The drawing below shows lagging strand template DNA bending, so that it faces in the same direction as the leading strand at the replication fork.



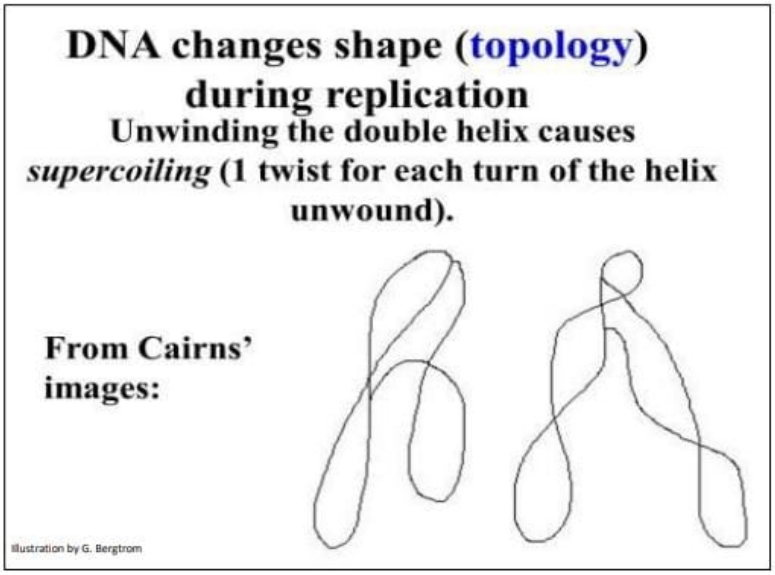
184 Processive Replication

The **replisome** structure cartooned at the replication fork consists of *clamp proteins*, primase, helicase, DNA polymerase and single-stranded binding proteins among others.

Newer techniques of visualizing replication by real-time fluorescence videography call the processive model into question, suggesting that the replication process is anything but smooth! Are lagging and leading strand replication not in fact coordinated? Alternatively, is the jerky movement of DNA elongation in the video an artifact, so that the model of smooth, coordinated replication integrated at a replisome still valid? Or is coordination defined and achieved in some other way? Check out the video yourself in the article here.

5. One More Problem with Replication

Cairns recorded many images of *E.coli* of the sort shown below.



The coiled, twisted appearance of the replicating circles were interpreted to be a natural consequence of trying to pull apart helically intertwined strands of DNA... or intertwined strands of any material! As the strands continued to unwind, the DNA should twist into a **supercoil** of DNA. Increased DNA unwinding would cause the phosphodiester bonds in the DNA to rupture,

fragmenting the DNA. Obviously, this does not happen. Experiments were devised to demonstrate supercoiling, and to test hypotheses explaining how cells *relax* the supercoils during replication. Testing these hypotheses revealed the **topoisomerase** enzymes. These enzymes bind and hold on to DNA, catalyze hydrolysis of phosphodiester bonds, control unwinding of the double helix, and finally catalyze the re-formation of the phosphodiester linkages. It is important to note that the topoisomerases are not part of a replisome, but can act far from a replication fork, probably responding to the tensions in overwound DNA. Recall that topoisomerases comprise much of the protein lying along eukaryotic chromatin.

185 Topoisomerases Relieve Supercoiling During Replication

We have considered most of the molecular players in replication. Below is a list of the key replication proteins and their functions (from here).

Enzyme	Function in DNA Replication
DNA Helicase	Also known as helix destabilizing enzyme. Unwinds the DNA double helix at the Replication Fork.
DNA Polymerase	Builds a new duplex DNA strand by adding nucleotides in the 5' to 3' direction. Also performs proof-reading and error correction.
DNA clamp	A protein which prevents DNA polymerase III from dissociating from the DNA parent strand.
Single-Strand Binding (SSB) Proteins	Bind to ssDNA and preven the DNA double helix from re-annealing after DNA helicase unwinds it thus maintaining the strand separation.
Topoisomerase	Relaxes the DNA from its super-coiled nature.
DNA Gyrase	Relieves strain of unwinding by DNA helicase; this is a specific type of topoisomerase
DNA Ligase	Re-anneals the semi-conservative strands and joins Okazaki Fragments of the lagging strand
Primase	Provides a starting point of RNA (or DNA) for DNA polymerase to begin synthesis of the new DNA strand.
Telomerase	Lengthens telomeric DNA by adding repetitive nucleotide sequences to the ends of eukaryotic chromosomes.

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9.3: DNA Repair

A. DNA Damage (Mutation) is a Fact of Life

We generally accept the notion that replication faithfully duplicates the genetic material. At the same time, evolution would not be possible without mutation, and mutation is not possible without at least some adverse consequences.

Germline mutations are heritable. When present in one, but especially in both alleles of a gene, such mutations can result in genetic disease (e.g., Tay-Sach's disease, cystic fibrosis, hemophilia, sickle-cell anemia, etc.). Rather than causing disease, some germline mutations may increase the *likelihood* of becoming ill (e.g., mutations of the BRCA2 gene greatly increase a woman's odds of getting breast cancer). **Somatic mutations** in actively dividing cells might result in benign "cysts" or malignant tumors (i.e., cancer). Other somatic mutations may play a role in dementia (Alzheimer's disease) or in some of the neuropathologies along the autism spectrum.

Since the complex chemistry of replication is subject to an inherent high rate of error, cells have evolved systems of DNA repair to survive high mutation rates. As we saw, DNA polymerases themselves have proofreading ability so that incorrectly inserted bases can be quickly removed and replaced. Beyond this, multiple mechanisms have evolved to repair mismatched base pairs and other kinds of damaged DNA that escape early detection. How often and where DNA damage occurs is random, as is which damage will be repaired and which will escape to become a mutation. For those suffering the awful consequences of unrepaired mutation, the balance between retained and repaired DNA, damage is to say the least, imperfect. However, evolution and the continuance of life itself rely on this balance.

B. What Causes DNA Damage

DNA is most exposed and therefore most vulnerable to damage, especially in eukaryotes. The simplest damage to DNA during replication is the point mutation, the accidental insertion of a 'wrong' nucleotide into a growing DNA strand. Other mutations, equally accidental, include DNA deletions, duplications, inversions, etc., any of which might escape repair. The causes of DNA damage can be chemical or physical, and include spontaneous intracellular events (e.g., oxidative reactions) and environmental factors (radiation, exogenous chemicals, etc.). Based on studies of different kinds of DNA damage, Thomas Lindahl estimated that DNA damaging events might be occurring at the rate of 10,000 per day! Lindahl realized that there must be some "fundamental DNA repair mechanisms" at work to protect cells against such a high rate of DNA damage. The discovery of the **base excision repair** mechanism earned Thomas Lindahl a share in the 2015 Nobel Prize in Chemistry. Environmental factors that can damage DNA include UV light, X-rays and other radiation, as well as chemicals (e.g., toxins, carcinogens, and even drugs, etc.). Both germline and somatic cells can be affected. While mutations can and do cause often debilitating diseases, it is instructive to keep the impact of mutations in perspective. Most mutations are actually *silent*; they do not cause disease. In addition, much DNA damage is repaired. Cells correct more than 99.9% of mistaken base changes before they have a chance to become mutations. That is why we think of replication as a "faithful" process. Let's look at some common types of DNA damage that are usually repaired:

- **Pyrimidine dimers,** typical of adjacent thymines (less often cytosines) in a single DNA strand, caused by UV exposure
- **Depurination;** the *hydrolytic* removal of guanine or adenine from the #1 C (carbon) of deoxyribose in a DNA strand
- **Deamination:** hydrolytic removal of amino (-NH2) groups from guanine (most common), cytosine or adenine
- **Oxidative damage** of deoxyribose with any base, but most commonly purines
- Inappropriate **methylation** of any bases, but most commonly purines
- **DNA strand breakage** during replication or from radiation or chemical exposure

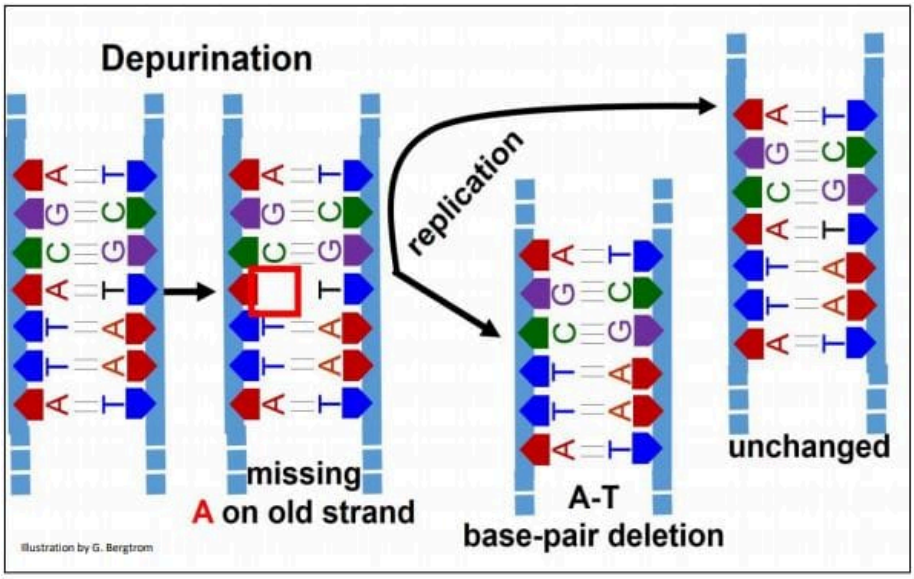
C. Some Molecular Consequences of Uncorrected DNA Damage

While bacteria suffer DNA damage, we will focus here on eukaryotes since they have evolved the most sophisticated mechanisms. Remember that unrepaired DNA damage **will** be passed on to daughter cells in mitosis, or **might** be passed on to the next generation if the mutation occurs in a germline cell.

Next, let us consider some *molecular consequences* of uncorrected DNA damage.

1. Depurination

This is the spontaneous *hydrolytic* removal of guanine or adenine from deoxyribose C#1 in a DNA strand. Its frequency of 5000 depurinations per cell per day emphases the high rate of DNA damage that demands a fix! If not repaired, depurination results in a single base-pair deletion in one chromosome after replication, leaving the DNA in the same region of the other chromosome unchanged. The effects of depurination are illustrated below.

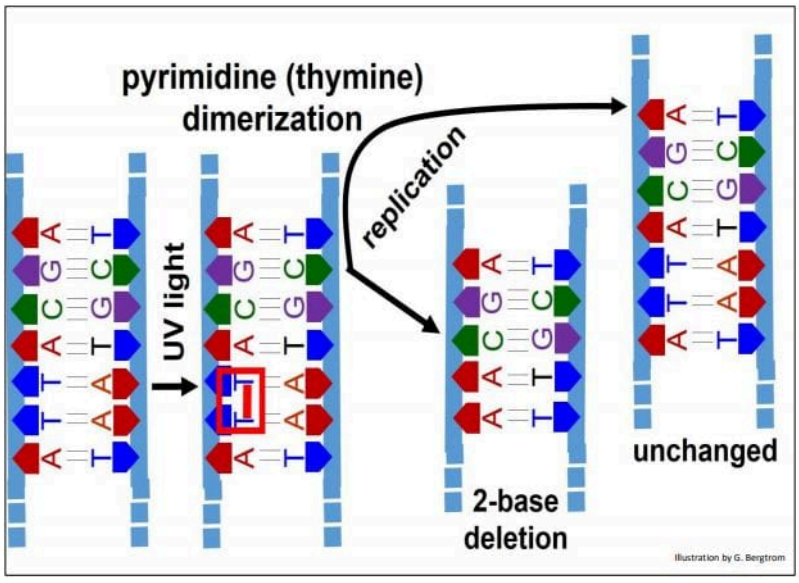


The replisome ignores the missing base during replication of the depurinated DNA region (an A in this example), jumping to the C in the depurinated template DNA. Unrepaired, one new double-stranded DNA will have a deletion, leaving the other new one with no mutation.

2. Pyrimidine Dimerization

UV light exposure of DNA can cause adjacent pyrimidines (commonly thymines; less often, cytosines) on a DNA strand to dimerize. Pyrimidine dimers form at a rate of a bit less than 100 per cell per day!

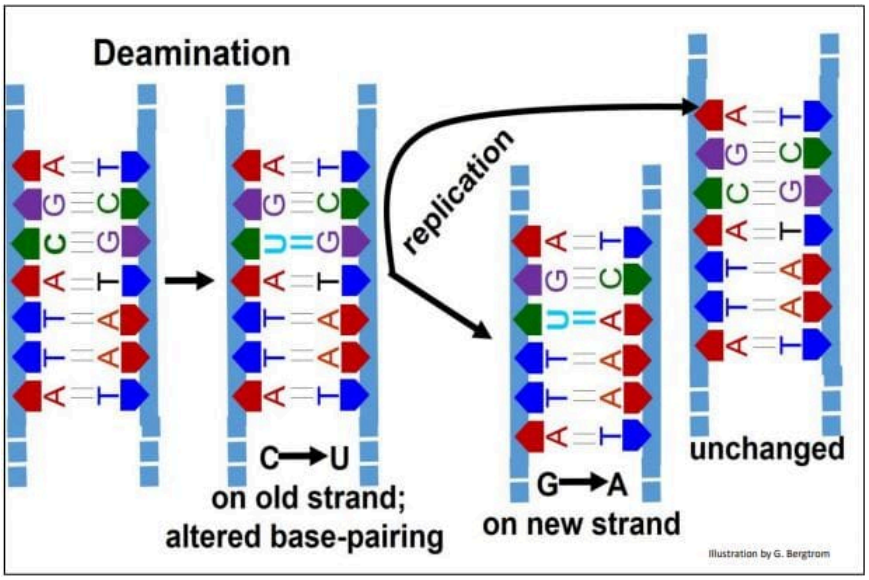
Uncorrected dimerization results in 2-base deletion in one chromosome while the other is unchanged (below).



You can predict that correction of this radiation-induced damage will either involve disrupting the dimers (in this case thymine dimers), or removal and replacement of the dimerized bases by monomeric bases.

3. Deamination

Deamination is the hydrolytic removal of amino (-NH2) groups from guanine (most common), cytosine or adenine, at a rate of 100 per cell per day. Deamination does not affect thymine (because it has no –amino groups!). Uncorrected deamination results in a base substitution on one chromosome (actually, a T-A pair substitution for the original C-G in this example) and no change on the other. Deamination of adenine or guanine results in unnatural bases (hypoxanthine and xanthine, respectively). These are easily recognized and corrected by DNA repair systems. The U-A base pair remains occasionally un-repaired. The consequences of deamination to base sequence are shown below.

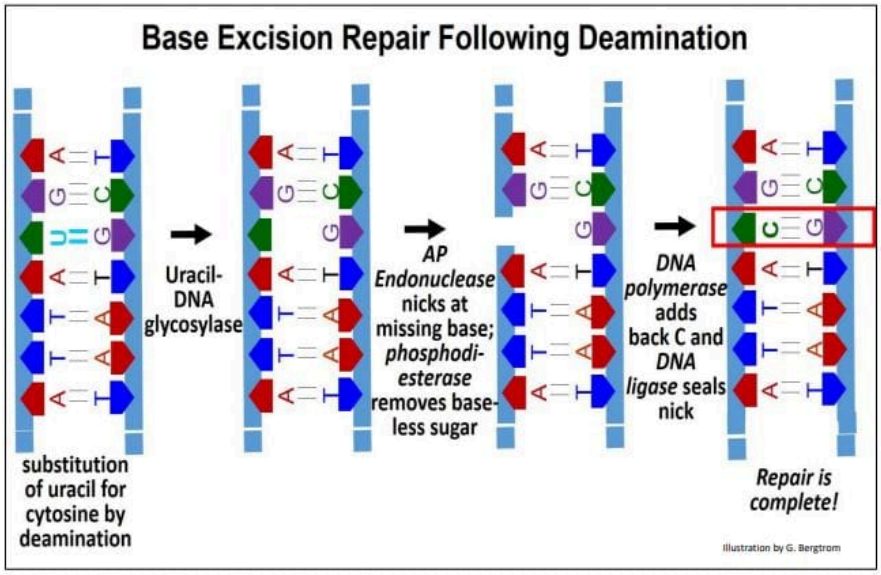


D. DNA Repair Mechanisms

Many enzymes and proteins are involved in DNA repair. Some of these function in normal replication, mitosis and meiosis, but were co-opted for DNA repair activities. These molecular co-optations are so vital to normal cell function that some repair activities and molecular players are highly conserved in evolution. Among different DNA repair pathways that have been identified, we will look at **Base Excision Repair**, **Nucleotide Excision Repair**, **Transcription Coupled Repair**, **Non-homologous End-Joining**, and **Homologous Recombination** (of these, the last is perhaps the most complex).

1. Base Excision Repair

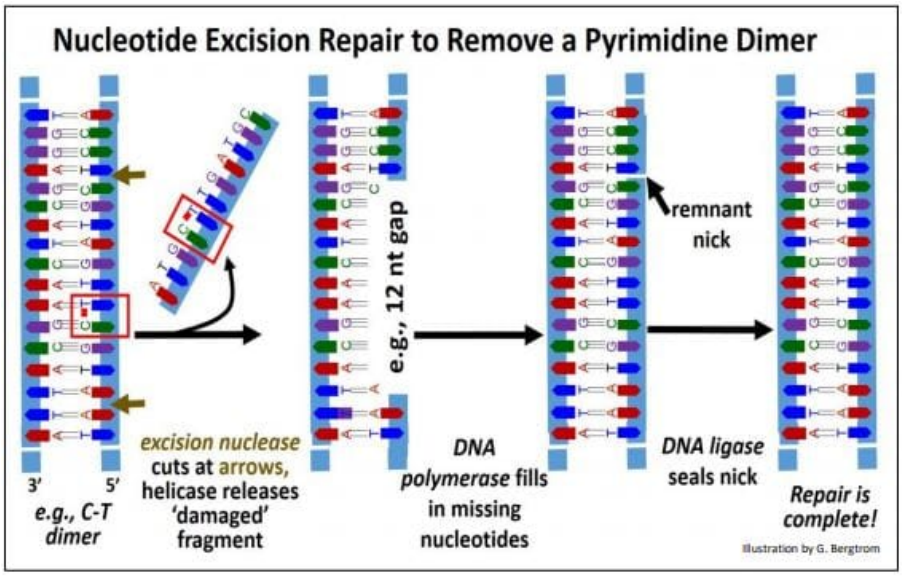
Upon detection and recognition of an incorrect base (e.g., oxidized bases, openring bases, deaminated Cs or As, bases containing C=C bonds saturated to C-C bonds...), specific **DNA glycosylases** catalyze hydrolysis of the damaged base from affected deoxyribose in the DNA. To learn more about the specific versions of this enzyme, click here. Events of **base excision repair** are summarized below.



After a DNA glycosylase removes an offending base, an **AP endonuclease** recognizes the deoxyribose with the missing base and nicks the DNA at that nucleotide. **Phosphodiesterase** next hydrolyzes the remaining phosphate-ester bond of 'base-less' sugar phosphate, removing it from the DNA strand. **DNA polymerase** then adds correct nucleotide to the 3' end of the nick. Finally, **DNA ligase III** (an ATP-dependent mammalian version of the original prokaryotic enzyme) seals the remaining nick in the strand. Thomas Lindahl (see above) discovered most of these enzymes.

2. Nucleotide Excision Repair

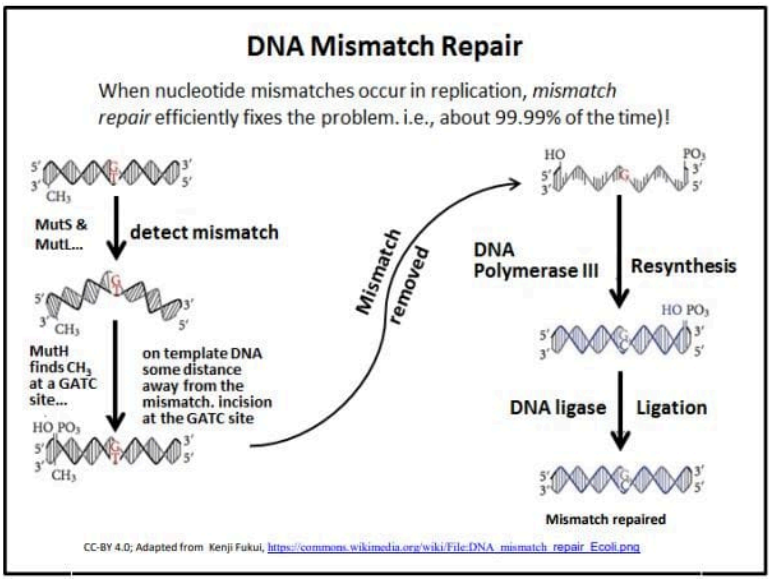
The discovery of **nucleotide excision repair** earned Aziz Sancar a share in the 2015 Nobel Prize in Chemistry. The results of this mechanism include the removal of thymidine dimers. The events of **nucleotide excision repair** are shown below for a pyrimidine dimer.



In this example, an **Excision Nuclease** recognizes a pyrimidine dimer and hydrolyzes phosphodiester bonds between nucleotides several bases away from either side of the dimer. A **DNA helicase** then unwinds and separates the DNA fragment containing the dimerized bases from the damaged DNA strand. Finally, **DNA polymerase** acts 5'-3' to fill in the gap and **DNA ligase** seals the remaining nick to complete the repair.

3. Mismatch Repair

DNA Mismatch Repair occurs when DNA polymerase proofreading misses an incorrect base insertion into a new DNA strand. This repair mechanism relies on the fact that double-stranded DNA shows a specific pattern of methylation. The discovery of the mismatch repair mechanism earned Paul Modrich a share in the 2015 Nobel Prize in Chemistry. These methylation patterns are related to epigenetic patterns of gene activity and chromosome structure that are expected to be inherited by daughter cells. When DNA replicates, the methyl groups on the template DNA strands remain, but the newly synthesized DNA is unmethylated. In fact, it will take some time for methylation enzymes to locate and methylate the appropriate nucleotides in the new DNA. In the intervening time, several proteins and enzymes can detect inappropriate base pairing (the mismatches) and initiate mismatch repair. The basic process is illustrated below.

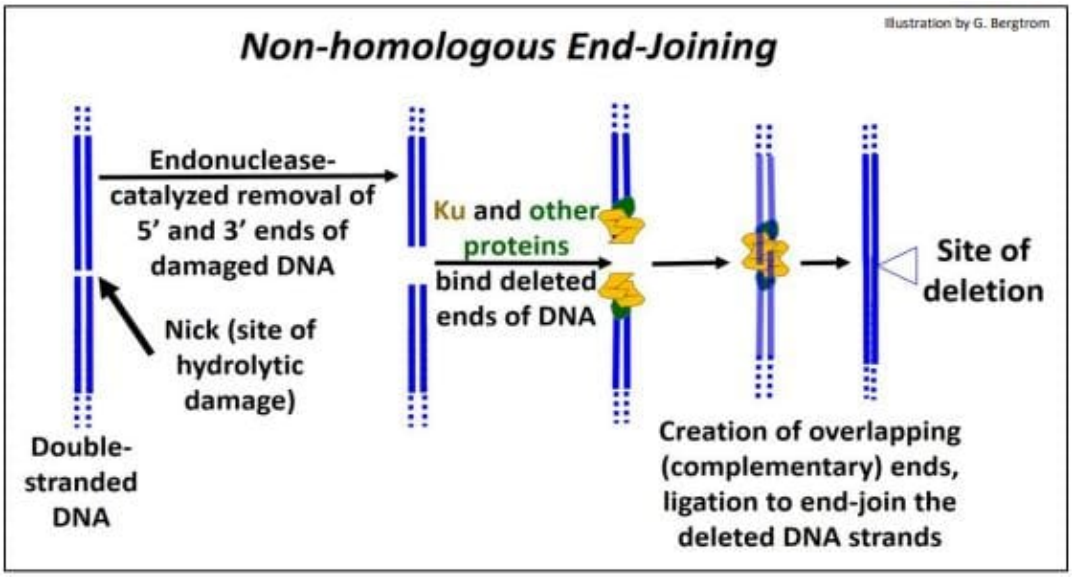


4. Transcription Coupled Repair (in Eukaryotes)

If an RNA polymerase reading a template DNA encounters a nicked template or one with an unusual base substitution, it might stall transcription and "not know what to do next". Thus at a loss, a normal transcript would not be made and the cell might not survive. No big deal in a tissue comprised of thousands if not millions of cells, right? Nevertheless, **Transcription Coupled Repair** exists! In this repair pathway, if **RNA polymerase** encounters a DNA lesion (i.e., damaged DNA) while transcribing a template strand, it will indeed stall. This allows time for **coupling proteins** to reach the stalled polymerase and enable repair machinery (e.g., by base, or nucleotide excision) to effect the repair. Once the repair is complete, the **RNA polymerase** 'backs up' along the template strand with the help of other factors, and resumes transcription of the corrected template.

5. Non-homologous End-Joining

DNA replication errors can cause **double stranded breaks**, as can environmental factors (ionizing radiation, oxidation, etc.). Repair by **non-homologous end-joining** deletes damaged and adjacent DNA and rejoins the 'cut' ends (shown below).



Once the site of a double-stranded break is recognized, nucleotides **hydrolyzed** from the ends of both strands at the break-site leave '*blunt ends*'. Next, several proteins (**Ku** among others) bring DNA strands together and further hydrolyze single DNA strands to create staggered (overlapping, or **complementary**) ends. The overlapping ends of these DNA strands form H-bonds. Finally, **DNA ligase** seals the H-bonded overlapping ends of DNA strands, leaving a repair with deleted bases.

In older people, there is evidence of more than 2000 'footprints' of this kind of repair per cell. How is this possible? This *quick-fix* repair often works with no ill effects because most of the eukaryotic genome does not encode genes or even regulatory DNA (whose damage would otherwise be more serious).

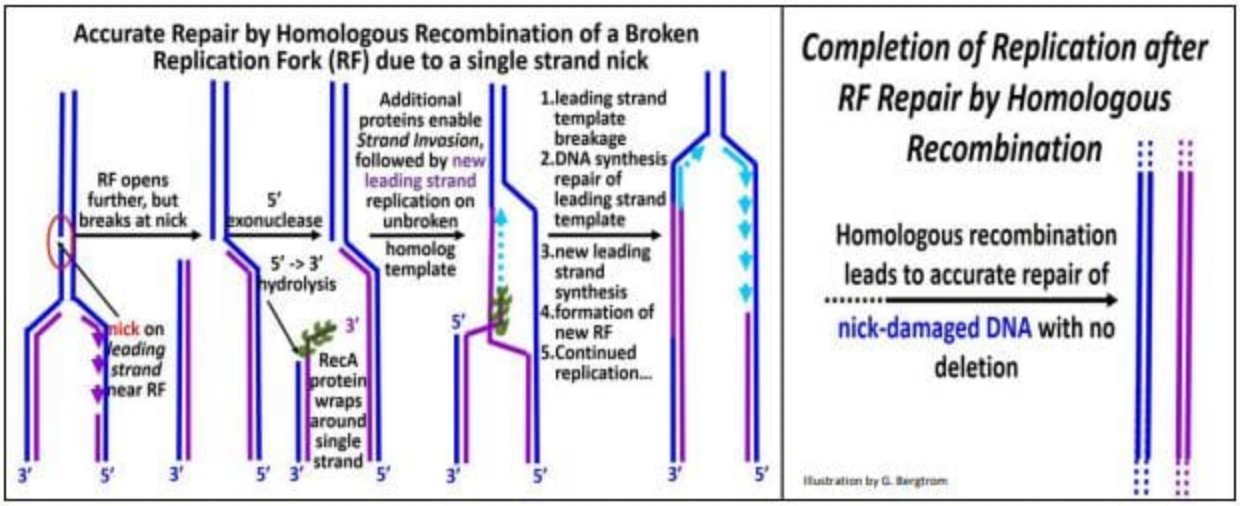
6. Homologous Recombination

Homologous recombination is a complex but normal and frequent part of meiosis in eukaryotes. You may recall that **homologous recombination** occurs in **synapsis** in the first cell division of **meiosis** (Meiosis I). During synapsis,homologous chromosomes align. This may lead to DNA breakage, an *exchange* of alleles, and ligation to reseal the now recombinant DNA molecules. Novel recombinations of variant **alleles** in the chromosomes of sperm and eggs ensure **genetic diversity** in species. The key point is that DNA breakage of DNA is required to exchange alleles between **homologous chromosomes**. Consult the genetics chapter in an introductory biology textbook, or the recombination chapter in a genetics text to be reminded of these events.

Cells use the same machinery to reseal DNA breaks during normal recombination and to repair DNA damaged by single or double stranded breakage. A single DNA strand nicked during replication can be repaired by recombination with strands of homologous DNA that are being replicated on the other strand. A double stranded break can be repaired using the same recombination machinery that operates on sister chromatids in meiosis. In both cases, the process accurately repairs damaged DNA **without any deletions**. These mechanisms are conserved in the cells of all species. This indicates an evolutionary imperative of accurate repair to the survival of species, no less than the imperative to maintain genetic diversity of species.

a) Repair of a Single-Stranded Break

A specific example of homologous recombination repair is the re-establishment of a replication fork damaged when a replisome reaches a break in one of the two strands of replicating DNA (illustrated below).



Such a break may have occurred prior to replication itself, and repair begins when the replication fork (RF) reaches the lesion. In the first step, a **5'-3' exonuclease** trims template DNA back along its newly synthesized complement. Next, **RecA** protein monomers (each with multiple DNA binding sites) bind to the single-stranded DNA to form a **nucleoprotein filament**. With the help of additional proteins, the 3' end of the 'filament' scans the 'other' replicating strand for homologous sequences. When such sequences are found, the RecA-DNA filament binds to the homologous sequences and the filament of new DNA 'invades' the homologous (i.e., opposite) double stranded DNA, separating its template and newly replicated DNA. After **strand invasion**, replication of a leading strand continues from the 3' end of the invading strand. A new RF is established as the leading strand template is broken and re-ligated to the original break; New lagging strand replication then resumes at the new (re-built) RF. The result is an accurate repair of the original damage, with no deletions or insertions of DNA.

RecA, a bacterial protein, is another of those evolutionarily conserved proteins. Its homolog in Archaea is called **RadA**. In Eukaryotes, the homolog is called **Rad51**, where it initiates **synapsis** during meiosis. Thus, it seems that a role for **RecA** and its conserved homologs in DNA repair predated its use in synapsis and crossing over in eukaryotes! For more about the functions of RecA protein and its homologs, click here.

b) Repair of a Double-Stranded Break

Homologous recombination can also repair a double-stranded DNA break with the aid of a number of enzymes and other proteins. Alternate repair pathways are summarized in the illustration on the next page. Here is a list of proteins involved in these homologous recombination pathways:

MRX, MRN: bind at double-stranded break; recruit other factors.

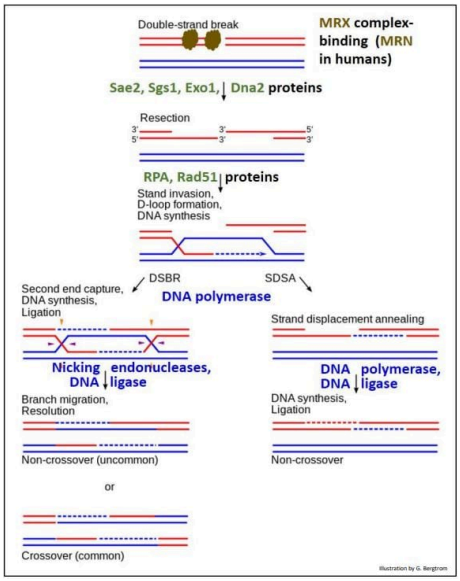
Sae2: an endonuclease (active when phosphorylated).

Sgs1: a helicase.

Exo1, Dna2: single strand exonucleases.

RPA, Rad51, DMC1: proteins that bind to overhanging DNA to form a nucleoprotein filament and initiate *strand invasion* at similar sequences.

The activities of other enzymes in the drawing are identified. Not shown in this illustration are two gene products that interact with some of the proteins that mediate the repair pathway. These are products of the BRCA1 and BRCA2 genes (the same ones that when mutated, increase the likelihood of a woman getting breast cancer). Expressed mainly in breast tissue, their wild-type (normal) gene products participate in homologous recombination repair of double-stranded DNA breaks. They do this by binding to Rad51 (the human RecA homolog!).



When mutated, the BRCA proteins function poorly and DNA in the affected cells is not efficiently repaired. This is the likely basis of the increased chance of getting breast cancer. It doesn't help matters that the normal BRCA1 protein also plays a role in mismatch repair... and that the mutated protein can't! To end this chapter, here is a bit of weird science! Read all about the genome of a critter, nearly 17% of which is comprised of foreign DNA, possibly the result of here.

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9.4: Key Words and Terms

base excision repair	initiation	replicons
bidirectional replication	initiator proteins	replisome
Central Dogma	lagging strand	S phase of the cell cycle
clamp proteins	leading strand	satellite DNA
condensation reactions	methylation	single-strand binding proteins
deamination	mutations	siRNA
density gradient centrifugation	nucleotide excision repair	supercoiling
depurination	Okazaki fragments	T2 phage
direct repeats	origin of replication	tardigrade
discontinuous replication	phosphate backbone	telomerase
DNA ligase	phosphodiester linkage	telomeres
DNA mismatch repair	primase	theta images
DNA polymerase I, II, III	primer	topoisomerases
DNA repair	processive replication	transcription-coupled repair
DNA sequence phylogeny	proofreading	transposase
elongation	RadA protein	VNTRs
env	RecA protein	
helicase	replication	
high-speed binder	replication fork	

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10: TRANSCRIPTION AND RNA PROCESSING

10.1: Introduction
10.2: Overview of Transcription
10.3: Details of Transcription
10.4: Details of Eukaryotic mRNA Processing
10.5: Ribosomal RNA Processing in Eukaryotic Nuclei
10.6: tRNA Processing in Eukaryotic Nuclei
10.7: RNA and Ribosome Export from the Nucleus
10.8: Key Words and Terms

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10.1: Introduction

Transcription, the synthesis of RNA based on a DNA template, is the central step of the Central Dogma proposed by Crick in 1958. The basic steps of transcription are the same as for replication: initiation, elongation and termination. Differences between transcription in prokaryotes and eukaryotes are in the details.

- *E.coli* uses a single **RNA polymerase** enzyme to transcribe all kinds of RNAs while eukaryotic cells use different RNA polymerases to catalyze **ribosomal RNA** (rRNA), **transfer RNA** (tRNA) and **messenger RNA** (mRNA) synthesis.
- In contrast to eukaryotes, some bacterial genes are part of **operons** whose mRNAs encode multiple polypeptides.
- Bacterial mRNAs are typically translated as they are being transcribed.
- Most RNA transcripts in prokaryotes emerge from transcription ready to use
- Eukaryotic transcripts synthesized as longer precursors undergo **processing** by **trimming**, **splicing**, or both!
- DNA in bacteria is virtually 'naked' in the cytoplasm while eukaryotic DNA is wrapped up in chromatin proteins in a nucleus.
- In bacterial cells the association of ribosomes with mRNA and the translation of a polypeptide can begin even before the transcript is finished. This is because these cells have no nucleus. In our cells, RNAs must exit the nucleus before they encounter *ribosomes* in the cytoplasm.

In this chapter, you will meet bacterial **polycistronic** mRNAs (transcripts of **operons** that encode more than one polypeptide) and the **split genes** of eukaryotes (with their **introns** and **exons**). We will look at some details of transcription of the three major classes of RNA and then at how eukaryotes process precursor transcripts into mature, functional RNAs. Along the way, we will see one example of how protein structure has evolved to interact with DNA.

Learning Objectives

1. Discriminate between the *three steps of transcription* in pro- and eukaryotes, and the factors involved in each.

2. State an hypothesis for why eukaryotes evolved complex *RNA processing* steps.

3. Speculate on why any cell in its right mind would have genes containing *introns* and *exons* so that their transcripts would have to be processed by *splicing*.

4. Articulate the differences between *RNA vs. DNA structure*.

5. Explain the need for *sigma factors* in bacteria.

6. Speculate on *why eukaryotes do not have operons*.

7. List structural features of proteins that bind and *recognize specific DNA sequences*.

8. Explain how proteins that *do not* bind specific DNA sequences *can* still bind to specific regions of the genome.

9. Formulate an hypothesis for why bacteria do not polyadenylate their mRNAs as much as eukaryotes do.

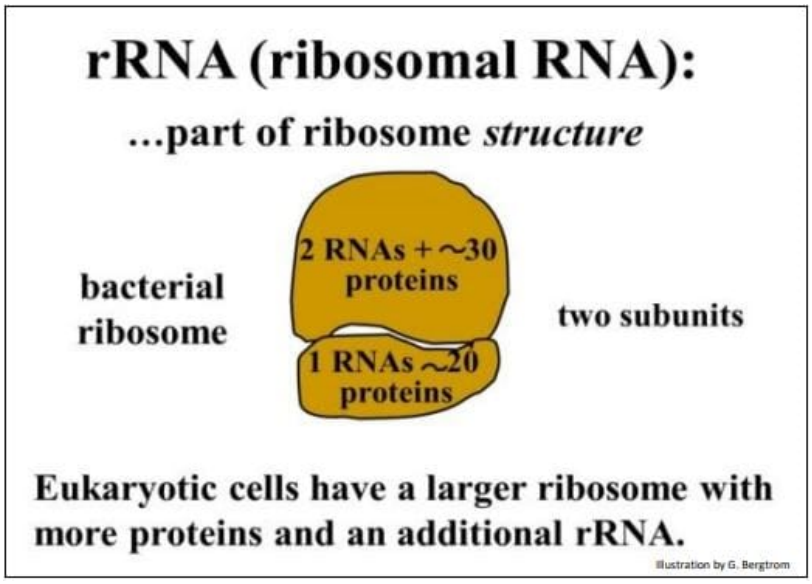
10. Formulate an hypothesis for why bacteria do not cap their mRNAs.

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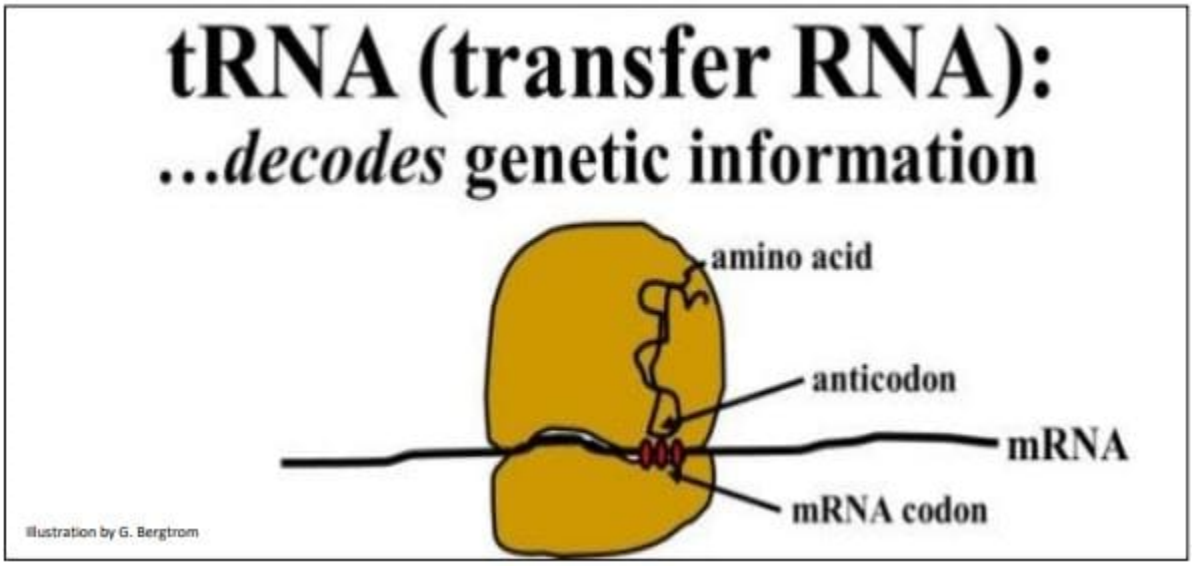
10.2: Overview of Transcription

A. The Major Types of Cellular RNA

All cells make three main kinds of RNA: ribosomal RNA (**rRNA**), transfer RNA (**tRNA**) and messenger RNA (**mRNA**). rRNA is a structural as well as enzymatic component of ribosomes, the protein-synthesizing machine in the cell. Quantitatively, rRNAs are by far the most abundant RNAs in the cell and mRNAs, the least. Three **rRNAs** and about 50 ribosomal proteins make up the two subunits of a bacterial ribosome, as illustrated below.



tRNAs are the *decoding devices* used in protein synthesis (**translation**) to convert nucleic acid sequence information into the amino acid sequences of polypeptides. tRNAs attached to amino acids are positioned on ribosomes based on codonanticodon recognition, as shown below.

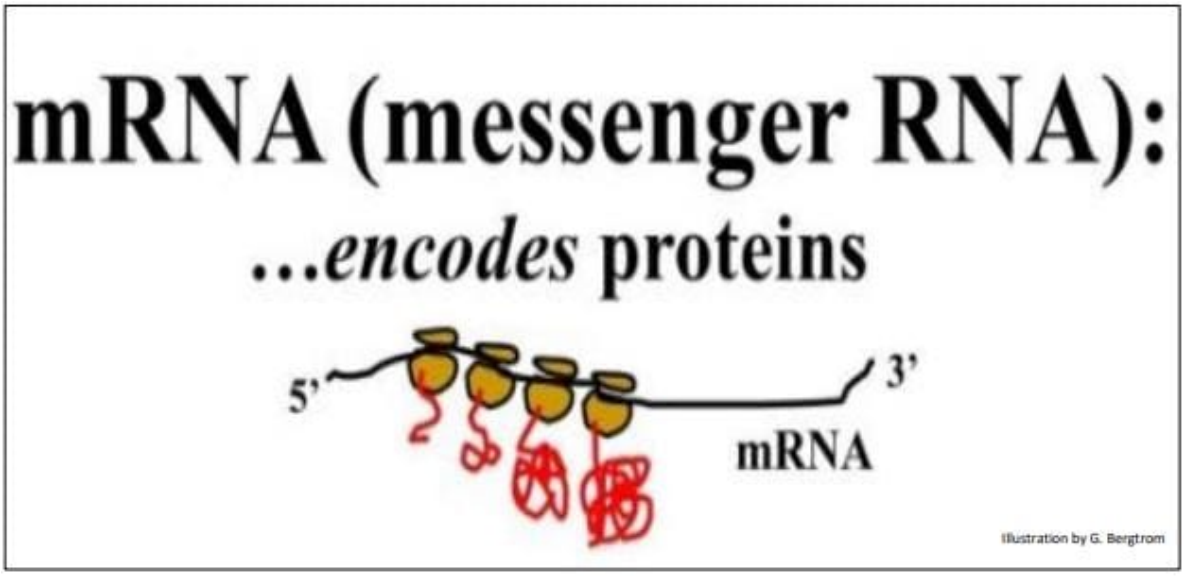


During translation, tRNAs decode base sequences in **messenger RNA** (**mRNAs**) into amino acid sequences of polypeptides.

In 2009, Venkatraman Ramakrishnan, Thomas A. Steitz and ADA Yonath received the Nobel Prize in Chemistry their studies on the structure and molecular biology of the ribosome. Dr. Yonath is one of five women to receive a Nobel Prize – the others were Marie Curie, Irène Joliot-Curie, Dorothy Hodgkin and Barbara Mclintock.

The fact that genes are inside the eukaryotic nucleus and that the synthesis of polypeptides encoded by those genes happens in the cytoplasm led to the proposal that there must be a messenger RNA (mRNA. Sidney Brenner eventually confirmed the existence of mRNAs. Check out his classic experiment in Brenner S (1961, *An unstable intermediate carrying information from genes to ribosomes for protein synthesis*. Nature 190:576-581).

Recall polypeptide synthesis by the formation of polyribosomes (polysomes) along a single mRNA, as illustrated below.

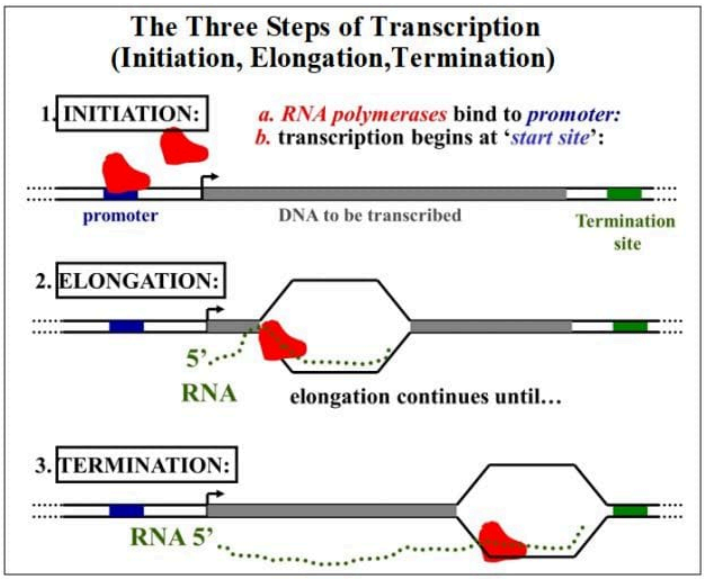


While mRNA is a small fraction of total cellular RNA, there are still smaller amounts of other RNAs such as the transient primers that we saw in DNA replication. We'll encounter still other kinds of low-abundance RNAs later.

B. Key Steps of Transcription

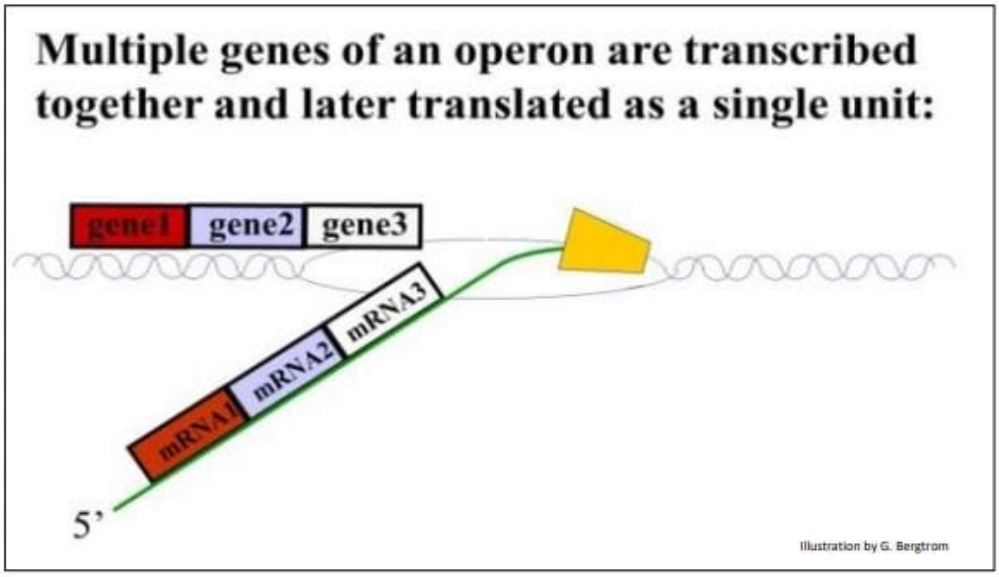
In transcription, an *RNA polymerase* uses the template DNA strand of a gene to catalyze synthesis of a complementary, antiparallel RNA strand. RNA polymerases use ribose nucleotide triphosphate (NTP) precursors, in contrast to DNA polymerases, which use *deoxyribose nucleotide* (dNTP) precursors. In addition, RNAs incorporate *uracil* (U) nucleotides into RNA strands instead of the thymine (T) nucleotides that end up in new DNA. Another contrast to replication - RNA synthesis does not require a primer. With the help of transcription initiation factors, RNA polymerase locates the *transcription start site* of a gene and begins synthesis of a new RNA strand from scratch. Finally, like replication, transcription is error-prone.

The basic steps of transcription are summarized on the next page. Here we can identify several of the DNA sequences that characterize a gene. The *promoter* is the binding site for RNA polymerase. It usually lies 5' to, or *upstream* of the transcription start site (the bent arrow). Binding of the RNA polymerase positions the enzyme to near the transcription start site, where it will start unwinding the double helix and begin synthesizing new RNA. The transcribed grey DNA region in each of the three panels are the **transcription unit** of the gene. Termination sites are typically 3' to, or downstream from the transcribed region of the gene. By convention, *upstream* refers to DNA 5' to a given reference point on the DNA (e.g., the transcription start-site of a gene). *Downstream* then, refers to DNA 3' to a given reference point on the DNA.



188 Transcription Overview: The Basic Mechanism of RNA Synthesis

In bacteria, some transcription units encode more than one kind of RNA. Bacterial operons are an example of this phenomenon. The resulting mRNAs can be translated into multiple polypeptides at the same time. In the illustration below, RNA polymerase is transcribing a single mRNA molecule encoding three separate polypeptides.



Bacterial transcription of the different RNAs requires only one RNA polymerase. Different RNA polymerases catalyze rRNA, mRNA and tRNA transcription in eukaryotes. Roger Kornberg received the Nobel Prize in Medicine in 2006 for his discovery of the role of **RNA polymerase II** and other proteins involved in eukaryotic messenger RNA transcription (like father-like son!!).

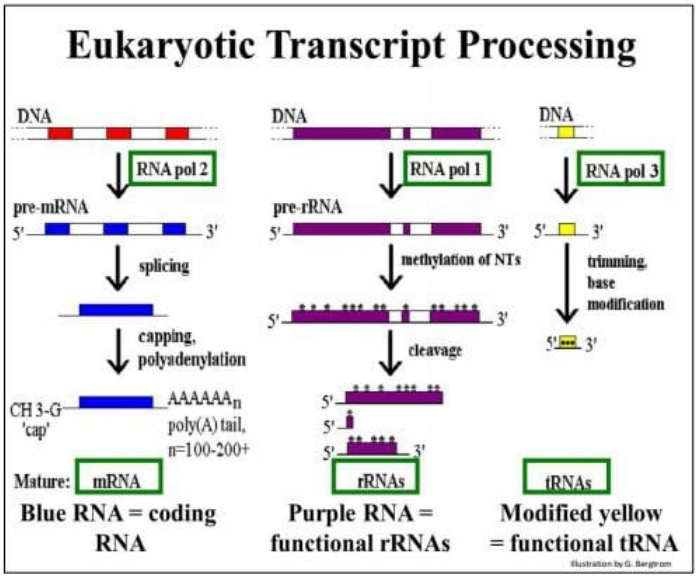
189 RNA Polymerases in Prokaryotes and Eukaryotes

While mRNAs, rRNAs and tRNAs are most of what cells transcribe, a growing number of other RNAs (e.g., *siRNAs, miRNAs, lncRNAs*...) are also transcribed. Some functions of these transcripts (including control of gene expression or other transcript use) are discussed elsewhere.

C. RNAs are Extensively Processed After Transcription in Eukaryotes

Many eukaryotic RNAs are processed (trimmed, chemically modified) from large precursor RNAs to mature, functional RNAs. These precursor RNAs (pre-RNAs, or primary transcripts) contain in their sequences the information necessary for their function in the cell.

Processing of the three major types of transcripts in eukaryotes is shown below.



To summarize the illustration:

1. Many eukaryotic genes are 'split' into coding regions (exons) and non-coding intervening regions (introns).
2. Transcription of split genes generates a primary mRNA transcript (pre-mRNA).
3. Primary transcripts are spliced to remove the introns from the exons; exons are then ligated into a continuous mRNA. In some cases, the same pre-mRNA is spliced into alternate mRNAs encoding related but not identical polypeptides!
4. Pre-rRNA is cleaved and/or trimmed (not spliced!) to make shorter mature rRNAs.
5. Pre-tRNAs are trimmed, some bases within the transcript are modified and 3 bases (not encoded by the tRNA gene) are enzymatically added to the 3'-end.

190 Post Transcriptional Processing Overview

The details of transcription and processing differ substantially in prokaryotes and eukaryotes. Let's focus first on details of transcription itself, and then RNA processing.

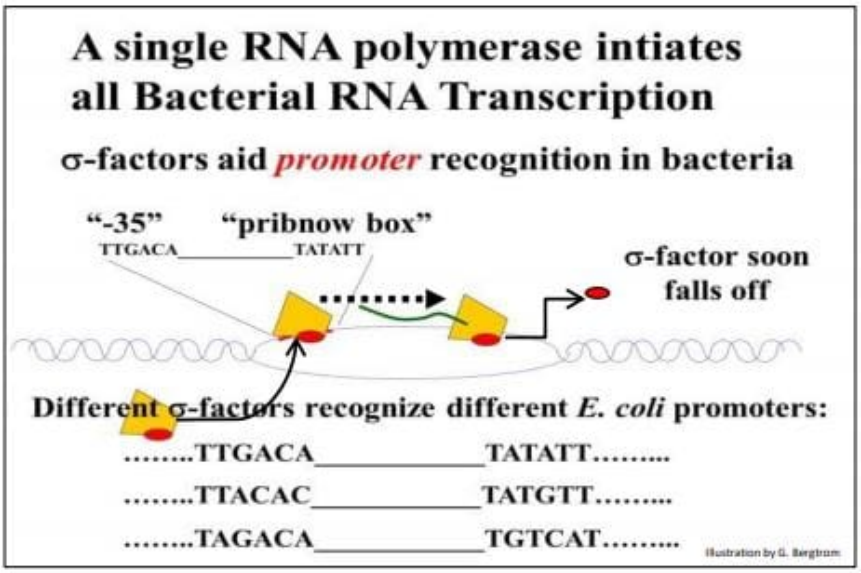
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10.3: Details of Transcription

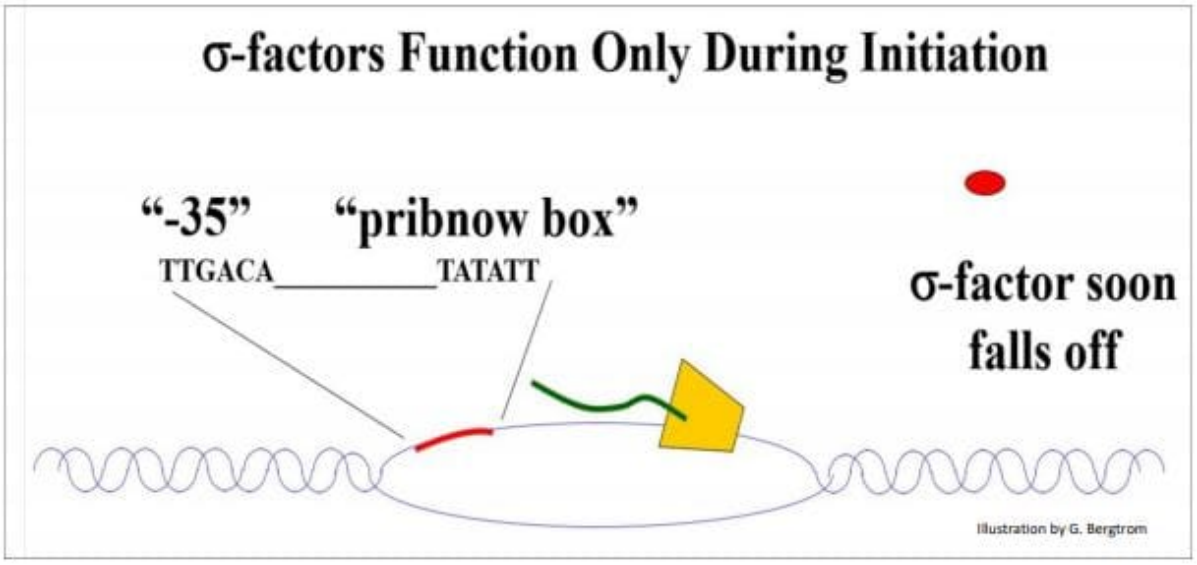
Find a well-written summary of transcription in prokaryotes and eukaryotes at an NIH website (Transcription in Prokaryotes and Eukaryotes). Here (and at this link), you will encounter proteins that bind DNA. Some proteins bind DNA to regulate transcription, inducing or silencing transcription of a gene. We will discuss their role in the regulation of gene expression later. Other proteins interact with DNA simply to allow transcription. These include one or more that, along with RNA polymerase itself, that must bind to the gene promoter to initiate transcription. We will look at bacterial transcription first.

A. Transcription in Prokaryotes

In E. coli, a single RNA polymerase transcribes all kinds of RNA, associating with one of several sigma factor proteins (σ **-factors**) to initiate transcription. It turns out that different promoter sequences and corresponding σ -factors play roles in the transcription of different genes (illustrated below).



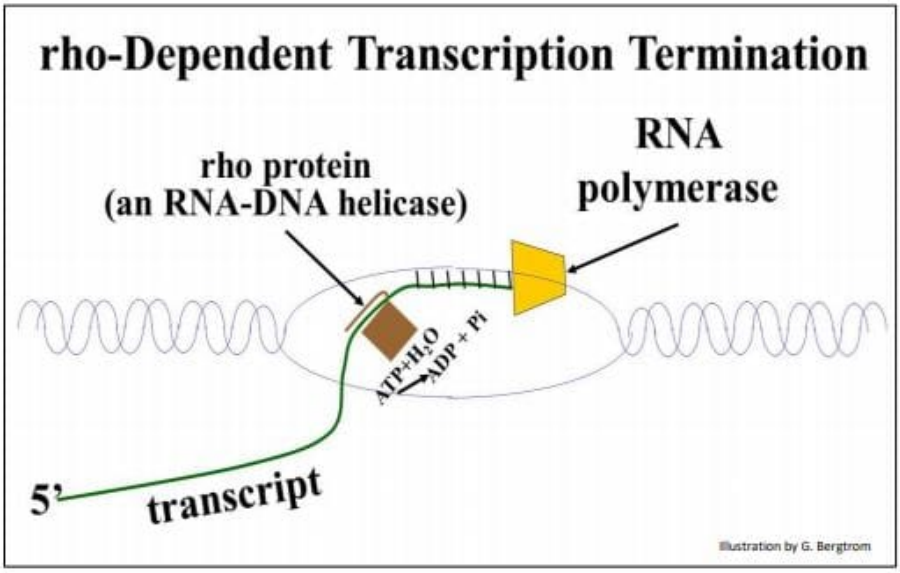
In the absence of the σ-factor, the *E. coli* RNA polymerase can transcribe RNA, but does so at a high rate, and from random sequences in the chromosome. In contrast, when the σ-factor is bound to the RNA polymerase, the complex seems to scan the DNA, recognize and then bind to the promoter sequence of a gene. In this case, the overall transcription rate is slower, but only genes are transcribed, rather than random bits of the bacterial genome! The *Pribnow box,* named for its discoverer, was the first promoter sequence characterized.



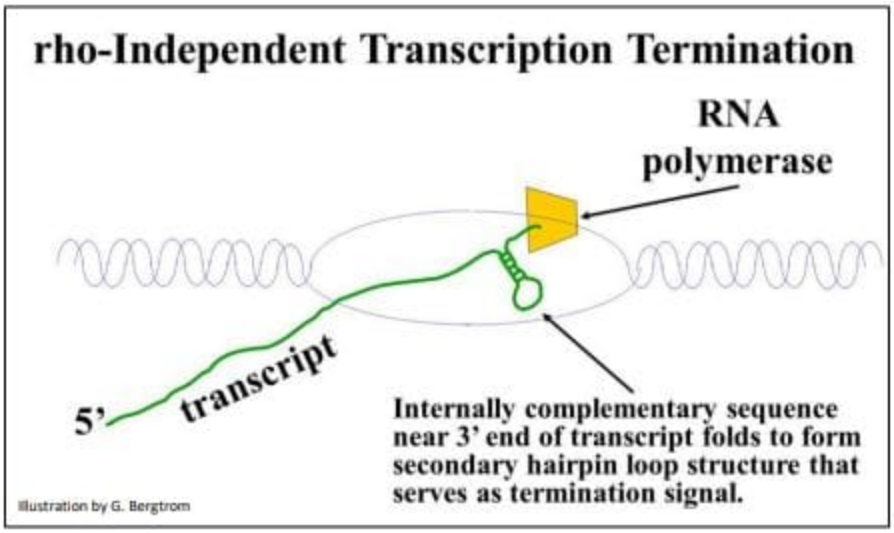
Elongation is the successive addition of nucleotides complementary to their DNA templates, forming phosphodiester linkages. The enzymatic reactions of elongation are similar to the DNA polymerase-catalyzed elongation during replication.

There are two ways that bacterial RNA polymerase 'knows' when it has reached the end of a transcription unit. In one case, as the RNA polymerase nears the 3' end of the nascent transcript, it transcribes a 72 base, C-rich region. At this point, a *termination factor* called the **rho** protein binds to the nascent RNA strand. *Rho* is an ATP-dependent helicase that breaks the H-bonds between the RNA and the template DNA strand, thereby preventing further transcription.

Rho-dependent termination is illustrated below.



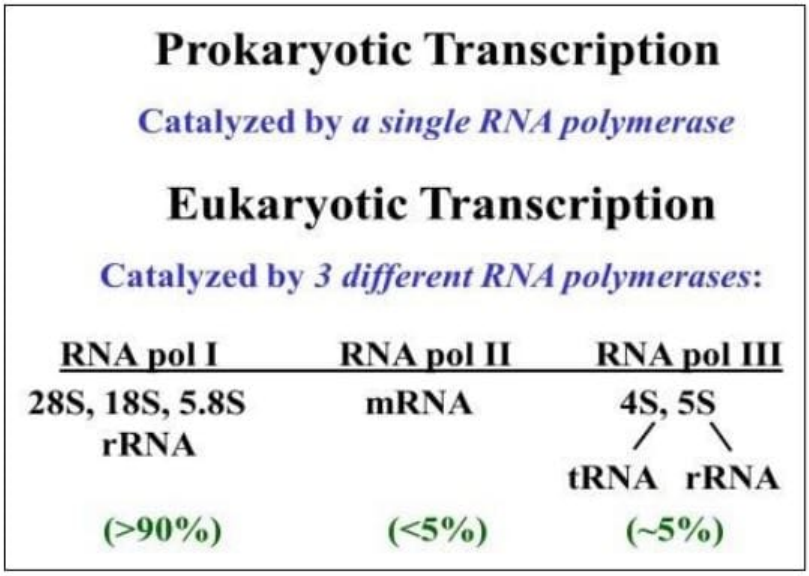
In the other mechanism of termination, the polymerase transcribes RNA whose *termination signal* assumes a secondary *hairpin loop* structure that causes the dissociation of the RNA polymerase, template DNA and the new RNA transcript. The role of the hairpin loop in **rho-independent termination** is illustrated below.



191 Details of Prokaryotic Transcription

B. Transcription in Eukaryotes

Whereas bacteria rely on a single RNA polymerase for their transcription needs, eukaryotes use three different RNA polymerases to synthesize the three major different kinds of RNA, as shown below.

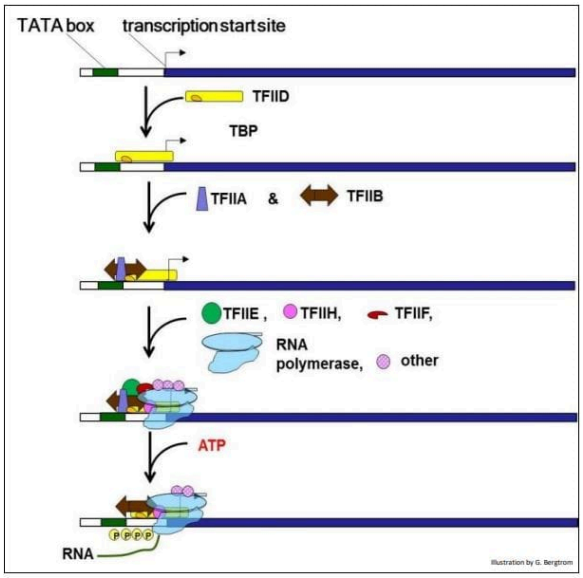


Note that catalysis of the synthesis of most of the RNA in a eukaryotic cell (rRNAs) is by RNA polymerase I. With the help of initiation proteins, each RNA polymerase initiates transcription at a promoter sequence. Once initiated, the RNA polymerases then catalyze the successive formation of phosphodiester bonds to elongate the transcript. Recall that mRNAs are the least abundant in eukaryotes as they are in bacterial cells.

Unfortunately, the details of the termination of transcription in eukaryotes are not as well understood as they are in bacteria. Therefore, we will focus on initiation, and then consider the processing of different eukaryotic RNAs into ready-to-use molecules.

1. Eukaryotic mRNA Transcription

The multiple steps of eukaryotic mRNA transcription are shown on the next page.



Transcription of eukaryotic mRNAs by *RNA polymerase II* begins with the sequential assembly of a eukaryotic *initiation complex* at a gene promoter. The typical eukaryotic promoter for a protein-encoding gene contains a TATA box DNA *sequence motif* as well as additional short upstream sequences. *TATA-binding* protein (TBP) first binds to the TATA box along with TFIID (*transcription initiation factor IID*).

This intermediate recruits *TFIIA* and *TFIIB*. Next, *TFGIIE*, *TFIIF* and *TFIIH*, several other initiation factors and RNA polymerase II bind to form the transcription **initiation complex**. Phosphorylation adds several phosphates to the aminoterminus of the RNA polymerase, after which some of the TF's dissociate from the initiation complex. The remaining RNA polymerase-TF complex can now start making the mRNA.

Unlike prokaryotic RNA polymerase, eukaryotic RNA Polymerase II does not have an inherent helicase activity. For this, eukaryotic gene transcription relies on the multi-subunit TFIIH protein, in which two subunits have helicase activity. Consistent with the closer relationship of *archaea* to eukaryotes (rather to prokaryotes), archaeal mRNA transcription initiation resembles that of eukaryotes, albeit requiring fewer initiation factors during formation of an initiation complex.

192 Eukaryotic mRNA Transcription

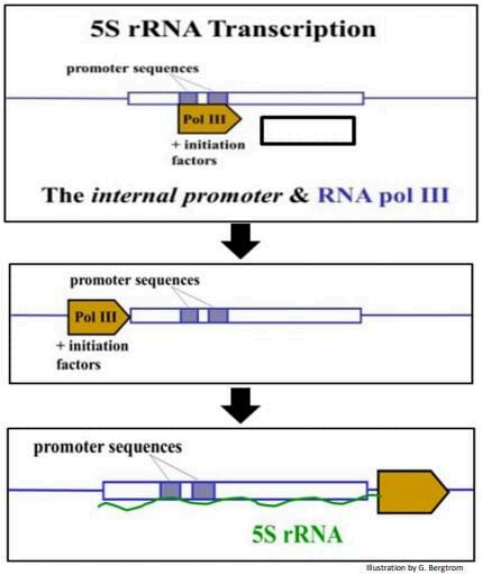
A significant difference between prokaryotic and eukaryotic transcription is that RNA polymerase and other proteins involved at a gene promoter do not see naked DNA. Instead, they must recognize specific DNA sequences through chromatin proteins. On the other hand, all proteins that interact with DNA have in common a need to recognize the DNA sequences to which they must bind..., within the double helix. In other words, they must see the bases within the helix, and not on its uniformly electronegative phosphate backbone surface. To this end, they must penetrate the DNA, usually through the **major groove** of the double helix. We will see that DNA regulatory proteins face the same problems in achieving specific shape-based interactions!

193 Recognition of Transcription factors at Promoters

2. Eukaryotic tRNA and 5SRNA Transcription

Transcription of 5S rRNA and tRNAs by *RNA Polymerase III* is unusual in that the promoter sequence to which it binds (with the help of initiation factors) is not upstream of the transcribed sequence, but lies within the transcribed sequence. After binding to this internal promoter, the polymerase re-positions itself to transcribe the RNA from the transcription start site so that the final transcript thus contains the promoter sequence!

5S rRNA by RNA polymerase III is shown below.



3. Transcription of the Other Eukaryotic rRNAs

tRNAs are also transcribed by RNA polymerase III in much the same way as the 5S rRNA. The other rRNAs are transcribed by RNA polymerase I, which binds to an upstream promoter along with transcription initiation factors. We know less of the details of this process compared to our understanding of mRNA transcription. We'll explore what we do know next. As already noted, transcription termination is not as well understood in eukaryotes as in prokaryotes. Coupled termination and polyadenylation steps common to most prokaryotic mRNAs are discussed in more detail below, with a useful summary at the NIH-NCBI website here.

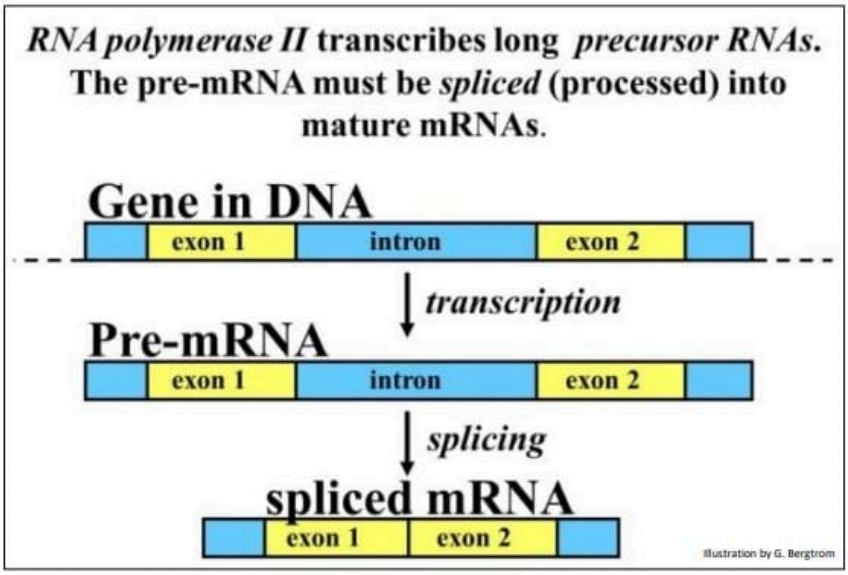
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10.4: Details of Eukaryotic mRNA Processing

Eukaryotic mRNA primary transcripts undergo extensive processing, including *splicing, capping* and, *polyadenylation*. The steps described here are considered in order of (sometimes overlapping!) occurrence. We begin with splicing, an mRNA phenomenon.

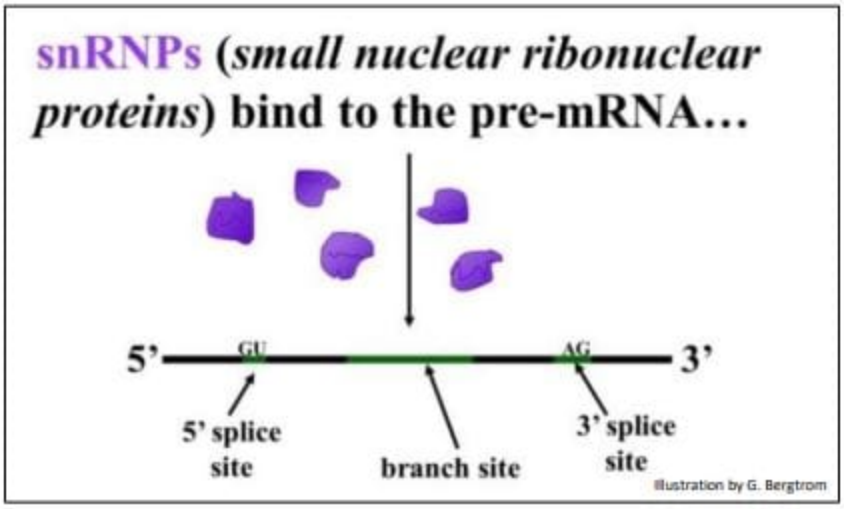
A. Spliceosomal Introns

Bacterial gene coding regions are continuous. The discovery of eukaryotic split genes with introns and exons came as quite a surprise. Not only did it seem incongruous for evolution to have stuck irrelevant DNA in the middle of coding DNA, no one could have dreamt up such a thing! For their discovery of split genes, by Richard J. Roberts and Phillip A. Sharp shared the Nobel Prize for Physiology in 1993. In fact, all but a few eukaryotic genes are split, and some have one, two (or more than 30-50!) **introns** separating bits of coding DNA, the **exons**. **Splicing** is summarized below.

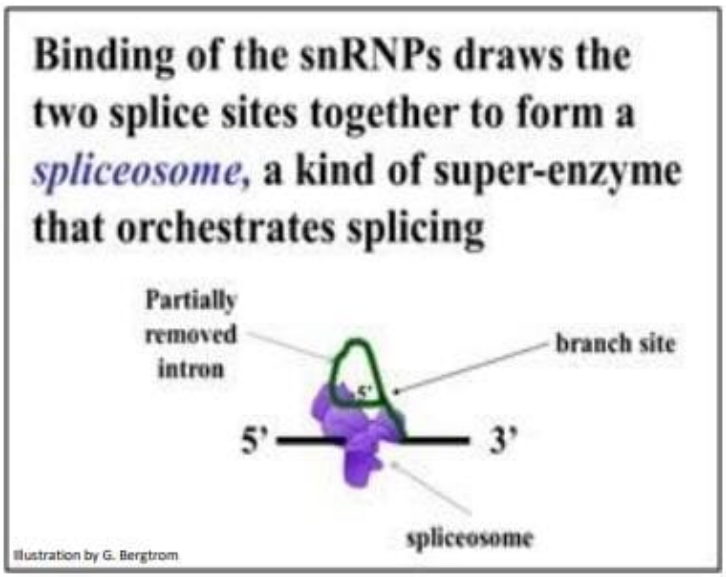


Splicing involves a number of *small ribonuclear proteins* (**snRNPs**). snRNPs are particles composed of RNA and proteins. They bind to specific sites in an mRNA and then direct a sequential series of cuts and ligations (the *splicing*) necessary to process the mRNAs.

The role of snRNPs in splicing pre-mRNAs is illustrated below.



snRNP binding to a pair of splice sites flanking an intron in a pre-mRNA forms the **spliceosome** that completes the splicing, including removal of the **lariat** (the intermediate structure of the intron). The last step is to ligate exons into a continuous mRNA with all its codons intact and ready for translation. Spliceosome action is summarized below.

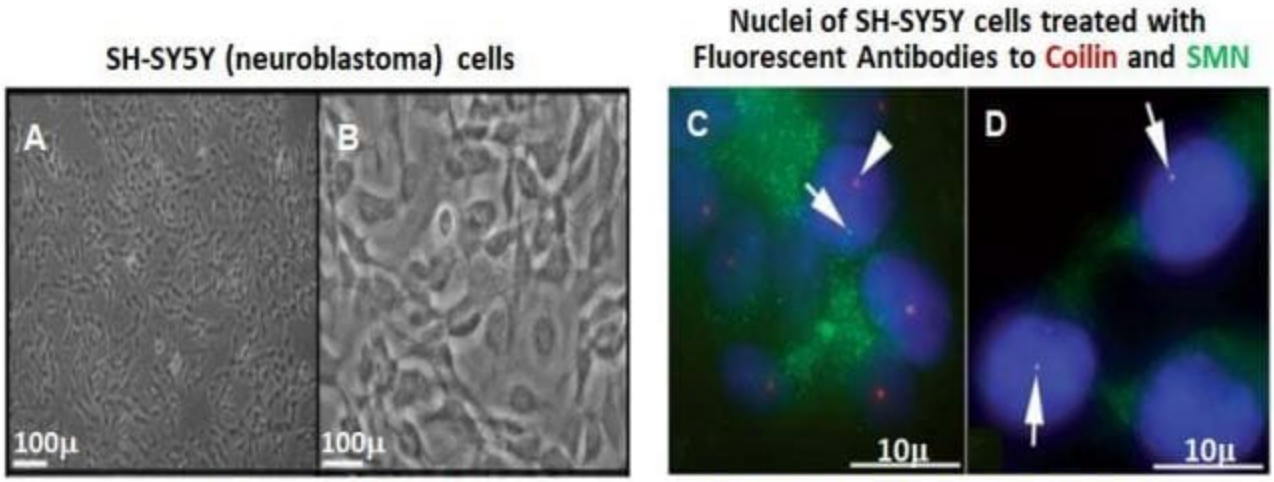


194 The Discovery of Split Genes

195 mRNA Splicing

B. Specific Nuclear bodies and their associated proteins facilitate the assembly and function of the SnRNPs

Recall the organization of nuclei facilitated by nuclear bodies. **Cajal bodies** (**CBs**) and **Gems** are nuclear bodies that are similar in size and have related functions in assembling spliceosomal SnRNPs. Some splicing defects correlate with mutations in the *coil* protein that associate with Cajal bodies; others correlate with mutations in SMN proteins normally associated with Gems. An hypothesis was that CBs and Gems interact in SnRNP and spliceosome assembly..., but how? Consider the results of an experiment in which antibodies to **coilin** and the **SMN** protein were localized in undifferentiated and differentiated neuroblastoma cells.



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A and C are undifferentiated cells in culture; B and D are cells that were stimulated to differentiate. In the fluorescence micrographs at the right, arrows point to fluorescent nuclear bodies. The coilin protein is associated with CBs and SMN is found in Gems. Therefore, we expect that fluorescent antibodies to coilin (green) will localize to CBs and antibodies to SMN protein (red) will bind to Gems. This is what happens in the nuclei of undifferentiated cells (panel C). But in panel D, the two antibodies colocalize, suggesting that the CBs and Gems aggregate in the differentiated cells. This would explain the need for both functional coilin and SMN protein to produce functional SnRNPs. The CBs and Gems may be aggregating in differentiated cells due to an observed increase in expression of the SMN protein. This could lead to more active Gems more able to associate with the CBs.

This and similar experiments demonstrate that different nuclear bodies do have specific functions. They are not random structural artifacts, have evolved to organize nuclear activities in time and space in ways that are essential to the cell.

C. Group I and Group II *Self-Splicing* Introns

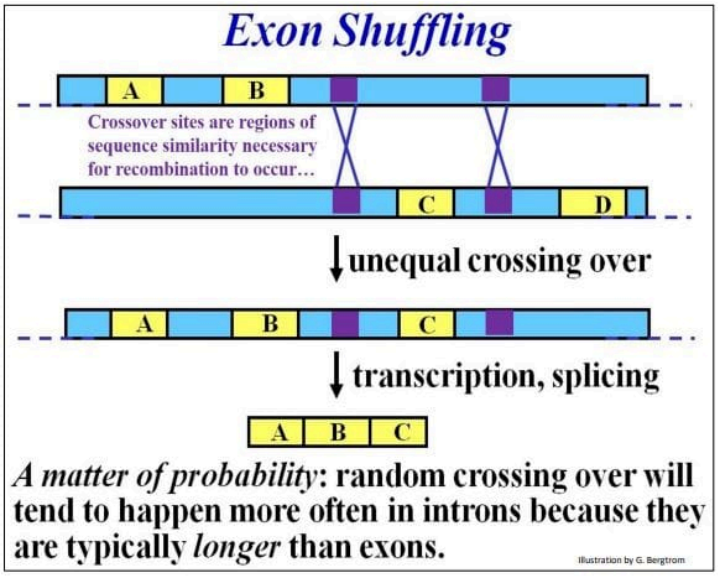
While Eukaryotic *Spliceosomal introns* are spliced using snRNPs as described above, *Group I* or *Group II* introns are removed by different mechanisms. **Group I introns** interrupt mRNA and tRNA genes in bacteria and in mitochondrial and chloroplast genes. They are occasionally found in bacteriophage genes, but rarely in nuclear genes, and then only in lower eukaryotes. Group I introns are *self-splicing*! Thus, they are *ribozymes* that do not require snRNPs or other proteins. Instead, they fold into a secondary stem-loop structure that positions catalytic nucleotides at appropriate splice sites, excise themselves, and re-ligate the exons. **Group II introns** in chloroplast and mitochondrial rRNA, mRNA, tRNA and some bacterial mRNAs can be quite long, form complex stem-loop tertiary structures, and self-splice, at least in a test tube! However, *Group II introns* encode proteins required for their own splicing *in vivo*. Like spliceosomal introns, they form a lariat structure at an A residue branch site. All this suggests that the mechanism of spliceosomal intron splicing evolved from that of *Group II* introns.

D. So, Why Splicing?

The puzzle implied by the question of course is why higher organisms have split genes in the first place. While the following discussion can apply to all splicing, it will reference mainly spliceosomal introns. Here are some answers to the question “Why splicing?”

- Introns in nuclear genes are typically longer (often much longer!) than exons. Since they are non-coding, they are large targets for mutation. In effect, noncoding DNA, including **introns can buffer the ill effects of random mutations**.
- You may recall that gene duplication on one chromosome (and loss of a copy from its homolog) arise from unequal recombination (non-homologous crossing over). It occurs when similar DNA sequences align during synapsis of meiosis. In an organism that inherits a chromosome with both gene copies, the duplicate can accumulate mutations as long as the other retains original function. The diverging gene then becomes part of a pool of selectable DNA, the grist of evolution, in the descendants of organisms that inherit the duplicated genes, increasing species diversity. Unequal recombination can also occur between similar sequences (e.g., in introns) in the same or different genes. Introns can also enable the sharing of exons between genes. After unequal recombination between introns flanking an exon, one gene will acquire another exon

while the other will lose it. Once again, as long as an organism retains a copy of the participating genes with original function, the organism can make the required protein and survive. Meanwhile, the gene with the extra exon may produce the same protein, but one with a new structural domain and function. Like a complete duplicate gene, one with a new exon and added function is in the pool of selectable DNA. Thus, this phenomenon of **exon shuffling increases species diversity**! The evidence indicates that exon shuffling has occurred, creating proteins with different overall functions that nonetheless share at least one domain and one common function. An example discussed earlier involves calcium-binding proteins that regulate many cellular processes. Structurally related calcium (Ca++) binding domains are common to many otherwise structurally and functionally unrelated proteins. Consider exon shuffling in the unequal crossover (non-homologous recombination) illustrated below.



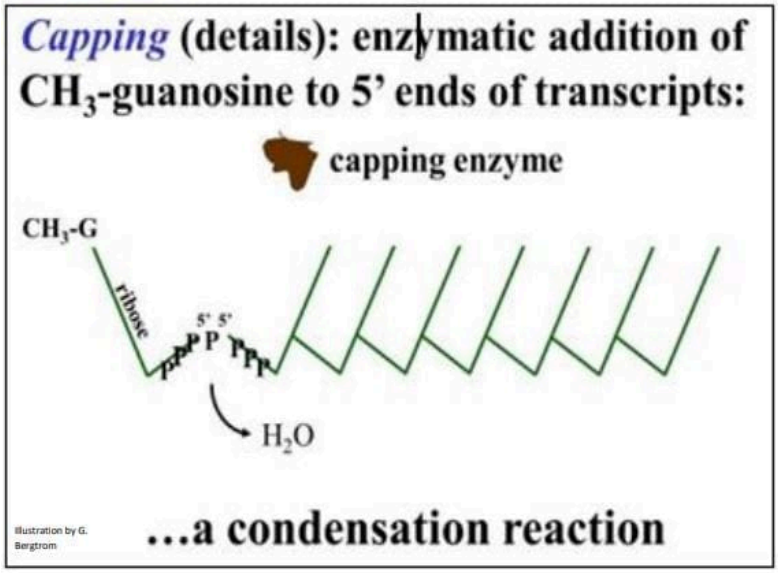
In this example, regions of strong similarity in different (non-homologous) introns in the same gene align during synapsis of meiosis. Unequal crossing over between the genes inserts exon C in one of the genes. The other gene loses the exon (not shown in the illustration). In sum, introns are *buffers against deleterious mutations*, and equally valuable, are potential targets for gene duplication and exon shuffling. This makes introns key players in creating *genetic diversity, the hallmark of evolution*.

196 Origin of Introns

197 Intron Evolution-What was selected here?

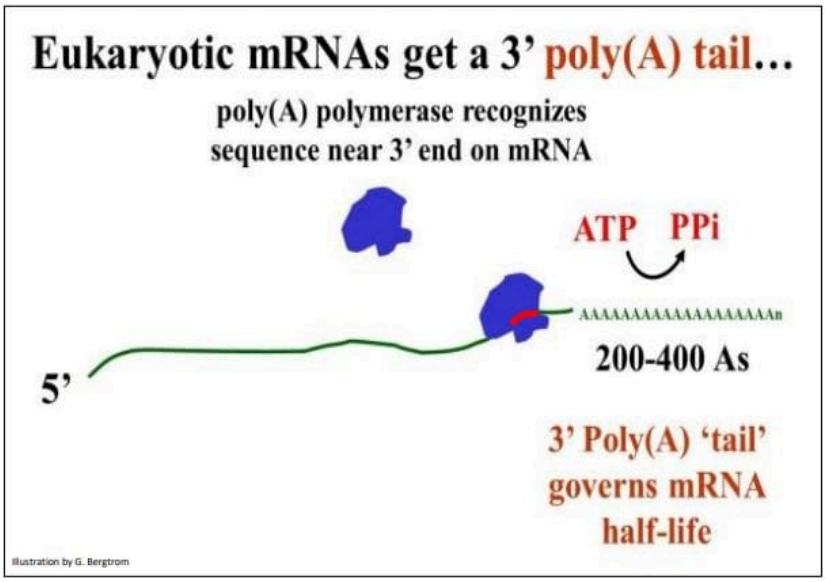
E. Capping

A **methyl guanosine cap** added 5'-to-5' to an mRNA functions in part to help mRNAs leave the nucleus and associate with ribosomes. The cap is added to an exposed 5' end, even as transcription and splicing are still in progress. A *capping enzyme* places a methylated guanosine residue at the 5'-end of the mature mRNA. The 5' cap structure is shown below (check marks are 5'-3' linked nucleotides).



F. Polyadenylation

After transcription termination, **poly(A) polymerase** catalyzes the addition of multiple AMP residues (several hundred in some cases) to the 3' terminus by the enzyme. The enzyme binds to an **AAUAA** sequence near the 3' end of an mRNA and begins to catalyze the addition of the adenosine monophosphates. The AAUAA *poly(A)* recognition site is indicated in red in the illustration of polyadenylation shown below.



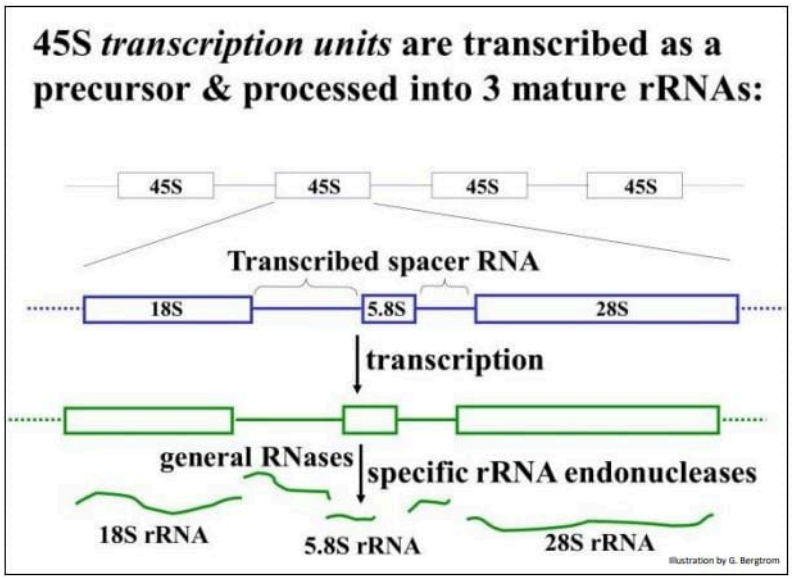
The result of *polyadenylation* is a 3' **poly (A) tail** whose functions include assisting in the transit of mRNAs from the nucleus and regulating the half-life of mRNAs in the cytoplasm. The poly (A) tail shortens each time a ribosome completes translating the mRNA.

198 mRNA 5' Capping and 3' Polyadenylation

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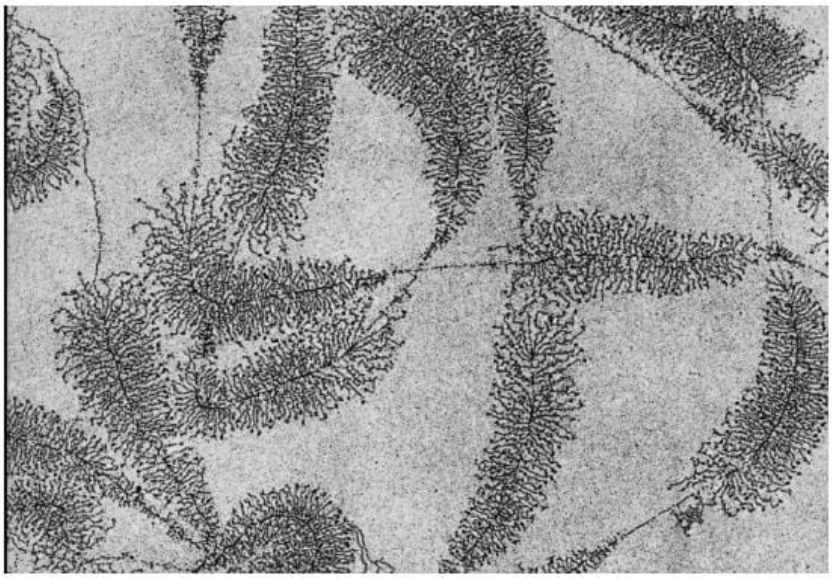
10.5: Ribosomal RNA Processing in Eukaryotic Nuclei

In most eukaryotes, a large rRNA gene in most eukaryotes transcribes a 45S precursor transcript containing (from shortest to longest) 5.8S rRNA, 18SrRNA and 28S rRNA. The 'S' stands for Svedberg, the biochemist who developed the *sedimentation velocity ultracentrifugation* technique to separate molecules like RNA by size. The higher the S value, the larger the molecule and therefore the faster it moves through the viscous sugar gradient during centrifugation. RNA Polymerase I transcribes 45S precursor rRNAs (preRNAs) from multiple large transcription units in the genome (shown below).



The 45S pre-rRNA is processed by cleavage. The many copies (200-400!) of the 45S gene in eukaryotic cells might be expected, since making proteins (and therefore ribosomes) will be an all-consuming cellular activity. In humans, 45S genes (45S rDNA) are distributed among five acrocentric chromosomes (those that have a centromere very near one end of the chromosome). The 45S rDNA in chromosomes is packed in the **nucleolus** inside nuclei.

Because these genes are present in so many copies and organized into a specific region of chromatin, it is possible to visualize 45S transcription in progress in electron micrographs such as the ones below.



The term *lampbrush* came from the shape of the 45S genes in the process of transcription; the RNAs extending from the DNA template look like an old-fashioned brush used to clean the chimney of a kerosene lamp.

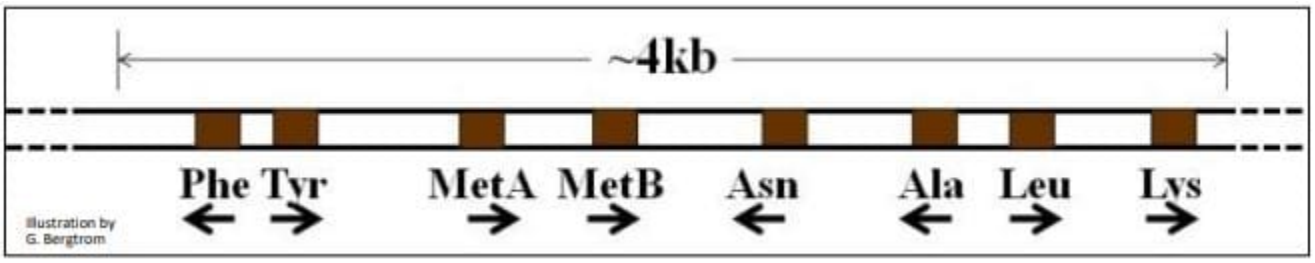
Multiple gene copies encode 5S rRNAs. However, unlike the 45S rRNA genes, 5S rRNA gene may be spread among many chromosomes (seven in *Neurospora crassa*, the bread mold). Or in the case of humans, 5S RNA gene copies are distributed along chromosome 1. The 5S rRNA genes are transcribed by RNA polymerase III with minimal posttranscriptional processing. As already noted, the promoters of the 5S genes are within the transcribed part of the genes, rather than upstream of their 5S transcription units.

199 rRNA Transcription and Processing

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10.6: tRNA Processing in Eukaryotic Nuclei

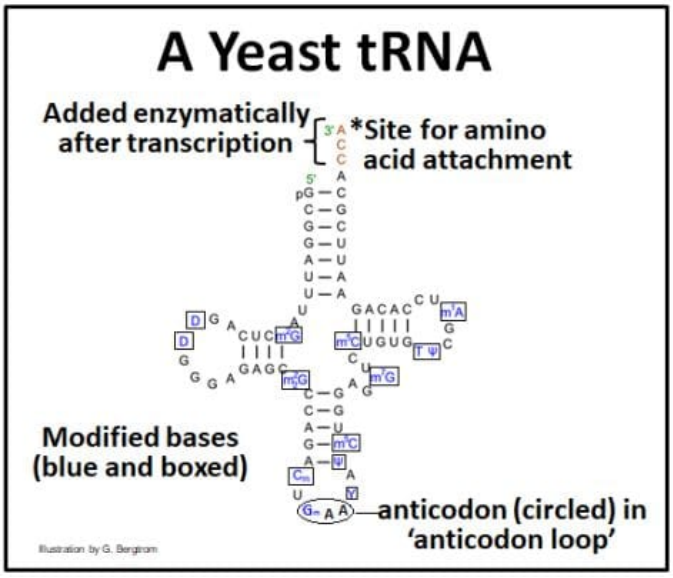
RNA polymerase III also transcribes tRNA genes from internal promoters, but unlike the 5S rRNA genes, tRNA genes tend to cluster in the genome (below).



tRNA primary transcripts are processed by

- trimming,
- enzymatic addition of -C-C-A base triplet at the 3' end
- the modification of bases internal to the molecule

A yeast tRNA showing these modifications is illustrated below.



The tRNA folds into several hairpin loops based on internal H-bond formation between complementary bases in the molecule. The 3'-terminal A residue of this (and every) tRNA will bind to an amino acid specific for the tRNA.

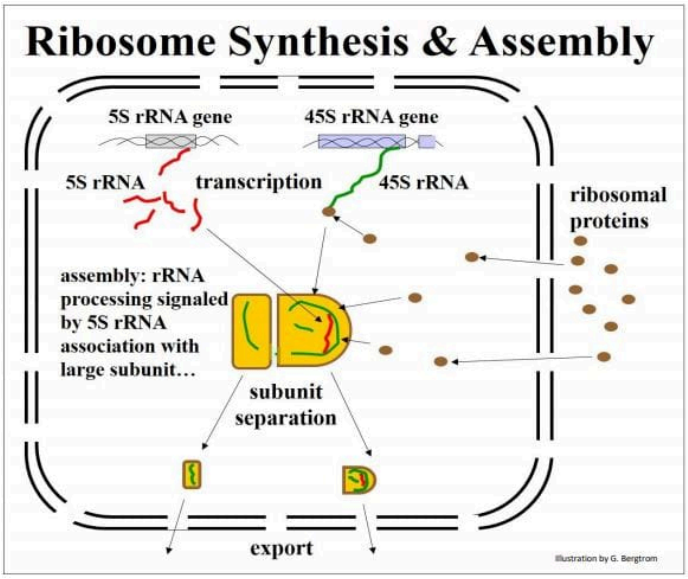
200 tRNA Transcription and Processing

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10.7: RNA and Ribosome Export from the Nucleus

A. rRNA and Ribosomes

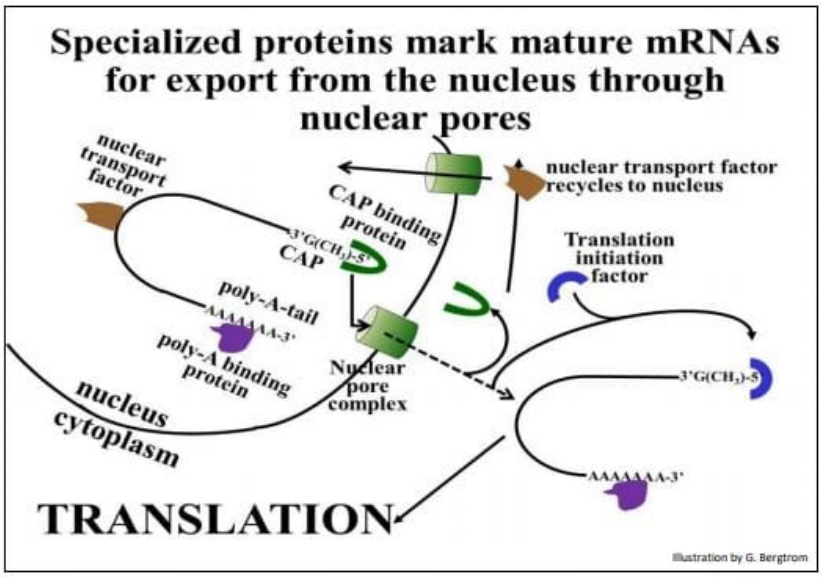
The synthesis and processing of rRNAs are coincident with the assembly of the ribosomal subunits, as shown below.



The 45S pre-rRNAs initially bind to ribosomal proteins in the nucleolus (that big nuclear body!) to initiate assembly and then and serve as a scaffold for the continued addition of ribosomal proteins to both the small and large ribosomal subunits. After the 5S rRNA added to the nascent large ribosomal subunit, processing (cleavage) of 45S rRNA is completed and the subunits are separated. The separated ribosomal subunits exit the nucleus o the cytoplasm where they will associate with mRNAs to translate new proteins. To better understand what is going on, try summarizing what you see here in the correct order of steps. You can also see this process animated at this link: here.

B. mRNA

The 5' methyl guanosine cap and the poly(A) tail collaborate to facilitate exit of mRNAs from the nucleus into the cytoplasm. We now understand that proteins in the nucleus participate in the export process. A *nuclear transport receptor* binds along the mature (or maturing) mRNA, a *poly-A-binding protein* binds along the poly-A tail of the message, and another protein binds at or near the methyl guanosine CAP itself. These interactions enable transport of the mRNA through nuclear pores. After the mRNA is in the cytoplasm, the nuclear transport receptor re-cycles back into the nucleus while a *translation initiation factor* replaces the protein bound to the CAP. The nuclear transport process is summarized in the illustration below.



See a more detailed description of mRNA transport from the nucleus at this link: here. The mature mRNA, now in the cytoplasm, is ready for translation. Translation is the process of protein synthesis mediated by ribosomes and a host of translation factors (including the initiation factor in the illustration above. The genetic code directs polypeptide synthesis during translation. Details of translation will be discussed shortly.

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10.8: Key Words and Terms

16S rRNA	internal promoters	rRNA
18S rRNA	introns	rRNA cleavage
23S rRNA	lariat	rRNA endonucleases
28S rRNA	mature RNA transcript	σ-factor
45S pre-rRNA	mRNA	SINEs
45S rRNA methylation	mRNA capping	snRNP
4S rRNA	mRNA polyadenylation	spacer RNA
5'-methyl guanosine capping	mRNA splicing	splice sites
5S rRNA	operons	spliceosome
8S rRNA	poly (A) polymerase	Svedburg unit
adenine	poly (A) tail	TATA binding protein
Alu	polycistronic RNA	TBP
branch sites	Pribnow box	termination
crossing over	promoter	TFIIB, TFGIIE, TFIIF, TFIIH
cytosine	recombination	transcription
DNA binding proteins	regulatory DNA sequence	transcription start site
E. coli RNA polymerase	regulatory factor	transcription unit
elongation	rho termination factor	translation
eukaryotic RNA polymerases	rho-independent termination	transposition
exon shuffling	ribonucleoproteins	transposons
exons	RNA polymerase I	tRNA
guanine	RNA polymerase II	tRNA processing
helitrons	RNA polymerase III	upstream v. downstream
helix-turn-helix motif	RNA processing	uracil
initiation	RNA secondary structure	

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11: THE GENETIC CODE AND TRANSLATION

11.1: Introduction

11.2: An Overview of the Genetic Code

11.3: Gene and Protein Colinearity and Triplet Codons

11.4: Translation

11.5: Key Words and Terms

Thumbanil: Diagram of RNA translation. (CC BY 3.0 - unported; Kelvinsong).

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11.1: Introduction

We begin this chapter with a look at how the **genetic code** was broken (deciphered). The very terms genetic code, broken and deciphered came from what was at the time, the recent history of the World War II. Winning WWII relied heavily on breaking enemy codes (recall the **Enigma machine**), and hiding strategic battle information from the enemy (recall the Navajo *code talkers*). We will look at the elegant experiments that first deciphered the amino acid meaning of a few 3-base **codons**, and then all 64 codons. Of these, 61 encode amino acids and three are **stop codons**. The same kinds of experiments that broke the genetic code also led to our under-standing of the mechanism of protein synthesis. Early studies indicated that genes and proteins are colinear, i.e., that the length of a gene was directly proportional to the polypeptide it encoded. It would follow then, that the lengths of mRNAs are also collinear with their translation products.

Colinearity suggested the obvious hypotheses that translation proceeded in three steps (**initiation**, **elongation** and **termination**), just like transcription itself. We now know that initiation is a complex process involving the assembly of a translation machine near the 5' end of the mRNA. This machine consists of ribosomes, mRNA, several **initiation factors** and a source of chemical energy. Since *mature* mRNAs are actually longer than needed to specify a polypeptide (even after splicing!), one function of initiation factors is to position the ribosome and associated proteins near a **start codon**. The start codon specifies the first amino acid in a new polypeptide. Once the **initiation complex** forms, elongation begins. Cycles of condensation reactions on the ribosome connect amino acids by peptide linkages, growing the chain from its amino-end to its carboxyl-end. Translation ends when the ribosome moving along the mRNA encounters a stop codon. We will look at how we came to understand the discrete steps of translation.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Compare and contrast the mechanisms and energetics of initiation, elongation and termination of translation and transcription.

2. Speculate on why the genetic code is universal (or nearly so).

3. Justify early thinking about a 4-base genetic code.

4. Justify early thinking about an *overlapping genetic code* (for example, one in which the last base of a codon could be the first base of the next codon in an mRNA.

5. Explain why all *tRNA structures* share some, but not other features.

6. Compare and Contrast the roles of ribosomal A, E and P sites in translation.

7. Trace the formation of an aminoacyl-tRNA and the bacterial Initiation Complex.

8. Describe the steps of translation that require chemical energy.

9. Formulate an hypothesis to explain why stop codons all begin with U.

10. Create a set of rules for inferring an amino acid sequence from a stretch of DNA sequence.

11. Speculate about why large eukaryotic genomes encode so few proteins.

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11.2: An Overview of the Genetic Code

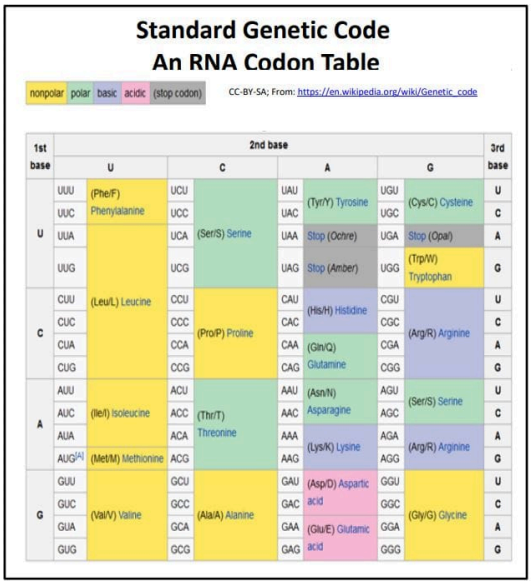
A. The (Nearly) Universal, Degenerate Genetic Code

The **genetic code** is the information for linking amino acids into polypeptides in an order based on the base sequence of 3-base code words (**codons**) in a gene and its messenger RNA (mRNA). With a few exceptions (some prokaryotes, mitochondria, chloroplasts), **the genetic code is universal** – it's the same in all organisms from viruses and bacteria to humans. The table of the **Standard Universal Genetic Code** on the next page shows the RNA version of triplet codons and their corresponding amino acids. There is a single codon for two amino acids (methionine and tryptophan), but two or more codons for each of the other 18 amino acids. For the latter reason, we say that the genetic code is **degenerate**. The three **stop codons** in the *Standard Genetic Code* 'tell' ribosomes the location of the last amino acid to add to a polypeptide. The last amino acid itself can be any amino acid consistent with the function of the polypeptide being synthesized. However, evolution has selected AUG as the **start codon** for all polypeptides, regardless of function, as well as for the placement of methionine within a polypeptide. Thus, all polypeptides begin life with a methionine at their amino-terminal end. As we will see in more detail, the mRNA translation machine is the **ribosome** and the *decoding device* is tRNA. Each amino acid attaches to a tRNA whose short sequence contains a 3-base *anticodon* that is complementary to an mRNA codon. Enzymatic reactions catalyze the **dehydration synthesis** (**condensation**) reactions that link amino acids in peptide bonds in the order specified by codons in the mRNA.

201 The Genetic Code Dictionary

B. Comments on the Nature and Evolution of Genetic Information

The near-universality of the genetic code from bacteria to humans implies that the code originated early in evolution. It is probable that portions of the code were in place even before life began. Once in place however, the genetic code was highly constrained against evolutionary change. The degeneracy of the genetic code enabled and contributed to this constraint by permitting base many base changes that do not affect the amino acid encoded in a codon.



The near universality of the genetic code and its resistance to change are features of our genomes that allow us to compare gene and other DNA sequences to establish evolutionary relationships between organisms (species), groups of organisms (genus, family, order, etc.) and even individuals within a species.

In addition to constraints imposed by a universal genetic code, some organisms show *codon bias*, a recent constraint on which universal codons an organism uses. Codon bias is seen in organisms preferably use A-T rich codons, or in organisms that favor codons richer in G and C. Interestingly, codon bias in genes often accompanies corresponding *genomic nucleotide bias*. An

organism with an AT codon bias may also have an AT-rich genome (likewise GC-rich codons in GC-rich genomes). You can recognize genome nucleotide bias in Chargaff's base ratios!

Finally, we often think of genetic information as genes for proteins. Obvious examples of *non-coding genetic information* include the genes for rRNAs and tRNAs, common to all organisms. The amount of these kinds of informational DNA (i.e., genes for polypeptides, tRNAs and rRNAs) as a proportion of total DNA can range across species, although it is higher in eukaryotes prokaryotes. For example, ~88% of the E. coli circular chromosome encodes polypeptides, while that figure is less ~1.5% for humans. Some less obvious informative DNA sequences in higher organisms are transcribed (e.g., introns). Other informative DNA in the genome is never transcribed. The latter include regulatory DNA sequences, DNA sequences that support chromosome structure and other DNAs that contribute to development and phenotype. As for that amount of truly non-informative (useless) DNA in a eukaryotic genome, that amount is steadily shrinking as we sequence entire genomes, identify novel DNA sequences and discover novel RNAs (topics covered elsewhere in this text).

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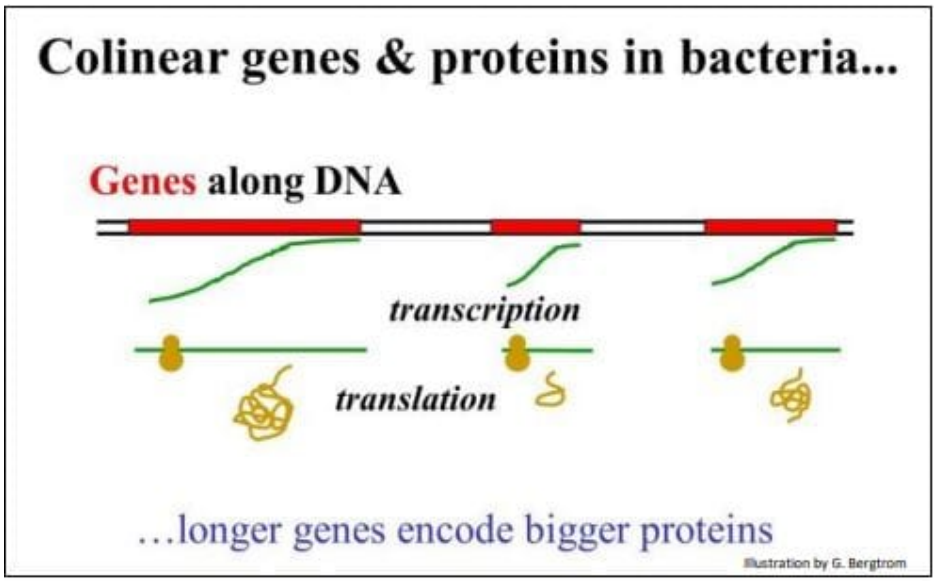
11.3: Gene and Protein Colinearity and Triplet Codons

Serious efforts to understand how proteins are encoded began after Watson and Crick used the experimental evidence of Maurice Wilkins and Rosalind Franklin (among others) to determine the structure of DNA. Most hypotheses about the genetic code assumed that DNA (i.e., genes) and polypeptides were **colinear**.

A. Colinearity

For genes and proteins, colinearity just means that the length of a DNA sequence in a gene is proportional to the length of the polypeptide encoded by the gene. The gene mapping experiments in *E. coli* already discussed certainly supported this hypothesis.

The concept of colinearity is illustrated below.

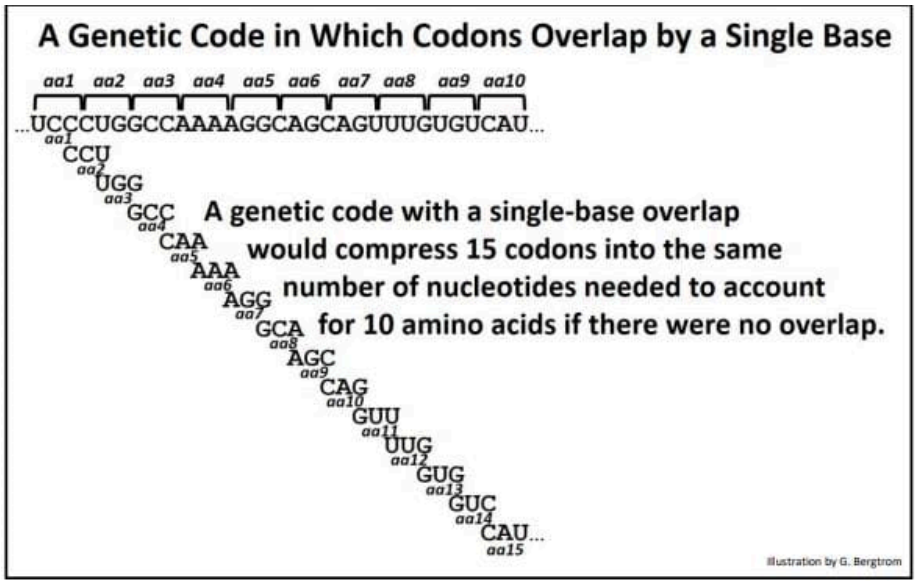


If the genetic code is collinear with the polypeptides it encodes, then a one-base **codon** obviously does not work because such a code would only account for four amino acids. A two-base genetic code also doesn't work because it could only account for 16 (4 2) of the twenty amino acids found in proteins. However, threenucleotide codons could code for a maximum of 43 or 64 amino acids, more than enough to encode the 20 amino acids. And of course, a 4-base code also works; it satisfies the expectation that genes and proteins are collinear, with the' advantage' that there would be 256 possible codons to choose from (i.e., 44 possibilities).

B. How is the Genetic Code 'Read' to Account for All of an Organisms' Gene?

George Gamow (a Russian Physicist working at George Washington University) was the first to propose **triplet codons** to encode the twenty amino acids, the simplest hypothesis to account for the colinearity of gene and protein, and for encoding 20 amino acids. One concern that was raised was whether there is enough DNA in an organism's genome to fit the all codons it needs to make all of its proteins? Assuming genomes did not have a lot of extra DNA laying around, how might genetic information be compressed into short DNA sequences in a way that is consistent with the colinearity of gene and polypeptide. One idea assumed 44 meaningless and 20 *meaningful* 3-base codons (one for each amino acid) and 44 *meaningless* codons, and that the *meaningful* codons in a gene (i.e., an mRNA) would be read and translated in an overlapping manner.

A code where codons overlap by one base is shown below.



You can figure out how compressed a gene could get with codons that overlapped by two bases. However, as attractive as an overlapping codon hypothesis was in achieving genomic economies, it sank of its own weight almost as soon as it was floated! If you look carefully at the example above, you can see that each succeeding amino acid would have to start with a specific base. A look back at the table of 64 triplet codons quickly shows that only one of 16 amino acids, those that begin with a C can follow the first one in the illustration. Based on amino acid sequences accumulating in the literature, virtually any amino acid could follow another in a polypeptide. Therefore, overlapping genetic codes are untenable. The genetic code must be non-overlapping!

Sidney Brenner and Frances Crick performed elegant experiments that directly demonstrated the non-overlapping genetic code. They showed that bacteria with a single base deletion in the coding region of a gene failed to make the expected protein. Likewise, deleting two bases from the gene. On the other hand, bacteria containing a mutant version of the gene in which three bases were deleted were able to make the protein. The protein it made was slightly less active than bacteria with genes with no deletions.

The next issue was whether there were only 20 **meaningful** codons and 44 **meaningless** ones. If only 20 triplets actually encoded amino acids, how would the translation machinery recognize the correct 20 codons to translate? What would prevent the translational machinery from 'reading the wrong' triplets, i.e., reading an mRNA *out of phase*? If for example, if the translation machinery began reading an MRNA from the second or third bases of a codon, it would likely encounter a meaningless 3-base sequence in short order.

One speculation was that the code was *punctuated*. That is, perhaps there were the chemical equivalent of commas between the meaningful triplets. The commas would be of course, additional nucleotides. In such a punctuated code, the translation machinery would recognize the 'commas' and would not translate *any* meaningless 3- base triplet, avoiding out-of-phase translation attempts. Of course, a code with nucleotide 'commas' would increase the amount of DNA needed to specify a polypeptide by a third!

Then, Crick proposed the *Commaless Genetic Code*. He divided the 64 triplets into 20 *meaningful* codons that encoded the amino acids, and 44 *meaningless* ones that did not. The result was such that when the 20 *meaningful* codons are placed in any order, any of the triplets read in overlap would be among the 44 *meaningless* codons. In fact, he could arrange several different sets of 20 and 44 triplets with this property! Crick had cleverly demonstrated how to read the triplets in correct sequence without nucleotide 'commas'.

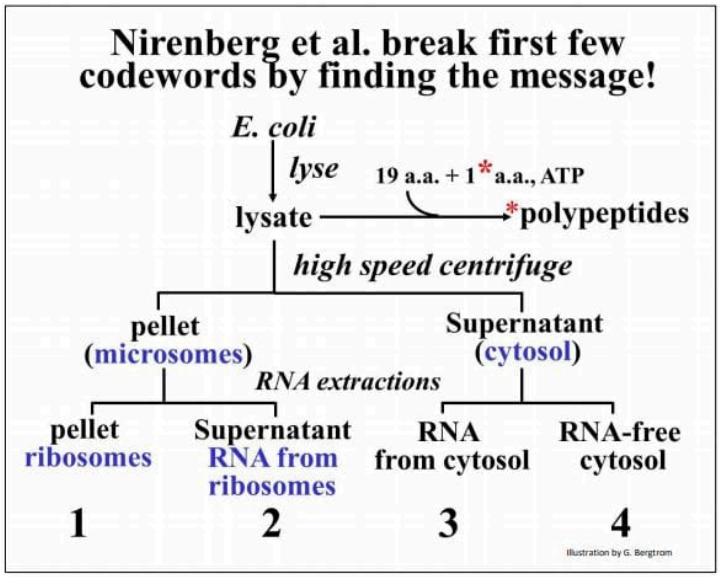
202 Speculations About a Triplet Code

As we know now, the genetic code is indeed 'commaless'... but not in the sense that Crick had envisioned. What's more, Thanks to the experiments described next, we know that ribosomes read the correct codons in the right order because they know exactly where to start!

C. Breaking the Genetic Code

When the genetic code was actually broken, it was found that 61 of the codons specify amino acids and therefore, that the code is **degenerate**. Breaking the code began when Marshall Nirenberg and Heinrich J. Matthaei decoded the first triplet. They fractionated *E. coli* and identified which fractions had to be added back together in order to get polypeptide synthesis in a test tube (*in vitro* translation).

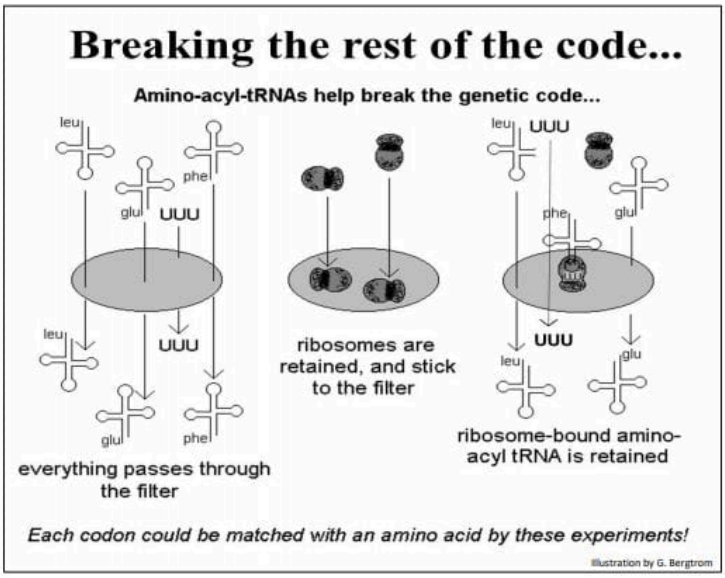
The cell fractionation is summarized below.



Check out the original work in the classic paper by Nirenberg MW and Matthaei JH [(1961) *The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribo-nucleotides*. Proc. Natl. Acad. Sci. USA 47:1588-1602]. The various cell fractions isolated by this protocol were added back together along with amino acids (one of which was radioactive) and ATP as an energy source. After a short incubation, Nirenberg and his coworkers looked for the presence of high molecular weight radioactive proteins as evidence of cell-free protein synthesis.

They found that all four final sub-fractions (1-4 above) must be added together to make radioactive proteins in the test tube. One of the essential cell fractions consisted of RNA that had been gently extracted from ribosome (fraction 2 in the illustration). Reasoning that this RNA might be mRNA, they substituted a synthetic poly(U) preparation for this fraction in their cell-free protein synthesizing mix, expecting poly(U) to encode a simple repeating amino acid.

They set up 20 reaction tubes, with a different amino acid in each..., and made only poly-phenylalanine. The experiment is illustrated below.



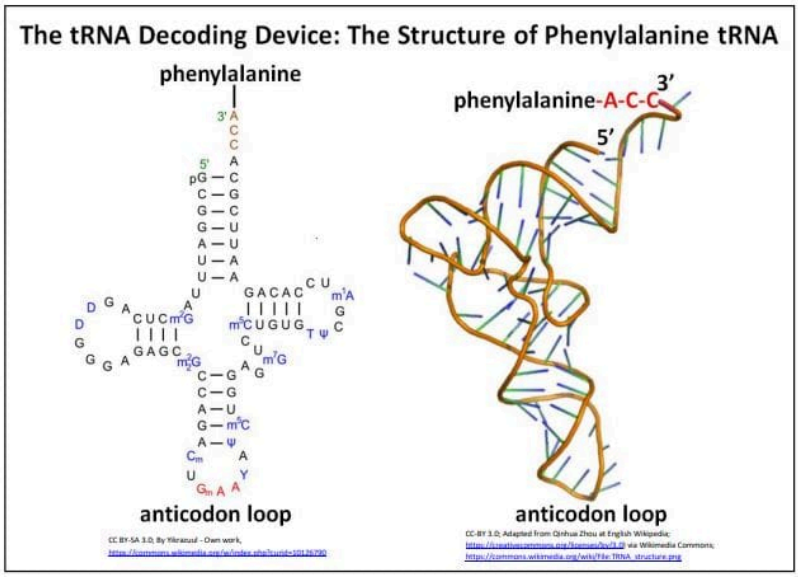
So, the triplet codon UUU means *phenylalanine*. Other polynucleotides were synthesized by G. Khorana, and in quick succession, poly(A) and poly(C) were shown to make poly-lysine and poly-proline in this experimental protocol. Thus AAA and CCC must encode lysine and proline respectively. With a bit more difficulty and ingenuity, poly di- and tri-nucleotides were also used in the cell free system to decipher several additional codons.

203 Deciphering the First Codon

M. W. Nirenberg, H. G. Khorana and R. W. Holley shared the 1968 Nobel Prize in Physiology or Medicine for their contributions to our understanding of protein synthesis. Deciphering the rest of the genetic code was based on Crick's realization that chemically, amino acids have no attraction for either DNA or RNA (or triplets thereof). Instead, he predicted the existence of an **adaptor molecule** that would contain nucleic acid and amino acid information *on the same molecule*. Today we recognize this molecule as tRNA, the genetic *decoding device*.

Nirenberg and Philip Leder designed the experiment that pretty much *broke* the rest of the genetic code. They did this by adding individual amino acids to separate test tubes containing tRNAs, in effect causing the synthesis of specific aminoacyl-tRNAs.

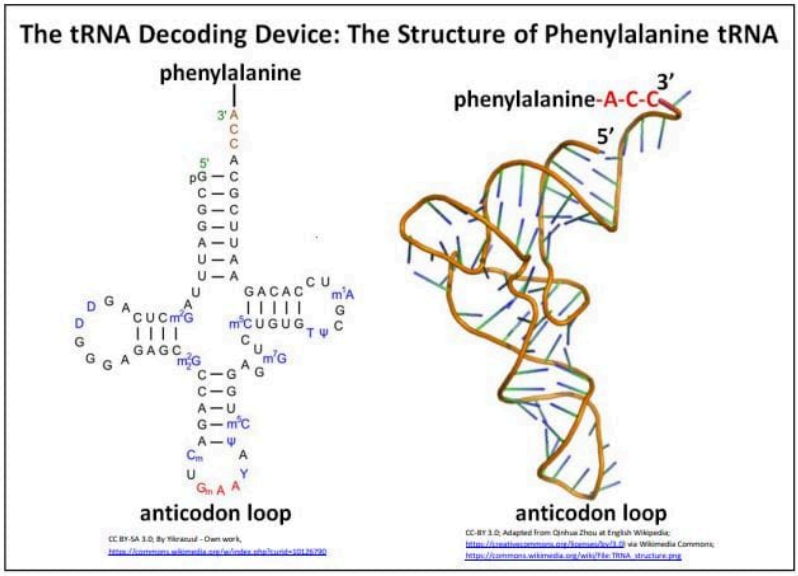
They then mixed their amino acid-bound tRNAs with isolated ribosomes and synthetic triplets. Since they had already shown that synthetic three-nucleotide fragments would bind to ribosomes, they hypothesized that triplet-bound ribosomes would in turn, bind appropriate amino acid-bound tRNAs. The experiment is shown below.



Various combinations of tRNA, ribosomes and aminoacyl-tRNAs were placed over a filter. Nirenberg and Leder knew that aminoacyl-tRNAs alone passed through the filter and that ribosomes did not. They predicted then, that triplets would associate with the ribosomes, and further, that this complex would bind the tRNA with the amino acid encoded by the bound triplet. This 3-part complex would also be retained by the filter, allowing the identification of the amino acid retained on the filter, and therefore the triplet code-word that had enabled binding the amino acid to the ribosome.

204 Deciphering all 64 Triplet Codons

After the code was largely deciphered, Robert Holley actually sequenced a yeast tRNA, and from regions of internal complementarity, predicted the folded structure of the tRNA. This first successful sequencing of a nucleic acid was possible because the tRNA was short, and contained several modified bases that facilitated the sequencing chemistry. Holley found the amino acid alanine at one end of the tRNA and he found one of the anticodons for an alanine codon roughly in the middle of the tRNA sequence. Holley predicted that this (and other) tRNAs would fold and assume a *stem-loop*, or *cloverleaf* structure with a central **anticodon loop**. The illustration below shows this structure for a phenylalanine tRNA along with subsequent computer-generated structures (below right) showing a now familiar "L"-shaped molecule with an **amino acid attachment site** at the 3'-end at the top of the molecule, and the *anticodon loop* at the other, bottom 'end'



205 tRNA Structure and Base Modifications

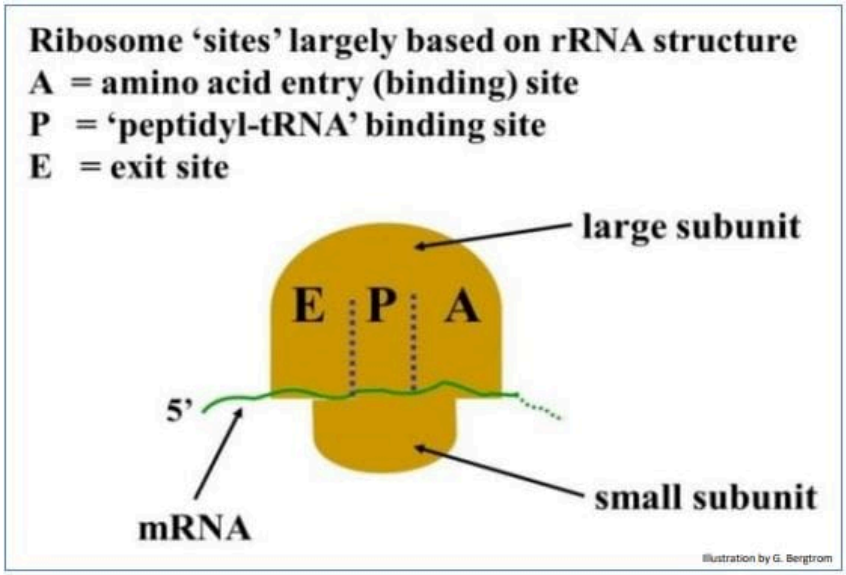
After a brief overview of translation, we'll break translation down into its 3 steps and see how aminoacyl-tRNAs function in the initiation and elongation steps of translation, as well as the special role of an *initiator* tRNA.

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11.4: Translation

A. Overview of Translation (Synthesizing Proteins)

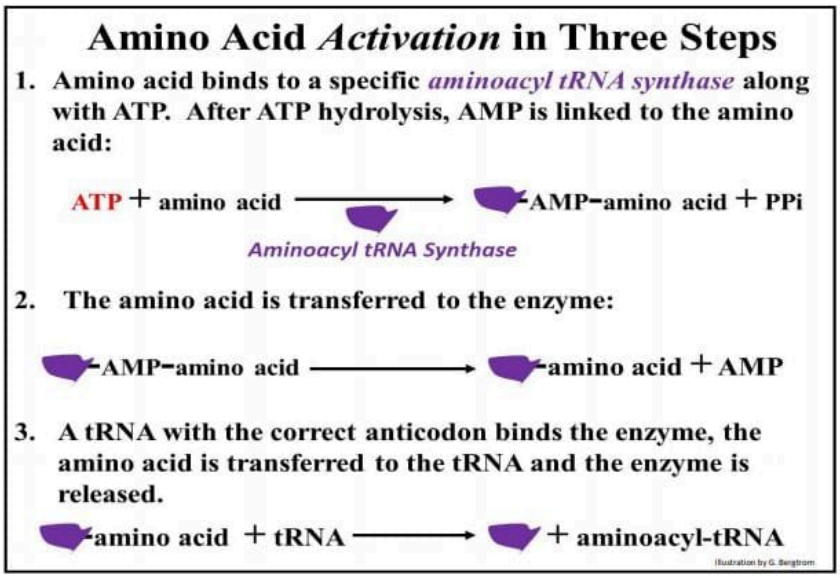
Like any polymerization in a cell, translation occurs in three steps: **initiation** brings a ribosome, mRNA and an *initiator* tRNA together to form an initiation complex. **Elongation** is the successive addition of amino acids to a growing polypeptide. **Termination** is signaled by sequences (one of the stop codons) in the mRNA and protein **termination factors** that interrupt elongation and release a finished polypeptide. The events of translation occur at specific **A**, **P** and **E** sites on the ribosome (see drawing below).



B. Translation - First Steps

1. Making Aminoacyl-tRNAs

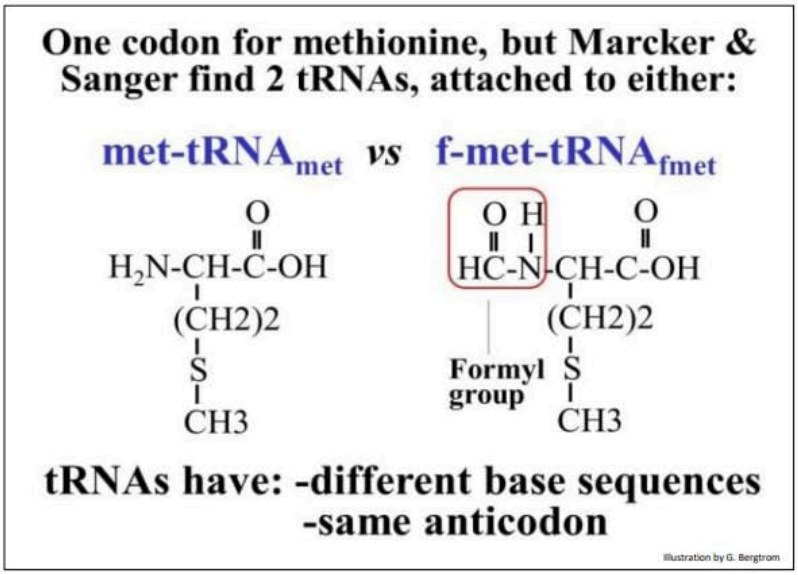
Translation is perhaps the most energy-intensive job a cell must do, beginning with the attachment of amino acids to their tRNAs. The basic amino-acylation reaction is the same for all amino acids. A specific **aminoacyl-tRNA** synthase attaches each tRNA to (**charges**) an appropriate amino acid. Charging tRNAs requires ATP and proceeds in three steps (shown below).



In the first step, ATP and an appropriate amino acid bind to the aminoacyl-tRNA synthase. ATP is hydrolyzed releasing a pyrophosphate (PPi) and leaving an enzyme-AMP-amino acid complex. Next, the amino acid is transferred to the enzyme, releasing the AMP. Finally, the tRNA binds to the enzyme, the amino acid is transferred to the tRNA and the intact enzyme is regenerated and released. The charged tRNA is ready to use in translation.

Several studies had already established that polypeptides are synthesized from their amino (N-) terminal end to their carboxyl (C-) terminal end. When it became possible to determine the amino acid sequences of polypeptides, it turned out that around 40% of *E. coli* proteins had an N-terminal methionine, suggesting that *all* proteins began with a methionine. It also turned out that, even though there is only one codon for methionine, two different tRNAs for methionine could be isolated. One of the tRNAs was bound to a methionine modified by *formylation*, called **formylmethionine-tRNA$_{fmet}$** (or **fmet-tRNAf** for short). The other was **methionine-tRNA$_{met}$** (**met-tRNA$_{met}$** for short), charged with an unmodified methionine.

Methionine and formylated methionine are shown below.



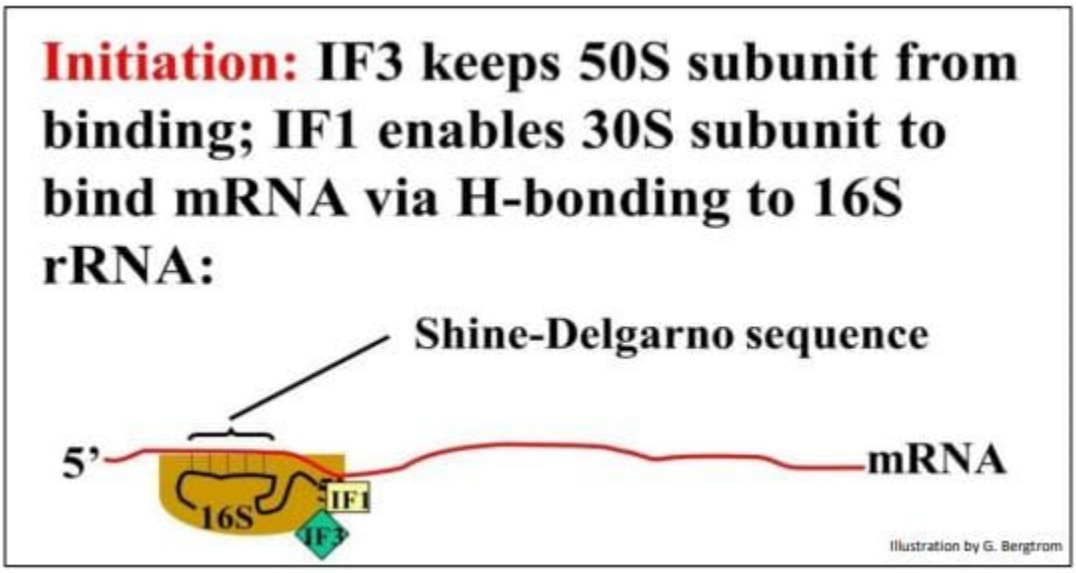
tRNA$_{met}$ and **tRNAf** each have an anticodon to AUG, the only codon for methionine, but have different base sequences encoded by different tRNA genes. tRNA$_{met}$ is used to insert methionine in the middle of a polypeptide. tRNAf is the initiator tRNA, and is only used to start new polypeptides with formylmethionine. In prokaryotes, methionine on met-tRNAf is formylated at its amino group to make the fmet-tRNAf. The formylating enzyme that does this does not recognize methionine on met-tRNA$_{met}$.

In *E. coli*, a *formylase* enzyme removes the formyl group from all N-terminal formyl methionines at some point after translation has begun. As we noted, the methionine itself (and sometimes more N-terminal amino acids) are also removed from about 60% of E. coli polypeptides. Eukaryotes have inherited both the initiator tRNAf and the tRNAmet, using only met-tRNAf during initiation. However, methionine on the eukaryotic initiator met-tRNAf is never formylated in the first place. What's more, methionine is absent from virtually all mature eukaryotic polypeptides.

Early in evolution, the need for an initiator tRNA must have ensured a correct starting point for translation on an mRNA and therefore growth of a polypeptide from one end to the other, that is, from its N- to its C-terminus. At one time, formylation of the N-terminal methionine may have served to block accidental addition of amino acids or other modifications at the N-terminus of a polypeptide. Today, formylation seems to be a kind of molecular appendix in bacteria. Since then, evolution (in eukaryotes at least) has selected other features to replace actual formylation as the protector of the N-terminus of polypeptides.

2. Initiation

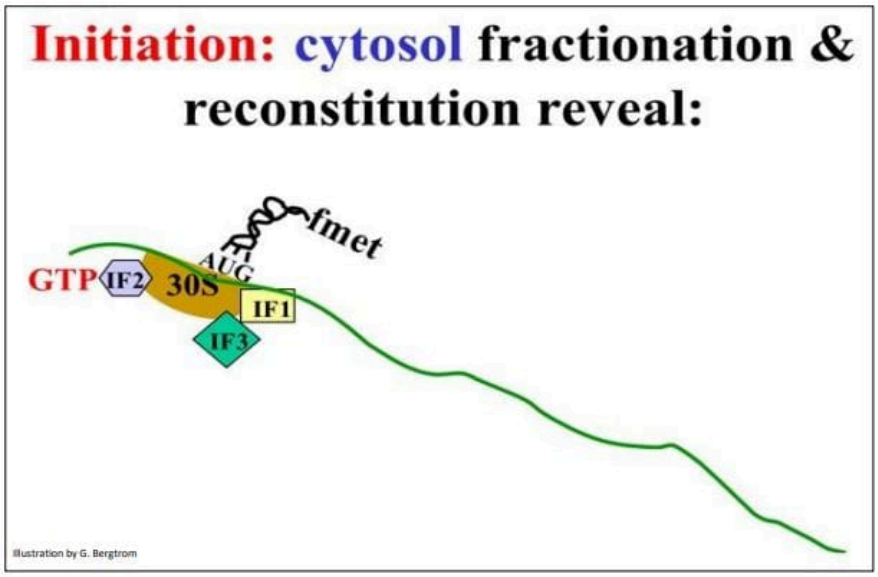
Now that we have charged the tRNAs, we can look more closely at the three steps of translation. Understanding translation initiation began with a molecular dissection of the components of E. coli cells required for cell-free (in vitro) protein synthesis, including cell fractionation, protein purification and reconstitution experiments. Initiation starts with when the **Shine-Delgarno** sequence forms Hbonds with a complementary sequence in the **16S rRNA** bound to 30S ribosomal subunit. The Shine-Delgarno sequence is a short nucleotide sequence in the 5' **untranslated region** (**5'-UTR**) of the messenger RNA, just upstream of the initiator AUG codon. This requires the participation of initiation factors **IF1** and **IF3**. In this event, IF1 and IF3 as well as the mRNA are bound to the 30S ribosomal subunit (below).



Demonstration of the binding of an mRNA to a ribosomal subunit required isolation and separation of the 30S ribosomal subunit, an RNA fraction of the cell, and the purification of initiation factor proteins from the bacterial cells. This was followed by reconstitution (adding the separated fractions back together) in the correct order show that mRNA would only bind to the 30S subunit in the presence of the two specific initiation factor proteins.

206 Translation Initiation: mRNA Associates with 30S Ribsomal Subunit

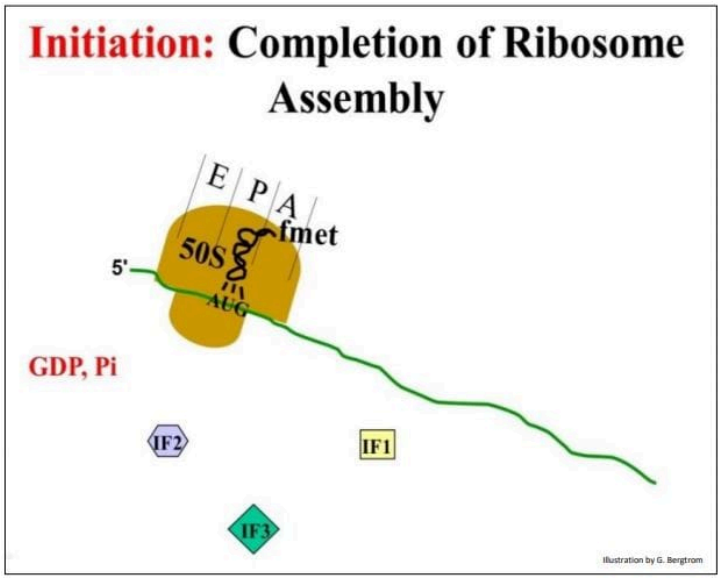
Next, with the help of GTP and another initiation factor (**IF2**), the initiator fmettRNAf recognizes and binds to the AUG start codon found in all mRNAs. Some call the resulting structure (shown below) the **Initiation Complex**, which includes the 30S ribosomal subunit, Ifs 1, 2 and 3, and the fmet-tRNAf.



207 Initiation Complex Formation

In the last step of initiation, the large ribosomal subunit binds to this complex. IFs 1, 2 and 3 disassociate from the ribosome and the initiator fmet-tRNA^{fmet} ends up in the P site of the ribosome.

Some prefer to call the structure formed at this point the initiation complex (below).



208 Adding the Large Ribosomal Subunit

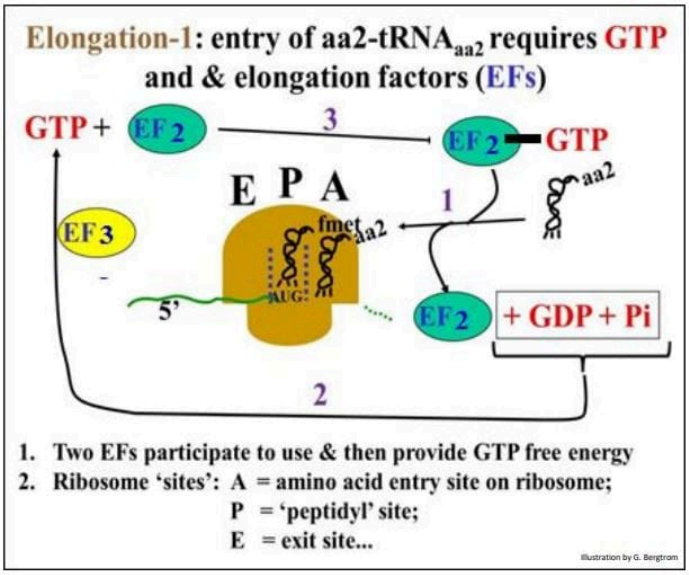
Initiation can happen multiple times on a single mRNA, forming the polyribosome, or polysome described in Chapter 1. Each of the complexes formed above will engage in the elongation of a polypeptide described next.

3. Elongation

Elongation is a sequence of protein factor-mediated condensation reactions and ribosome movements along an mRNA. As you will see, polypeptide elongation requires a considerable input of free energy.

a) Elongation 1

The first step in elongation is the entry of the next aminoacyl-tRNA (**aa2- tRNAaa2**), which requires the free energy of GTP hydrolysis. The energy is supplied by the hydrolysis of GTP bound elongation factor 2 (**EF2-GTP**). The aa2-tRNAaa2 enters the ribosome based on codon-anticodon interaction at the **A** site as shown below.

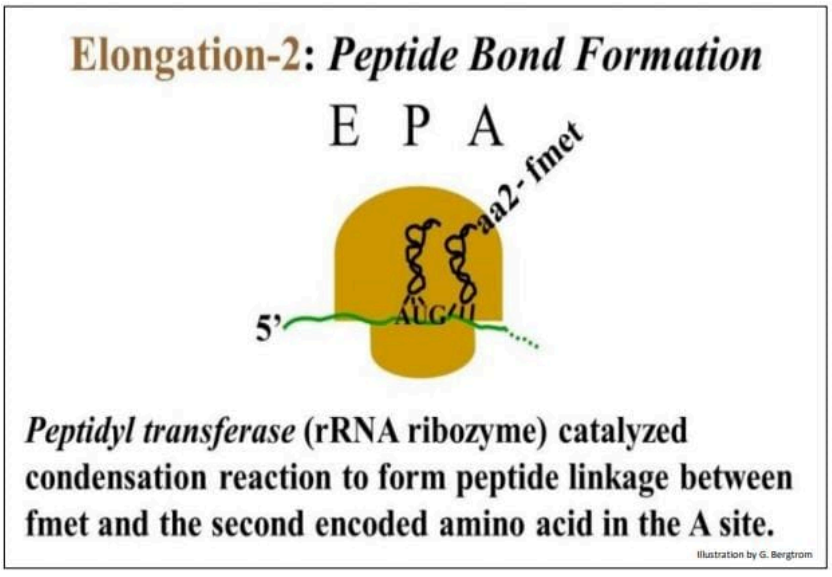


The GDP dissociates from EF2 as aa2-tRNAaa2 binds the anticodon in the A site. To keep elongation moving along, elongation factor (EF3) rephosphorylates the GDP to GTP, which can re-associate with free EF2.

209 Elongation: Elongation Factors and GTP

b) Elongation 2

Peptidyl transferase, a **ribozyme** component of the ribosome itself, links the incoming amino acid to a growing chain in a condensation reaction.

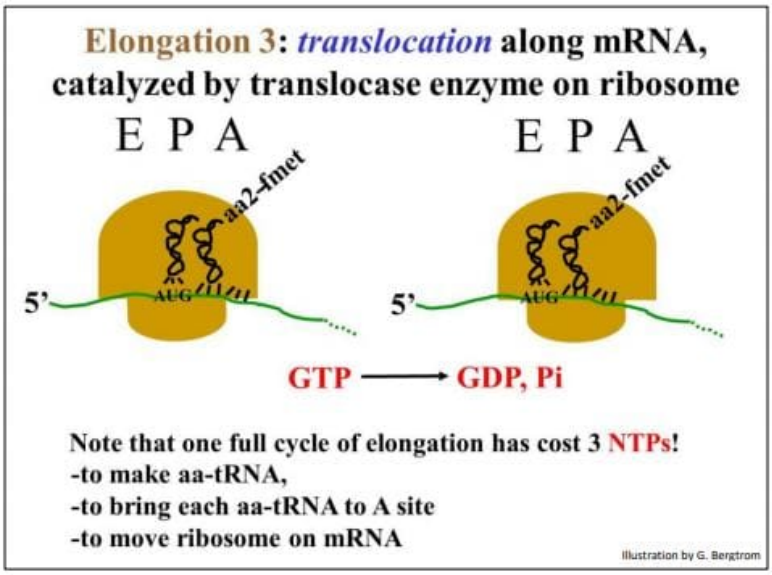


In this reaction, the fmet is transferred from the initiator tRNAf in the P site to aa2-tRNAaa2 in the A site, forming a peptide linkage with aa2.

c) Elongation 3

Translocase catalyzes GTP hydrolysis as the ribosome moves (translocates) along the mRNA. After translocation, the next mRNA codon shows up in the A site of the ribosome and the first tRNA (in this example, tRNAf) ends up on the E site of the ribosome.

The movement of the ribosome along the mRNA is illustrated below.



The tRNAf, no longer attached to an amino acid, will exit the E site as the next (3rd) aa-tRNA enters the empty A site, based on a specific codon-anticodon interaction (assisted by elongation factors and powered by GTP hydrolysis) to begin another cycle of elongation. Note that in each cycle of elongation, an ATP is consumed to attach each amino acid to its tRNA, and two GTPs are hydrolyzed in the cycle itself. In other words, at the cost of three NTPs, protein synthesis is the most expensive polymer synthesis reaction in cells!

211 Elongation: Translocase Moves Ribosomes along mRNA

212 Adding the Third Amino Acid

213 Big Translation Energy Costs

As polypeptides elongate, they eventually emerge from a groove in the large ribosomal subunit. As noted, a formylase enzyme in *E. coli* cytoplasm removes the formyl group from the exposed initiation fmet from all growing polypeptides. While about 40% of *E. coli* polypeptides still begin with methionine, specific proteases catalyze the hydrolytic removal of the amino-terminal methionine (and sometimes even more amino acids) from the other 60% of polypeptides. The removal of the formyl group and one or more N-terminal amino acids from new polypeptides are examples of post-translational processing.

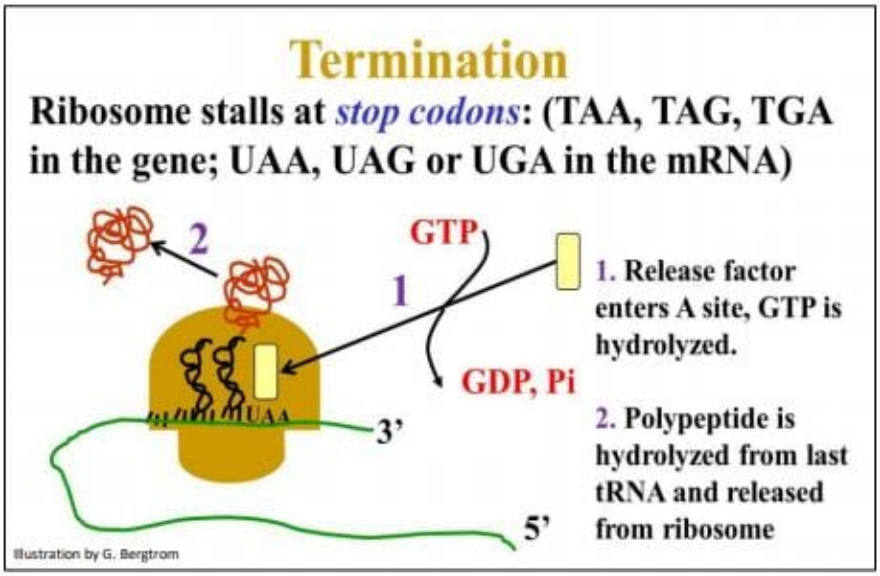
214 The Fates of fMet and Met: Cases of Post-Translational Processing

4. Termination

Translation of an mRNA by a ribosome ends when translocation exposes one of the three stop codons in the A site of the ribosome. Stop codons are not situated some distance from the 3' end of an mRNA. The region between a stop codon to the end of the mRNA is called the **3' untranslated region** of the messenger RNA (**3'UTR**).

Since there is no aminoacyl-tRNA with an anticodon to the stop codons (UAA, UAG or UGA), the ribosome actually stalls and the translation slow-down is just long enough for a protein **termination factor** to enter the A site. This interaction causes release of the new polypeptide and the disassembly of the ribosomal subunits from the mRNA. The process requires energy from yet

another GTP hydrolysis. After dissociation, ribosomal subunits can be reassembled with an mRNA for another round of protein synthesis. Translation termination is illustrated below.



215 Translation Termination

We have seen some examples of post-translational processing (removal of formyl groups in *E. coli*, removal of the N-terminal methionine from most polypeptides, etc.) Most proteins, especially in eukaryotes, undergo one or more additional steps of posttranslational processing before becoming biologically active. We will see examples in upcoming chapters.

Let's conclude this chapter with a "we thought we knew everything" moment! A recent study reports that ribosomes can sometimes **re-initiate** translation in the 3' UTR of an mRNA using AUG codons upstream of the normal start codon of the mRNA. There is evidence that the resulting short polypeptides may be functional! Click here to read more: here.

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11.5: Key Words and Terms

64 codons	genetic code	ribonucleoprotein
adapter molecules	initiation	ribosome
amino terminus	initiation complex	small ribosomal subunit
aminoacyl tRNA	initiation factors	start codon
aminoacyl tRNA synthase	large ribosomal subunit	termination
anticodon	meaningful codons	termination factor
AUG	mRNA, tRNA	translocation
bacterial bound ribosomes	nascent chains	triplets
Carboxyl-terminus	ochre, amber, opal	tRNA v. tRNAaa
colinearity	peptide linkage	UAG, UUA, UGA
comma-less genetic code	peptidyl transferase	universal genetic code
degenerate genetic code	polypeptide	UUU
elongation	polysome	Wobble Hypothesis
free v. bound ribosomes	reading phase	

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12: REGULATION OF TRANSCRIPTION AND EPIGENETIC INHERITANCE

12.1: Introduction
12.2: Gene Regulation in Prokaryotes
12.3: The Problem with Unregulated (Housekeeping) Genes in All Cells
12.4: Gene Regulation in Eukaryotes
12.5: Epigenetics
12.6: Key Words and Terms

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12.1: Introduction

Cells regulate their metabolism in several ways. We have already that allosterically regulated enzymes monitor the cellular levels of metabolites. Recall that glycolytic intermediates rise and fall in cells based on cellular energy needs, binding to or dissociating from **allosteric sites**. Allosteric enzymes respond to interaction with **allosteric effectors** with an increase or decrease in catalytic activity.

Cells can also control absolute levels of enzymes and other proteins by turning genes on and off, typically by controlling transcription. **Transcription regulation** usually starts with extracellular environmental signaling. The signals are chemicals in the in the air, in the water, or in the case of multicellular organisms, in blood, lymph or other extracellular fluids. Bacterial and protist genes often respond to environmental **toxins** or fluctuating **nutrient levels**. Familiar **signal molecules** in higher organisms include hormones released at the appropriate time in a sequential **developmental program** of gene expression, or in response to nutrient levels in body fluids.

Some signal molecules get into cells binding to **specific intracellular receptors** to convey their instructions. Others bind to cell surface **receptors** that transduce their 'information' into intracellular molecular signals. When signaling leads to gene regulation, responding cells ultimately produce **transcription factors**. These in turn recognize and bind to specific *regulatory DNA sequences* associated with the genes that they control. DNA sequences that bind transcription factors are relatively short. They can lie **proximal** (close) to the transcription start site of a gene, and/or in the case of eukaryotes, **distal** to (far from) it. We will see that binding some regulatory DNA sequences are **enhancers**, turning on or increasing gene transcription. Others are **silencers**, down-regulating, or suppressing transcription of a gene. Finally, DNA regulatory sequences are hidden behind a thicket of chromatin proteins in eukaryotes. When patterns of gene expression in cells change during development, chromatin is re-organized, cells differentiate, and new tissues and organs form. To this end, new patterns of gene expression and chromatin configuration in a cell must be remembered in its descendants. CMB3e 257

In this chapter, we look at the path from cell recognition of a signal molecule to the interaction of regulatory proteins with DNA in both prokaryotic and eukaryotic cells. We also consider how eukaryotic cells *remember* instructions that alter chromatin configuration and patterns of gene expression, topics in the field of **epigenetics**.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Compare and contrast *transcription factors* and so-called *cis-acting* elements.
2. Discuss the role of *DNA bending* in the regulation of gene expression.
3. Explain the benefits of organizing bacterial genes into *operons*, and *why* some bacterial genes are not part of operons
4. Compare and contrast regulation of the *lac* and *trp* operons in *E. coli*.
5. Define and describe *regulatory genes* and *structural genes* in *E. coli*.
6. Discuss why a fourth gene was suspected in *lac operon* regulation.
7. Distinguish between gene repression and de-repression and between *positive* and *negative* gene regulation, using examples. For example, explain how it is possible to have repression by positive regulation.
8. Draw and label all functional regions of prokaryotic and eukaryotic genes.
9. Compare and contrast different mechanisms of gene regulation in eukaryotic cells.
10. Describe the *transcription initiation complex* of a regulated gene in eukaryotes.
11. Define and articulate differences between *gene expression* and *transcription regulation*.
12. Define a gene
13. Distinguish between the roles of enhancers and other cis-acting elements in transcription regulation.
14. Compare and contrast the *genome* and the *epigenome*.

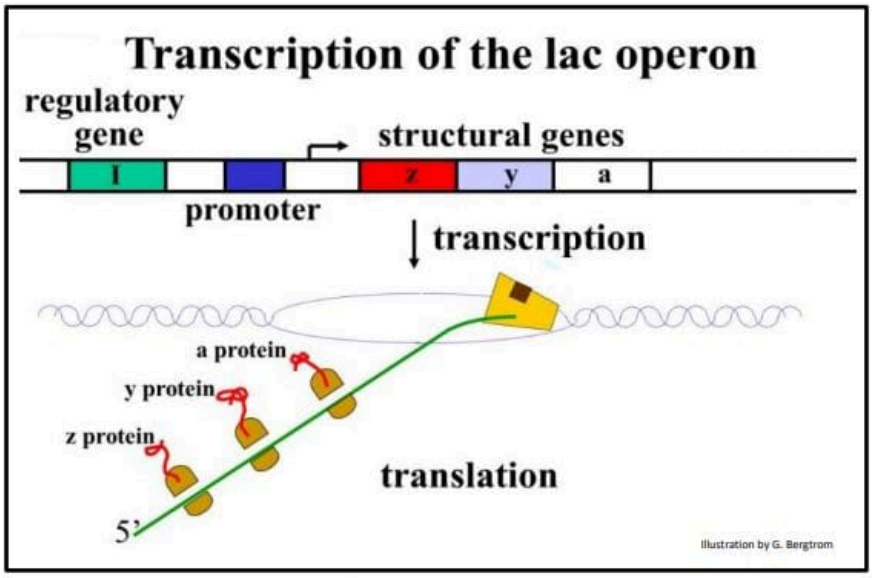
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12.2: Gene Regulation in Prokaryotes

Many prokaryotic genes are organized in **operons**, linked genes transcribed into a single mRNA encoding two or more proteins. Operons usually encode proteins with related functions. Regulating the activity of an operon (rather than multiple single genes encoding single proteins) allows better coordination of the synthesis of several proteins at once. In *E. coli*, the regulated **lac operon** encodes three enzymes involved in the metabolism of **lactose** (an alternative nutrient to glucose). Regulation of an operon (or of a single gene for that matter) can be by **repression** or by **induction**. When a small metabolite in a cell binds to a regulatory **repressor** or **inducer** protein, the protein undergoes an allosteric change that allows it to bind to a regulatory DNA sequence..., or to un-bind from the DNA. We will see examples of such regulation in the **lac** and **trp** operons. Lac operon gene regulation is an example of **gene repression** as well as **induction**. Trp (tryptophan) operon regulation is by **gene repression**. In both the operons, changes in levels of intracellular metabolites reflect the metabolic status of the cell and elicit appropriate changes in gene transcription. We will look at the regulation of both operons.

216 Overview of Prokaryotic Gene Regulation

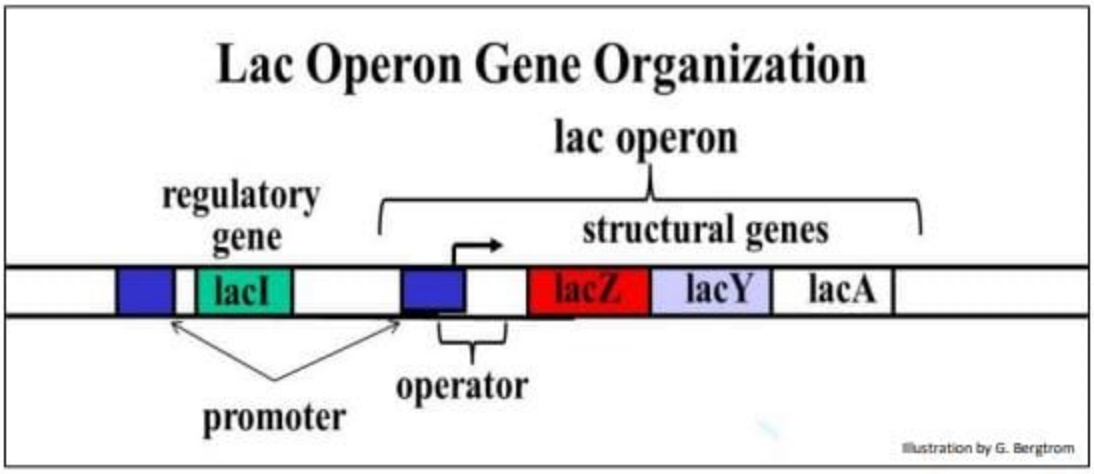
The mRNA transcribed from the **lac operon** is simultaneously translated into those three enzymes, as shown below.



A. Mechanisms of Control of the Lac Operon

In the animal digestive tract (including ours), genes of the *E. coli* **lac operon** regulate the use of *lactose* as an alternative nutrient to glucose. Think cheese instead of chocolate! The operon consists of lacZ, lacY, and lacA genes that were called **structural genes**. By definition, structural genes encode proteins that participate in cell structure and metabolic function. As already noted, the *lac operon* is transcribed into an mRNA encoding the Z, Y and A proteins.

Let's take a closer look at the structure of the lac operon and the function of the Y, Z and A proteins (below).



The lacZ gene encodes **β-galactosidase**, the enzyme that breaks lactose (a disaccharide) into *galactose* and glucose. The lacY gene encodes lactose **permease**, a membrane protein that facilitates lactose entry into the cells. The role of the lacA gene (a **transacetylase**) in lactose energy metabolism is not well understood. The **I gene** to the left of the lac Z gene is a **regulatory gene** (to distinguish it from structural genes). Regulatory genes encode proteins that interact with regulatory DNA sequences associated with a gene to control transcription. The **operator** sequence separating the I and Z genes is a transcription regulatory DNA sequence.

The *E. coli* lac operon is usually silent (repressed) because these cells prefer glucose as an energy and carbon source. In the presence of sufficient glucose, a **repressor protein** (the I gene product) is bound to the **operator**, blocking transcription of the lac operon. Even if lactose is available, cells will not be use it as an alternative energy and carbon source when glucose levels adequate. However, when glucose levels drop, the lac operon is active and the three enzyme products are translated. We will see how limiting glucose levels induce maximal lac operon transcription by both **derepression** and direct **induction**, leading to maximal transcription of the lac genes only when necessary (i.e., in the presence of lactose and absence of glucose). Let's look at some of the classic experiments that led to our understanding of E. coli gene regulation in general, and of the lac operon in particular.

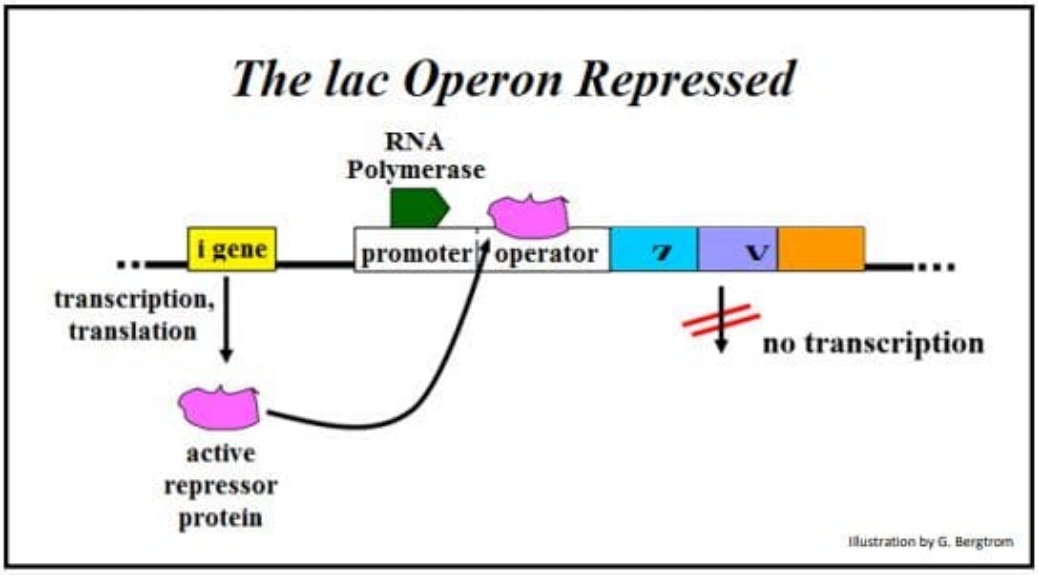
In the late 1950s and early 1960s, Francois Jacob and Jacques Monod were studying the use of different sugars as carbon sources by *E. coli*. They knew that wild type *E. coli* would **not** make the β-galactosidase, β-galactoside permease or β-galactoside transacetylase proteins when grown on glucose. Of course, they also knew that the cells would switch to lactose for growth and reproduction if they were deprived of glucose! They then searched for and isolated different E. coli mutants that could not grow on lactose, even when there was no glucose in the growth medium. Here are some of the mutants they studied:

1. One mutant failed to make active β-galactosidase enzyme but made permease.
2. One mutant failed to make active permease but made normal amounts of β-galactosidase.
3. Another mutant failed to make transacetylase..., but could still metabolize lactose in the absence of glucose. Hence the uncertainty of its role in lactose metabolism.
4. Curiosly, one mutant strain failed to make any of the three enzymes!

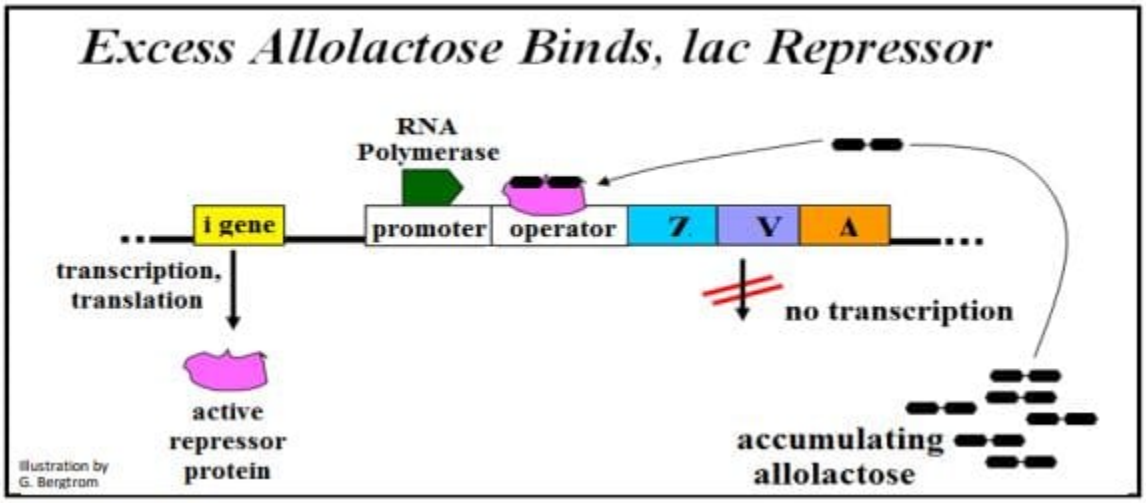
Since double mutants are very rare and triple mutants even rarer, Jacob and Monod inferred that the activation of all three genes in the presence of lactose were controlled together in some way. In fact, it was this discovery that defined the operon as a set of genes transcribed as a single mRNA, whose expression could therefore be effectively coordinated. They later characterized the repressor protein produced by the lacI gene. Jacob, Monod and Andre Lwoff shared the Nobel Prize in Medicine in 1965 for their work on bacterial gene regulation. We now know that *negative* and *positive* regulation of the *lac operon* (described below) depend on two regulatory proteins that together, control the rate of lactose metabolism.

1. Negative Regulation of the Lac Operon by Lactose

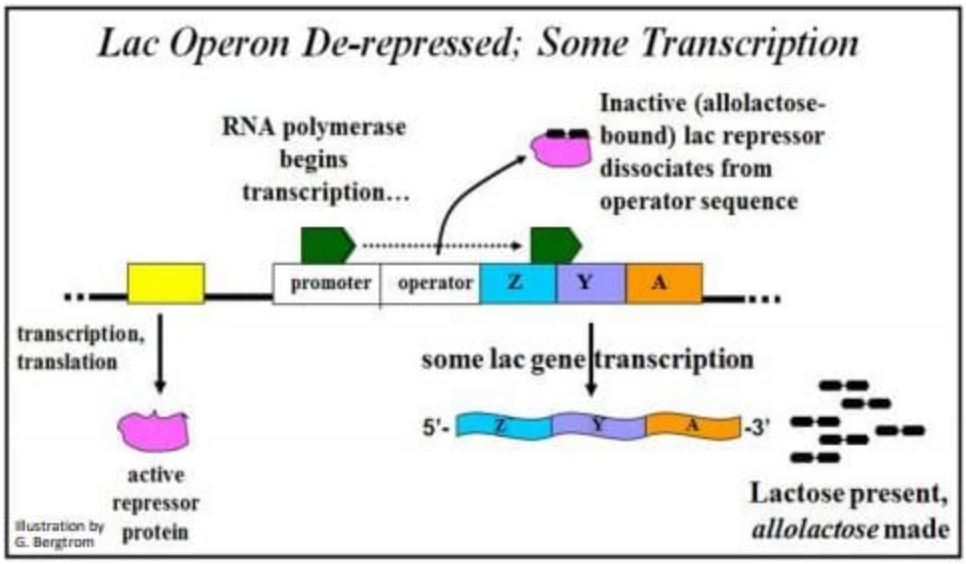
Refer to the illustration below to identify the players in lac operon derepression.



The repressor protein product of the I gene is always made and present in *E. coli* cells. I gene expression is not regulated! In the absence of lactose in the growth medium, the repressor protein binds tightly to the operator DNA. While **RNA polymerase** is bound to the promoter and ready to transcribe the operon, the presence of the repressor bound to the operator sequence close to the Z gene physically blocks its forward movement. Under these conditions, little or no transcript is made. If cells are grown in the presence of lactose, the lactose entering the cells is converted to **allolactose**. Allolactose binds to the repressor sitting on the operator DNA to form a 2-part complex, as shown below.

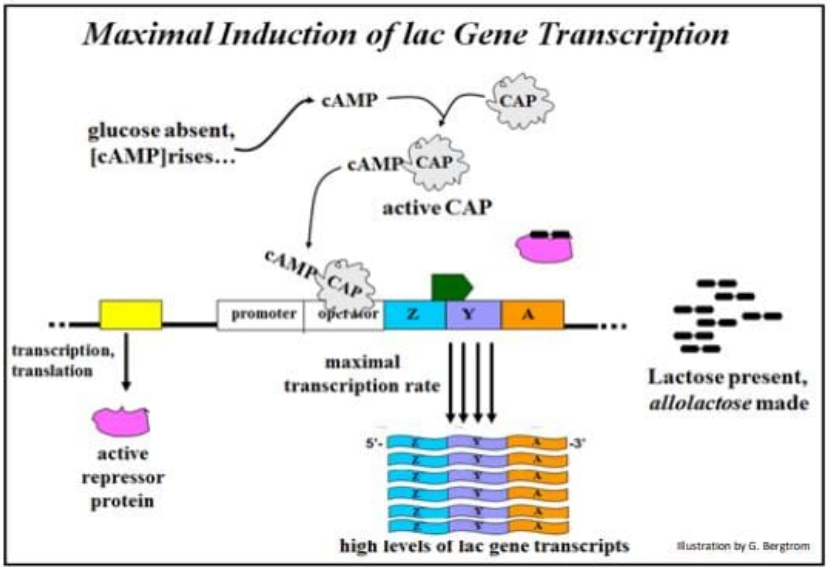


The allosterically altered repressor dissociates from the operator and RNA polymerase can transcribe the *lac* operon genes as illustrated below



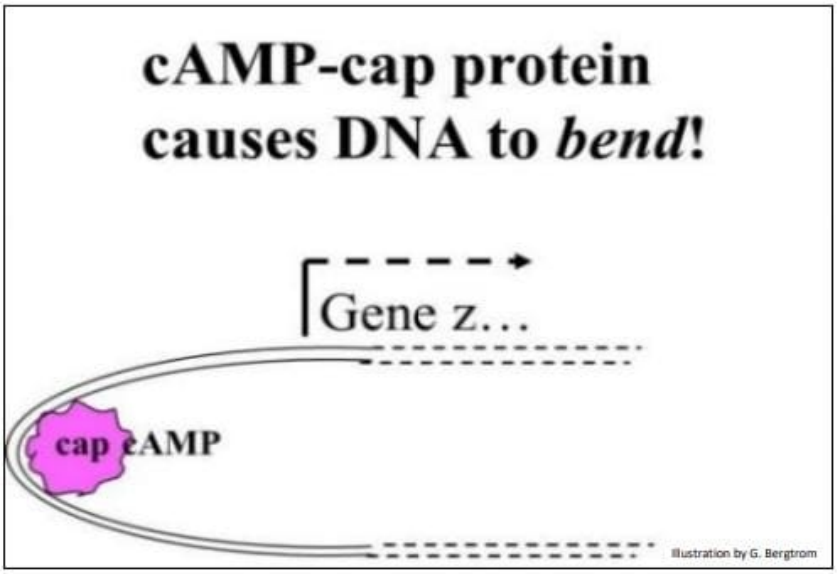
2. Positive Regulation of the Lac Operon; Induction by Catabolite Activation

The second control mechanism regulating lac operon expression is mediated by CAP (cAMP-bound **catabolite activator protein** or cAMP receptor protein). When glucose is available, cellular levels of cAMP are low in the cells and CAP is in an inactive conformation. On the other hand, if glucose levels are low, cAMP levels rise and bind to the CAP, activating it. If lactose levels are also low, the cAMP-bound CAP will have no effect. If lactose is present and glucose levels are low, then allolactose binds the lac repressor causing it to dissociate from the operator region. Under these conditions, the cAMP-bound CAP can bind to the operator in lieu of the repressor protein. In this case, rather than blocking the RNA polymerase, the activated Camp-bound CAP induces even more efficient lac operon transcription. The result is synthesis of higher levels of lac enzymes that facilitate efficient cellular use of lactose as an alternative to glucose as an energy source. Maximal *activation* of the lac operon in high lactose and low glucose is shown below.



217 Regulation of the Lac Operon

cAMP-bound CAP is an **inducer** of transcription. It does this by forcing the DNA in the promoter-operator region to bend. And since bending the double helix loosens H-bonds, it becomes easier for RNA polymerase to find and bind the promoter on the DNA strand to be transcribed…, and for transcription to begin. cAMP-CAPinduced bending of DNA is illustrated below.



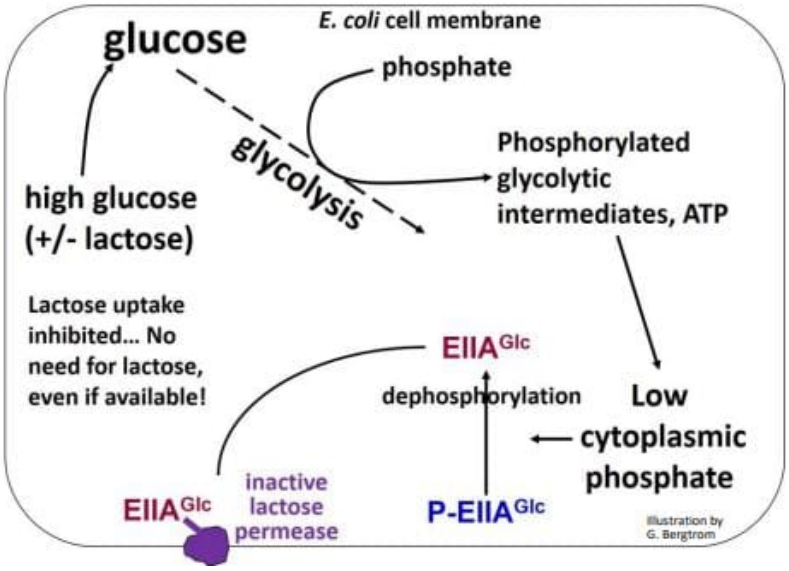
3. Lac Operon Regulation by Inducer Exclusion and Multiple Operators

In recent years, additional layers of lac operon regulation have been uncovered. In one case, the ability of *lac permease* to transport lactose across the cell membrane is regulated. In another, additional operator sequences have been discovered to interact with a multimeric repressor to control lac gene expression.

a) Regulation of Lactose use by Inducer Exclusion

When glucose levels are high (even in the presence of lactose), phosphate is consumed to phosphorylate glycolytic intermediates, keeping cytoplasmic phosphate levels low. Under these conditions, unphosphorylated EIIAGlc binds to the *lactose permease* enzyme in the cell membrane, preventing it from bringing lactose into the cell.

The role of phosphorylated and unphosphorylated $EIIA^{Glc}$ in regulating the lac operon are shown below.



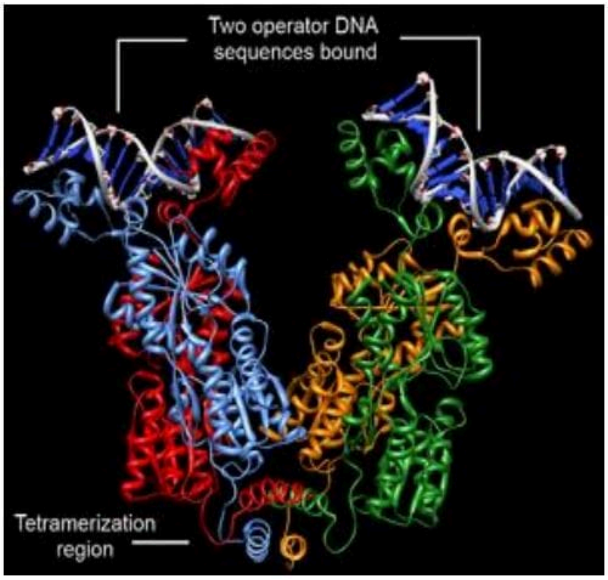
High glucose levels block lactose entry into the cells, effectively preventing allolactose formation and the derepression of the lac operon. Inducer exclusion is thus a logical way for the cells to handle an abundance of glucose, whether or not lactose is present. On the other hand, if glucose levels are low in the growth medium, phosphate concentrations in the cells rise sufficiently for a

specific kinase to phosphorylate the EIIAGlc. Phosphorylated EIIAGlc then undergoes an allosteric change and dissociates from the lactose permease, making it active so that more lactose can enter the cell. In other words, the inducer is not “excluded” under these conditions!

The kinase that phosphorylates $EIIA^{Glc}$ is part of a phosphoenolpyruvate (PEP)- *dependent phosphotransferase* system (PTS) cascade. When extracellular glucose levels are low, the cell activates the PTS system in an effort to bring whatever glucose is around into the cell. But the last enzyme in the PTS phosphorylation cascade is the kinase that phosphorylates $EIIA^{Glc}$. Phosphorylated $EIIA^{Glc}$ dissociates from the lactose permease, re-activating it, bringing available lactose into the cell from the medium.

b) Repressor Protein Structure and Additional Operator Sequences

The lac repressor is a tetramer of identical subunits (below).



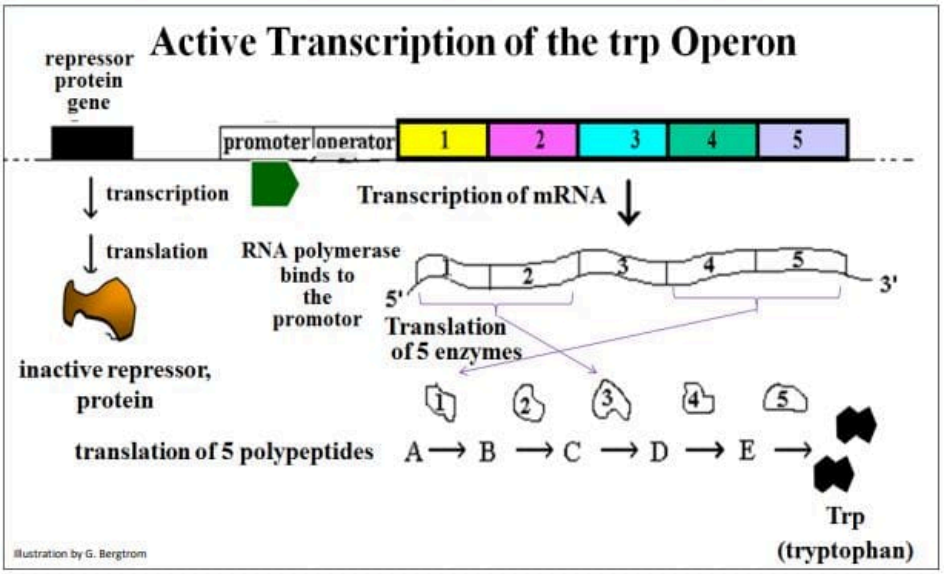
Each subunit contains a *helix-turn-helix* motif capable of binding to DNA. However, the operator DNA sequence downstream of the promoter in the operon consists of a pair of *inverted repeats* spaced apart in such a way that they can only interact two of the repressor subunits, leaving the function of the other two subunits unknown... that is, until recently!

Two more operator regions were recently characterized in the lac operon. One, called $\mathbf{O_2}$, is within the *lac z gene* itself and the other, called $\mathbf{O_3}$, lies near the end of, but within the *lac I* gene. Apart from their unusual location within actual genes, these operators, which interact with the remaining two repressor subunits, went undetected at first because mutations in the O2 or the O3 region individually do not contribute substantially to the effect of lactose in derepressing the lac operon. Only mutating both regions at the same time results in a substantial reduction in binding of the repressor to the operon.

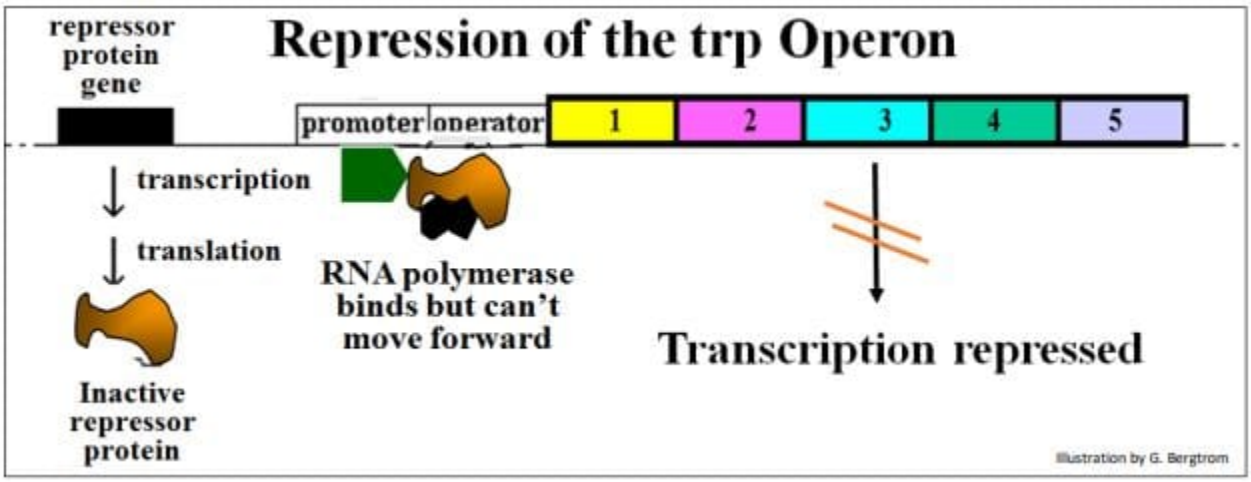
B. Mechanism of Control of the Tryptophan Operon

If ample tryptophan (**trp**) is available, the tryptophan synthesis pathway can be inhibited in two ways. First, recall how feedback inhibition by excess trp can allosterically inhibit the trp synthesis pathway. A rapid response occurs when tryptophan is present in excess, resulting in rapid feedback inhibition by blocking the first of five enzymes in the trp synthesis pathway. The *trp operon* encodes polypeptides that make up two of these enzymes.

Enzyme 1 is a *multimeric* protein, made from polypeptides encoded by the *trp5* and *trp4* genes. The trp1 and trp2 gene products make up **Enzyme 3**. If cellular tryptophan levels drop because the amino acid is rapidly consumed (e.g., due to demands for proteins during rapid growth), E. coli cells will continue to synthesize the amino acid, as illustrated below.



On the other hand, if tryptophan consumption slows down, tryptophan accumulates in the cytoplasm. Excess tryptophan will bind to the trp repressor. The trp-bound repressor then binds to the trp operator, blocking RNA polymerase from transcribing the operon. The repression of the trp operon by trp is shown below.



In this scenario, tryptophan is a **co-repressor**. The function of a co-repressor is to bind to a repressor protein and change its conformation so that it can bind to the operator.

219 Repression of the Tryptophan (TRP) Operon

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12.3: The Problem with Unregulated (Housekeeping) Genes in All Cells

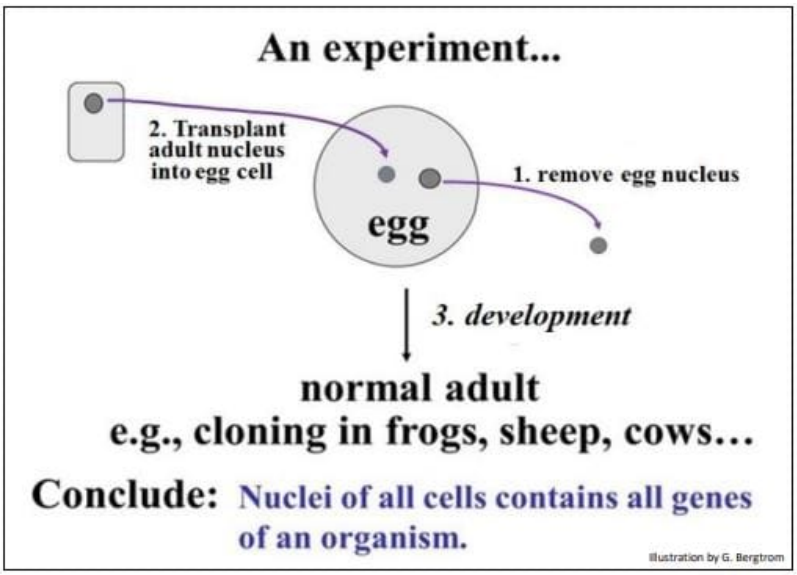
Before we turn our attention to the regulation of gene expression in eukaryotes, consider for a moment the expression of **constitutive**, or **housekeeping**, genes that are always active. The requirement that some genes are always "on" raises questions about cellular priorities of gene expression. Constitutive gene products are sets of many polypeptides that form large macromolecular complexes in cells, or enzyme sets that participate in vital biochemical pathways. How do cells maintain such polypeptides in stoichiometrically reasonable amounts? Or, can their levels rise or fall transiently without much effect? Recent studies suggest that transcription of *housekeeping* genes is in fact, not at all coordinated! Nevertheless, we also saw that the efficiency of glycolysis relied on the evolution of allosteric regulatory mechanisms to control the activities of glycolytic enzymes rather than their transcription. While this takes care some element of metabolic control, a problem remains. Recall that protein synthesis is energy-intensive, each peptide linkage costing three NTPs (not to mention the waste of an additional NTP per phosphodiester linkage made in transcription of an mRNA!). The overproduction of proteins under any circumstances would seem to be a waste of energy. We may not know just how expensive it is to express housekeeping genes. But whatever they are, these energy expenses are the cost of evolving complex structures and biochemical pathways vital to their everyday function and survival. Now back to our focus on regulated gene expression... in eukaryotes.

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12.4: Gene Regulation in Eukaryotes

A. The Difference between Eukaryotic and Prokaryotic Gene Regulation

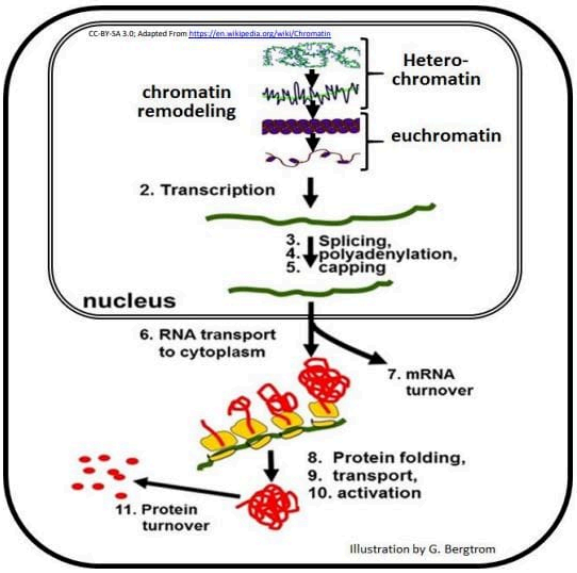
Let's Recall an experiment described earlier and illustrated below.



Results of this experiment provided the evidence that even very different cells of an organism contain the same genes. In fact, in any multicellular eukaryotic organism, every cell contains the same DNA (genes). Therefore, the different cell types in an organism differ not in which genes they contain, but which sets of genes they express! Looked at another way, cells differentiate when they turn on new genes and turn off old ones. Thus, gene regulation produces different sets of gene products during differentiation, leading to cells that look and function differently in the organism.

220 An Experiment: All of an Organism's Cells Have the Same Genome

Compared to prokaryotes, many steps in eukaryotes lie between transcription of an mRNA and the accumulation of a polypeptide end product. Eleven of these steps are shown in the pathway from gene to protein below.



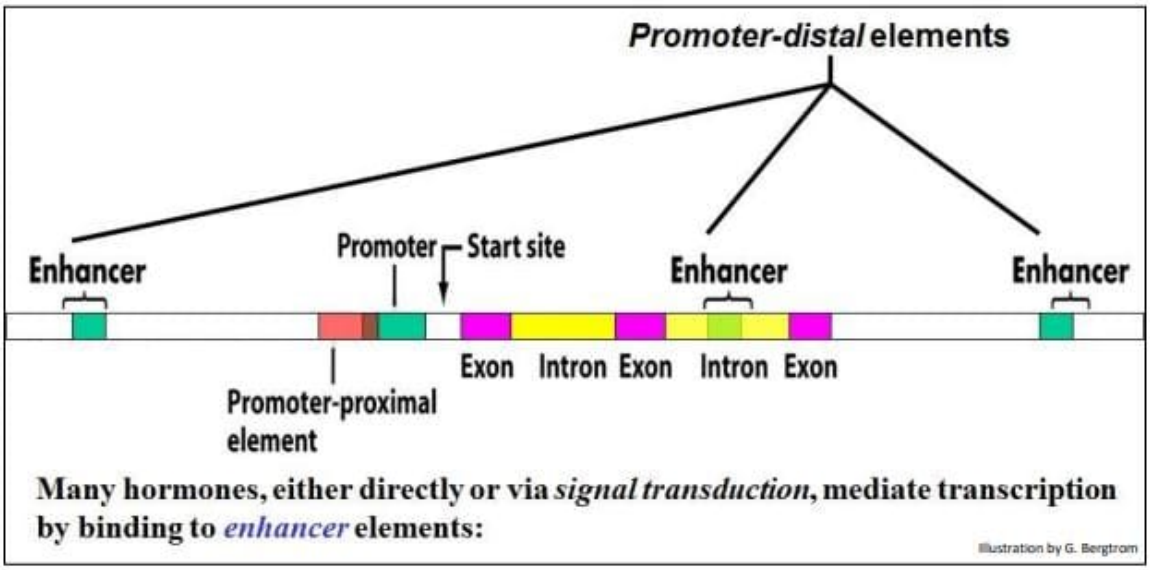
Theoretically, cells could turn on, turn off, speed up or slow down any of the steps in this pathway, changing the steady state concentration of a polypeptide in the cells. While regulation of any of these steps is possible, the expression of a single gene is typically controlled at only one or a few steps. A common form of gene regulation is at the level of transcription initiation, similar to transcriptional control in bacteria, in principle if not in detail.

221 Many Options for Regulating Eukaryotic Genes

B. Complexities of Eukaryotic Gene Regulation

Gene regulation in eukaryotes is more complex than in prokaryotes. This is in part because their genomes are larger and because they encode more genes. For example, the E. coli genome houses about 5,000 genes, compared to around 25,000 genes in humans. Furthermore, eukaryotes can produce even more than 25,000 proteins by alternative splicing of mRNAs and in at least a few cases, by initiating transcription from alternative start sites in the same gene. And of course, the activity of many more genes must be coordinated without the benefit of multigene operons! Finally, eukaryotic gene regulation is made more complicated because all nuclear DNA is wrapped in protein in the form of chromatin.

All organisms control gene activity with **transcription factors** that bind to specific DNA sequences (**cis regulatory elements**). In eukaryotes, these elements can be **proximal to** (near) the promoter of a gene, or **distal to** (quite far from) the gene they regulate. A eukaryotic map showing the components of a typical gene and its associated cis-acting regulatory elements is shown below.

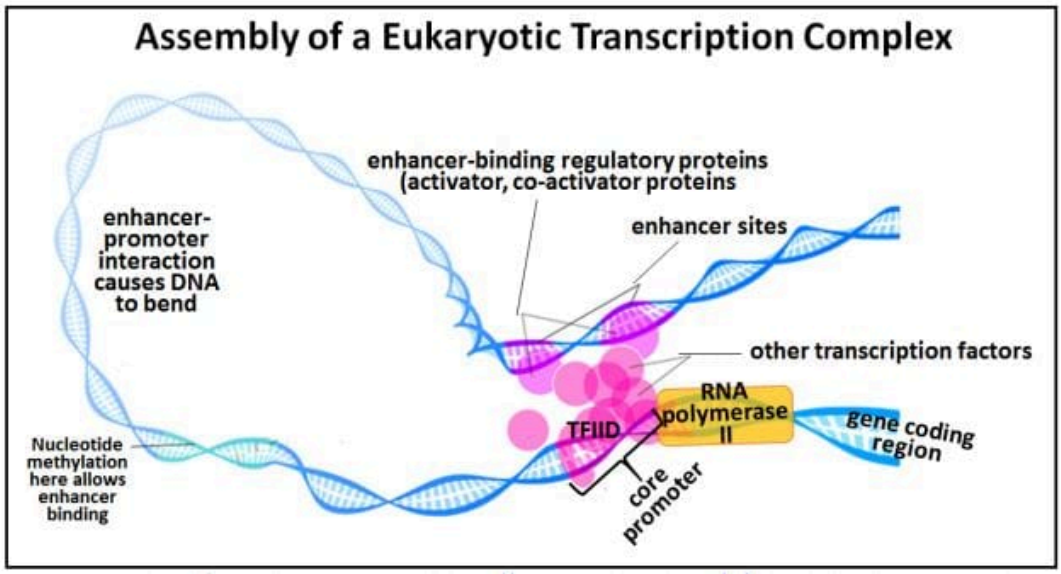


Enhancers are typical *distal* cis elements that recognize and bind transcription factors to increase the rate of transcription of a gene. Oddly enough, these short DNA elements can be in the 5' or 3' non-translated region of the gene, or even within introns, and can lie thousands of base pairs away from the genes they control. Note that enhancer elements are even in introns can also be very far from the start-site of transcription of a gene.

Upstream regulatory regions of eukaryotic genes (to the left of a gene promoter as shown above) often have distal binding sites for more than a few transcription factors, some with positive (*enhancing*) and others with negative (*silencing*) effects. Of course, which of these DNA regions are active in controlling a gene depends on which transcription factor(s) are present in the nucleus. Sets of positive regulators will work together to coordinate and maximize gene expression when needed, and sets of negative regulators will bind negative regulatory elements to silence a gene.

222 Transcription Factors Bind DNA Near and Far

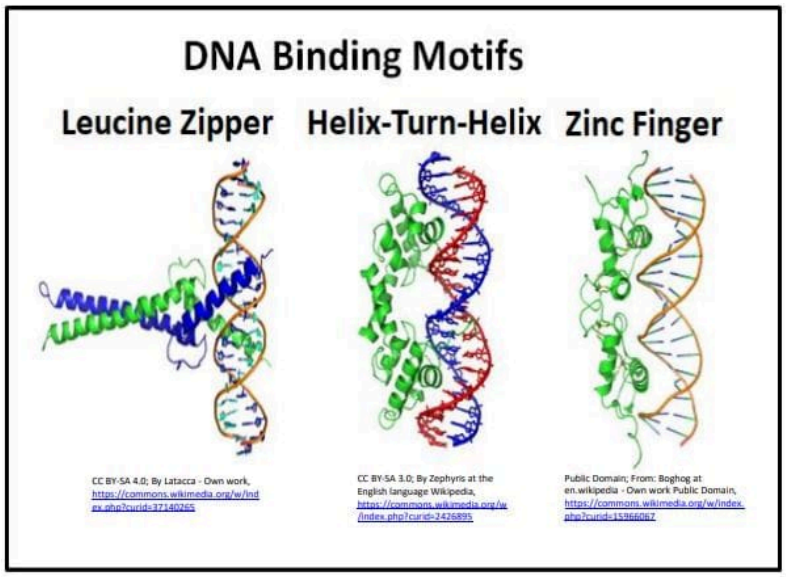
We saw that in eukaryotes, the initiation of transcription involves many transcription factors and RNA polymerase II acting at a gene promoter to form a **transcription preinitiation complex**. **TFIID**, or **TATA binding protein** is one of the first factors to bend, causing the DNA in the promoter region to bend, much like the CAP protein in bacteria. TFIID also recruits other transcription factors to the promoter. As in bacteria, bending the DNA loosens H-bonds between bases, facilitating unwinding the double helix near the gene. Bending eukaryotic DNA also brings distal regulatory proteins bound to enhancer sequences far from the promoter together with the proteins bound to more proximal regulatory elements, as shown in the drawing below.



Nucleotide methyation sites may facilitate regulatory protein-enhancer binding. When such regulatory proteins, here called *activators* (i.e., of transcription), bind to their enhancers, they acquire an affinity for protein cofactors that enable recognition and binding to other proteins in the transcription initiation complex. This attraction stabilizes the bend in the DNA that then makes it easier for RNA polymerase II to initiate transcription

223 Assembling a Eukaryotic Transcription Initiation Complex

It is worth reminding ourselves that it is shape and allosteric change that allow DNAprotein interactions (in fact, any interactions of macromolecules). The lac repressor we saw earlier is a transcription factor with *helix-turn-helix* DNA binding motifs. This motif and two others (*zinc finger*, and *leucine zipper*) characterize DNA binding proteins are illustrated below.



DNA-binding motifs in each regulatory protein shown here bind one or more regulatory elements 'visible' to the transcription factor in the major groove of the double helix.

224 Transcription Factor Domains/Motifs Bind Specific DNA Sequences

We will look next at some common ways in which eukaryotic cells are signaled to turn genes on or off, or to increase or decrease their rates of transcription. As we describe these models, remember that eukaryotic cells regulate gene expression in response to changes in extracellular environments. These can be unscheduled, unpredictable changes in blood or extracellular fluid composition (ions, small metabolites), or dictated by changes in a long-term genetic program of differentiation and development. Changes in gene expression even obey circadian (daily) rhythms, the ticking of a clock. In eukaryotes, changes in gene expression, expected or not, are usually mediated by the timely release of chemical signals from specialized cells (e.g., hormones, cytokines, growth factors, etc.). We will focus on some betterunderstood models of gene regulation by these chemical signals.

C. Regulation of Gene Expression by Hormones that enter Cells and Those That Don't

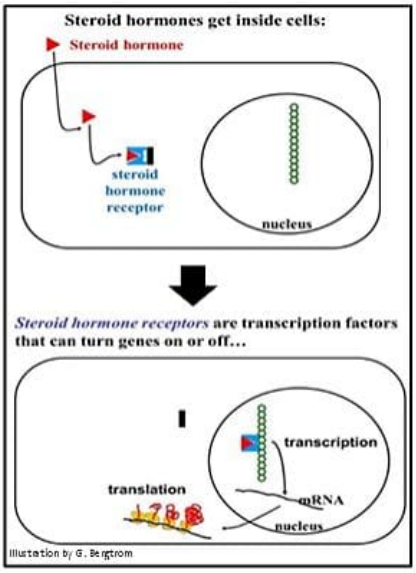
Gene-regulatory (cis) elements in DNA and the transcription factors that bind to them have co-evolved. But not only that! Eukaryotic organisms have evolved complete pathways that respond to environmental or programmed developmental cues and lead to an appropriate cellular response. Chemicals that regulate genes in prokaryotes are not usually signals communicated by other cells. In eukaryotes, chemicals released by some cells signal other cells to respond, thus coordinating the activity of the whole organism. Hormones released by cells in endocrine glands are well-understood signal molecules; hormones affect *target cells* elsewhere in the body.

225 Chemicals That Control Gene Expression

1. How Steroid Hormones Regulate Transcription

Steroid hormones cross the cell membranes to have their effects. Common steroid hormones include testosterone, estrogens, progesterone, glucocorticoids and mineral corticoids. Once in target cell, such hormones bind to a **steroid hormone** receptor protein to form a **steroid hormone-receptor complex**. The receptor may be in the cytoplasm or in the nucleus, but in the end, the hormone-receptor complex must bind to DNA regulatory elements of a gene to either enhance or silence transcription. Therefore, a steroid hormone must cross the plasma membrane, and may also need to cross the nuclear envelope.

Follow the Binding of a steroid hormone to a cytoplasmic receptor below.



Here the hormone (the triangle) enters the cell. An allosteric change in the receptor releases a protein subunit called Hsp90 (the black rectangle in the illustration). The remaining hormone-bound receptor enters the nucleus.

The fascinating thing about Hsp90 is that it was first discovered in cells subjected to heat stress. When the temperature gets high enough, cells shut down most transcription and instead transcribe Hsp 90 and/or other special *heat shock* genes. The resulting **heat shock proteins** seem to protect the cells against metabolic damage until temperatures return to normal. Since most cells never experience such high temperatures, the evolutionary significance of this protective mechanism is unclear. As we now know, heat shock proteins have critical cellular functions, in this case blocking the DNA-binding site of a hormone receptor until a specific steroid hormone binds to it.

Back to hormone action! No longer associated with the Hsp90 protein, the receptor bound to its hormone cofactor binds to a cis-acting transcription control element in the DNA, turning transcription of a gene on or off. The hormone receptors for some steroid hormones are already in the nucleus of the cell, so the hormone must cross not only the plasma membrane, but also the nuclear envelope in order to access the receptor.

As for steroid hormone functions, we already saw that glucocorticoids turn on the genes of gluconeogenesis. Steroid hormones also control sexual development and reproductive cycling in females, salt and mineral homeostasis in the blood, metamorphosis in arthropods, etc., all by regulating gene expression.

226 Steroid Hormones Regulate Gene Transcription

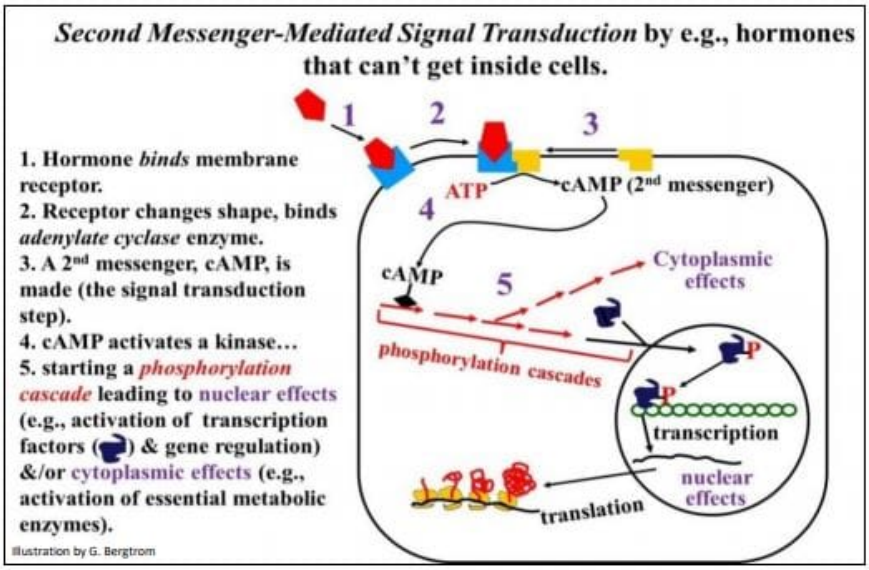
2. How Protein Hormones Regulate Transcription

Protein hormones are of course large and soluble, with highly charged surfaces. Other hormones might be relatively small (e.g., adrenalin), but are charged. Large or highly charged signal molecules cannot get across the phospholipid barrier of the plasma membrane. To have any effect at all, they must bind to receptors on the surface of cells. These receptors are typically membrane glycoproteins.

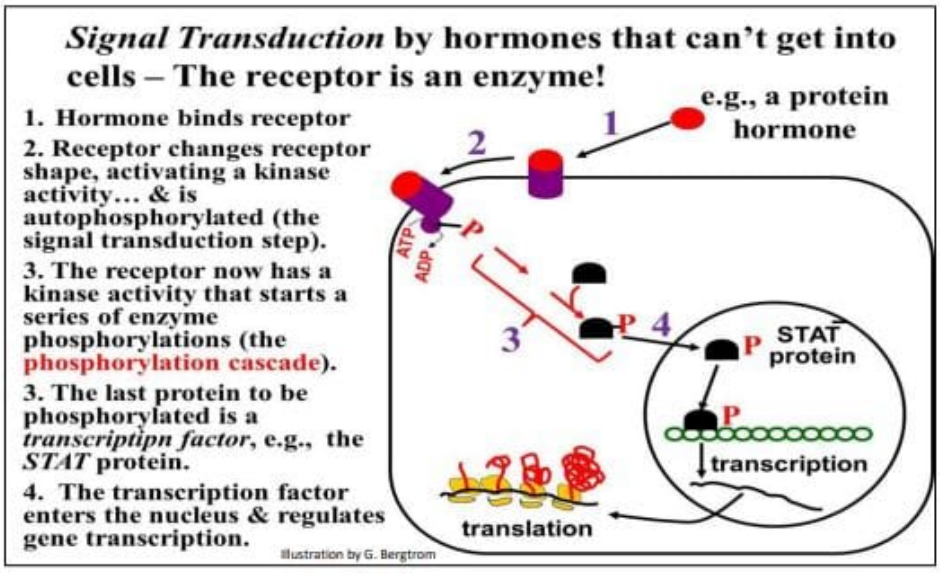
The information (signals) carried by protein hormones must be conveyed into the cell indirectly, by a process called *signal transduction*. There are two well-known pathways of signal transduction, each of which involves activating pathways of protein phosphorylation in cytoplasm. The **phosphorylation cascade** that results activates a transcription factor that binds to regulatory DNA, turning a gene on or off.

Binding of a hormone to a cell surface receptor leads to an allosteric change in the receptor. This in turn activates other proteins either in the plasma membrane or in the cytoplasm, leading to the synthesis of a cytoplasmic **second messenger**. The second messenger typically binds to a protein kinase in the cytoplasm, launching a series of protein phosphorylations, or a phosphorylation cascade. The last in the series of proteins to be phosphorylated is an activated transcription factor that will bind to a cis-regulatory DNA sequence.

cAMP was the first second messenger metabolite to be discovered. It mediates many hormonal responses, controlling both gene activity and enzyme activity. cAMP forms when the hormone-receptor in the membrane binds to and activates a membrane-bound adenylate cyclase enzyme. The cAMP produced then binds to a protein kinase, the first of several in a phosphorylation cascade. Signal transduction mediated by cAMP is summarized in the illustration below.



A different kind of signal transduction involves a hormone receptor that is itself the protein kinase. The role of **enzyme-linked hormone receptors** in signal transduction is summarized below.

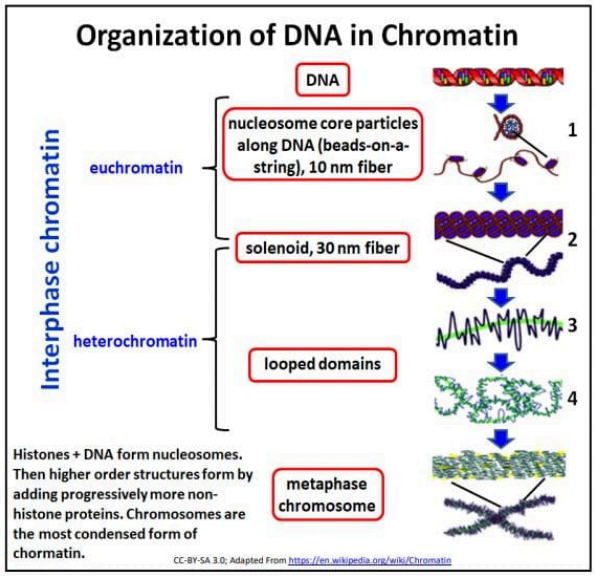


Binding of the signal protein (e.g. hormone) to the enzyme-linked receptor causes an allosteric change that activates the receptor kinase, starting phosphorylation cascade resulting in an active transcription factor. We look at signal transduction in more detail in another chapter.

227 Signal Transduction Can Lead to Gene Regulation

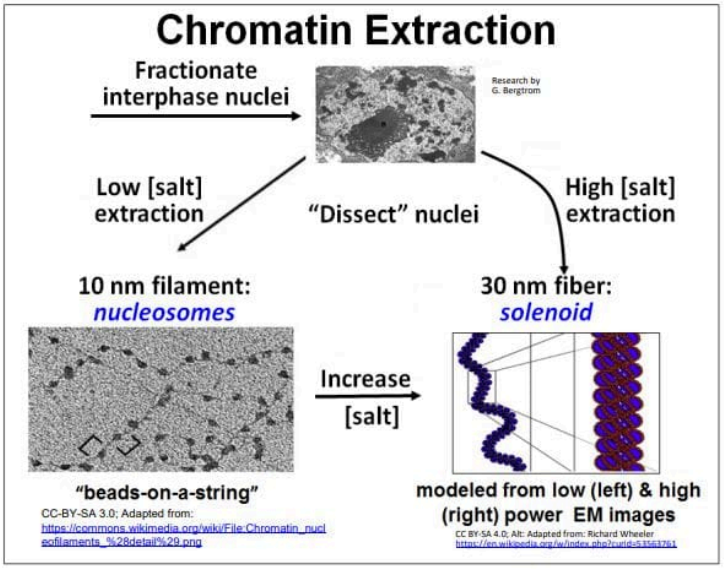
D. Regulating Eukaryotic Genes Means Contending with Chromatin

Consider again the illustration of the different levels of chromatin structure (below).



Transcription factors bind specific DNA sequences by detecting them through the grooves (mainly the major groove) in the double helix. The drawing above reminds us however, that unlike the nearly naked DNA of bacteria, eukaryotic (nuclear) DNA is coated with proteins that, in aggregate are by mass, greater than the mass of DNA that they cover. The protein-DNA complex of the genome is of course, chromatin.

Again, as a reminder, DNA coated with histone proteins forms the 9 nm diameter beads-on-a-string structure in which the beads are the **nucleosomes**. The association of specific non-histone proteins causes the nucleosomes to fold over on themselves to form the **30 nm solenoid**. As we saw earlier, it is possible to selectively extract chromatin. Take a second look at the results of typical extractions of chromatin from isolated nuclei below.



Further accretion of non-histone proteins leads to more folding and the formation of **euchromatin** and **heterochromatin** characteristic of non-dividing cells. In dividing cells, the chromatin further condenses to form the **chromosomes** that separate during either *mitosis* or *meiosis*.

Recall that biochemical analysis of the 10 nm filament extract revealed that the DNA wraps around histone protein octamers, the nucleosomes or beads in this beads-on-astring structure. Histone proteins are highly conserved in the eukaryotic evolution (they are not found in prokaryotes). They are also very basic (many *lysine* and *arginine* residues) and therefore very positively charged. This explains why they are able to arrange themselves uniformly along DNA, binding to the negatively charged *phosphodiester* backbone of DNA in the double helix.

Since the DNA in euchromatin is less tightly packed than it is in heterochromatin, perhaps active genes are to be found in euchromatin and not in heterochromatin. Experiments in which total nuclear chromatin extracts were isolated and treated with the enzyme deoxyribonuclease (DNAse) revealed that the DNA in active genes was degraded more rapidly than non-transcribed DNA. More detail on these experiments can be found in the two links below.

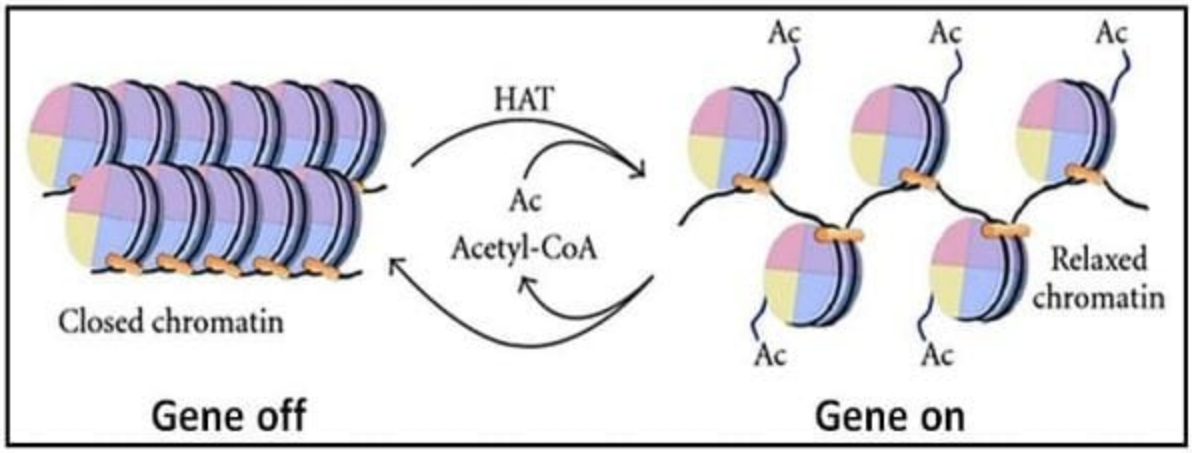
228 Question: Is Euchromatic DNA Transcribed?

229 Experiment and Answer: Euchromatin *is* Transcribed

The results of such experiments are consistent with the suggestion that active genes are more accessible to DNAse because they are in less coiled, or less condensed chromatin. DNA in more condensed chromatin is surrounded by more proteins, and thus is less accessible to, and protected from DNAse attack. When packed up in chromosomes during mitosis or meiosis, all genes are largely inactive.

Regulating gene transcription must occur in non-dividing cells or during the interphase of cells, where changing the shape of chromatin (**chromatin remodeling**) in order to silence some and activate other genes is possible. Changing chromatin conformation involves chemical modification of chromatin proteins and DNA. For example, chromatin can be modified by histone acetylation, de-acetylation, methylation and phosphorylation, reactions catalyzed by *histone acetyltransferases* (HAT enzymes), de-acetylases, methyl transferases and kinases, respectively. For example, acetylation of lysines near the amino end of histones H2B and H4 tends to unwind nucleosomes and open the underlying DNA for transcription. De-acetylation then, promotes condensation of the chromatin in the affected regions of DNA. Likewise, methylation of lysines or arginines (the basic amino acids that characterize histones!) of H3 and H4 can open DNA for transcription, while demethylation has the opposite effect. In one case, di-methylation of a lysine in H3 can suppress transcription. These chemical modifications affect recruitment of other proteins that alter chromatin conformation and ultimately activate or block transcription.

This reversible and its effect on chromatin are illustrated below.



Nucleosomes themselves can be moved, slid and otherwise repositioned by complexes that hydrolyze ATP for energy to accomplish the physical shifts. Some cancers are associated with mutations in genes for proteins involved in chromatin remodeling. This is no doubt, because failures of normal remodeling could adversely affect normal cell cycling and normal replication. In fact, a single, specific pattern of methylation may mark DNA in multiple cancer types (check out Five Cancers with the Same Genomic Signature - Implications).

E. Regulating all the Genes on a Chromosome at Once

Recall that X chromosomes in human female somatic cells is inactivated, visible in the nucleus as a Barr body. One of the two X chromosomes in female fruit flies is also inactivates. However, both males and females of Drosophila (presumably also us!) require X chromosome gene expression during embryogenesis. Given the difference in X chromosome **gene dosage** between males and females, do males get by with fewer X chromosome gene products than females?

Experiments looking at the expression of X chromosome gene in male and female flies revealed similar levels of gene products. It turns out that the activity of a nuclear body called **HLB** (**H**istone **L**ocus **B**ody) is required for increase in X chromosome gene transcription. A protein, called **CLAMP** (**C**hromatin-**L**inked **A**daptor for **M**ale-specific lethal (MSL) **P**rotein), was shown to bind to **GAGA** nucleotide repeats lying between the genes for histones 3 and 4. As there are about 100 repeats of the fivegene histone locus on X chromosomes, and thus about 100 repeats of the GAGA repeats. Therefore, many CLAMP proteins bind to the HLBs, where they recruit many MSL proteins. The MSL protein complexes that form then globally increase male X chromosome gene expression, compensating for the lower X gene dosage in males. Read the original research here (L.E. Reider et al. (2018) Genes & Development 31:1-15). And finally, there is emerging evidence that the HLB action may also be involved in inactivation of an entire female X chromosome later in embryogenesis in females!

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12.5: Epigenetics

Aristotle thought that an embryo emerged from an amorphous mass, a “less fully concocted seed with a nutritive soul and all bodily parts”. The much later development of the microscope led to more detailed (if inaccurate) descriptions of embryonic development. In 1677, no less a luminary than Anton von Leeuwenhoek, looking at a human sperm with his microscope, thought he saw a miniature human inside! The tiny human, or **homunculus**, became the epitome of **preformation** theory.

William Harvey, also in the 17 th century, described changes in morphology in the developing embryos of chickens (and other animals). Harvey coined the term **epigenesis** to counter the notion that any tiny adult structures in eggs or sperm simply grew bigger during embryonic gestation. Meanwhile, other experiments were leading embryologists to the conclusion that the physical and chemical environment of an embryo strongly affected development. Thus temperature, pH, and in the case of chicken eggs, position of incubation, affect embryonic development. In a series of very elegant experiments reported in 1924, Hans Speeman reported that cells associated with differentiation of one region of an embryo could be transplanted to a different part of the same embryo, or to another embryo entirely, where it would induce new tissue development. He won the 1935 Nobel Prize in Physiology and Medicine for his discovery of embryonic **organizers** that induced morphogenesis.

Other embryologists (including Conrad Waddington) demonstrated that cells killed by freezing or boiling still induced morphogenesis after being placed on an embryo. Thus, actual chemicals influence embryogenesis. The fact that differences in physical or chemical environment could affect embryonic development led many to conclude that environment played the dominant role and that genes played only a minor one in an organism’s ultimate phenotype. Unlike most of his fellow embryologists, Waddington believed in a more equitable role of genes and environment in determining phenotype. Adapting the term epigenesis, he coined the term epigenetics to describe the impact of environment on embryonic development (1942, The Epigenotype. Endeavour. **1**: 18–20).

At the time, the concept of epigenetics led to a nature vs. nurture controversy. We now understand that differences in environmental influence can cause individuals with the same genes (genotype) to vary in appearance (phenotype). A modern version of thenature vs. nurture argument has more to do with complex traits, for example how much do genetics vs. environment influence intelligence, psychology and behavior. There is much to-do and little evidence to resolve these questions..., and likely too many factors affecting these traits to separate them experimentally.

These days, the field of epigenetics looks closely at protein interactions in eukaryotes affecting gene expression. These interactions change the structure NOT of genes (or DNA), but of the proteins (and other molecules) that affect how DNA and genes are used. As we have seen, the control of transcription involves transcription factors that recognize and bind to regulatory sequences in DNA such as enhancers or silencers. These proteinDNA interactions often require selective structural changes in the conformation of the chromatin surrounding genes. These changes can be profound and stable, and they are not easily undone.

An example of epigenetics is inheritance of chromatin protein alterations that accompany gene expression changes in development. Given an appropriate signal, say a hormone at the right time, a few cells respond with chromatin rearrangements and the expression of a new set of genes. The new pattern of gene expression defines a cell that has differentiated. Hundreds, even thousands of such changes accompany progress from fertilized egg to fully mature eukaryotic organism. Every one of these changes in a cell is passed on to future generations of cells by mitosis, accounting for different tissues and organs in the organism. Hence, the many different *epigenomes* representing our differentiated cells are *heritable*.

To sum up, epigenetics is the study of when and how undifferentiated cells (embryonic and later, adult stem cells) acquire their epigenetic characteristics and then pass on their epigenetic information to progeny cells. As we’ll see shortly, epigenetic inheritance is not limited to somatic cells, but can span generations! First, let’s look at this brief history of our changing understanding of evolution.

Jean-Baptiste Lamarck proposed (for instance) that when a giraffe’s neck got longer so that it could reach food higher up in trees, that character would be inherited by the next giraffe generation. According to Lamarck, evolution was *purposeful*, with the goal of improvement.

Later, Darwin published his ideas about evolution by *natural selection*, where nature selects from pre-existing traits in individuals (the raw material of evolution). The individual that just randomly happens to have a useful trait then has a survival (and reproductive) edge in an altered environment.

Later still, the rediscovery of Mendel's genetic experiments, it became increasingly clear that it is an organism's genes that are inherited, are passed down the generations, and are the basis of an organism's traits. By the start of the 20th century, Lamarck's notion of purposefully acquired characters was discarded.

Epigenetic inheritance implies an epigenetic blueprint in addition to our DNA blueprint. This means that, in addition to passing on the genes of a male and female parent, epigenomic characteristics (which genes are expressed and when) are also passed to the next generation. Waddington suspected as much early on, calling the phenomenon *genetic assimilation*, and once again created controversy! Does genetic assimilation make Lamarck right after all? Prominent developmental biologists accused Waddington of promoting purposeful evolution. Waddington and others denied the accusation, trying to explain how epigenetic information might be heritable, without leading to *purposeful* evolution.

Is there in fact, an *epigenetic code*? Data from the small Swedish town of Överkalix led to renewed interest in epigenetic phenomena. Consider the meticulous harvest, birth, illness, death and other demographic and health records collected and analyzed by L. O. Bygren and colleagues at Sweden's Karolinska Institute.

A sample of Bygren's data is shown in the table below.

Grand-parent	Food supply	Grandson relative risk of death from cardio disease and diabetes	Granddaughter relative risk of death from cardio disease and diabetes
Grandfather	poor	-35%	No change
Grandfather	abundant	+67%	No change
Grandmother	poor	No change	-49%
Grandmother	abundant	No change	+113%

It looked to the good doctor as if environment was influencing inheritance! It is as if the environment was indeed causing an *acquired change* in the grandparent that is passed not to one, but through two generations... and in a sex-specific way!

23.0 Epigenetic Inheritance: First Inkling

This phenomenon was subsequently demonstrated experimentally with the exposure of pregnant rats to a toxin. Rat pups born to exposed mothers suffered a variety of illnesses. This might be expected if the toxic effects on the mother were visited on the developing pups, for example through the placenta. However, when the diseased male rat pups matured and mated with females, the pups in the new litter grew up suffering the same maladies as the male parent. This *even though the pregnant females in this case were NOT exposed to the toxins*. Because the original female was already pregnant when she was exposed, the germ line cells (eggs, sperm) of her litter had not suffered mutations *in utero*. This could only mean that epigenetic patterns of gene expression caused by the toxin in pup germ line cells (those destined to become sperm & eggs) *in utero* were retained during growth to sexual maturity, and then passed on to their progeny, even while gestating in a normal unexposed female.

For some interesting experimental findings on how diet influences epigenetic change in Drosophila click here. For recent evidence for a role of male DNA methylation in trans-generational epigenetic inheritance, check out this link.

These days, the term epigenetics describes heritable changes in chromatin modifications and gene expression. We now know that epigenetic configurations of chromatin that are most stable include patterns of histone modification (acetylation, phosphorylation, methylation...) or DNA (methylation, phosphorylation...). Such changes can convert the 30nm fiber to the 10nm 'beads-on-a-string' nucleosome necklace... and vice versa. Such changes in chromatin (chromatin remodeling) lead to altered patterns of gene expression, whether during normal development or when deranged by environmental factors (abundance or limits on nutrition, toxins/poisons or other life-style choices). The active study of DNA methylation patterns even has its own name, methylomics! Check out Epigenetics Definitions and Nomenclature for more epigenetic nomenclature.

Let's close this chapter with a question and some observations. Can you be sure that your smoking habit will not affect the health of your children or grandchildren? What about your eating habits? Drinking? It is not a little scary to know that I have a gullible germline epigenome that can be influenced by my behavior, good and bad. And that my children (and maybe grandchildren) will inherit my epigenetic legacy long before they get my house and my money. And that may not be all... epigenetic memory in C. elegans can stretch to 14 generations! Read about epigenetic inheritance resulting from Dad's cocaine use at Sins of the Father and about multigenerational epigenetic inheritance at Epigenetic Memory in Caenorhabditis elegans.

231 Experimental Demonstration of Germ-Line Epigenetic Inheritance

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12.6: Key Words and Terms

10 nm fiber	galactose	pseudogene
3' non-transcribed DNA	galactoside	PTS
30 nm solenoid fiber	gene activation	regulatory genes
5' non-transcribed DNA	gene derepression	second messenger
adaptive immune system	gene expression	nucleosomes
adult stem cells	gene induction	O_1 and O_2 lac operators
allolactose	gene regulation	operator
antisense RNA	gene repression	operon regulation
basic v. non-basic proteins	HAT enzymes	PEP-dependent P-transferase system
beads-on-a-string	helix-turn-helix motif	phage DNA
β-galactosidase	heterochromatin	phophodiester backbone
cAMP	histone acetylation	phosphorlyation
cAMP receptor protein	histone kinases	pluripotent cells
CAP protein	histone methyl transferases	polycistronic mRNA
CAT box	histone methylation	positive regulation
catabolite activator protein	histone phosphorlyation	promoter
chromatin remodeling	housekeeping genes	proximal regulatory element
cis-acting elements	inducer exclusion	signal transduction
condensed chromatin	interphase	steroid hormone
developmental program	introns	structural genes
differential gene	lac operon	TATA box
distal regulatory element	lacl gene	tetrametric lac repressor
DNA bending	lactose	totipotent cells
DNAse	lactose permease	transcription factors
embryonic stem cells	lactose repressor	transcription regulation
enhancers	lac Z, lac Y, lac A genes	transcription start site
environmental signals	leucine zipper motif	translation regulation
epigenome	levels of chromatin structure	trp operon
euchromatin	major groove	trp repressor
exons	minor groove	zinc finger motif
extended chromatin	miRNA (micro RNA)	
fully differentiated cells	negative regulation	

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13: POST TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

13.1: Introduction
13.2: Post-transcriptional Control of Gene Expression
13.3: Eukaryotic Regulation of Translation
13.4: Key Words and Terms

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13.1: Introduction

The metabolic potential of cells is flexible, depending on various mechanisms that ultimately determine the levels and activities of proteins that dictate a cell's metabolic state. We have seen some of these regulatory mechanisms:

- the *regulation of transcription* by extracellular chemical signals or developmental chemical prompts
- the control of enzyme or other protein activity by allosteric regulation or chemical modification (e.g., phosphorylation or dephosphorylation).

In this chapter, we look at different kinds of **post-transcriptional regulation**, events somewhere between mRNA transcription and controls on the activity of finished proteins. These control mechanisms are most diverse in eukaryotes.

Like other pathways for regulating gene expression, post-transcriptional regulation begins with extracellular chemical signaling. Responses include changes in the rate of polypeptide translation, and changes in macromolecular **turnover rate** (e.g., changes in the **half-life** of specific RNAs and proteins in cells). Regardless of mechanism, each upor down-regulation of gene expression contributes to changes in the **steady state** of a particular RNA or protein required for proper cell function. In considering posttranscriptional regulation, we will see how cells use specific proteins and different noncoding RNA transcripts to target unwanted proteins or RNAs for degradation.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Explain what it is about C. elegans makes it a model organism for studying development and the regulation of gene expression.
2. Compare and contrast the origins and functions of miRNA and siRNA.
3. Search for examples of miRNAs, siRNAs, lncRNAs and circRNAs that regulate the expression of specific genes and explain their mechanisms.
4. Explain how a riboswitch functions to control bacterial gene expression.
5. Explain the origins and roles of bacterial CRISPR-Cas immune system components.
6. Explain how eif2 activity is modulated to coordinate red cell heme and globin levels.
7. Describe how eukaryotic cells degrade unwanted proteins and speculate on how bacteria might do so
8. answer the questions "How did junk DNA arise?" and "Does junk DNA have value?"

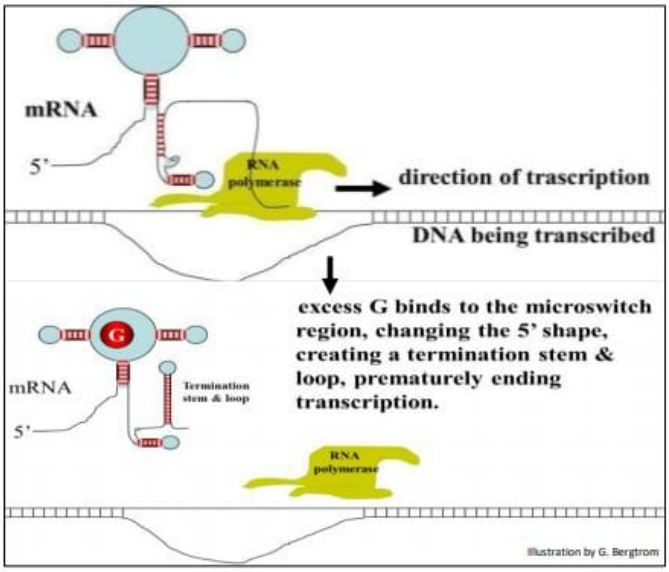
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13.2: Post-transcriptional Control of Gene Expression

Not too long ago we thought that very little of the eukaryotic genome was ever transcribed. We also thought that the only non-coding RNAs were tRNAs and rRNAs. Now we know that other RNAs play roles in gene regulation and the degradation of spent cellular DNA or unwanted foreign DNA. These are discussed in detail below.

A. Ribosomes

The *riboswitches* is a bacterial transcription mechanism for regulating gene expression. While this mechanism is not specifically post-transcriptional, it is included here because the action occurs after transcription initiation and aborts completion of an mRNA. When the mRNA for an enzyme in the guanine synthesis pathway is transcribed, it folds into stem-&-loop structures. Enzyme synthesis will continue for as long as the cell needs to make guanine. But if guanine accumulates in the cell, excess guanine will bind stem-loop elements near the 5' end of the mRNA, causing the RNA polymerase and the partially completed mRNA dissociate from the DNA, prematurely ending transcription. The basis of guanine riboswitch regulation of expression of a guanine synthesis pathway enzyme is shown below.



232 Riboswitches Interrupt Bacterial Transcription

The ability to form folded, stem-loop structures at the 5' ends of bacterial mRNAs seems to have allowed the evolution of translation regulation strategies. Whereas guanine interaction with the stem-loop structure of an emerging 5' mRNA can abort its own transcription, similar small metabolite/mRNA and even protein/mRNA interactions can also regulate (in this case prevent) translation. As we will see shortly, 5' mRNA folded structures also play a role in eukaryotic translation regulation.

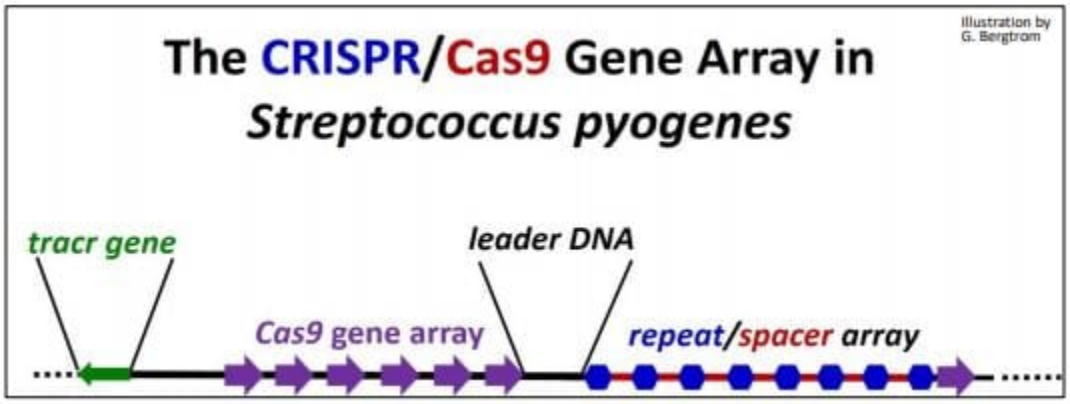
233 Small Metabolites Also Regulate Bacterial mRNA translation

B. CRISPR/Cas: RNA-Protein Complex of a Prokaryotic Adaptive Immune System

In higher organisms, the *immune system* is adaptive. It remembers prior exposure to a pathogen, and can thus mount a response to a second exposure to the same pathogen. The discovery of an '*adaptive immune system*' in many prokaryotes (bacteria, archaebacteria) was therefore something of a surprise.

CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeat) RNAs are derived from phage transcripts that have interacted with CRISPR-Associated (Cas) proteins. They make up the **CRISPR/Cas** system that seems to have evolved to fight

of viral infection by targeting phage DNA for destruction. When viral DNA gets into a cell during a phage infection, it can generate a CRISPR/Cas gene array in the bacterial genome, with **spacer** DNA sequences separating repeats of the CRISPR genes. These remnants of a phage infection are the *memory* of this p*rokaryotic immune system*. When a phage attempts to re-infect a previously exposed cell, spacer RNAs and Cas genes are transcribed. After Cas mRNA translation, the Cas protein and spacer RNAs will engage and target the incoming phage DNA for destruction to prevent infection. Thus, the CRISPR/Cas systems (there is more than one!) *remember* prior phage attacks, and transmit that memory to progeny cells. The CRISPR/Cas9 system in *Streptococcus pyogenes* is one of the simplest of these immune defense systems (illustrated below).



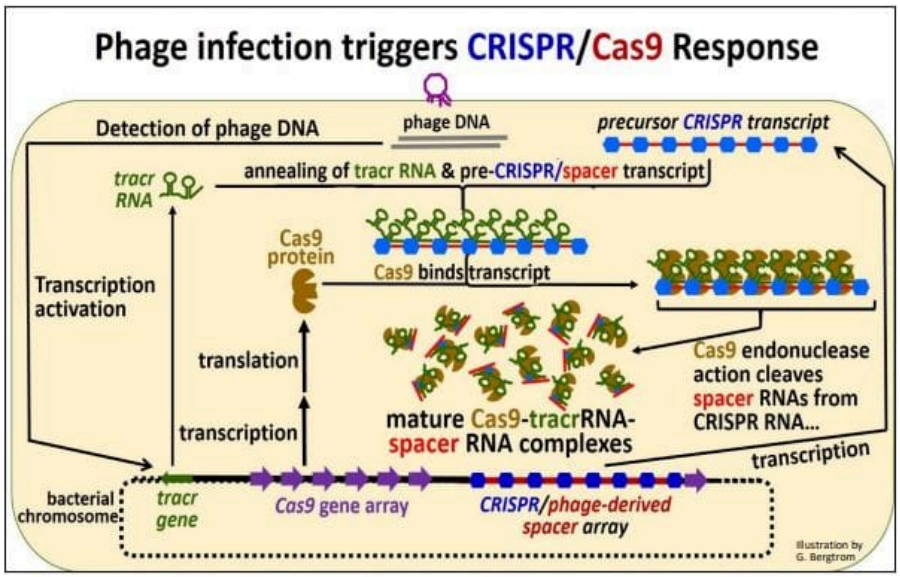
The CRISPR/Cas gene array consists of the following components:

- Cas: Genes native to host cells
- CRISPR: 24-48 bp repeats native to host cells
- Spacer DNA: DNA between CRISPR repeats: typically, phage DNA from prior phage infection or plasmid transformation
- leader DNA: Contains promoter for CRISPR/spacer RNA transcription
- tracr gene: Encodes transcription activator (tracr) RNA (not all systems)

Let's look at CRISPR/Cas in action.

1. The CRISPR/Cas Immune Response

Consider the mechanism of action of this prokaryotic immune system. The action begins when infectious phage DNA gets into the cell, as drawn below.



Let's summarize what has happened here:

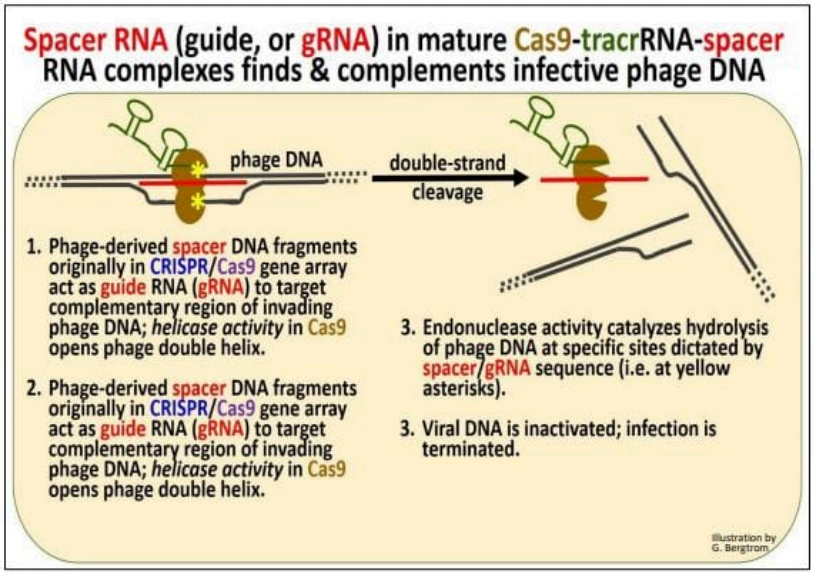
a) Incoming phage DNA was detected after phage infection.

b) Then the tracr and Cas genes are transcribed along with the CRISPR/spacer region. Cas mRNAs are translated to make the Cas protein. Remember, the spacer DNAs in the CRISPR region are the legacy of a prior phage infection.

c) CRISPR/spacer RNA forms hydrogen bonds with a complementary region of the tracr RNA as the two RNAs associate with Cas proteins.

d) Cas protein endonucelases hydrolyze spacer RNA from CRISPR RNA sequences. The spacer RNAs remain associated with the complex while the actual, imperfectly palindromic CRISPR sequences (shown in blue in the illustration above) fall off.

In the next steps, *phage-derived spacer* RNAs, now called **guide RNAs** (or **gRNAs**) 'guide' mature *Cas9/tracrRNA/spacer* RNA complexes to new incoming phage DNA resulting from a phage attack. The association of the complex with the incoming phage DNA and subsequent events are illustrated below.



Once again, let's summarize:

a) Spacer (i.e., gRNA) in the complex targets incoming phage DNA.

b) Cas helicase unwinds incoming phage DNA at complementary regions.

c) gRNA H-bonds to incoming phage DNA.

d) Cas endonucleases create a double-stranded break (hydrolytic cleavage) at specific sites in incoming phage DNA. Because precise site DNA strand cleavage is guided by RNA molecules, CRISPR/Cas endonucleases are classified as type V restriction enzymes.

e) The incoming phage DNA is destroyed and a new phage infection is aborted.

Check out here to learn more about how bacteria acquire spacer DNAs, and therefore how this primitive adaptive immune system 'remembers') in the first place

2. Using CRISPR/Cas to Edit/Engineer Genes

Early studies demonstrated the reproducible cleavage of incoming phage DNA at specific nucleotides. Several labs quickly realized that it might be possible to adapt the system to cut DNA at virtually any specific nucleotide in a target DNA! It has turned out that the system works both in vivo and in vitro, allowing virtually unlimited potential for editing genes and RNAs in a test

tube... or in any cell. Here is the basic process:

a) Engineer gDNA with a Cas-specific DNA sequence that targets a desired target in genomic DNA.

b) Fuse the gDNA to tracr DNA to make a single guide DNA (sgDNA) so that it can be made as a single guide transcript (sgRNA).

c) Engineer a CRISPR/Cas9 gene array that substitutes this sgDNA for its original spacer DNAs.

d) Place engineered array in a plasmid next to regulated promoters.

e) Transform cells by 'electroporation' (works for almost any cell type!)

f) Activate the promoter to transcribe the CRISPR/Cas9 genes...

The applications are powerful... and controversial!

3. The Power and the Controversy

The application of gene editing with CRISPR/Cas systems has already facilitated studies of gene function in vitro, in cells and in whole organisms. Click here for a description of CRISPR/Cas applications already on the market! The efficiency of specific gene editing using CRISPR/Cas systems holds great promise for understanding basic gene structure and function, for determining the genetic basis of disease, and for accelerating the search for gene therapies. Here are just a few examples of how CRISPR/Cas approaches are being applied.

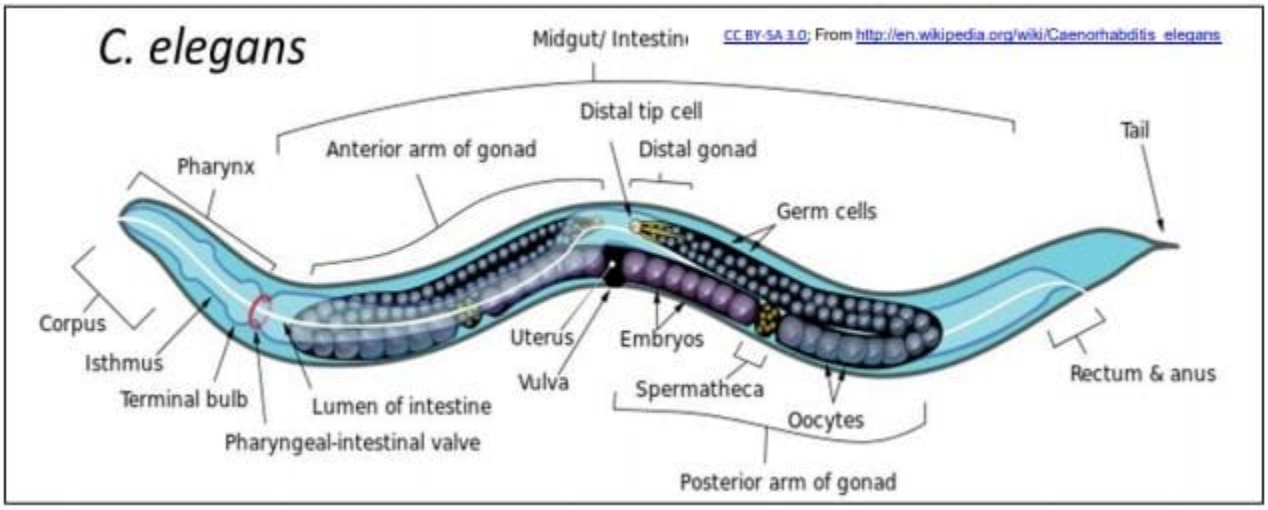
- One can engineer an sgRNA with desired mutations targeting specific sites in chromosomal DNA. Then clone sgRNA into the CRISPR/Cas9 array on a plasmid. After transformation of appropriate cells, the engineered CRISPR/Cas9 forms a complex with target DNA sequences. Following nicking of both strands of the target DNA, DNA repair can insert the mutated guide sequences into the target DNA. The result is loss or acquisition of DNA sequences at *specific, exact sites*, or **Precision Gene Editing**. It is the ability to do this in living cells that has excited the basic and clinical research communities.
- Before transforming cells, engineer the **CRISPR/Cas9** gene array on the plasmid to eliminate both **endonuclease** activities from the Cas protein. Upon transcription of the array in transformed cells, the *CRISPR/Cas9-sgRNA* still finds an *sgRNA*-targeted gene. However, lacking CAS protein endonuclease activities, the complex that forms just sits there **blocking transcription**. This technique is sometimes referred to as **CRISPRi** (*CRISPER interference*), by analogy to *RNAi*. Applied to organisms (and not just in vitro or to cells), it mimics the much more difficult **knockout** mutation experiments that have been used in studies of behavior of cells or organisms rendered unable to express a specific protein.
- There are now several working CRISPR/Cas systems capable of *Precision Gene Editing*. They are exciting for their speed, precision, their prospects for rapid, targeted gene therapies to fight disease, and their possibilities to alter entire populations (called **Gene Drive**). By inserting modified genes into the germline cells of target organisms, gene drive can render harmless entire malarial mosquito populations, to eliminate pesticide resistance in e.g. insects, eliminate herbicide resistance in undesirable plants, or genetically eliminate invasive species. For more information, click Gene drive; for an easy read about this process and the controversies surrounding applications of CRISPR technologies to mosquitoes in particular, check out J. Adler, (2016) *A World Without Mosquitoes*. Smithsonian, 47(3) 36-42, 84.
- It is even possible to delete an entire chromosome from cells. This bit of global genetic engineering relies on identifying multiple unique sequences on a single chromosome and then targeting these sites for CRISPR/Cas. When the system is activated, the chromosome is cut at those sites, fragmenting it beyond the capacity of DNA repair mechanisms to fix the situation. Click here to learn more.

If for no other reason than its efficiency and simplicity, precision gene editing with CRISPR/Cas techniques has raised ethical issues. Clearly, the potential exists for abuse, or even for use with no beneficial purpose at all. It is significant that, as in all discussions of biological ethics, scientists are very much engaged in the conversation. Despite the controversy, we will no doubt continue to edit genes with CRISPR/Cas, and we can look for a near future Nobel Prize for its discovery and application! If you still have qualms, maybe RNA editing will be the answer. Check out the link at Why edit RNA? for an overview of the possibilities!

Finally, "mice and men" (and women and babies too) have antibodies to Cas9 proteins, suggesting prior exposure to microbial CRISPR/Cas9 antigens. This observation may limit clinical applications of the technology! See Uncertain Future of CRISPR-Cas9 Technology.

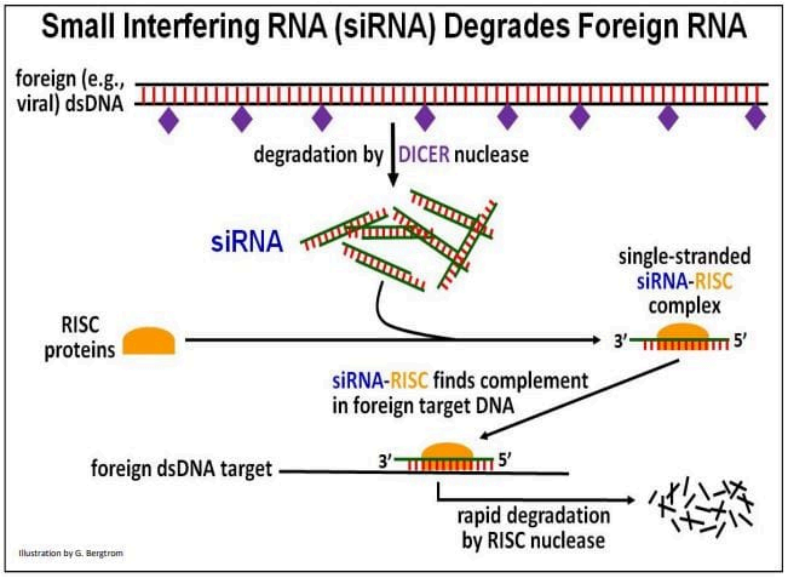
C. The Small RNAs: miRNA and siRNA in Eukaryotes

Micro RNAs (miRNAs) and small interfering RNAs (siRNAs) are found in *C. elegans*, a small nematode (roundworm) that quickly became a model for studies of cell and molecular biology and development. The particular attractions *C. elegans* are that (a) its genome has ~21,700 genes, comparable to the ~25,000 genes in a human genome!; (b) it uses the products of these genes to produce an adult worm consisting of just 1031 cells organized into all of the major organs found in higher organisms; (c) It is possible to trace the embryonic origins of every single cell in its body! *C. elegans* is illustrated below.



1. Small Interfering RNA (siRNA)

siRNA was first found in plants as well as in *C. elegans*. However, siRNAs (and miRNAs) are common in many higher organisms. siRNAs were so-named because they *interfere* with the function of other RNAs foreign to the cell or organism. Their action was dubbed *RNA interference* (**RNAi**). For their discovery of siRNAs, A. Z. Fire and C. C. Mello shared the 2006 Nobel Prize in Physiology or Medicine. The action of siRNA targeting foreign DNA is illustrated below.



When cells recognize foreign double-stranded RNAs (e.g., some viral RNA genomes) as alien, the **DICER** a *nuclease* called hydrolyzes them. The resulting short double-stranded hydrolysis products (the **siRNAs**) combine with **R**NAi **I**nduced **S**ilencing **C**omplex, or **RISC** proteins. The *antisense* siRNA strand in the resulting **siRNA-RISC** complex binds to complementary regions of foreign RNAs, targeting them for degradation. Cellular use of RISC to control gene expression in this way may have derived

from the use of RISC proteins by miRNAs as part of a cellular defense mechanism, to be discussed next.

Custom-designed siRNAs have been used to disable expression of specific genes in order to study their function *in vivo* and *in vitro*. Both siRNAs and miRNAs are being investigated as possible therapeutic tools to interfere with RNAs whose expression leads to cancer or other diseases.

234 siRNA Post Transcriptional Regulation

235 Did siRNA Coopt RISC strategy to Trash Corrupt or Worn out RNA?

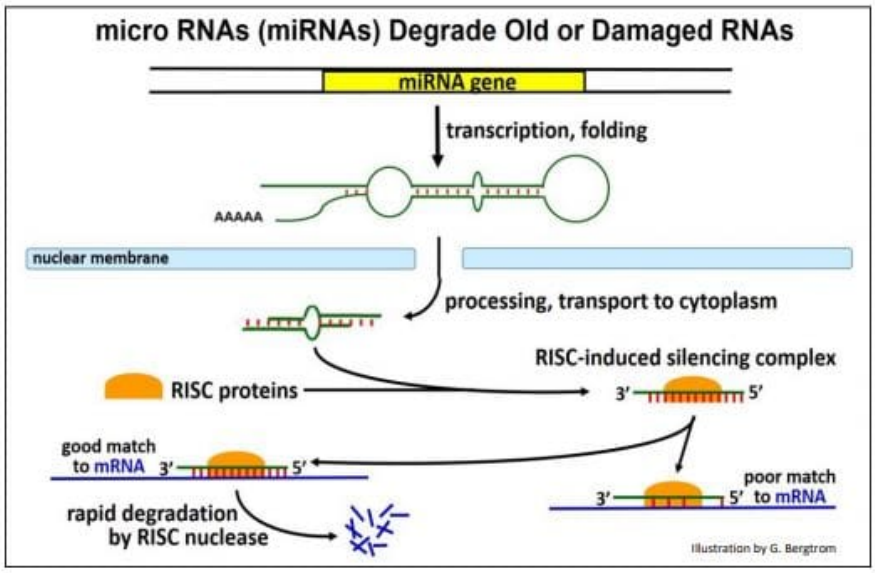
For an example check out a Youtube video of unexpected results of an RNAi experiment at this link. In the experiment described, RNAi was used to block embryonic expression of the **orthodenticle** (**odt**) gene that is normally required for the growth of horns in a dung beetle. The effect of this *knock-out mutation* was, as expected, to prevent horn growth. What was unexpected however, was the development of an eye in the middle of the beetle's head ('third eye' in the micrograph).

The 3rd eye not only looks like an eye, but is a functional one. This was demonstrated by preventing normal eye development in *odt*-knockout mutants. The 3rd eye appeared..., and was responsive to light! Keep in mind that this was a beetle with a 3rd eye, not *Drosophila*! To quote Justin Kumar from Indiana University, who though not involved in the research, stated that "...lessons learned from *Drosophila* may not be as generally applicable as I or other Drosophilists, would like to believe ... The ability to use RNAi in non-traditional model systems is a huge advance that will probably lead to a more balanced view of development."

2. Micro RNAs (miRNA)

miRNAs target unwanted *endogenous* cellular RNAs for degradation. They are transcribed from genes now known to be widely distributed in eukaryotes. The pathway from pre-miRNA transcription through processing and target mRNA degradation is illustrated on the next page.

As they are transcribed, pre-miRNAs fold into a stem-loop structure that is lost during cytoplasmic processing. Like SiRNAs, mature miRNAs combine with RISC proteins. The **RISC** protein-miRNA complex targets old or no-longer needed mRNAs or mRNAs damaged during transcription.



An estimated 250 miRNAs in humans may be sufficient to H-bond to diverse target RNAs; only targets with strong complementarity to a RISC protein-miRNA complex will be degraded.

236 miRNA Post-Transcriptional Regulation

D. Long Non-Coding RNAs

Long non-coding RNAs (**lncRNAs**) are a yet another class of eukaryotic RNAs. They include transcripts of antisense, intronic, intergenic, pseudogene and retroposon DNA. Retroposons are one kind of transposon, or mobile DNA element; pseudogenes are recognizable genes with mutations that make them non-functional. While some lncRNAs might turn out to be incidental transcripts that the cell simply destroys, others have a role in regulating gene expression.

A recently discovered lncRNA is XistAR that, along with the Xist gene product, is required to form *Barr bodies*. Barr bodies form in human females when one of the X chromosomes in somatic cells is inactivated. For a review of lncRNAs, see Lee, J.T. (2012. *Epigenetic Regulation by Long Noncoding RNAs*; Science 338, 1435-1439).

An even more recent article (at lncRNAs and smORFs) summarizes the discovery that some long non-coding RNAs contain short open reading frames (**smORFs**) that are actually translated into short peptides of 30+ amino acids! Who knows? The human genome may indeed contain more than 21,000-25,000 protein-coding genes!

E. Circular RNAs (circRNA)

Though discovered more than 20 years ago, circular RNAs (circRNAs) are made in different eukaryotic cell types. Click Circular RNAs (circRNA) to learn more about this peculiar result of *alternative* splicing. At first circRNAs were hard to isolate. When they were isolated, circRNAs contained “scrambled” exonic sequences and were therefore thought to be nonfunctional errors of mRNA splicing.

In fact, circRNAs are fairly stable. Their levels can rise and fall in patterns suggesting that they are functional molecules. Levels of one circRNA, called **circRims1**, rise specifically during neural development. In mice, other circRNAs accumulate during synapse formation, likely influencing how these neurons will ultimately develop and function. Thus, circRNAs do not seem to be ‘molecular mistakes’. In fact, *errors in their own synthesis* may be correlated with disease! Speculation on the functions of circRNAs also includes roles in gene regulation, particularly the genes or mRNAs from which they themselves are derived.

F. "Junk DNA" in Perspective

Not long ago, we thought that less than 5% of a eukaryotic genome was transcribed (i.e., into mRNA, rRNA and tRNA), and that much of the non-transcribed genome served a structural function... or no function at all. The latter, labeled junk DNA, included non-descript intergenic sequences, pseudogenes, ‘dead’ transposons, long stretches of intronic DNA, etc. Thus, junk DNA was DNA we could do without. Junk DNAs were thought to be accidental riders in our genomes, hitchhikers picked up on the evolutionary road.

While miRNA genes are a small proportion of a eukaryotic genome, their discovery, and that of more abundant lnc RNAs suggest a far greater amount of functional DNA in the genome. Might there be in fact, no such thing as “junk DNA”? The debate about how much of our genomic DNA is a relic of past evolutionary experiments and without genetic purpose continues. Read all about it at Junk DNA - not so useless after all and Only 8.2% of human DNA is functional.

Perhaps we need to re-think what it means for DNA to be “junk” or to be without “genetic purpose”. Maintenance of more than 90% of our own DNA with no known genetic purpose surely comes at an energy cost. At the same time, all of that DNA is grist for future selection, a source of the diversity required for long-term survival. The same natural selection that picks up ‘hitchhiker’ DNA sequences, as we have seen, can at some point, put them to work!

G. The RNA Methylome

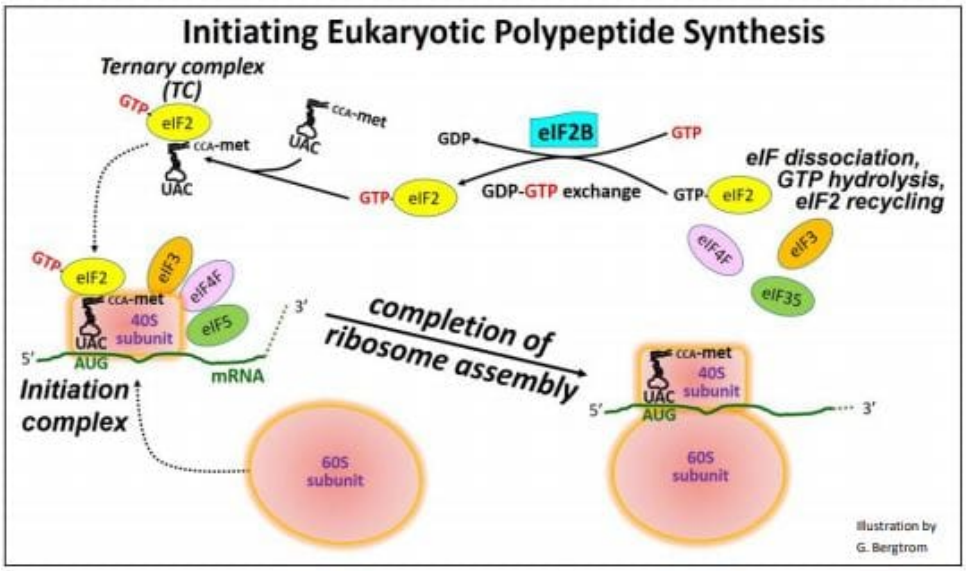
Call this an *RNA epi-transcriptome* if you like! Recall that methyl groups direct cleavage of ribosomal RNAs from eukaryotic 45S pre-RNA transcripts. tRNAs among other transcripts, are also post-transcriptionally modified. Known since the 1970s, such modifications were thought to be non-functional. But are they?

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13.3: Eukaryotic Regulation of Translation

A. The basics of Eukaryotic mRNA Translation

The basic features of translation initiation in eukaryotes are shown below.

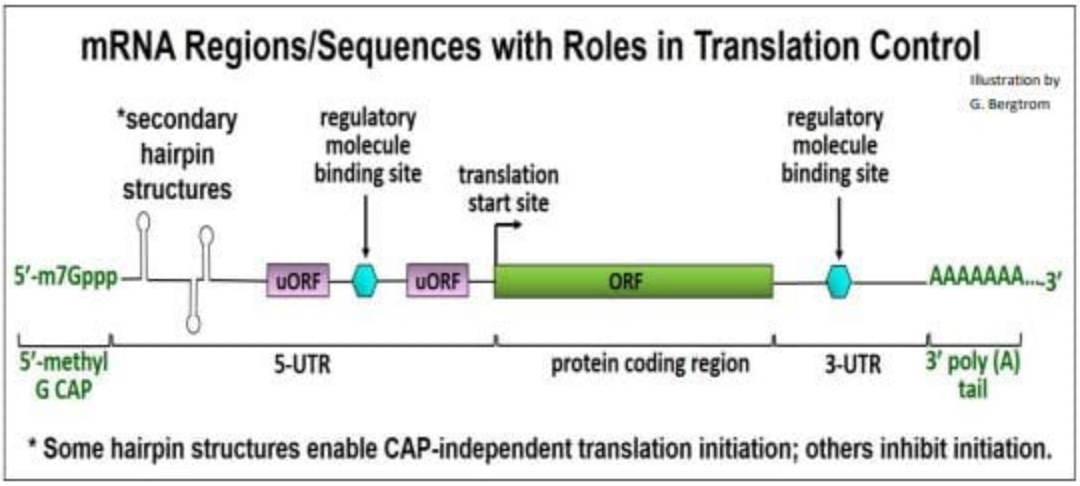


In many respects, the overall process is similar to prokaryotic translation initiation described elsewhere. The 40S ribosomal subunit itself can bind to and scan an mRNA, seeking the start site of an ORF (open reading frame) encoding a polypeptide. When GTP-bound *eukaryotic initiation factor 2* (**GTP-eIF2**) binds met-tRNAf, it forms a *ternary complex* (**TC**). The TC can associate with the scanning 40S subunit. When a TC-associated scanning subunit encounters the start site of the ORF, scanning stalls. Additional eIFs enable formation of the *initiation complex*, positioning the initiator tRNA anticodon over the start site AUG in the mRNA. The initiation complex then recruits the large (60S) ribosomal subunit. Binding of the 60S ribosomal subunit to the initiation complex causes the release of all the eIFs and hydrolysis of the GTP on eIF2. The GDP remains bound to eIF2. For protein syntheses to continue, new GTP must replace GDP on eIF2. Another initiation factor, **eIF2B**, facilitates this GTP/GDP swap, recycling GTP-eIF2 for use in initiation. The regulation of translation is superimposed on these basic processes.

B. Translation Regulation

Since mRNAs are made to be translated, it is likely that by default, they are! We know that CAP and poly(A) tails on mRNAs are required for efficient translation because mRNAs engineered to lack one and/or the other are poorly translated. Also, there is little evidence to that cells modify the process of capping or polyadenylation, or the structures themselves.

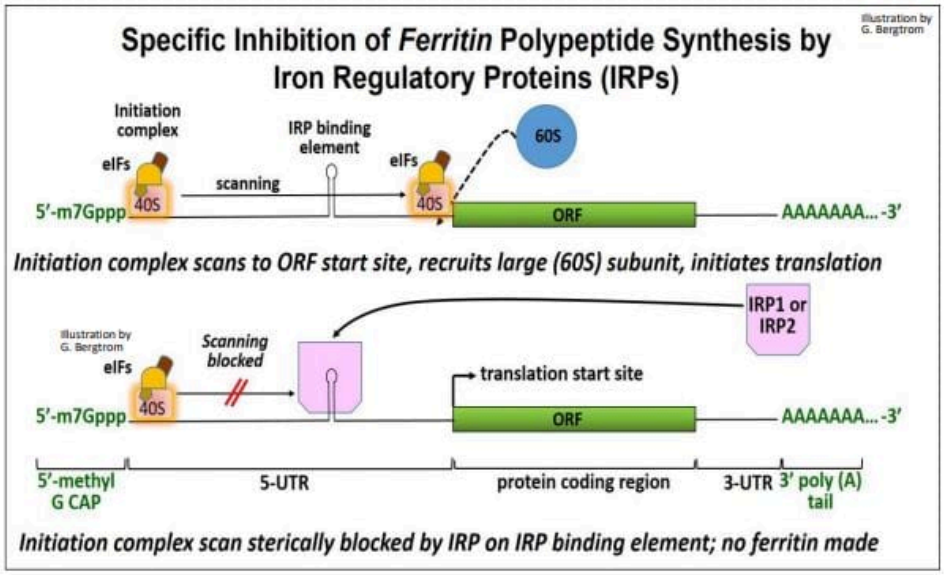
Translation regulation typically targets initiation. It may be global, affecting the synthesis of many polypeptides at once, or specific, affecting a single polypeptide. Global regulation involves changes in the activity of eukaryotic initiation factors (eIFs) that would typically affect all cellular protein synthesis. Specific regulation involves binding sequences or regions on one or a few mRNAs that recognize and bind specific regulatory proteins and/or other molecules. That binding controls translation of only those mRNAs, without affecting general protein biosynthesis. mRNA structural features involved in translation and in translation regulation are illustrated below.



We will consider three examples of translational control of gene expression.

1. Specific Translation Control by mRNA Binding Proteins

Ferritin is a cellular iron-storage protein made up of heavy and light chain polypeptides. Translation of ferritin in iron-deficient cells is inhibited. In the absence of ferritin production, ferritin-iron complexes release iron for metabolic use. The 5'-UTR of mRNAs for both chains contain stem-loop binding sites that specifically recognize **iron regulatory proteins** (IRP1, IRP2). When ferritin mRNAs are bound to IRPs, translation initiation is blocked. The inhibition of ferritin translation by IRPs is illustrated below.



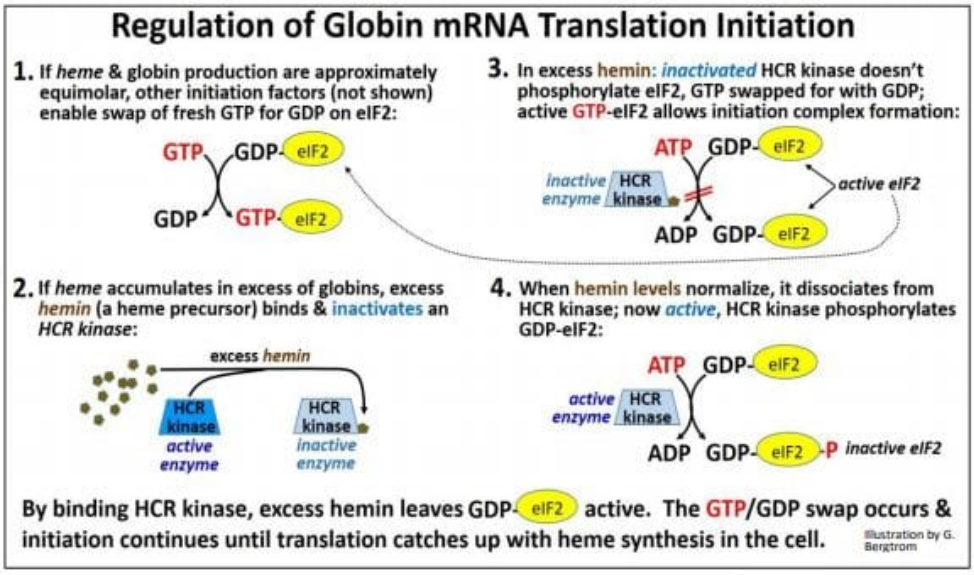
Normally, the initiation complex scans the 5'-UTR of an mRNA. When it finds the normal translation start site, it can bind the large subunit and begin translating the polypeptide. In iron-deficient cells, scanning by the initiation complex is thought to be physically blocked by steric hindrance.

2. Coordinating Heme & Globin Synthesis

Consider that *reticulocytes* (the precursors to *erythrocytes*, the red blood cells in mammals) synthesize **globin** proteins. They also synthesize **heme**, an iron-bound porphyrin-ring molecule. Each globin must bind to a single heme to make a hemoglobin protein subunit. Clearly, it would not do for a reticulocyte to make too much globin protein and not enough heme, or vice versa. It turns out that **hemin** (a precursor to heme) regulates the initiation of translation of both α and β globin mRNAs. Recall that, to sustain globin mRNA translation, the **GDP-eIF2** generated after each cycle of translation elongation must be exchanged for

fresh GTP. This is facilitated by the **eIF2B** initiation factor. **eIF2B** can exist in phosphorylated (inactive) or un-phosphorylated (active) states. Making sure that globin is not under- or overproduced relative to heme biosynthesis involves controlling levels of active vs. inactive eIF2B by **hemin**. Hemin accumulates when there is not enough globin polypeptide to combine with heme in the cell. Excess hemin binds and inactivates an **HCR kinase**, preventing phosphorylation of eIF2B. Since unphosphorylated eIF2B is active, it facilitates the GTP/GDP swap needed to allow continued translation. Thus, ongoing initiation ensures that globin mRNA translation can keep up with heme levels. In other words, if hemin production gets ahead of globin, it will promote more globin translation.

When globin and heme levels become approximately equimolar, hemin is no longer in excess. It then dissociates from the active HCR kinase. The now- active kinase catalyzes eIF2B phosphorylation. *Phospho-eIF2B* is inactive, and cannot facilitate the GTP/GDP swap on eIF2. Globin mRNA translation initiation, thus blocked, allows a lower rate of globin polypeptide translation to keep pace with heme synthesis. The regulation of globin mRNA translation initiation by hemin is shown below.

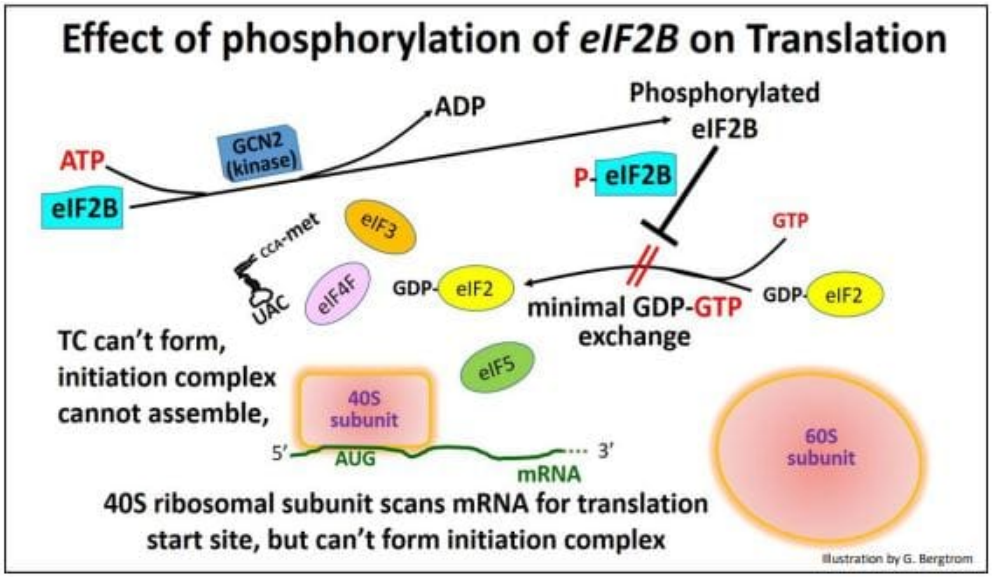


237 Translation Regulation of Globin Polypeptide Synthesis

3. Translational Regulation of Yeast GCN4

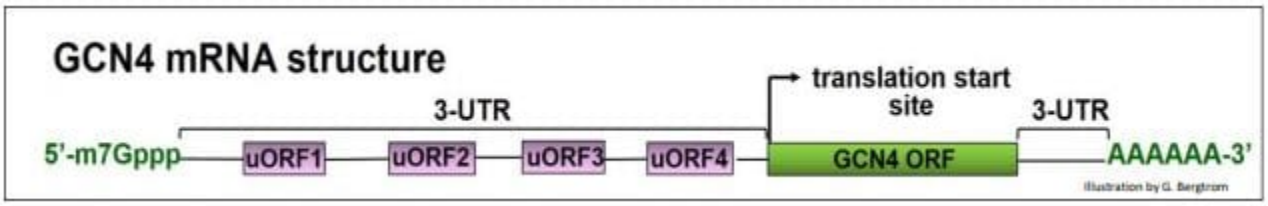
Like the coordination of heme and globin production, the regulation of the GCN4 protein is based on controlling the ability of the cells to swap GTP for GDP on eIF2. However, this regulation is quite a bit more complex, despite the fact that yeast is a more primitive eukaryote! GCN4 is a **global transcription factor** that controls the transcription of as many as 30 genes in pathways for the synthesis of 19 out of the 20 amino acids! The discovery that amino acid starvation caused yeast cells to increase their production of amino acids in the cells led to the discovery the *General Amino Acid Control* (**GAAC**) mechanism involving GCN4. GCN is short for *General Control Nondepressible*, referring to its global, positive regulatory effects. It turns out that the GCN4 protein is also involved in stress gene expression, glycogen homeostasis, purine biosynthesis..., in fact in the action of up to 10% of all yeast genes! Here we focus on the GAAC mechanism.

Yeast cells provided with ample amino acids do not need to synthesize them. Under these conditions, GCN4 is present at basal (i.e., low) levels. When the cells are starved of amino acids, GCN4 levels increase as much as ten-fold within two hours, resulting in an increase in general amino acid synthesis. This rapid response occurs because amino acid starvation signals an increase in the activity of **GCN2**, a *protein kinase*. The GCN2 kinase catalyzes phosphorylation of GDPeIF2. As we have already seen, phosphorylated eIF2B cannot exchange GTP for GDP on the eIF2, in this case with the results shown below.

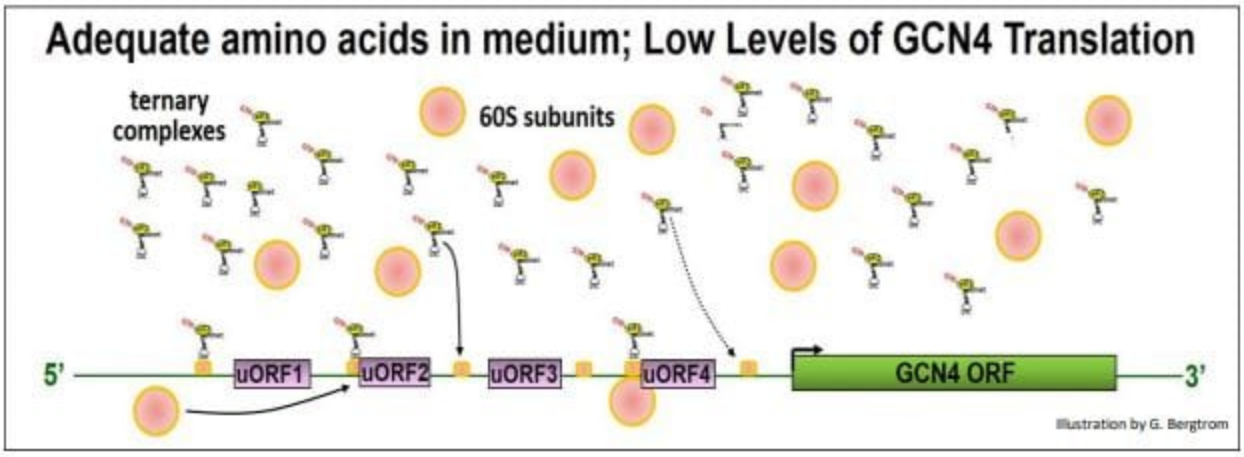


There is a paradox here. You would expect a slowdown in GTP-eIF2 regeneration to inhibit overall protein synthesis, and it does. However, the reduced levels of GTP-eIF2 somehow also stimulate translation of the GCN4 mRNA, leading to increased transcription of the amino acid synthesis genes. In other words, amino acid starvation leads yeast cells to use available substrates to make their own amino acids in order that protein synthesis can continue... at the same time as initiation complex formation is disabled!

Let's accept that paradox for now, and look at how amino acid starvation leads to increased translation of the GCN4 protein and the up-regulation of amino acid biosynthesis pathways. To begin with, we are going to need to understand the structure of GCN4 mRNA. In the illustration below, note the 4 short **uORFs** in the 5'UTR of the RNA; these play a key role in GCN4 translation regulation.

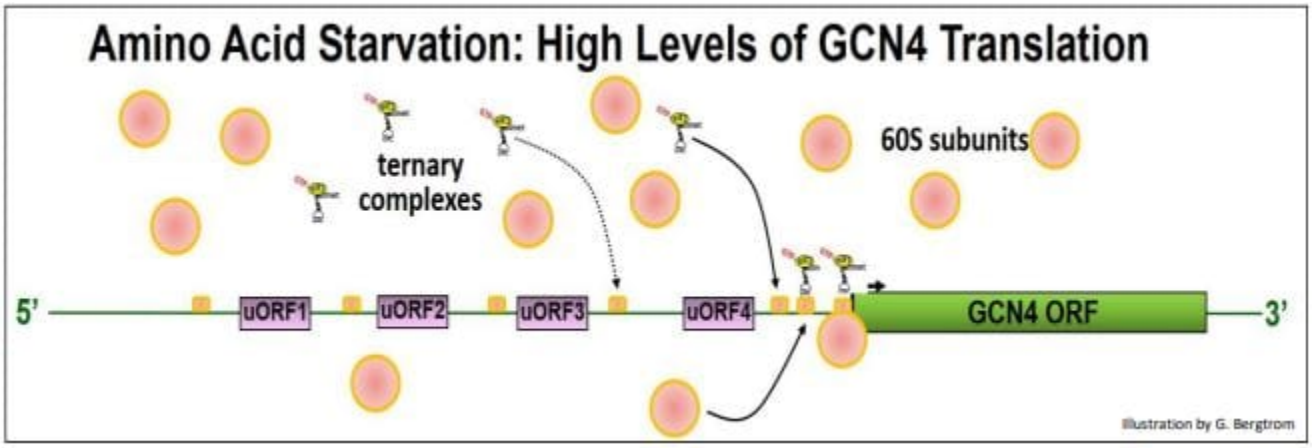


We noted earlier that when a Ternary Complex (TC)-associated 40S ribosomal subunit scans an mRNAs and find the ORF start sites for its polypeptide, initiation complexes form, 60S ribosomal subunits bind and translation starts. GCN4 mRNA has four uORFs in its 5' UTR. While uORFs encode only a few amino acids before encountering a stop codon, they can also be recognized during scanning. When TCs and 40S subunits are plentiful, they seem to engage uORFs in preference to the GCN4 coding region ORF, as illustrated below.



Under these conditions, active eIF2B allows the GTP/GDP swap on GDP-eIF2, leading to efficient GTP-eIF2 recycling and high TC levels. TCs bind small subunits during scanning and/or at the start sites of uORFs, forming initiation complexes that then bind 60S ribosomal subunits and begin uORF translation. The effect is to slow down scanning past the uORFs, thereby inhibiting initiation complex formation at the actual GCN4 ORF.

What happens in amino acid-starved cultures of yeast cells, when GTP-eIF2 cannot be efficiently regenerated and TCs are in short supply? To review, amino acid starvation signals an increase in GCN2 kinase activity resulting in phosphorylation and inactivation of eIF2B. Inactive phospho-eIF2 will not facilitate the GTP/GDP swap at GDP-eIF2, inhibiting overall protein synthesis. The resulting reduction in GTP-eIF2 also lowers the levels of TC and TC-associated 40S subunits. The illustration below shows how this phenomenon up-regulates GCN4 translation, even as the translation of other mRNAs has declined.



C. Regulating Protein Turnover (Half-Life)

We have already seen that organelles have a finite life span, or half-life. Recall that lysosomes participate in destroying worn out mitochondria and their molecular components. We also saw the role of small RNAs (especially miRNA) in destroying old, damaged or otherwise unwanted RNAs from cells. All cell structures and molecules have a finite half-life, defined as the time it takes for half of them to disappear in the absence of new synthesis of the structure or molecule. As we already know, the steady-state level of any cellular structure or molecule exists when the rate of its manufacture or synthesis is balanced by the rate of its turnover. Of course, steady state levels of things can change. For example, the level of gene expression (the amount of a final RNA or protein gene product in a cell) can change if rates of transcription, processing or turnover change. We should also expect the same for the steady-state levels of cellular proteins. Here we consider the factors that govern the half-life of cellular proteins.

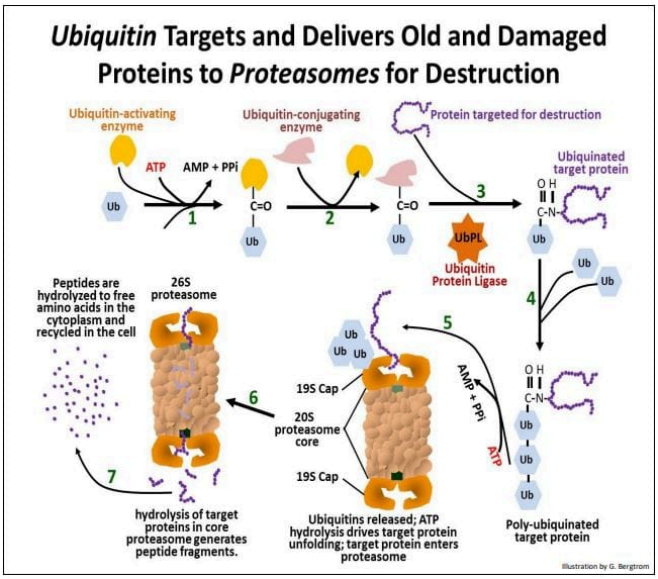
The half-life of different proteins seems to be inherent in their structure. Thus, some amino acid side chains are more exposed at the surface of the protein and are thus more susceptible to change or damage over time than others. Proteins with fewer 'vulnerable' amino acids should have a longer half-life than those with more of them. Proteins damaged by errors of translation, folding, processing gone awry or just worn out from use or 'old age' will be targeted for destruction. All molecules have a half-life!

The mechanism for detecting and destroying unwanted old, damaged or misbegotten proteins involves a 76-amino acid polypeptide called ubiquitin that targets the protein for destruction, delivering it to a large complex of polypeptides called the proteasome. Here is what happens:

1. The first step is to activate an ubiquitin. This starts when ATP hydrolysis fuels the binding of ubiquitin to an **ubiquitin-activating enzyme**.
2. An **ubiquitin-conjugating enzyme** then replaces the ubiquitin-activation enzyme.
3. The protein destined for destruction replaces the ubiquitin-conjugating enzyme.
4. Several more ubiquitins then bind to this complex.
5. The **poly-ubiquinated protein** delivers its protein to one of the 19S 'CAP' structures of a proteasome.
6. After binding to one of the CAP structures of a proteasome, the poly-ubiquinated target proteins dissociate and the ubiquitins are released and recycled as the target protein unfolds (powered by ATP hydrolysis). The unfolded protein then enters a 20S core proteasome.

The target protein is digested to short peptide fragments by proteolytic enzymes in the interior of the proteasome core. The fragments are release from the CAP complex at the other end of the proteasome and digested down to free amino acids in the cytoplasm. There is a mind-boggling variety of proteins in a cell..., and there are as many as 600 different ubiquitin proteins, encoded by as many genes! Presumably, each ubiquitin handles a subclass of proteins based on common features of their structure.

With its complex quaternary structure, the 26S proteasome is smaller than a eukaryotic small ribosomal subunit (40S), but is still one of the largest cytoplasmic particles... and without the benefit of any RNA in its structure! The illustration on the next page details the role of ubiquitin in the degradation of a worn out protein by a proteasome. Click on Proteasome in Action to see an animated version of the illustration.



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13.4: Key Words and Terms

19S proteasome cap complex	gene editing	RISC endonuclease
20S proteasome complex	global transcription factor	RISC proteins
amino acid starvation	globin	RNA interface
Barr Bodies	gRNA	RNA turnover rates
C. Elegans	GTP/GDP swap	RNAi
Cas	GTP-eiF2 recycling	RNA-induced silencing complex
Cas helicase activity	half life	sgRNA
Cas9 endonuclease	HCR kinase	siRNA (small interfering RNA)
circRNA	heme	small RNAs
circular RNA	hemin	smORF
CRISPR	HRC kinase	spacer RNA
CRISPR interference	initiation complex scanning	steady state
CRISPR/Cas	iron regulatory protein	*Streptococcus pyogenes*
CRISPR/Cas9 gene array	IRP	tracr
CRISPRi	Junk DNA	tracr gene
dicer	IncRNA	tracr RNA
eiF2 phosphorlyation	long non-coding RNA	translation elongation
eiF2B	micro RNA	ubiquitin
$EIIA^{Glc}$	miRNA	ubiquitination
Ferritin	mRNA scanning	uORF
GAAC	proteasome	XistAR
GCn2	protein turnover rates	Yeast GCN4
GDP-eiF2	riboswitch	
Gene Drive	RISC	

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14: REPETITIVE DNA - A EUKARYOTIC GENOMIC PHENOMENON

14.1: Introduction
14.2: The Complexity of Genomic DNA
14.3: The 'Jumping Genes' of Maize
14.4: Transposons Since McClintock
14.5: On the Evolution of Transposons, Genes, and Genomes
14.6: Roles of Transposition in Evolution and Diversity
14.7: Coping with the Dangers of Rampant Transposition
14.8: Key Words and Terms

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14.1: Introduction

Because of their small size, bacterial genomes have few **repetitive DNA** sequences. In contrast, repetitive DNA sequences make up a large part of a eukaryotic genome. Much of this repeated DNA consists of identical or nearly identical sequences of varying length repeated many times in a genome. Examples include **satellite DNA** (minisatellite and microsatellite DNA) and **transposons**, or **transposable elements**. Here we look at experiments that first revealed the existence and proportion of repeated DNA in genomes. Next we describe Barbara McClintock's even earlier (and pretty amazing!) discovery of transposable elements. After we describe the different classes of transposons and different mechanisms of **transposition**, we tackle the question of why they and other repetitive DNAs even exist. Elsewhere we introduced the notion of **junk DNA** as DNA sequences that serve no known purpose. Is repeated DNA *junk* DNA? Are transposable elements *junk*? We are now learning that transposons and other repetitive DNAs can have specific functions, from regulating gene expression to reshaping genomes to increasing genetic diversity in evolution. So, far from being 'junk', much redundant DNA exists in genomes because of evolutionary selection.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Compare and contrast renaturation kinetic data.
2. Explain *CoT curves* and *DNA complexity*
3. List physical and chemical properties of *main band* and *satellite DNAs*
4. Outline an experiment to determine if a given sequence of DNA is repetitive or not.
5. Summarize how Barbara McClintock revealed the genetics of maize mosaicism.
6. Outline the experiments suggesting that the Ds gene moves from one lovus to another in the maize genome.
7. Compare and contrast *cut-&-paste* and *replicative transposition*.
8. Compare the behaviors of *autonomous* and *non-autonomous* transposons.
9. List the difference between *Mu phage* infection and transposition.
10. Describe the common structural features of transposons.
11. Compare the mechanisms of LINE and SINE transposition.
12. Speculate on how species avoid potentially lethal consequences of transposition.
13. Speculate on which came first in evolution: DNA transposons, RNA transposons or retroviruses, and explain your reasoning.

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14.2: The Complexity of Genomic DNA

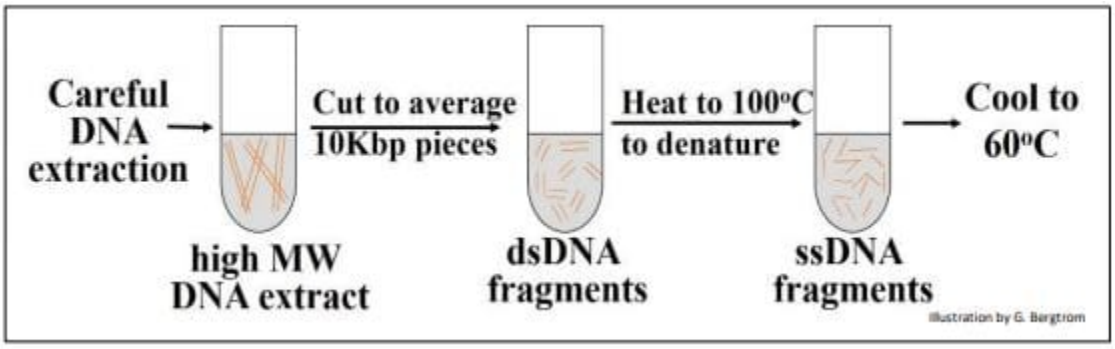
By the 1960s, when Roy Britten and Eric Davidson were studying eukaryotic gene regulation, they knew that there was more than enough DNA to account for the genes needed to encode an organism. It was also likely that DNA was more structurally complex than originally thought. They knew that **cesium chloride** (**CsCl**) **density gradient centrifugation** separated molecules based on differences in density and that fragmented DNA would separate into a main and a minor band of different density in centrifuge tube. The minor band was dubbed **satellite DNA**, recalling the *Sputnik* satellite recently launched by Russia. DNA bands of different density could not exist if the proportions of A, G, T and C in DNA (already known to be species-specific) were the same throughout a genome. Instead, there must be regions of DNA that are richer in A-T than G-C pairs and vice versa. Analysis of satellite bands that moved further on the gradient (i.e., were more dense) than the main band were indeed richer in GC content. Those that lay above the main band were more AT-rich.

Consider early estimates of how many genes it might take to make a human, mouse, chicken or petunia: about 100,000! We know now that it takes fewer! Nevertheless, even with inflated estimates of the number of genes it takes to make a typical eukaryote, their genomes contain 100-1000 times more DNA than necessary to account for 100,000 genes. How then to explain this extra DNA? Britten and Davidson's elegant **renaturation kinetics** experiments revealed some physical characteristics of genes and so-called 'extra' DNA. Let's look at these experiments in some detail.

A. The Renaturation Kinetic Protocol

The first step in a renaturation kinetic experiment is to shear DNA isolates to an average size of 10 Kbp by pushing high molecular weight DNA through a hypodermic needle at constant pressure. The resulting double-stranded fragments (**dsDNA**) is then heated to 100°C to **denature** (separate) the two strands. The solutions are then cooled to 600C to allow the single stranded DNA (**ssDNA**) fragments to slowly re-form complementary double strands. At different times after incubation at 60oC, the partially renatured DNA was sampled and ssDNA and dsDNA were separated and quantified.

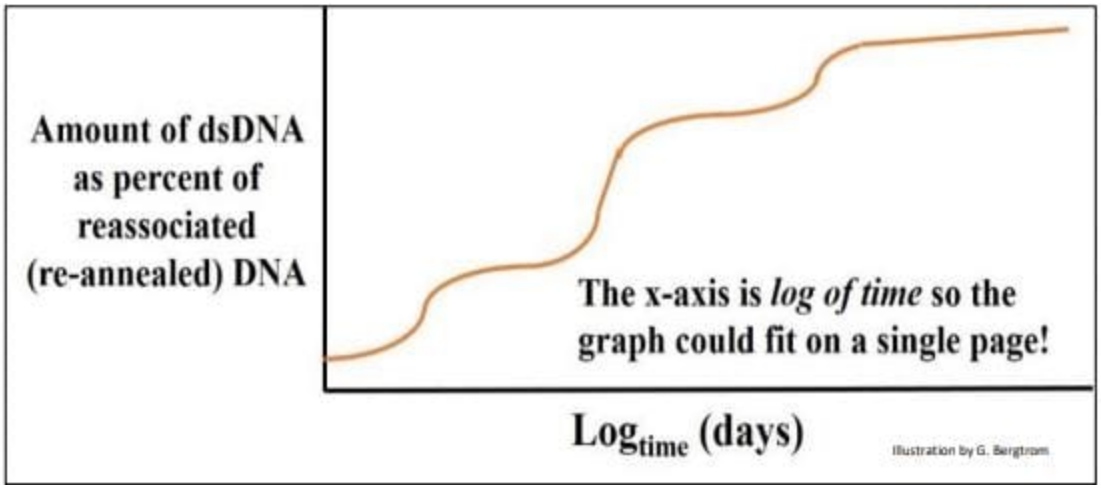
The experiment is summarized in the drawing below.



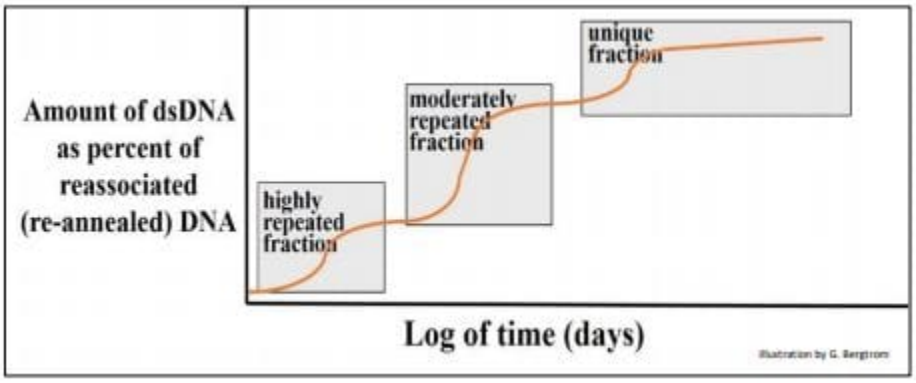
The amount, or percent of DNA that had renatured over time could be graphed.

B. Renaturation Kinetic Data

A plot of dsDNA formed at different times (out to many days!) is shown below for a renaturation kinetics experiment using rat DNA.



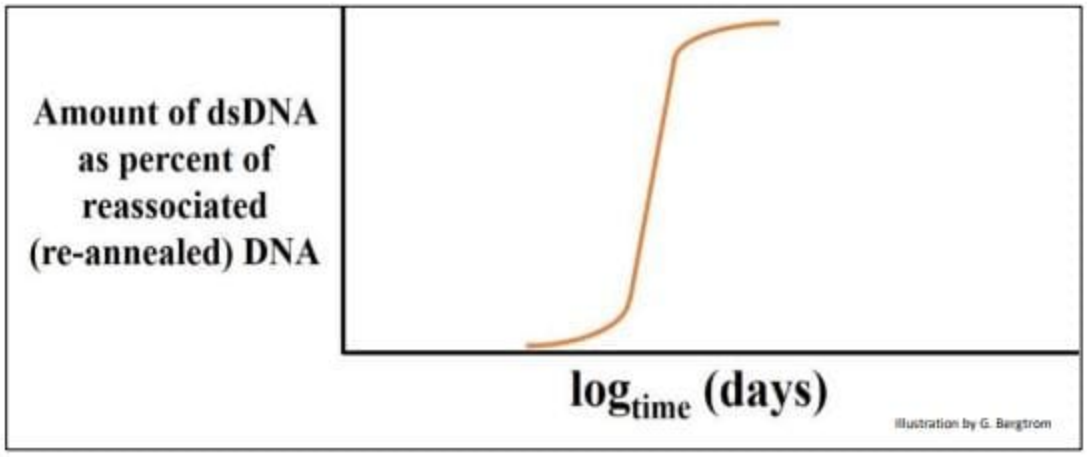
In this example, the DNA fragments could be placed in three main groups with different overall rates of renaturation. Britten and Davidson reasoned that the dsDNA that had formed most rapidly was composed of sequences that must be more highly repetitive than the rest of the DNA. The rat genome also had a lesser amount of more moderately repeated dsDNA fragments that took longer to anneal than the highly repetitive fraction, and even less of a very slowly re-annealing DNA fraction. The latter sequences were so rare in the extract that it could take days for them to re-form double strands, and were classified as non-repetitive, unique (or nearly unique) sequence DNA, as illustrated below.



It became clear that the rat genome (in fact most eukaryotic genomes) consists of different classes of DNA that differ in their redundancy. From the graph, a surprisingly a large fraction of the genome was repetitive to a greater or lesser extent.

238 Discovery of Repetitive DNA

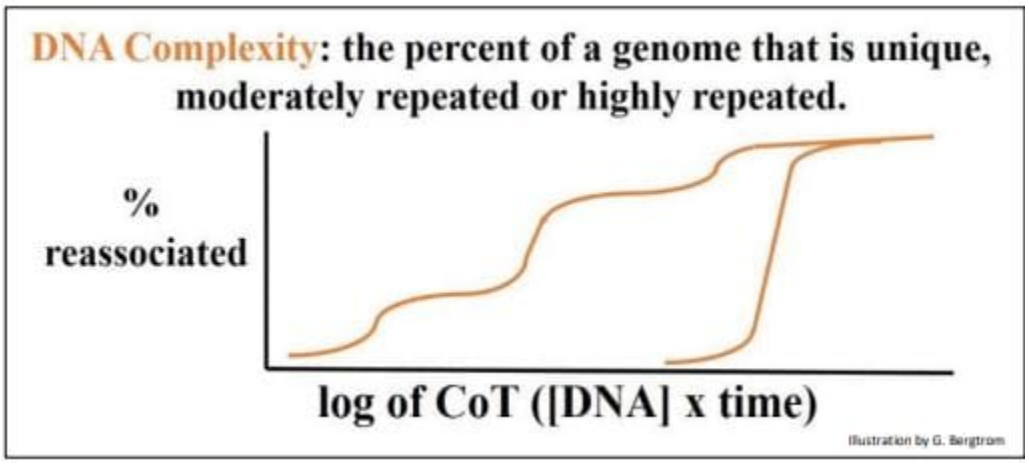
When renaturation kinetics were determined for E. coli DNA, only one ‘redundancy class’ of DNA was seen, as is shown below.



Based on E. coli gene mapping studies and the small size of the E. coli 'chromosome', the reasonable assumption was that there is little room for 'extra' DNA in a bacterial genome, and that the single class of DNA on this plot must be unique-sequence DNA.

C. Genomic Complexity

Britten and Davidson defined the relative amounts of repeated and unique (or singlecopy) DNA sequences in an organism's genome as its **genomic complexity**. Thus, prokaryotic genomes have a lower genomic complexity than eukaryotes. Using the same data as is in the previous two graphs, Britten and Davidson demonstrated the difference between eukaryotic and prokaryotic *genome complexity* by a simple expedient. Instead of plotting the fraction of dsDNA formed vs. time of renaturation, they plotted the percent of re-associated DNA against the **concentration of the renatured DNA multiplied by the time that DNA took to reanneal** (the **CoT** value). When **CoT** values from rat and *E. coli* renaturation data are plotted on the same graph, you get the **CoT curves** in the graph below.



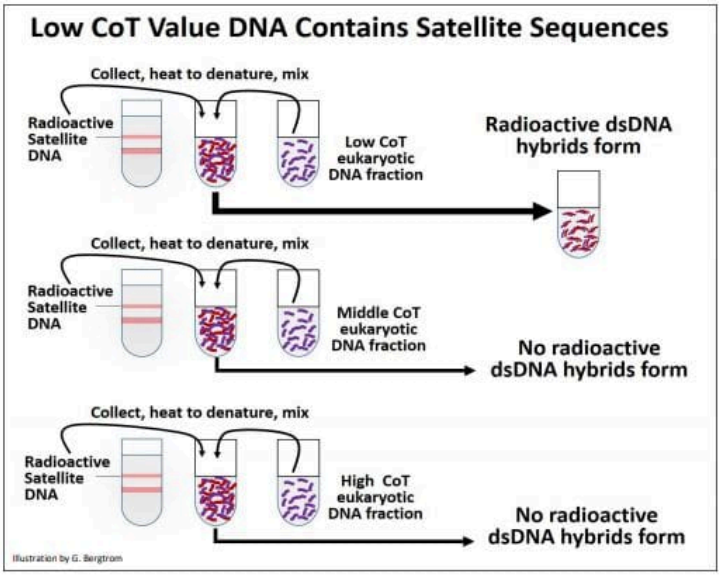
This deceptively simple extra calculation (from the same data!) allows comparison of the complexities of any number of genomes. These CoT curves tell us that ~100% of the bacterial genome consists of unique sequences, compared to the rat genome with its three DNA redundancy classes. Prokaryotic genomes are indeed largely composed of unique (non-repetitive) sequence DNA that must include single-copy genes (or operons) that encode proteins, ribosomal RNAs and transfer RNAs.

239 CoT Curves and DNA Complexity Explained!

D. Functional Differences between CoT Classes of DNA

The next question of course was what kinds of sequences are repeated and which are 'unique' in eukaryotic DNA? Eukaryotic satellite DNAs, transposons and ribosomal RNA genes were early suspects. To begin to answer these questions, satellite DNA

was isolated from the CsCl gradients, made radioactive and then heated to separate the DNA strands. In a separate renaturation kinetic experiment, rat DNA was sampled at different times. The isolated Cot fractions were once again denatured and mixed with heat-denatured radioactive satellite DNA. The mixture was then cooled to allow renaturation. The experimental protocol is illustrated below.



The results of this experiment showed that radioactive satellite DNA only annealed to DNA from the *low Cot fraction* (highly repeated) fraction of DNA. Satellite DNA is thus highly repeated in the eukaryotic genome. In similar experiments, isolated rRNAs made radioactive formed RNA-DNA hybrids when mixed and cooled with the denatured *middle CoT fraction* of eukaryotic DNA. Thus, rRNA genes were moderately repetitive. With the advent of recombinant DNA technologies, the redundancy of other kinds of DNA were explored using cloned genes (encoding rRNA, proteins, transposons and other sequences) to probe DNA fractions obtained from renaturation kinetics experiments. Results of such experiments are summarized in the table below.

Repetitive DNAs in Eukaryotes

Class	Type	Copy number; % of Genome e.g. Mammals	Sub-type	Organization */or Properties	Unit Length (bp)	Location	Function (if known), Examples
Highly Repetitive	*Satellite DNAs*	Up to 10^6; 10-15%	*Microsatellite*	Tandem repeats (*VNTRs)	2-8 bp X 5-50	Centromeres, Heterochromatin, Dispersed	Spindle fiber attachment, gene regulation
			Minisatellite	Tandem repeats (*VNTRs)	10-60 bp X ?	Dispersed	Gene Regulation
			Telomeres (sub-category of minisattelite DNAs)	Tandem Repeats	4-6 bp	Chromosome ends	Prevent chromosome shortening during replication
Moderately Repetitive	*Transposable Elements*	10-10^6; 20-45% (>80% in Maize!)	*DNA Transposons*	Move via DNA intermediates ("cut-&-Paste")	Up to 7,000 bp	Dispersed	P-element, Mariner, Ac, Ds
			Retrotransposons: LTR elements, LINES & SINES	Move via RNA intermediates	80-400 bp (SINEs),	Dispersed	Alu
					Up to 7000 bp (LTR, LINEs)	Dispersed	L1
	rRNA genes	<1%	*45S rRNA genes*	Tandem 45S rDNA repeats	13.7 Kbp	Nucleolus	Translation
			5S rRNA genes	Tandem 5SrDNA repeats	120 bp	Dispersed	Translation
Unique Sequence	*Transcribed genes, introns, intergenic DNA*	Single copy sequences; ~50% in aggregate	*Protein-coding genes (introns + exons)*	~25000 in humans	variable	Dispersed	Just about everything else!

** VNTR: Variable Number of Tandem Repeats*

The table compares properties (lengths, copy number, functions, percent of the genome, location in the genome, etc.) of different kinds of repetitive sequence DNA. The observation that most of a eukaryotic genome is made up of repeated DNA, and that transposons can be as much as 80% of a genome was a surprise!

240 Identifying Different Kinds of DNA Each CoT Fraction

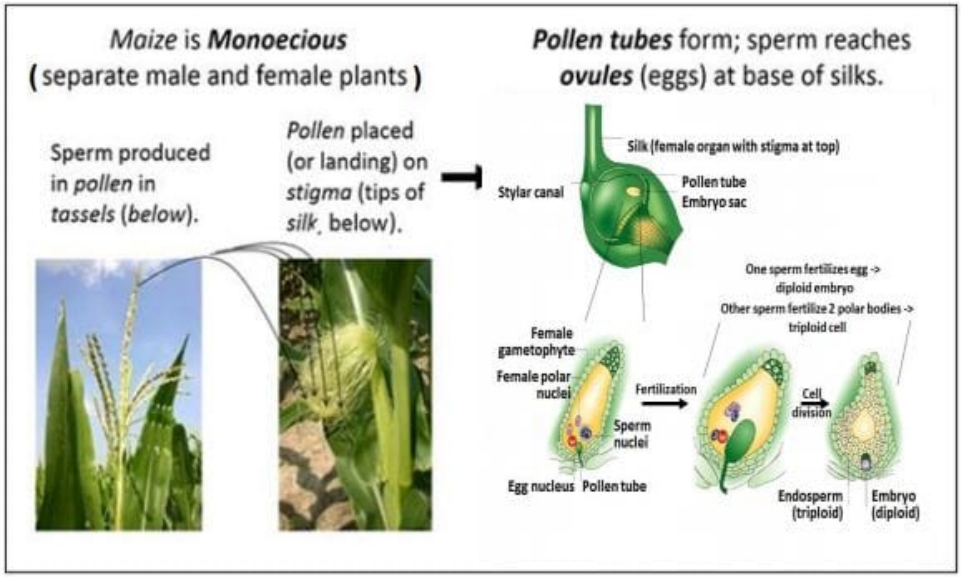
241 Some Repetitive DNA Functions

We'll focus next on the different kinds of *transposable elements*

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14.3: The 'Jumping Genes' of Maize

Barbara McClintock's report that bits of DNA could jump around and integrate themselves into new loci in DNA was so dramatic and arcane that many thought the phenomenon was either a one-off, or not real! Only with the subsequent discovery of transposons in bacteria (and in other eukaryotes) were McClintock's jumping genes finally recognized for what they were! As we describe her experiments, keep in mind that McClintock's research and intuitions about gene regulation and **epigenetic** inheritance came long before molecular technologies made it possible to prove and give names to these phenomena. To begin our tale of transposons, look at the illustration of maize reproduction below.



The different colors of corn seeds (ker nels) result from **anthocyanin** pigments that are expressed differentially by cells of the *aleurone* tissue. Mclintock was studying the inheritance of color variation, which ranged from colorless (white or yellow due to an absence of *anthocyanins*) to brown, purple, spotted or streaked.

The **mosaic** of kernel colors are vividly shown the corncobs in the photograph below



Clearly, kernel color is inherited. The inheritance of colorless and purple seed color did indeed follow Mendelian rules, but the genetics of mosaicism did not. Mosaic color patterns after genetic crosses were not consistent, implying that the mutations responsible for kernel color were not due to mutations in germ cells. Rather, genes controlling anthocyanin synthesis must be undergoing mutations in somatic cells that would become (or already were) the ones in which the pigments were produced.

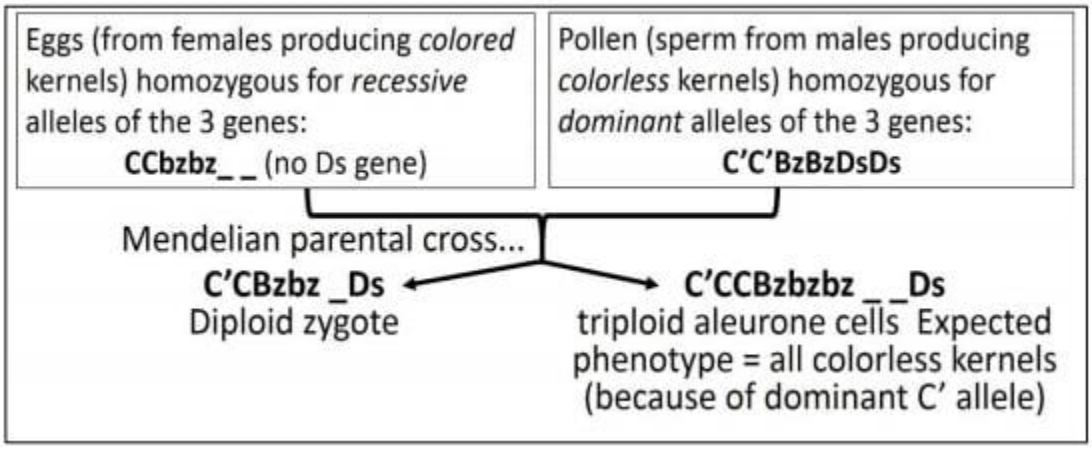
A. Discovering the Genes of Mosaicism; the Unstable Ds Gene

McClintock was looking for a genetic explanation for seed color variation in the 1940s and early 1950s. DNA structure had only recently been published. Gene cloning and DNA sequencing were decades into the future! Her only available technologies were based on understanding Mendelian allelic assortment in traditional breeding studies. Nevertheless, since seed color is expressed in cells derived from endosperm, she knew that the inheritance of kernel color **phenotype** must be studied against a **triploid** genetic background. McClintock was also aware of proposals that the variegated color phenotype might result when an 'unstable mutation' that produced colorless kernels 'reverted' in some cells but not others to create a spotted or streaked phenotype. Just what made for an 'unstable mutation' was of course, unknown. McClintock ultimately identified three genes involved in seed kernel coloration. Two of the genes initially studied by McClintock controlled the presence vs. absence of kernel color. These are the **C** and **Bz** genes:

1. **C'** is the dominant *inhibitor allele*, so-called because if even one copy was present, the kernels were **colorless** (yellow), regardless of the rest of the genetic background
2. **Bz** and **bz** are dominant and recessive alleles of the Bz gene, respectively. In the absence of a C' (dominant) allele, the presence of a Bz allele would lead to purple kernels. If the bz allele was present without both C' and Bz alleles, the kernels would be dark brown.
3. The gene required to get variegated kernel color was the **Ds** (**Dissociator**) gene. Without a viable **Ds** gene, kernels were either colored or colorless depending on the possible genotypes dictated by the C and Bz alleles.

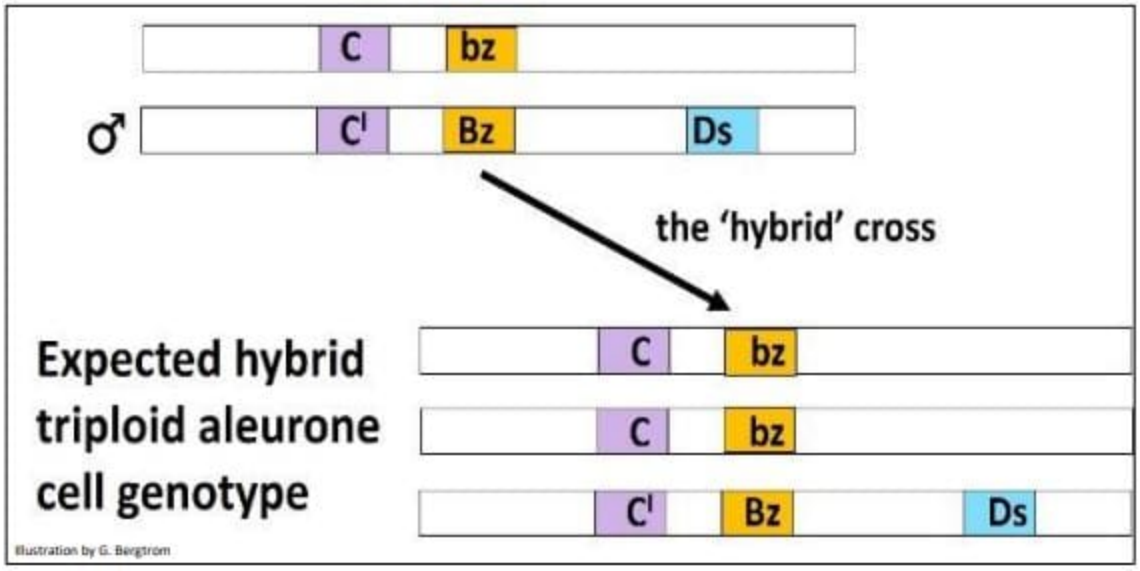
In other words, it must be the Ds gene that suffers 'unstable mutations. Because the **Ds** gene effect was random and only affected some aleurone layer cells, it was suspected to be a region of *chromosomal instability* (prone to damage or breakage) in some cells but not others. Let's look at what McClintock did to figure out what was going on in corn kernel color genetics.

Having already demonstrated crossing-over in maize (actually, another remarkable achievement!), McClintock **mapped** the C', Bz and Ds genes to *Chromosome 9*. She then selectively mated corn with the genotypes shown in the protocol below.

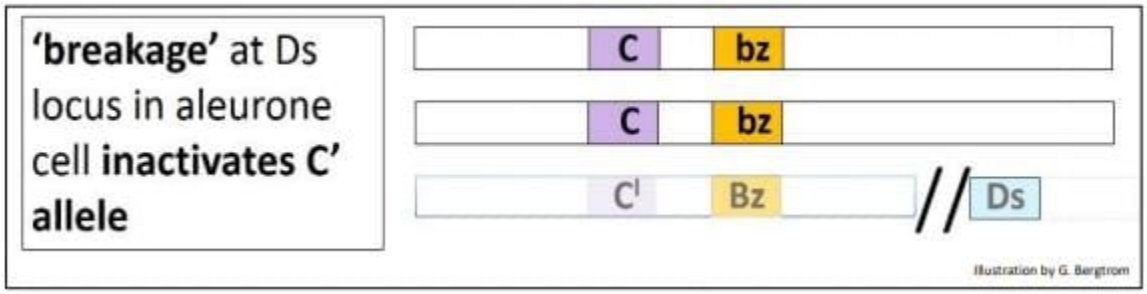


Remember that triploid cell genotypes are being considered in this illustration! You can refer to the phenotypic effects of the allelic backgrounds of three genes as we follow McClintock's cross. Her cross of a homozygous recessive with a homozygous dominant plant should ring a bell! Let's look more closely at this cross.

The expected triploid genotypes from the cross are shown below.



Aleurone cells resulting from this cross should all be colorless (yellow) because of the presence of the dominant C' allele. However, while there were indeed many **colorless** kernels on the *hybrid* cob, there were also many **mosaic** kernels with dark spots/streaks against a colorless background. McClintock's interpretation of events is illustrated below



According to McClintock, if some aleurone layer cells in some kernels suffered chromosome breakage at the **Ds** (**Dissociator**) locus (indicated by the double slash, //), the C' allele is inactivated. Without a functional C' allele, the **operative** genotype in the affected cells is **CCbzbz**. These cells then revert to making the brow pigment as directed by the bz allele. When these cells divide, they create clusters of brown cells surrounded by cells with an unbroken chromosome and thus an active C' allele, creating the appearance of pigment spots or streaks in the kernel, against the otherwise colorless background in the surrounding cells.

243 Variegated Maize Kernels result from "Loss" of the Ds Gene

B. The Discovery of Mobile Genes: the Ac/Ds System

The experiments just described were reproducible using a single breeding stock of maize. But when McClintock tried to repeat the experiments by crossing the homozygous dominant males with homozygous recessive females from a different breeding stock, all the kernels of the progeny cobs were colorless, as if the Ds gene had not caused any chromosomal damage.

It seemed that the **Ds** gene contributed by the male was unable to function (i.e., 'break') in females of this new breeding stock. McClintock hypothesized that the female in the original cross must have contributed a factor that could somehow activate the Ds gene to break, and that this factor, yet another gene, was absent or inactive in the females of the new breeding stock. McClintock called the new factor the **activator**, or **Ac** gene. Based on the dependence of **Ds** on the **Ac** locus, McClintock recognized that these 'genes' were part of as a 2-element, **Ac/Ds system** influencing mosaicism in maize kernels.

She then demonstrated that Ac-dependent Ds 'breakage' was in some cases also associated with inactivation of a normal Bz gene, leading to a loss of purple color kernels. It was at this point that McClintock concluded that far from simply 'breaking' the chromosome at a fragile Ds locus, the Ds gene had actually moved to (or into) the Bz gene, disrupting its function. Again, this

could not happen in the absence of an active Ac gene. McClintock had discovered the first transposon, earning the 1983 Nobel Prize in Physiology or Medicine! With the advent of recombinant DNA technologies, we now know that:

1. The **Ds** element is a transposon missing a gene for a **transposase** enzyme required for transposition.
2. The **Ac** element has this gene and is capable of independent transposition.
3. **Ac** provides the transposase needed to mobilize itself and the Ds element
4. The sequence similarity of Ds and Ac elements support their common ancestory.

The basic features of the maize Ac/Ds system are:

1. Ac is 4563 bp long.
2. Ds is a truncated version of Ac.
3. There are **eleven bp inverted repeats** at either end of the Ac and Ds element.
4. There are **eight bp direct repeats** (NOT inverted repeats) of 'target DNA' at the site of insertion of either transposon.

Look for these features as we describe prokaryotic and eukaryotic transposons.

244 Discovery of Mobile Elements and the Ac-Ds System

245 The Ac-Ds System Today

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14.4: Transposons Since McClintock

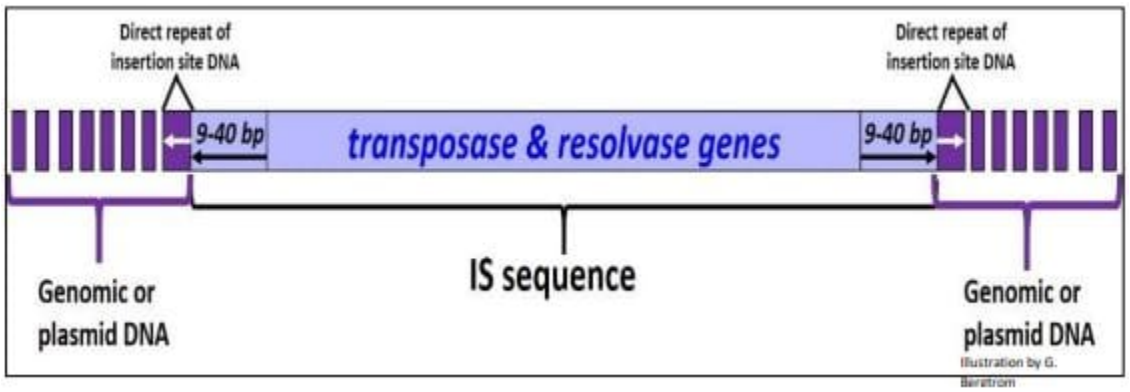
Transposons exist everywhere we look in prokaryotes and account for much of eukaryotic repetitive DNA. As such, they can be a large proportion of eukaryotic genomes, including some that no longer even transpose. Transposons were once considered useless or **junk DNA**, with no obvious function..., or **selfish genes** with no other purpose than selfreplication. But in light of some new evidence, perhaps not!

As you will see, mechanisms of transposition share many features with DNA replication, recombination and repair, and even viral infection. As you study these mechanisms, keep in the back of your mind that transposition is often triggered by cellular stress.

A. Insertion Sequences (IS Elements)

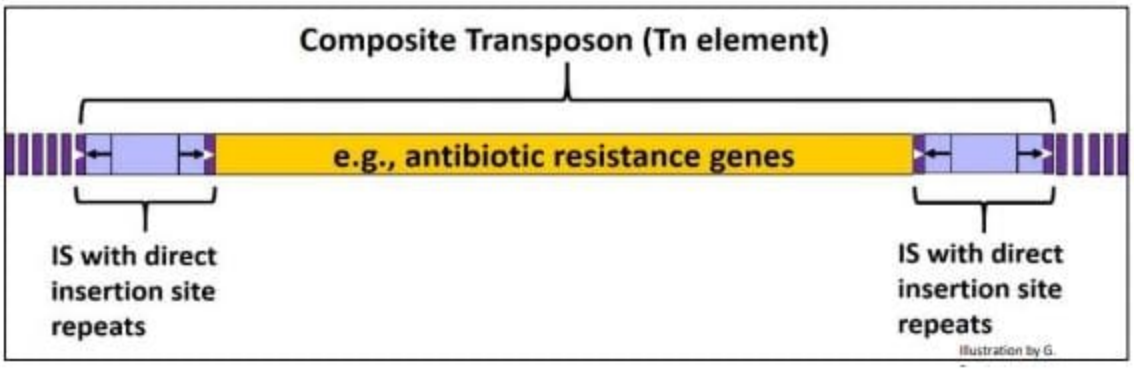
Bacterial **IS elements** were the first mobile elements described after those in maize. As we'll see, they share some structural features of eukaryotic transposons. Discovered in the late 1960s, many have been identified (IS1, IS2..., IS10 etc.). Some are inserted into well-known genes (e.g., those of the lac operon), but most are not, likely because there is little 'extra' DNA in the compact bacterial genome. Without extra non-coding DNA to buffer against damaging mutations, few bacterial cells would live to tell a tale of transposition! It should surprise us that IS elements can be made to transpose in the lab, but are generally silent in nature.

Members of the IS element family vary in length from about 750 to 1425 bp. Within this stretch of DNA lie **transposase** and **resolvase** genes whose products are necessary for mobility. At either end of the IS element are **inverted repeats**, and when found in either genomic or plasmid DNA, the IS sequence itself is flanked by **direct repeats** of host genome or plasmid DNA that result from the mechanism of transposition. Again, because of their compact genomes, bacteria can only tolerate low copy numbers of IS elements in their genome or on plasmids (less than ten copies and as few as one!). A typical IS element is illustrated below.



B. Composite Transposons: Tn Elements

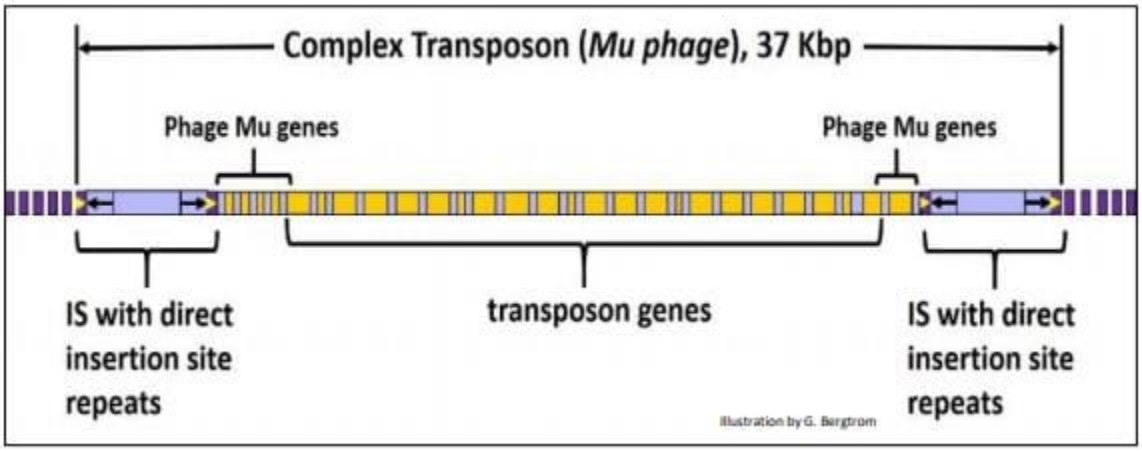
If a pair of IS elements should lie close to each other, separated by a short stretch of genomic or plasmid DNA, they can transpose together, carrying the DNA between them as part of a **composite transposon**, or **Tn element**. If some of the DNA between IS elements in a Tn element contains antibiotic resistance genes, its transposition can carry and spread these genes to other DNA in the cell. Tn elements (like IS elements) are present in low copy number. A generic Tn element is drawn below.



Antibiotic resistance genes have the medical community worried; their spread has led to antibiotic-resistant pathogens that cause diseases that are increasingly hard and even impossible to treat. Earlier we saw genetic 'transformation' of streptococcal cells that pick up virulence genes in DNA from dead cell. We routinely transform cells with plasmids as part of recombinant DNA experiments. But bacteria can transfer plasmid DNA between themselves quite naturally. During bacterial conjugation, an **F** (**fertility**) plasmid normally transfers DNA between compatible bacterial mating types (review bacterial conjugation elsewhere in this text for more details). An F plasmid containing a Tn element harboring an antibiotic resistance gene can thus is passed from donor to recipient during conjugation. The Tn element can transpose into to the recipient bacterial genome. In this way, transposition is a major pathway for the transfer and spread of antibiotic resistance.

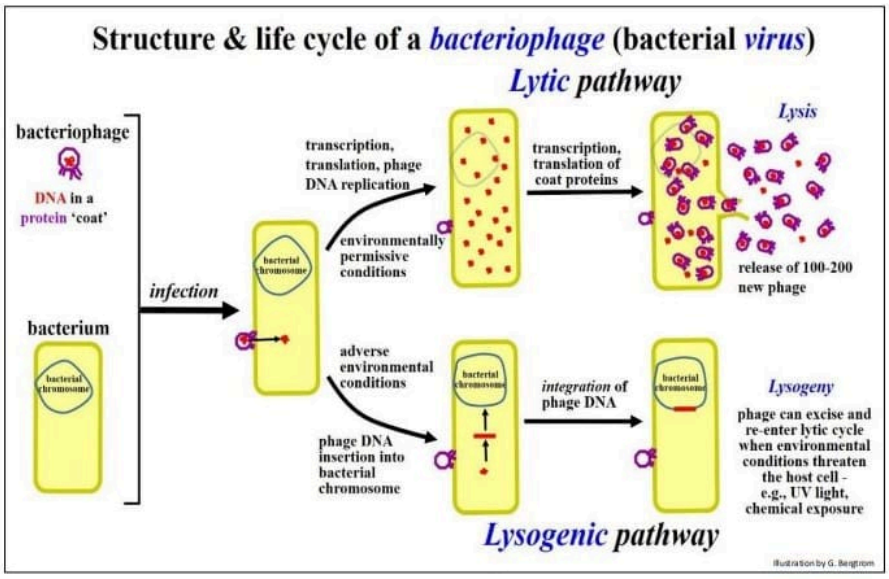
C. Complex Transposons

Bacterial **Complex Transposons** also contain other genes in addition to those required for mobility. Some complex transposons resemble a bacteriophage, or as in the case of **phage Mu**, actually are phage! In fact, Mu can function either as an infectious phage that reproduces in an infected cell, or as a transposon in the bacterial genome. Transposon genes in Mu phage are illustrated below.

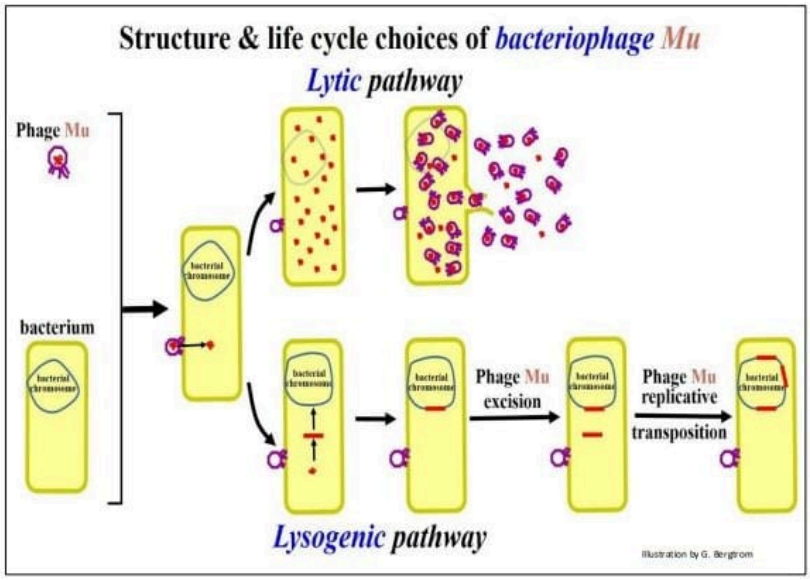


After infecting a bacterium, Mu can enter the lytic phase of its life cycle, replicating its DNA, producing and ultimately releasing new infectious phage 'particles' by lysing the host bacterial cell. Alternatively, like other phage, Mu can undergo **lysogeny**, inserting its DNA into the host cell chromosome. Integrated copies of Mu might excise and re-enter the lytic phase to produce more phage, particularly if some environmental stress threatens host bacterial survival. But, a third lifestyle choice, transposition, is available to Mu once the phage integrates into the bacterial chromosome. The three lifestyle options for Mu phage are illustrated in the next few pages.

Lytic and lysogenic lifestyle options for Mu phage are shown below.



Mu phage DNA can act as a transposable element while in the lysogenic pathway, as shown below



264 Bacterial Mobile Elements

As we turn to a description of eukaryotic transposons, look for similarities to bacterial IS and Tn elements.

D. Overview of Eukaryotic Transposable Elements

There are two classes transposons in eukaryotes:

Class I (Retrotransposons) move/'jump' by transcription of RNA at one locus, followed by reverse transcription and integration of the cDNA back into genomic DNA at a different location. Retrotransposons may be derived from (or be the source of) retroviruses since active retroviruses excise from and integrate into DNA much like retrotransposons. Retroposons are a sub-class of retrotransposons (see below).

Class II (DNA Transposons) move by one of two mechanisms. In the cut-&-paste pathway, the transposon leaves one locus and integrates at another. In the replicative pathway, the original transposon remains in place while new copies are mobile. The table below shows the distribution and proportion of genomes represented by different classes/types of transposable elements.

Proportions of DNA and RNA transposons in Different Organisms:

Organism	Class I Transposons (Retroposons) as proportion of all transposons	Retroposons as % of Genome	Class II Transposons (DNA Transposons) as proportion of all transposons	DNA transposons as % of Genome	All Transposons as % of genome
Bacteria (e.g., *E. coli*)	--------	--------	100%	~3%	~3%
Yeast	100%	~3.5%	--------	--------	~3.5%
Corn (e.g., *Z. maize*)	>50%	~30-45%	<50%	~40%	~70-95%
Protozoa (*T. Vaginalis*)	--------	--------	100%	~66%	~66%
Frog (e.g., *R. esculenta*)	~25%	~19%	~75%	~58%	~77%
Mouse (*M. Musculus*)	~95%	~38%	~5%	~2%	~40%
Mosquito (e.g., *A. aegypti*)	~30%	~14%	~70%	~33%	~47%
Us! (*Homo sapiens*)	>90%	~40%	<10%	<5%	~42-45%
Flatworm (*C. elegans*)	~5%	~0.5%	~95%	~11-12%	~12%
Fruit fly (*D. Melanogaster*)	<80%	~3%	>20%	~1%	~4%
Rice (e.g., *O. sativa*)	~15%	~1%	~85%	~5%	~6%

The table confirms that bacteria contain few transposons, while eukaryotes vary widely in transposon load (transposons as a percentage of genomic DNA), from as low as 4% to more than 70%.

The table below summarizes transposable elements by class, sub-type, size, genomic distribution, mechanism of transposition, etc.

Types of Transposable Elements

Class	Type	Sub-Types	Basis of Mobility	Organismic Distribution (e.g.)	Examples	All, as % of Genome	Length (bp)	Genomic Location; Special Features
PROKARYOTIC Mobile Elements	*DNA transposons*	IS (Insertion Elements)	Cut-&-Paste	*E. coli*	IS1, IS2, IS3...	3%	1000-2000bp	Intergenic DNA; usually insertion-site specific
		Composite Tn	Cut-&-Paste		Tn5		~5900bp	Source of antibiotic resistance genes
		Complex Tn	Replicative, cointegrate formation		Mu		~37Kbp	Can function as bacteriophage or transposon
EUKARYOTIC Class II (DNA) transposons: **Move via DNA intermediates	*DNA transposons*	----------	Cut-&-Paste	*D. melanogaster*	P-element, Mariner	both = ~1%	1000-7000bp	Dispersed
				C. elegans,	Tc1 (Mariner)	~11-12%		
				Z. maize	Ac, Ds	~40%		
				H. Sapiens (human)	Mariner	~2-6%		
		----------	Replicative, cointegrate formation	*O. Sativa* (rice) [Also plants, bacteria]	*Miniature Inverted Repeat Transposable Elements* (MITEs)	~6% (rice)	<500bp	Mostly associated with genes; transcribed into small RNAs
EUKARYOTIC Class I (RNA) transposons: **Move via RNA intermediates	*retro-transposons, retroposons*	*LTR retrotransposons*	Reverse-transcription & integration (original copy not excised)	*S. cerevisiae* (yeast)	Ty	~3%	Up to 7000bp	Dispersed; *(retrovirus-like, but no envelope protein genes)*
				D. melanogaster	Copia	~3%		
		LINEs (NON-LTR retrotransposons)		*H. Sapiens* (human)	L1	~5%	~6000bp	Interspersed
				Z. maize	*Cin-1*	~45%		
		SINEs (NON-LTR retrotransposons, or retroposons)		*H. Sapiens* (human)	Alu	~40%	80-400 bp	

* In so-called ***Cut-&-Paste*** transposition, DNA transposons move from one location to another. In ***Replicative*** transposition, DNA transposons replicated a copy of the element that moves. ***Retrotransposons*** are active if their transcripts are translated so that appropriate enzymes can integrate their cDNA copies into genomic DNA.

** Many transposons are inactive, having been silenced by mutations or other factors. Active eukaryotic Class I or II transposons can be ***autonomous*** or ***non-autonomous***. ***Autonomous*** transposons have all of the structural and functional features necessary for transposition (e.g., the maize **Ac** element). ***Non-autonomous*** transposons have all the structural features of autonomous transposons (e.g., inverted repeats and other DNA needed for transposition), except they lack or can't transcribe genes for enzymes required for mobility (e.g., the maize **Ds** element). Therefore, they can only transpose with the assistance of an actively transposing autonomous element that can provide the required enzymes.

Between the two tables above, we can conclude the following:

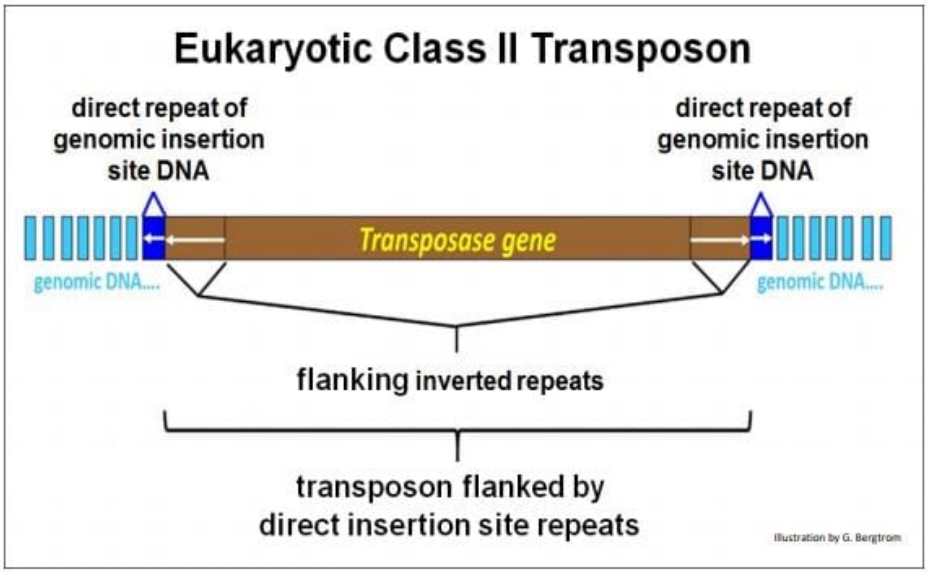
- Transposon load is not correlated with evolutionary complexity of organisms.
- Shared Transposons have different evolutionary histories in different organisms.
- Where transposons remain active, they continue to shape genomic landscapes, especially in organisms with a high transposons load.

We will revisit some of these conclusions later, after looking at the structure and mechanism of mobility of different transposable elements.

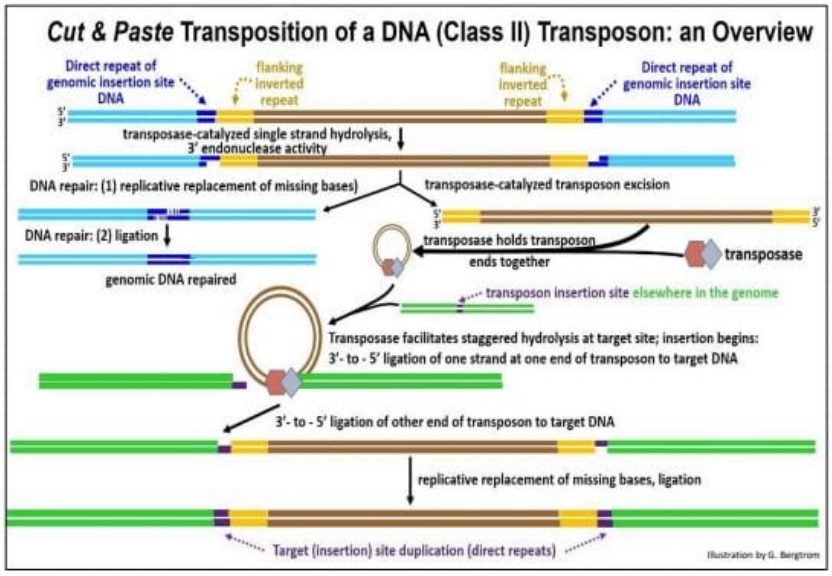
247 Introduction to Eukaryotic Transposons

E. The Structure of Eukaryotic DNA (Class II) Transposons

Active eukaryotic DNA transposons share structural features with bacterial mobile elements, including genes required for transposition, flanking inverted repeats and flanking insertion-site direct repeats of host cell DNA. The characteristic structure of a eukaryotic *DNA transposon* is shown below.



Class II transposons can 'jump' by **cut-and-paste** or **replicative** mechanisms. **Cut-and-paste** transposition removes a copy from one location and moves (*transposes*) it to another location. As its name suggests, *replicative* transposition leaves a copy of the original transposon in place while inserting a new copy elsewhere in the genome. Transposition by the *cut-and-paste* mechanism is diagrammed below.

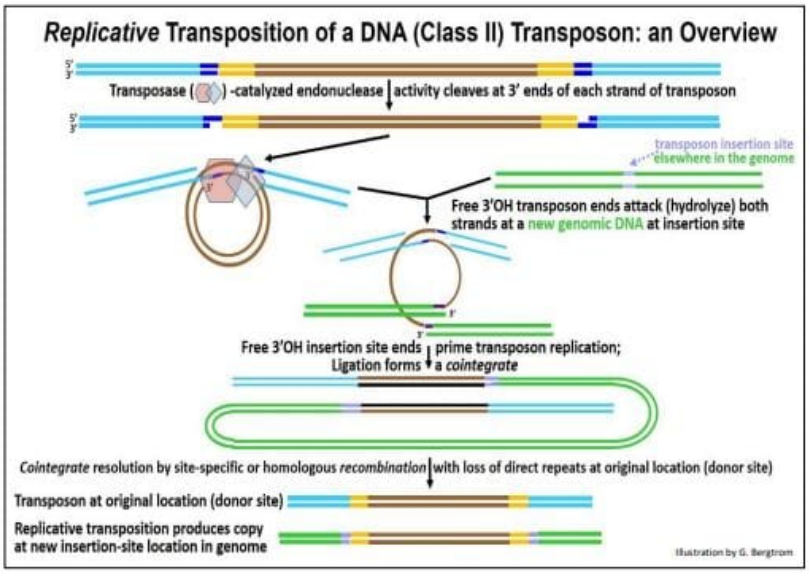


Note that after transcription of the transposase gene, the enzyme nicks the DNA and trims the 3'OH ends to create a staggered cut to excise the transposon. The transposase actually brings the transposon ends together during the cut step and mediates its insertion at a new DNA site. After ligation of the 3'OH ends of the transposon to the 5'OH at the insertion site, replication replaces the missing bases, generating the direct repeats of host cell genomic DNA at the insertion site. A final ligation step completes transposition.

In the **replicative transposition**, the transposon also nicks and trims the DNA at its source (original) insertion site. But, unlike

the cut and paste mechanism, the source transposon is not excised.

Details of the replicative mechanism of transposition is summarized below.



After nicking the 3' ends of the transposon at the insertion site, transposase holds the transposon ends together while catalyzing a hydrolytic attack of DNA at a new insertion site. This is followed by priming of transposon strand replication from the 3'OH ends of the insertion site DNA strands. A **cointegrate** structure forms in which each transposon copy has been made by semi-conservative replication. The cointegrate is resolved by one of two recombinational mechanisms. The result leaves copies of the transposon at both the original site and the new insertion site.

Let's compare and contrast the features of cut-&-paste and replicative DNA transposition. The common features are that:

- Transposon-encoded *transposase* binds, brings transposon ends together and catalyzes single-stranded cleavage (hydrolysis) leaving 'staggered ends'.
- Transposase holds the transposon ends together for the remaining steps.

The differences between the two mechanisms are that in *cut & paste* transposition, the transposon is completely excised and then transposed to a new site in genomic DNA. In contrast, after single stranded cleavage in replicative transposition, transposase-bound free 3' ends of the transposon hydrolyze both strands of stranded DNA at a new insertion site. After ligation of the 3' ends of transposon strands to 5' ends of cut genomic DNA insertion-site ends, the remaining 3' ends of the insertion site DNA ends prime replication of the transposon, forming the **cointegrate**, which is followed by its resolution by one of two recombination pathways.

248 Eukaryotic Class II (DNA) Transposition

F. The Structures of Eukaryotic RNA (Class I) Transposons

Like DNA transposons, all RNA transposons leave insert-site footprints, i.e., direct repeats of genomic DNA flanking the element. Unlike DNA transposons, active eukaryotic Class I transposons move via an RNA intermediate. Also unlike DNA transposons, they lack terminal inverted repeats.

The mobility of the RNA intermediate of all retrotransposons requires a promoter that recognizes a **reverse transcriptase** enzyme as well as endonuclease and integrase enzymes (to be described below). **Autonomous** Class I RNA transposons include **LTR retrotransposons** (e.g., the yeast **Ty** element) as well as **Non-LTR retrotransposons**). The latter include the **autonomous LINEs** (**L**ong **I**nterspersed **N**uclear **E**lements). The **autonomous** LTR and Non-LTR LINEs contain and express genes needed for enzymes required for transposition. On the other hand, **SINEs** (a sub-class of Non-LTR retrotransposons) lack genes for enzymes required for transposition and therefore can't transpose independently. Thus, they are **non-autonomous** retrotransposons that rely on "true" (autonomous) retrotransposon activity for mobility. SINEs are sometimes called **retroposons** to distinguish them

from the autonomous retrotransposons.

249 Introduction to Features of Retrotransposition

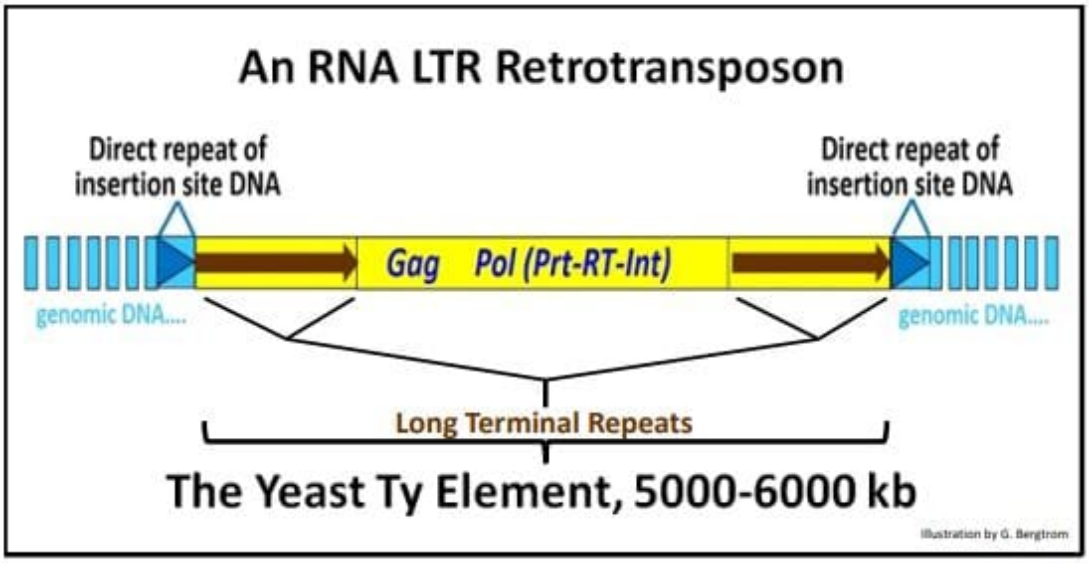
Next we take a closer look at Retrotransposon structures and the genes and enzyme activities required for retrotransposition

1. LTR retrotransposons: The Yeast Ty Element

The Ty transposon harbors several genes needed for transposition. These include:

- the **Gag** gene that encodes *group-specific antigen*, a protein that forms a viruslike particle that will contain reverse-transcribed transposon DNA,
- the **RT** gene that encodes the *reverse transcriptase* that will make reversetranscribed copies of retrotransposon transcript RNAs.
- the **Prt** gene that encodes a protease that will break down the virus-like particle as the retrotransposon enters the nucleus.
- the **Int** gene that encodes the *integrase* required for integration of the retrotransposon into a genomic DNA insertion site.

A representative Ty element is shown below as it would exist integrated into yeast genomic DNA.



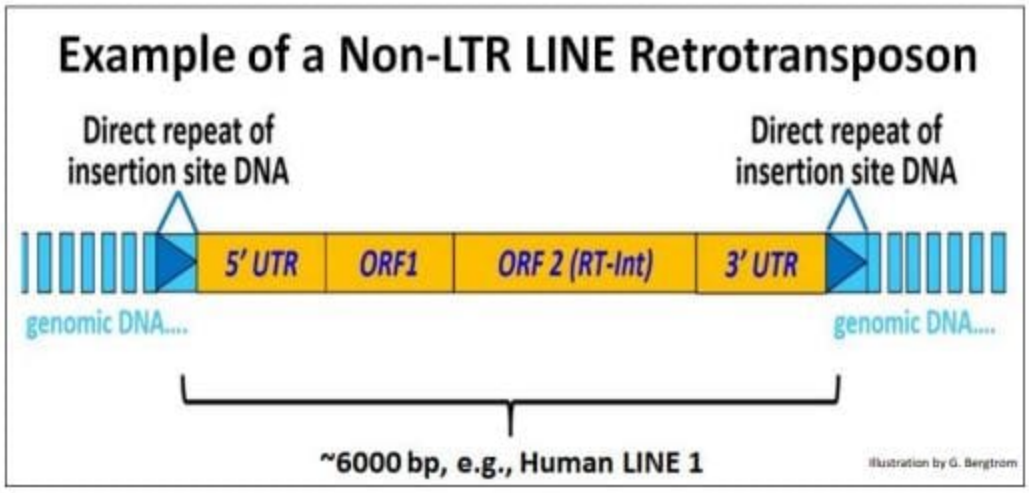
In fact, many of the events in Ty transposition occur in the cytoplasmic “virus-like particle” in yeast cells. To see more, click here. Note that the Pol region in the illustration above consists of overlapping open reading frames (ORFs) encoding the Prt, RT and Int genes. The ready-to-move transposon consists only of the region of DNA symbolized in yellow.

250 LTR Retrotransposons- the TY Element

2. Non-LTR Retrotransposons: LINEs

LINEs (**L**ong **I**nterspersed **N**uclear **E**lements) also encode enzymes needed for transposition and like other transposons, generate target-site direct repeats flanking the inserted element. But they do not have the long terminal repeats! Instead, their ORFs (genes) are flanked by 5’ and a 3’ **untranslated regions** (**UTRs**).

The structure of the human L1 Line is drawn below.



The 5' UTR contains a promoter from which cellular RNA polymerase II can transcribe the downstream genes (see the **Transcription** chapter). The second of these (ORF2) encodes the reverse transcriptase and an integrase activity essential for transposition of the LINE. All Class I (RNA-intermediate) autonomous transposons share the following features:

a) **a Promoter** in the 5' UTR from which they can be transcribed.

b) **a Reverse Transcriptase** that generates a cDNA copy of the transposable element.

c) **RNAse H** (an endonuclease) that degrades that transcript after reverse transcription.

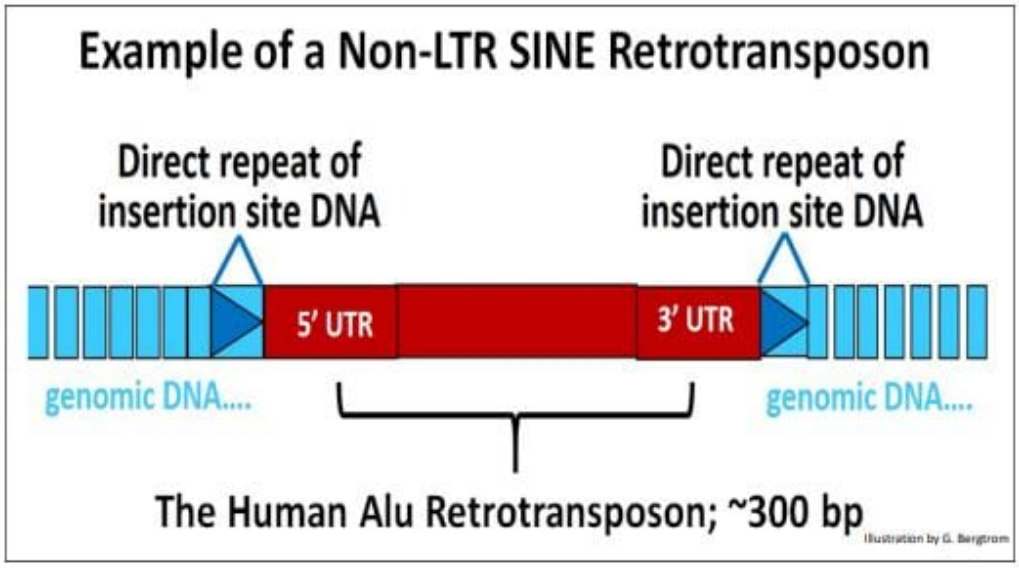
d) **Integrase** (like a transposase) that catalyzes insertion of the retrotransposon copy at insertion sites.

251 Non-LTR Retrotransposons: LINEs

3. Non-LTR SINE retrotransposons

Non-LTR SINE retrotransposons typically lack genes, but their non-genic DNA is nonetheless flanked by 5' and 3' UTRs. RNA polymerase III, which also transcribes transfer RNAs, also transcribes SINEs. However, to transpose, they rely on the concurrent activity of a Non-LTR transposon (a LINE) to provide the requisite enzymatic activities.

A typical SINE (e.g., the **Alu** element) is shown below.



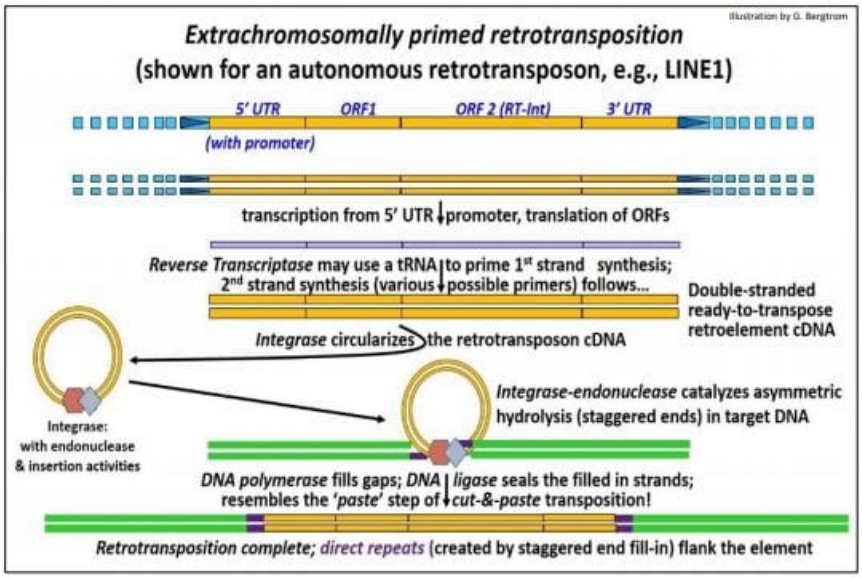
G. Mechanisms of Retrotransposition

There are two mechanisms of retrotransposition: **Extrachromosomally Primed Retrotransposition** (LTR retrotransposons for example) and **Insertion Target-Site Primed Retrotransposition** (non-LTR Retrotransposons like LINEs and SINEs). These will be considered next.

1. Extrachromosomally Primed Retrotransposition (e.g., of a LINE)

As its name suggests, in *extrachromosomally primed retrotransposition*, a circular reverse transcript of the retrotransposon attacks, nicks and integrates into a genomic insertion site. In this mechanism, reverse transcriptase creates a cDNA copy of a transcribed retro-element. Integrase/endonuclease then binds the cDNA copy, holding the ends together, in effect circularizing it. This isolable ribonucleoprotein resembles an **intasome**, a structure similar to the nucleoprotein complex that catalyzes the integration of retroviral cDNAs during lysogeny.

Extrachromosomally primed retrotransposition is illustrated below.

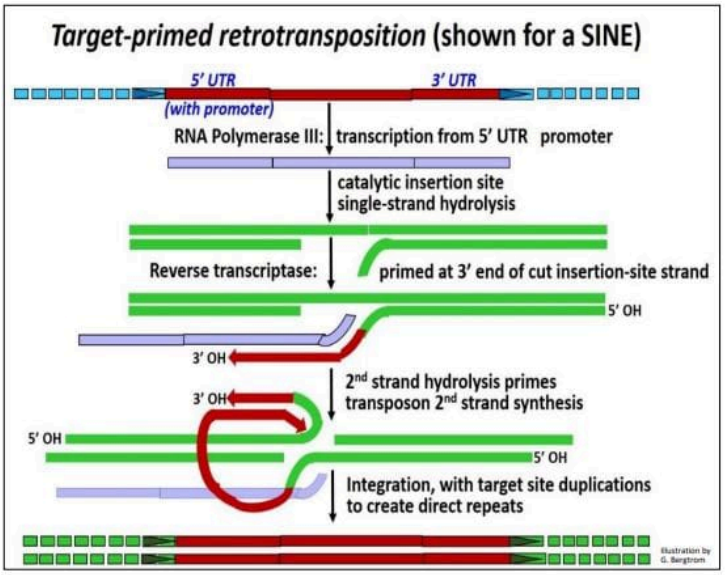


The three-dimensional structure of a retroviral *intasome* interacting with DNA and nucleosomes was recently determined (for more, see Retroviral Intasome 3D Structure). In this form, the retrotransposon attacks DNA at an insertion site, creating staggered ends. After insertion, the gaps in the DNA are filled in. Ligation seals the retrotransposon in its new location, creating direct insertion site repeats.

253 Extrachromosomally Primed Retrotransposition

2. Target-Site Primed SINE Retrotransposition (e.g., of a SINE)

A key feature of *target-site primed retrotransposition* (*retroposition*) is the absence of an integrase-bound, circular double-stranded reverse transcript. In SINE transposition, RNA polymerase III (the same enzyme that catalyzes tRNA and 5S rRNA transcription) transcribes the SINE. If a LINE is concurrently transcribed, its enzymes will be made. When its *integrase-endonuclease* catalyzes hydrolysis of one strand of DNA at a new insertion site, the 3'OH end of this strand can prime reverse transcription of the one SINE cDNA strand by the LINE *reverse transcriptase*. After hydrolysis of the second target site DNA strand, its 3'-OH end primes replication of the second strand of the SINE cDNA. Integrase completes insertion of the copy-SINE in its new genomic location. The *target-site primed retrotransposition* mechanism of retrotransposition is illustrated below.



254 Target Primed Retrotransposition

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14.5: On the Evolution of Transposons, Genes, and Genomes

We noted that transposons in bacteria carry antibiotic resistance genes, a clear example of benefits of transposition in prokaryotes. Of course, prokaryotic genomes are small, as is the typical bacterial transposon load. Yeast species also have low transposon load. But, what can we make of the high transposon load in eukaryotes?

To many, the fact that genes encoding proteins typically represent only 1-2% of a eukaryotic genome meant that the rest of the genome was informationally non-essential. Even though transposons turn out to be much of the non-coding DNA in some eukaryotic genomes, they seemed to serve no purpose other than their own replication. For many organisms, large amounts of transposon DNA were dubbed selfish DNA and their genes, selfish genes.

Are transposons just junk DNA, some kind of invasive or leftover *genomic baggage*? Given their propensity to jump around and potential to raise havoc in genomes, how do we tolerate and survive them? Is the sole 'mission' of transposons really just to reproduce themselves? Or are transposons tolerated because they are neither *selfish* nor junk? By their sheer proportions and activity in eukaryotic genomes, we will see that transposons have dispersed into, and re-shaped genomic landscapes. Do the consequences of transposition (relocation dispersal through a genome, structural alteration and mutation of genes) have any functional or evolutionary value? While all of these questions are a reasonable response to the phenomena of jumping genes, a rational hypothesis would be that, like all genetic change, transposons began by accident. But, their spread and ubiquity in genomes of higher organisms must in the long term have been selected by virtue of some benefit that they provide to their host cells and organisms. Let's briefly looks at the evolutionary history of transposons to see if this assumption has some merit.

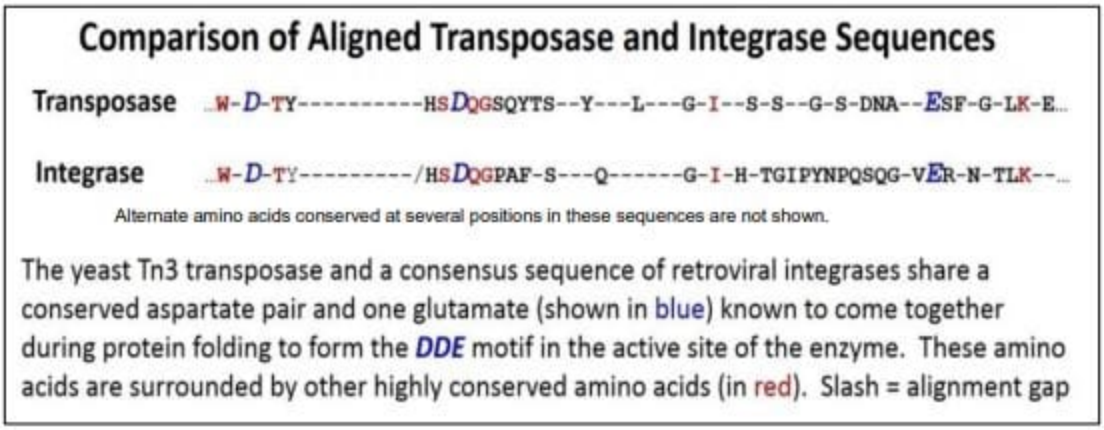
A. A Common Ancestory DNA and RNA (i.e., *All*) Transposons

Transposases catalyze the cut-and-paste as well as the replicative transposition of Class II (DNA) transposons. *Integrases* catalyze insertion of reverse-transcribed retrotransposons. Bottom line: both enzymes end by catalyzing insertion of transposons into new DNA locations. So, it should not be surprising that class I and II transposons enzymes share similar amino acid sequence and domain structures. These similarities support a common ancestry of class I and II transposons. Sequence comparisons of transposable elements themselves reveal that they comprise distinct families of related elements.

This allows us to speculate on the origins of these families in different species. For example, the TC1/*mariner* (DNA) transposon is found in virtually all organisms examined (except diatoms and green algae). Based on sequence analysis, there is even an insertion element in bacteria related to the *mariner* element. This amount and diversity of conservation bespeaks an early evolution of the enzymes of transposition, and of transposition itself, within and even between species. Linear descent, or the 'vertical" transmission of transposons from parents to progeny, is the rule. However, the presence of similar transposons in diverse species is best explained by interspecific DNA ("horizontal") transfer. That is, a transposon in one organism must have been the 'gift' of an organism of a different species! This is further discussed below. Clearly, *moveable genes* have been a part of life for a long time, speaking more to an adaptive value for organisms than to the parasitic action of a selfish, rogue DNA!

B. Retroviruses and LTR Retrotransposons Share a Common Ancestry

The 'integration' domain of retrotransposons and retroviruses share significant similarities as shown below.



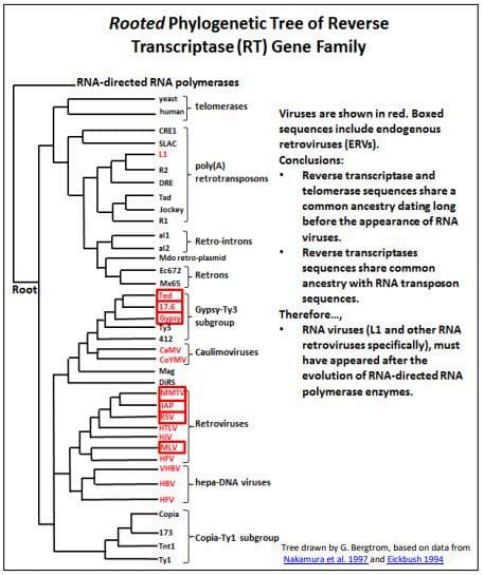
The question raised by these observations is: Did transposons (specifically retrotransposons) arise as defective versions of integrated retrovirus DNA (i.e., reverse transcripts of retroviral RNA)? Or, did retroviruses emerge when retrotransposons evolved a way to leave their host cells. To approach this question, let's first compare mechanisms of retroviral infection and retrotransposition.

In addition to the structural similarities between the enzymes encoded by retrotransposon and retroviral RNAs, LTR retrotransposons and retroviruses both contain flanking long terminal repeats. However, retrotransposition occurs within the nucleus of a cell while retroviruses must first infect a host cell before the retroviral DNA can be replicated and new viruses produced (check out Visualizing Retroviral Infection to see how immunofluorescence microscopy using antibodies to singlestranded cDNAs was used to track the steps of HIV infection!). A key structural difference between retrotransposons and most retroviruses is an **ENV** gene-encoded protein envelope surrounding retroviral DNA. After infection, the incoming retrovirus sheds its envelope proteins and viral RNA is reverse transcribed. After the reverse transcripts enter the nucleus, transcription of genes and translation of enzymes here.

- Retroviral DNA, like any genomic DNA, is mutable. If a mutation inactivates one of the genes required for infection and retroviral release, it could become an LTR retrotransposon. Such a genetically damaged retroviral *integrate* might still be transcribed and its mRNAs translated. If detected by its own reverse transcriptase, the erstwhile viral genomes would be copied. The cDNAs, instead of being packaged into infectious viral particles, would become a source of so-called *endogenous retroviruses* (**ERVs**). In fact, ERVs exist, making up a substantial portion of the mammalian genome (8% in humans)... and do in fact, behave like LTR retrotransposons!
- Yeast TY elements transcribe several genes during retrotransposition (see the list above), producing not only reverse transcriptase and integrase, but also a protease and a structural protein called **Gag** (*Group-specific antigen*). All of the translated proteins enter the nucleus. Mimicking the retroviral ENV protein, the Gag protein makes up most of a coat protein called **VLP** (**virus-like particle**). VLP encapsulates additional retrotransposon RNA in the cytoplasm, along with the other proteins. Double-stranded reverse transcripts (cDNAs) of the viral RNA are then made within the VLPs. But, instead of bursting out of the cell, the encapsulated cDNAs (i.e., new retrotransposons) shed their VLP coat and re-enter the nucleus, where they can now integrate into genomic target DNA. Compare this to the description of retroviral infection. During infection, retroviral envelope proteins attach to cell membranes and release their RNA into the cytoplasm. There, reverse transcriptase copies viral RNA into double-stranded cDNAs that then enter the nucleus where they can integrate into host cell DNA. When transcribed, the integrated retroviral DNA produces transcripts that are translated in the cytoplasm into proteins necessary to form an infectious viral particle. The resulting viral RNAs are encapsulated by an ENV (envelope) protein encoded in the viral genome. Of course, unlike VLP-coated retrotransposon RNAs, the enveloped viral RNAs do eventually lyse the host cell, releasing infectious particles. Nevertheless, while VLP coated Ty elements are not infectious, they sure do look like a retrovirus!

Common mechanisms of retrovirus and retrotransposon replication and integration clearly support their common ancestry, but they do not indicate origins. On the one hand, the origin of ERVs from retroviruses might imply an origin of retrotransposons from retroviruses. On the other, transposons have been around since the earliest prokaryotic cells, but that retrotransposons arose with eukaryotes. In that case, Type II (DNA) transposable elements were around before retroviruses.

The phylogenetic analysis below is based on comparisons of retroviral and retrotransposon reverse transcriptase gene DNA sequences.



Comparisons of aligned DNA sequences permit evolutionary analyses that reflect phylogenetic relationships of genes (in this case, retrotransposon and viral genes), in much the same way the evolutionary biologists historically demonstrated evolutionary relationships of plants and animals by comparing their morphological characteristics. The data in the analysis supports the evolution of retroviruses from retrotransposon ancestors. From the 'tree', TY3 and a few other retrotransposons share common ancestry with Ted, 17.6 and Gypsy ERVs (boxed) in the "Gypsi-TY3 subgroup". Further, this sub-group shares common ancestry with more distantly related retroviruses (e.g., MMTV, HTLV...), as well as the even more distantly related (older, longer diverged!) Copia-TY1 transposon sub-group. This and similar analyses suggest strongly that retroviruses evolved from a retrotransposon lineage [For a review of retroposon/retrovirus evolution, check Lerat P. & Capy P. (1999, Retrotransposons and retroviruses: analysis of the envelope gene. Mol. Biol. Evol. 19(9): 1198-1207).

C. Transposons Can Be Acquired by "Horizontal Gene Transfer"

As noted, transposons are inherited **vertically**, meaning that they are passed from cell to cell or parents to progeny by reproduction. But they also may have spread between species by **horizontal gene transfer**. This just means that organisms exposed to DNA containing transposons might inadvertently pick up such DNA and become transformed as the transposon becomes part of the genome. Accidental mobility of transposons between species would have been rare, but an exchange of genes by horizontal gene transfer would have accelerated with the evolution of retroviruses. Once again, despite the potential to disrupt the health an organism, retroviral activity might also have supported a degree genomic diversity useful to organisms.

255 Transposon Evolution

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14.6: Roles of Transposition in Evolution and Diversity

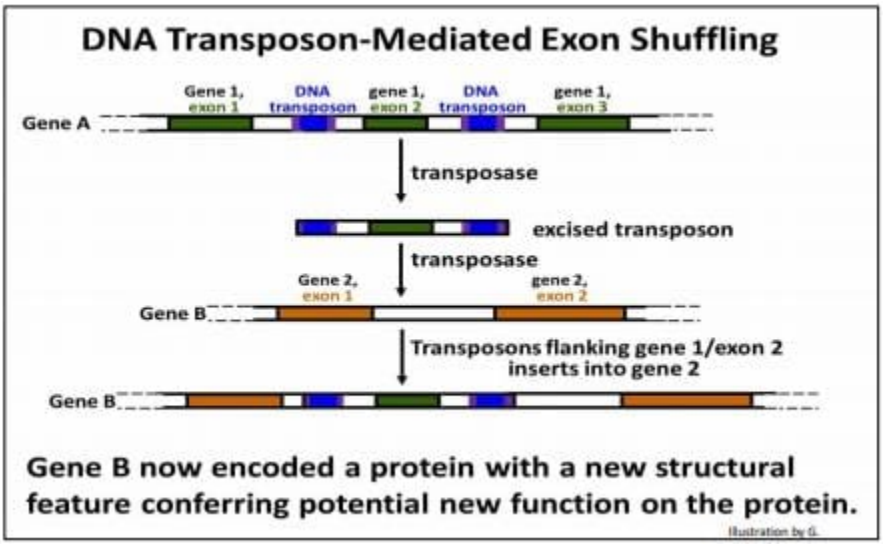
A. Transposons and Exon Shuffling

A role for unequal recombination in moving exons in and out of different eukaryotic split genes was described earlier. This kind of **exon shuffling** could happen when short DNA sequences in two different introns misalign during meiotic synapsis, allowing for unequal crossing over. Expression of a gene with a 'new' exon produces a protein with a new domain and a new activity. If the event is not harmful, diversity is increased!

When found in introns, transposons are long regions of DNA similarity that can stabilize synapsis, increasing the chances of unequal recombination and exon shuffling. For example, **Alu** (SINE) elements are often found within introns, where they can integrate with no ill effect. The similarity of Alu elements in the introns of unrelated genes does seem to account for exon shuffling by unequal crossing over between the different genes that share domains and specific functions as a result. Another way in which transposons facilitate germ line cell exon shuffling is more direct. Imagine a pair of transposons in introns of a gene on either side of an exon. Should such transposons behave like the two outer IS elements in a bacterial Tn element (discussed above), they might be excised as a single, large transposon containing an exon. The paired transposons flanking the exon might then insert in an intron of a completely different gene! This possibility is illustrated on the next page.

Transposon-mediated exon shuffling can explain insertion of exon-encoded domains of *epidermal growth factor* (**EGF**) into several unrelated genes. The mitogen EGF was discovered because it stimulated skin cells to start dividing. The gene for **TPA** (*tissue plasminogen activator*, a blood-clot dissolving *protease*) shares EGF gene domains. TPA is a treatment for heart attack victims that, if administered rapidly after the attack, can dissolve the clot and allow coronary artery blood flow to heart muscle to resume. Other genes that contain EGF domains include those for Neu and Notch proteins, both involved in cellular differentiation and development.

Some exon shuffling events may have been mediated by LINE transposition and by a special group of recently discovered transposons called **helitrons**. Helitrons replicate by a *rolling circle* mechanism. If you are curious about helitrons, do a google search to learn more about them, and what role they may have had in refashioning and reconstructing genomes in evolution. The general pathway of exon shuffling involving paired proximal DNA transposons is illustrated below.



In the generic example shown above, exon 2 of gene A has been inserted, along with flanking transposons, into another gene (gene B).

B. Transposon Genes and Immune System and Genes Have History

Several important eukaryotic genes may have been derived from transposons. Perhaps the most intriguing example of this is to be found in the complex vertebrate immune system. Our immune system includes **immunoglobulins** (**antibodies**). You inherited genes for immunoglobulin proteins from your parents.

These genes contain multiple variant **V**, **D**, and **J** regions linked to a **C** region. V, D, J and C are defined as **V**ariable, **J**oining, **D**iversity and **C**onstant DNA regions, respectively. They will recombine to create many diverse V-D-J-C immunoglobulin antibody molecules (the D region is not always included in the final recombined gene). These gene rearrangements occur during the maturation of certain stem cells in bone marrow that will become immune cells (B or T lymphocytes). In response to a challenge by foreign substances called **antigens**, cells will be selected that contain **rearranged immunoglobulin genes** coding for immunoglobulins that can recognize, bind and eliminate the invading antigens.

A discussion of the molecular biology of the immune system is beyond our scope here. Suffice it to say that the recombinational pathway of immunoglobulin gene rearrangements includes enzymatic activities very similar to those of transposition. In fact, the so-called RAG1 enzyme active in immunoglobulin gene rearrangement is closely related to genes in a family of transposons (*transib*) found in invertebrates and fungi. Thus, it looks like genes of the immune system might have their origins in a transposon!

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14.7: Coping with the Dangers of Rampant Transposition

Most organisms do not have the high transposon load that we have. For those like us, and given a general tendency of transposons to insert at random into new DNA loci, how come we exist at all? Isn't the danger of transposition into essential gene sequences magnified by the possibility of multiple simultaneous transpositions of elements generated by cut-and-paste and especially replicative mechanisms? Indeed, transposons have been found in genes that are inactive as a result.

An obvious explanation for our survival of transposon activity is that most transposition is into the >90% percent of the genome that does not code for proteins. Another is that eukaryotic organisms have two copies of every gene, so that if one is inactive, the other may sustain us. Beyond this, several mechanisms exist to silence a transposon after transposition has occurred, mitigating the dangers of rampant transposition. As long as a transposition is not lethal (e.g., because its integration disrupts an activity essential to life), the cell and organisms can survive the event. In time, mutations at the ends of CMB3e 344 transposons or in genes responsible for transposition would render them inactive. Finally, there may be a more direct curb on transposition. The s*mall interfering RNA*s (**siRNAs**) we encountered earlier could complement and target viral RNAs for destruction (see the Transcription chapter for more information on **siRNAs**). There is some evidence that siRNAs similarly target transposon transcripts.

Summing up, transposon activity is moderated by mutational loss of function and/or by more direct mechanisms that limit transposition and thus genetic damage. If an accumulation of transposons to a high load, as occurs in many species, were deleterious, they would be limited or eliminated from genomes. Instead, persistent transposons and acts of transposition are largely neutral, increasing options for diversity in the selection of new genotypes and phenotypic characteristics. We also know now that transposons can function in genetic regulation. Thus, transposons are neither selfish nor junk DNA. Check out these links for more: Not junk after all? and Eulogy for Junk DNA.

256 Transposons- Junk or Not

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14.8: Key Words and Terms

Alu	Gag	non-homologous recombination
anthocyanins	genomic complexity	Non-LTR transposons
antibiotic resistance genes	heterochromatin	protease
antibodies	immune system	protein coat
autonomous transposon	immunoglobulin genes	prt
bacterial composite transposons	integrase	renaturation kinetics
bacterial IS elements	inverted repeats	Repetitive DNA
bacterial Tn elements	jumping genes	replicative transposition
bacteriophage	L1	resolvase
centromere	LINE	retrotransposon
chromatin	LTR (long terminal repeats)	retrovirus
chromosomes	LTR transposons	RNA transposon
Class I transposon	lysis	satellite DNA
Class II transposon	lysogeny	SINE
cointegrate	lytic pathway of phage	spindle fibers
CoT curves	maize AC (activator) gene	telomeres
cut-and-paste transposition	maize Ds (dissociator) gene	transposase
density gradient centrifugation	mariner	triploid endosperm
direct repeats	McClintock	Ty
DNA sequence phylogeny	mosaicism	viral infection
exon shuffling	Mu phage	
fertility (F) plasmids	non-autonomous transposon	

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15: DNA TECHNOLOGIES

Learning Objectives

1. Suggest molecular techniques to design experiments (e.g., how would you use cDNA or a PCR product to clone a gene).
2. Determine when to make or use a cDNA library or a genomic library
3. Outline an experiment to purify rRNA from eukaryotic cells.
4. Outline an experiment to isolate a cDNA for a human protein and clone it so you can manufacture insulin for the treatment of disease.
5. Explain why you might want to clone and express a human growth hormone gene.
6. List components needed to make a cDNA library using purified poly(A) RNA.
7. List the components needed to make a genomic library from isolated genomic DNA.
8. Compare PCR and genomic cloning as strategies for isolating a gene.
9. Outline a strategy for using fly DNA to obtain copies of a human DNA sequence.
10. Ask a question that requires screening a genomic library to obtain a gene you want to study
11. Ask a question that requires using a microarray to obtain a gene you want to study

15.1: Overview

15.2: Make and Screen a cDNA Library

15.3: DNA Sequencing

15.4: Genomic Libraries

15.5: The Polymerase Chain Reaction (PCR)

15.6: Genomic Approaches- The DNA Microarray

15.7: Ome-Sweet Ome

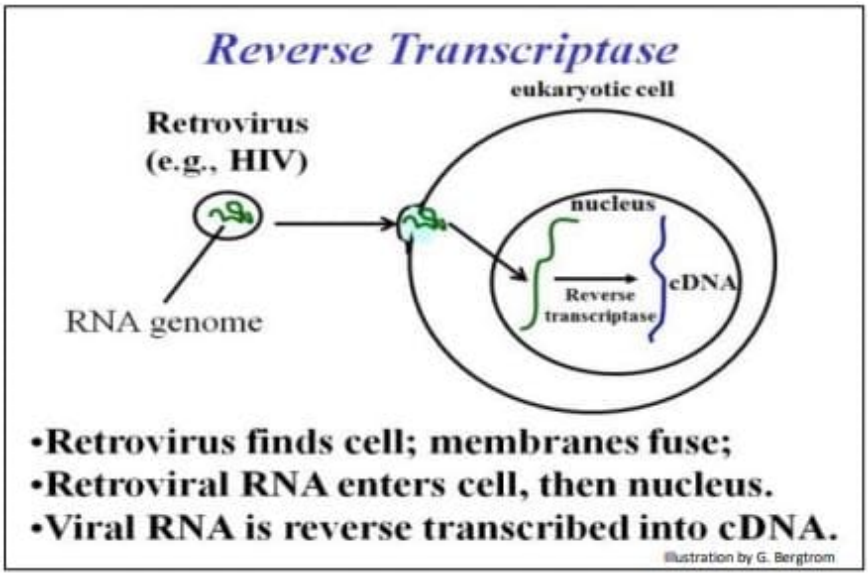
15.8: From Genetic Engineering and Genetic Modification

15.9: Key Words and Terms

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15.1: Overview

We start this chapter by looking at technologies that led to **genetic engineering**. The ability of make **recombinant DNA** is such a seminal technology that just realizing it could be done and then doing it in a test tube for the first time earned Paul berg a half-share in the 1980 Nobel Prize in Chemistry (the other half was shared by Walter Gilbert and Frederick Sanger for studies that enabled efficient **DNA sequencing**). First we'll look at cDNA synthesis, the synthesis of DNA copies from RNA, something retroviruses routinely do as part of the pathway of their reproduction. The retrovirus injects its RNA into target cells where it transcribes a **reverse transcriptase** enzyme. The enzyme reversetranscribes a copy DNA (the cDNA) complementary to the viral RNA. The First steps in retroviral infection is summarized in the illustration below.



The same reverse transcriptase enzyme makes a double stranded cDNA, or (ds)cDNA, which then replicates. These cDNAs are transcribed into new viral RNA genomes and mRNAs for viral proteins. The latter encapsulate the RNA genomes into new viruses. Reverse transcriptase is now a laboratory tool, used to reverse transcribe cDNA from virtually any RNA sequence. It, along with many viral, bacterial and even eukaryotic enzymes and biomolecules, are now part of our recombinant DNA and genetic engineering toolkit.

We will see how a **cDNA library** is made and screened for a **cDNA clone**, and how a cloned cDNA can fish an entire gene out of a **genomic library**. Next we will see how the **polymerase chain reaction** (**PCR**) can produce (amplify) millions of copies of a single gene (or other DNA sequence) from as little DNA as is found in a single cell. Apart from its well-publicized use in forensics, PCR is another important laboratory tool for fetching, amplifying and studying sequences of interest. These venerable technologies illustrate important principles of cloning and sequence analysis. Of course, the analysis of traditionally cloned and amplified DNA sequences has been used to study the evolution and expression of individual genes. And sometimes we are misled! For example, knowing that a genetic mutation is associated with an illness usually leads to a search for how the mutation might cause the illness. But, as researchers in any discipline keep warning us, correlation is not causation! In fact, we know that many phenotypes, including genetic disease, are not the result of a single mutant gene. Autism is just one example.

The newer fields of **genomics** and **proteomics** leverage a growing battery of powerful tools to study many genes and their regulatory networks at the same time. The **molecular networking** made possible by genomics and proteomics (and other colorful holistic terms we'll discuss later) promise to get us past naïve and often incorrect notions of causation. We may be soon able to identify *many* correlations that might sum up to causation or propensity to genetic illness.

YouTube Video

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Figure 15.1.1: Copy and Paste Caption here. (Copyright; author via source)

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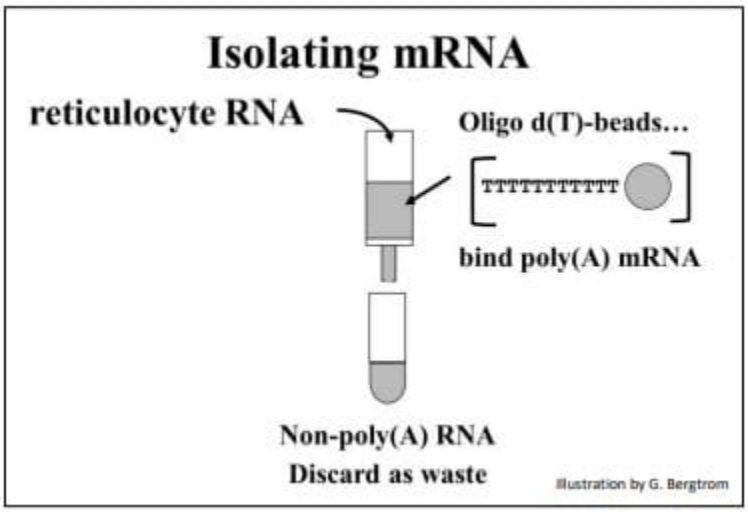
15.2: Make and Screen a cDNA Library

The first step in making a cDNA library is to isolate cellular mRNA. This mRNA extract should represent all of the transcripts in the cells at the time of isolation, or the cell's **transcriptome**. This term is used by analogy to genome. However, a genome is all of the genetic information of an organism. In contrast, a transcriptome (usually eukaryotic) reflects all of the genes expressed in a given cell type at a moment in time. Reversetranscribed cDNAs from an mRNA extract are also referred to as a transcriptome..., and likewise, a cDNA library. A cDNA library is a tube full of bacterial cells that have taken up (i.e., been **transformed** with) plasmids recombined with cDNAs. cDNA libraries made from mRNAs taken from different cell types or the same cells grown under different conditions are in effect, different transcriptomes. Each reflects mRNAs transcribed in cells at the moment of their extraction. When cells in a cDNA library are spread out on a nutrient agar petri dish, each cell grows into a colony of cells; each cell in the colony is a clone of a starting cell. cDNA libraries can be used isolate and sequence the DNA encoding a polypeptide that you are studying.

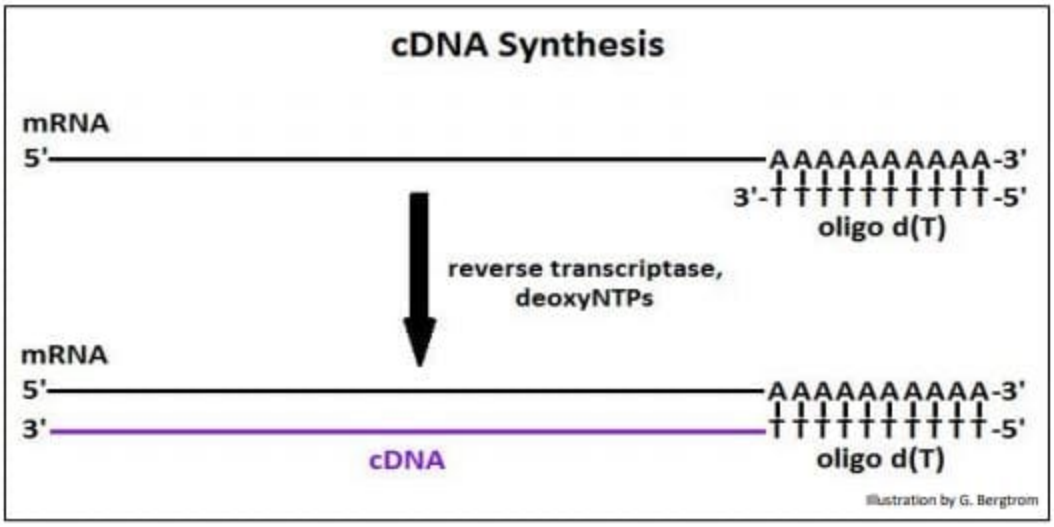
Recall that the mature mRNA in eukaryotic cells has been spliced. This means that cDNAs from eukaryotic cells do not include introns. Introns, as well as sequences of enhancers and other regulatory elements in and surrounding a gene must be studied in genomic libraries, to be discussed later. Here we look at how to make a cDNA library.

A. cDNA Construction

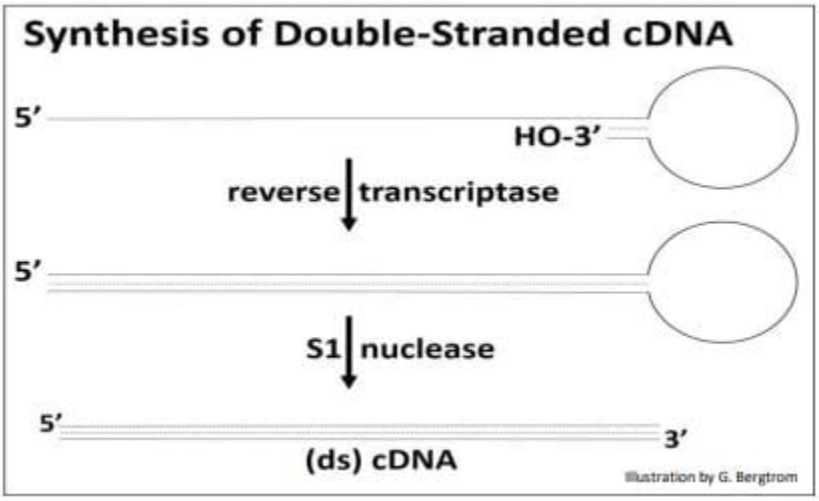
mRNA is only a few percent of a eukaryotic cell; most is rRNA. But that small amount of mRNA can be separated from other cellular RNAs by virtue of their 3' poly(A) tails. Simply pass a total RNA extract over an **oligo-d(T) column** (illustrated below).



The strings of thymidine (T) can H-bond with the poly(A) tails of mRNAs, tethering them to the column. All RNAs without a 3' poly(A) tail will flow through the column as waste. A second buffer is passed over the column to destabilize the A-T H-bonds to allow **elution** of an mRNA fraction. When free' oligo d(T) is added to the eluted mRNA, it forms H-bonds with the poly(A) tails of the mRNAs, serving as a primer for the synthesis of cDNA copies of the poly(A) mRNAs originally in the cells. Finally, four deoxynucleotide DNA precursors and **reverse transcriptase** (originally isolated from chicken retrovirus-infected cells) are added to start reverse transcription. The synthesis of a cDNA strand complementary to an mRNA is shown below.



After heating to separate the cDNAs from the mRNAs, the cDNA is replicated to produce double-stranded, or (ds)cDNA, as illustrated below.



Synthesis of the second cDNA strand is also catalyzed by reverse transcriptase! The enzyme recognizes DNA as well as RNA templates, and has the same 5'-to-3' DNA polymerizing activity as DNA polymerases. After 2nd cDNA strand synthesis, **S1 nuclease** (a *single-stranded endonuclease* originally isolated from an East Asian fungus!) is added to open the loop of the (ds) cDNA structure and trim the rest of the single-stranded DNA. What remains is the (ds) cDNA.

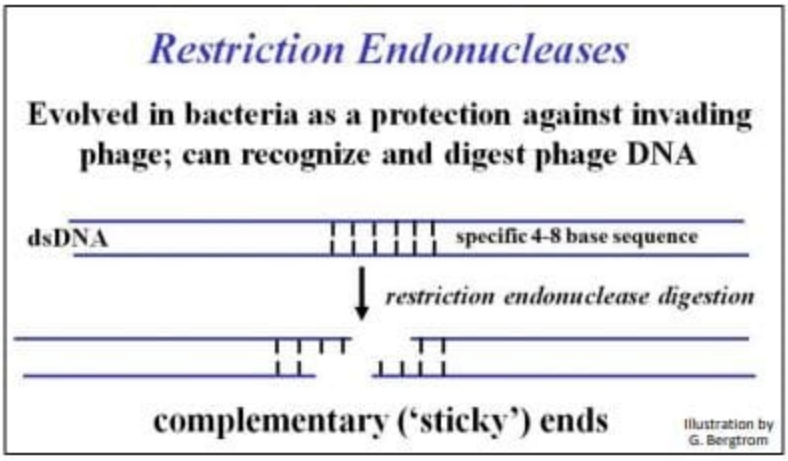
258 Isolate mRNA and Make cDNA

259 Reverse Transcriptase

B. Cloning cDNAs into Plasmid Vectors

To understand cDNA cloning and other aspects of making recombinant DNA, we need to talk a bit more about the recombinant DNA tool kit. In addition to *reverse transcriptase* and S1 nuclease, other necessary enzymes in the 'kit' include **restriction endonucleases** (*restriction enzymes*) and **DNA ligase**. The natural function of restriction enzymes in bacteria is to recognize specific restriction site sequences in phage DNA (most often **palindromic** DNA sequences), hydrolyze it and thus avoid infection.

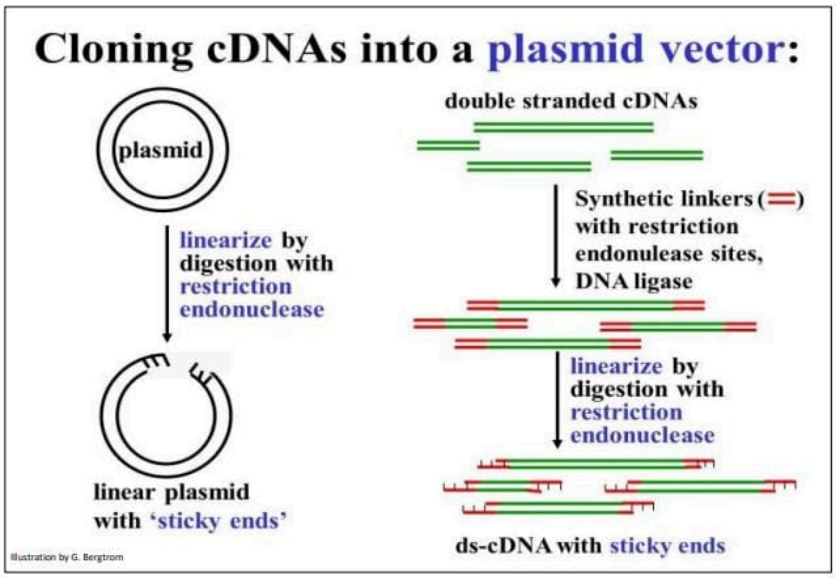
Restriction enzymes that make a scissors cut through the two strands of the double helix leaves *blunt ends*. Restriction enzymes that make a staggered cut on each strand at their restriction site leave behind *complementary* ('sticky') ends (below).



If you mix two of double-stranded DNA fragments with the same sticky ends from different sources (e.g., different species), they will form H-bonds at their complementary ends, making it easy to recombine plasmid DNA with (ds)cDNA, that have the same complementary 'sticky ends'. Using the language of recombinant DNA technologies, let's look at how plasmid vectors and cDNAs can be made to recombine.

1. Preparing Recombinant Plasmid Vectors Containing cDNA Inserts

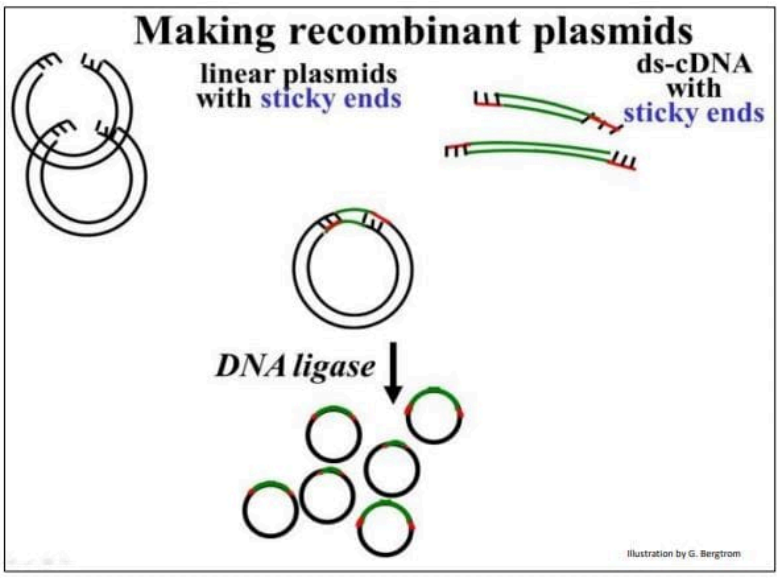
Vectors are *carrier* DNAs engineered to recombine with foreign DNAs of interest. When a recombinant vector with its foreign **DNA insert** gets into a host cell, it can replicate many copies of itself, enough in fact for easy isolation and study. cDNAs are typically inserted into plasmid vectors that are usually purchased "off-the-shelf". They can be *cut* with a restriction enzyme at a suitable location, leaving those sticky ends. On the other hand, it would not do to digest (ds)cDNA with restriction endonucleases since the goal is not to clone cDNA fragments, but entire cDNA molecules. Therefore, it will be necessary to attach **linkers** to either end of the (ds)cDNAs. Plasmid DNAs and cDNA-linker constructs can then be digested with the same restriction enzyme to produce compatible 'sticky ends'. Steps in the preparation of vector and (ds)cDNA for recombination are shown below.



To prepare for recombination, a plasmid vector is digested with a **restriction enzyme** to open the DNA circle. To have compatible *sticky ends*, double-stranded cDNAs to be inserted are mixed with *linkers* and **DNA ligase** to put a linker DNA at both ends of the (ds) cDNA. DNA ligase is another tool in the recombinant DNA toolkit. Linkers are short, synthetic double-stranded DNA oligomers containing restriction sites recognized and cut by the same restriction enzyme as the plasmid. Once the linkers are attached to the ends of the plasmid DNAs, they are digested with the appropriate restriction enzyme. This leaves both the (ds)cDNAs and the plasmid vectors with complementary sticky ends.

2. Recombining Plasmids and cDNA Inserts and Transforming Host Cells

The next step is to mix the cut plasmids with the digested linker-cDNAs in just the right proportions so that the most of the cDNA (linker) ends will anneal (form Hbonds) with the most of the sticky plasmid ends. Adding **DNA ligase** to the plasmid/linker-cDNA mixture forms phosphodiester bonds between plasmid and cDNA insert, completing the recombinant circle of DNA, as shown below.

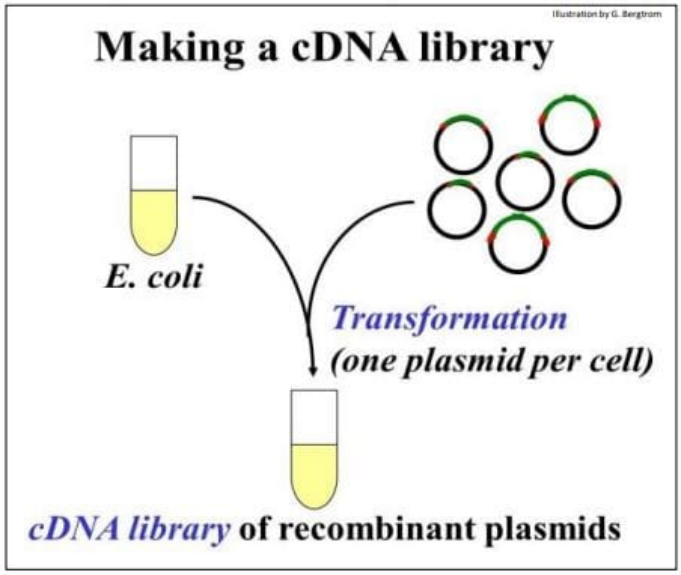


In early cloning experiments, an important consideration was to generate plasmids with only one copy of a given cDNA insert, rather than lots of re-ligated plasmids with no inserts or lots of plasmids with multiple inserts. Using betterengineered vector and linker combinations, this issue became less important.

261 Recombine a cDNA Insert with a Plasmid Vector

3. Transforming Host Cells with Recombinant Plasmids

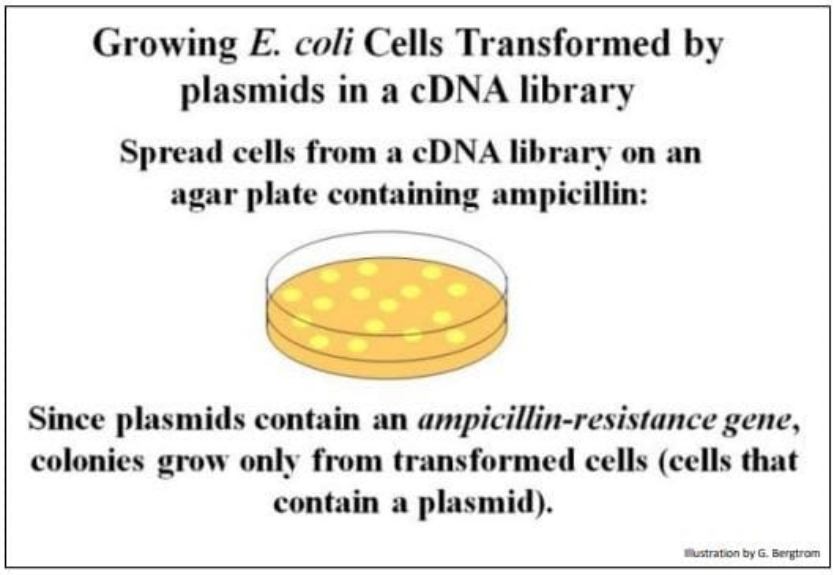
The recombinant DNA molecules are now ready for 'cloning'. They are added to E. coli (sometimes other host cells) *made permeable* so that they can be easily **transformed**. Recall that transformation as defined by Griffith is bacterial uptake of foreign DNA leading to a genetic change. The **transforming principle** in cloning is the recombinant plasmid! The transformation step is shown below.



The tube full of transformed cells is the **cDNA Library.**

262 Making the cDNA Library.

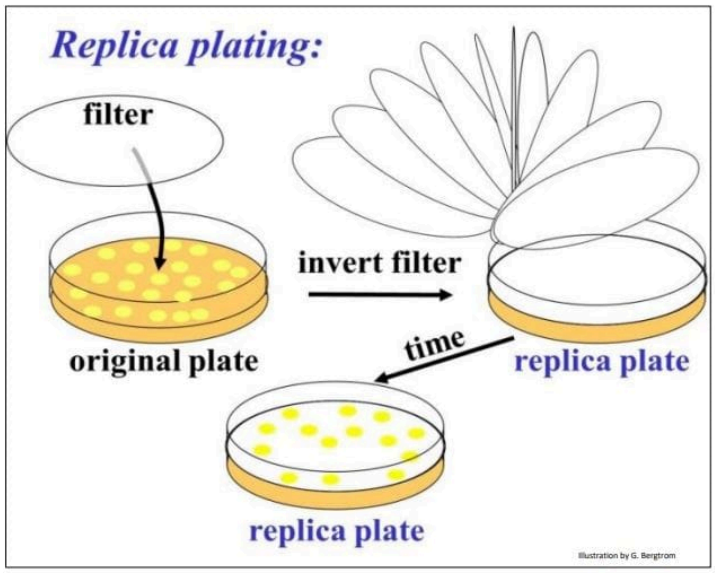
After all these treatments, not all plasmid molecules in the mix are recombinant; some cells in the mix haven't even taken up a plasmid. So when the recombinant cells are plated on agar, how do you tell which of the colonies that grow came from cells that took up a recombinant plasmid? Both the **host strain** of *E. coli* and plasmid vectors used these days were further engineered to solve this problem. One such plasmid vector carries an **antibiotic resistance gene**. In this case, ampicillin-sensitive cells would be transformed with recombinant plasmids containing the *resistance gene*. When these cells are plated on media containing **ampicillin** (a form of penicillin), they grow, as illustrated below.



Untransformed cells (cells that failed to take up a plasmid) lack the ampicillin resistance gene and thus, do not grow on ampicillin-medium. But, there is still a question. How can you tell whether the cells that grew were transformed by a recombinant plasmid containing a cDNA insert? It is possible that some of the transformants contain only non-recombinant plasmids that still have the ampicillin resistance gene!

To address this question, plasmids were further engineered with a **streptomycin resistance gene**. But this antibiotic resistance gene was also engineered to contain restriction enzyme sites in the middle of the gene. Thus, inserting a cDNA in this plasmid would disrupt and inactivate the gene. Here is how this second bit of genetic engineering enabled growth only of cells transformed

with a recombinant plasmid containing a cDNA insert. We can tell *transformants* containing recombinant plasmids apart from those containing non-recombinant plasmids by the technique of **replica plating** shown (illustrated below).

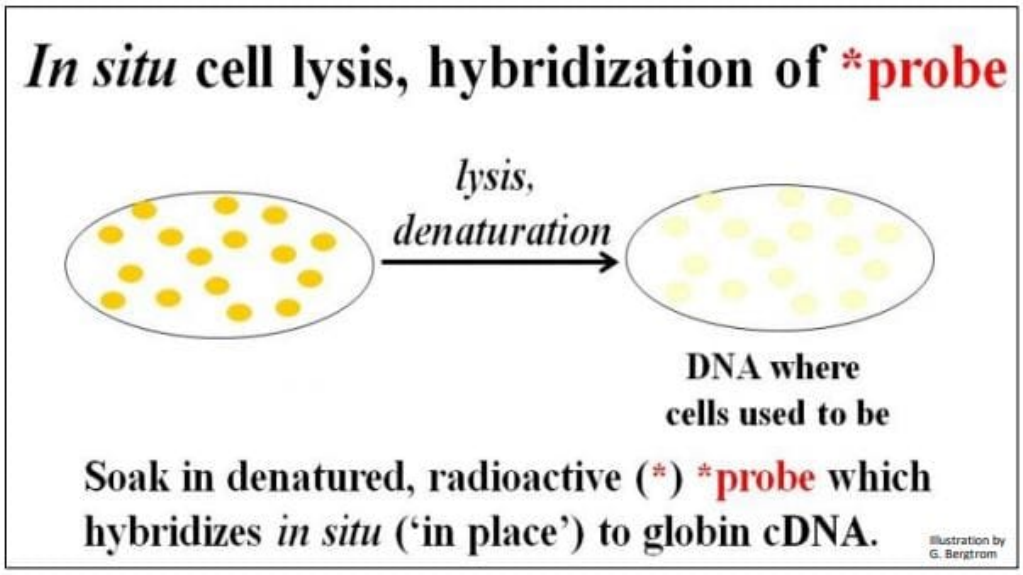


After colonies grow on the ampicillin agar plate, lay a filter over the plate. The filter will pick up a few cells from each colony, in effect becoming a replica (mirror image) of the colonies on the plate. Place the *replica filter* on a new agar plate containing streptomycin; the new colonies that grow on the filter must be streptomycin-resistant, containing only non-recombinant plasmids. Colonies containing recombinant plasmids, those that did not grow in streptomycin are easily identified on the original ampicillin agar plate. In practice, highly efficient recombination and transformation procedures typically reveal very streptomycinresistant cells (i.e., colonies) after replica plating. In this case, ampicillin-resistant cells constitute a good cDNA library, ready for screening.

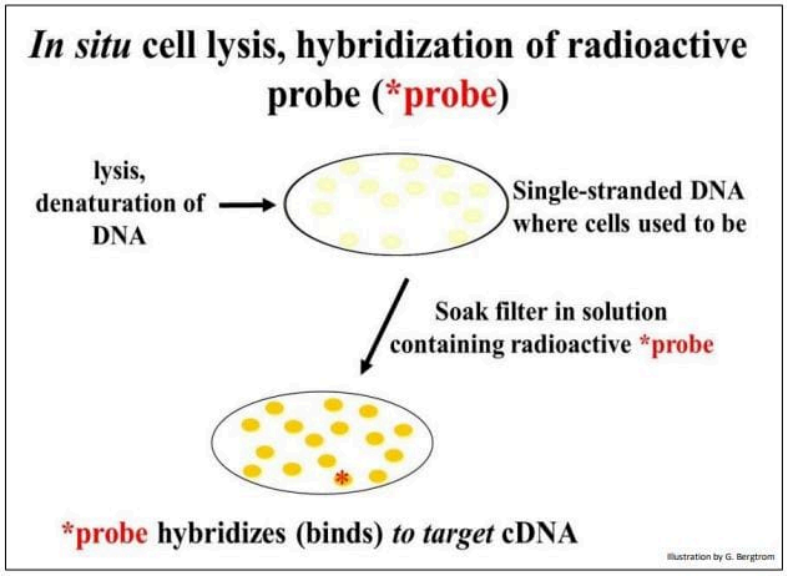
263 Making a Replica Plate Filter

4. Identifying Colonies Containing Plasmids with Inserts of Interest

The next step is to **screen** the colonies from the cDNA library for those containing the specific cDNA that you're after. This typically begins preparing multiple replica filters like the one above. Remember, these filters are replicas of bacterial cells containing recombinant plasmids that grow on ampicillin but not streptomycin. The number of replica filters that must be screened can be calculated from assumptions and formulas for estimating how many colonies must be screened to represent an entire transcriptome (i.e., the number of different mRNAs in the original cellular mRNA source). Once the requisite number of replica filters are made, they are subjected to **in situ lysis** to disrupt cell walls and membranes. The result is that the cell contents are released and the DNA is denatured (i.e., becomes single-stranded). The DNA then adheres to the filter *in place* (*in situ*, where the colonies were). The result of *in situ lysis* is a filter with faint traces of the original colony (below).

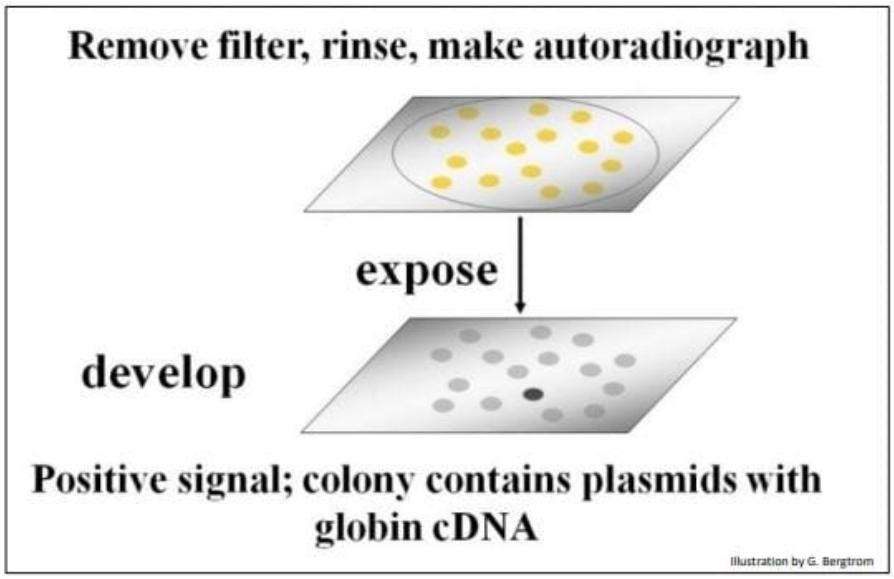


Next, a molecular **probe** is used to identify DNA containing the sequence of interest. The probe is often a **synthetic oligonucleotide** whose sequence was inferred from known amino acid sequences. These oligonucleotides are made radioactive and placed in a bag with the filter(s). DNA from cells that contained recombinant plasmids with a cDNA of interest will bind the complementary probe. The results of *in situ* lysis and **hybridization** of a radioactive probe to a replica filter are shown below.



264 Probing a Replica Plate Filter

The filters are rinsed to remove un-bound radioactive oligomer probe, and then placed on X-ray film. After a period of exposure, the film is developed. Black spots will form on the film from radioactive exposure, creating an autoradiograph of the filter. The black spots in the autoradiograph correspond to colonies on a filter that contain a recombinant plasmid with your target cDNA sequence (below).



Once a positive clone is identified on the film, the corresponding recombinant colony is located on the original plate. This colony is grown up in a liquid culture and the plasmid DNA is isolated. At that point, the cloned plasmid DNA can be sequenced and the amino acid sequence encoded by its cDNA can be inferred from the genetic code dictionary to verify that the cDNA *insert* in fact encodes the protein of interest. Once verified as the sequence of interest, a cloned plasmid cDNA can be made radioactive or fluorescent, and used to

- probe for the genes from which they originated.
- identify and quantitate the mRNA even locate the transcripts in the cells.
- quantitatively measure amounts of specific mRNAs.

Isolated plasmid cDNAs can even be expressed in suitable cells to make the encoded protein. These days, diabetics no longer receive pig insulin, but get synthetic human insulin human made from expressed human cDNAs. Moreover, while the introduction of the *polymerase chain reaction* (**PCR**, see below) has superseded some uses of cDNAs, they still play a role in genome-level and transcriptome-level studies.

265 Pick a Clone From a Replica Filter and Play With It!

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15.3: DNA Sequencing

A. A Brief History of DNA Sequencing

RNA sequencing came first, when Robert Holley sequenced a tRNA in 1965. The direct sequencing of tRNAs was possible because tRNAs are small, short nucleic acids, and because many of the bases in tRNAs are chemically modified after transcription. An early method for DNA sequencing developed by Walter Gilbert and colleagues involved DNA fragmentation, sequencing of the small fragments of DNA, and then aligning the overlapping sequences of the short fragments to assemble longer sequences. Another method, the DNA synthesis-based 'dideoxy' DNA sequencing technique, was developed by Frederick Sanger in England. Sanger and Gilbert both won a Nobel Prize in Chemistry in 1983 for their DNA sequencing efforts. However, because of its simplicity, Sanger's method quickly became the standard for sequencing all manner of cloned DNAs.

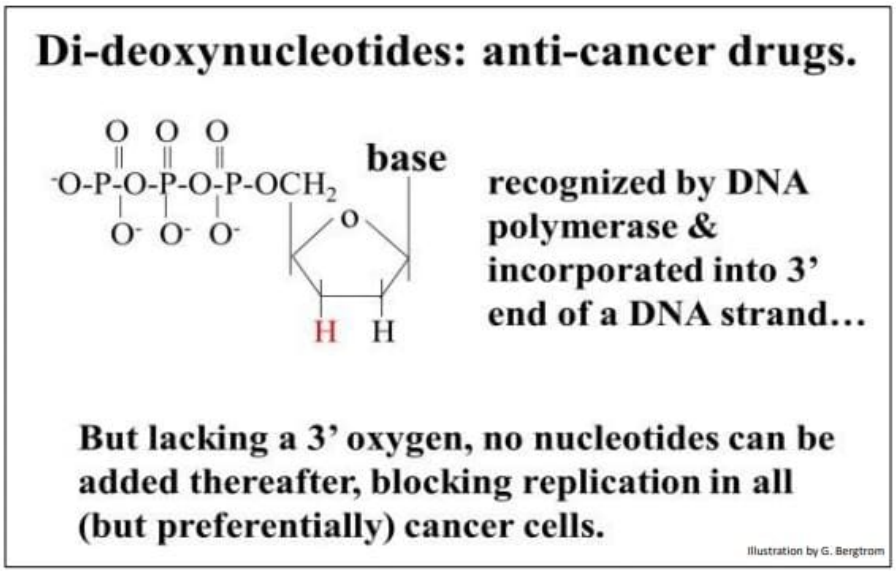
The first complete genome to be sequenced was that of a bacteriophage (bacterial virus) called φX174. At the same time as the advances in sequencing technology were occurring, so were some of the early developments in recombinant DNA technology. Together these led to more efficient and rapid cloning and sequencing of DNA from increasingly diverse sources. The first focus was of course on genes and genomes of important model organisms, such as *E. coli*, *C. elegans*, yeast (*S. cerevisiae*)..., and of course us! By 1995, Craig Venter and colleagues at the *Institute for Genomic Research* had completed the sequence of an entire bacterial genome (*Haemophilus influenzae*) and by 2001, Venter's private group along with Frances Collins and colleagues at the NIH had published a first draft of the sequence of the human genome. Venter had proven the efficacy of a whole-genome sequencing approach called *shotgun sequencing*, which was much faster than the gene-by-gene, fragment-by-fragment 'linear' sequencing strategy being used by other investigators (more later!). Since Sanger's dideoxynucleotide DNA sequencing method remains a common and economical methodology, let's consider the basics of the protocol.

B. Details of DiDeoxy Sequencing

Given a template DNA (e.g., a plasmid cDNA), Sanger used in vitro replication protocols to demonstrate that he could:

1. Replicate DNA under conditions that randomly stopped nucleotide addition at every possible position in growing strands.
2. *Separate and then detect* these DNA fragments of replicated DNA.

Recall that DNA polymerases catalyze the formation of *phosphodiester bonds* by linking the α *phosphate* of a **nucleotide triphosphate** to the free 3' OH of a deoxynucleotide at the end of a growing DNA strand. Recall also that the ribose sugar in the deoxynucleotide precursors of replication lack a 2' OH (hydroxyl) group. Sanger's trick was to add **dideoxynucleotide triphosphates** to his in vitro replication mix. The ribose on a **dideoxynucleotide triphosphate** (ddNTP) lacks a 3' OH, in addition to the 2' OH group (as shown below).

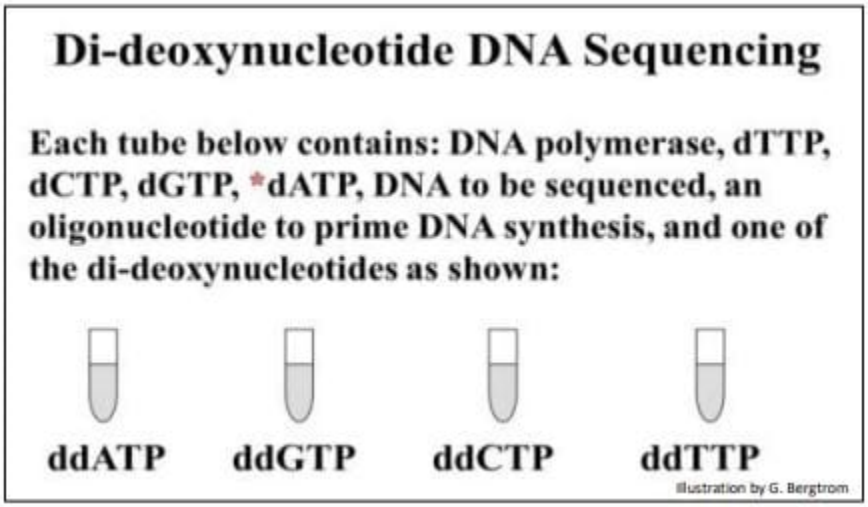


Adding a dideoxynucleotide to a growing DNA strand stops replication. No further nucleotides can add to the 3'-end of the replicating DNA strand because the 3'–OH necessary for the dehydration synthesis of the next phosphodiester bond is absent! Because they can stop replication in actively growing cells, ddNTPs such as dideoxyadenosine (tradename, **cordycepin**) are anti-cancer chemotherapeutic drugs.

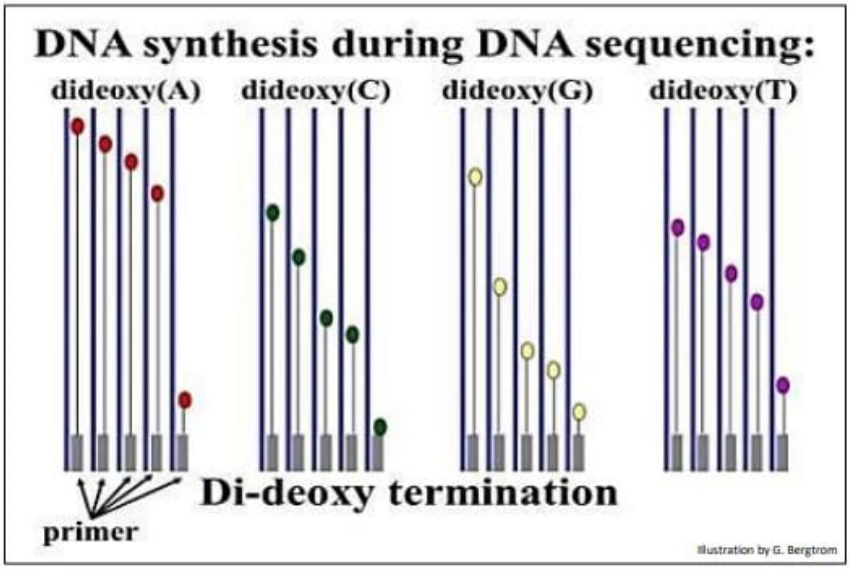
266 Treating Cancer with Dideoxynucleosides

A look at a manual DNA sequencing protocol reveals what is going on in the sequencing reactions. Four reaction tubes are set up, each containing the template DNA to be sequenced, a *primer* of known sequence and the four required deoxynucleotide precursors necessary for replication.

The set-up for manual DNA sequencing is shown below.

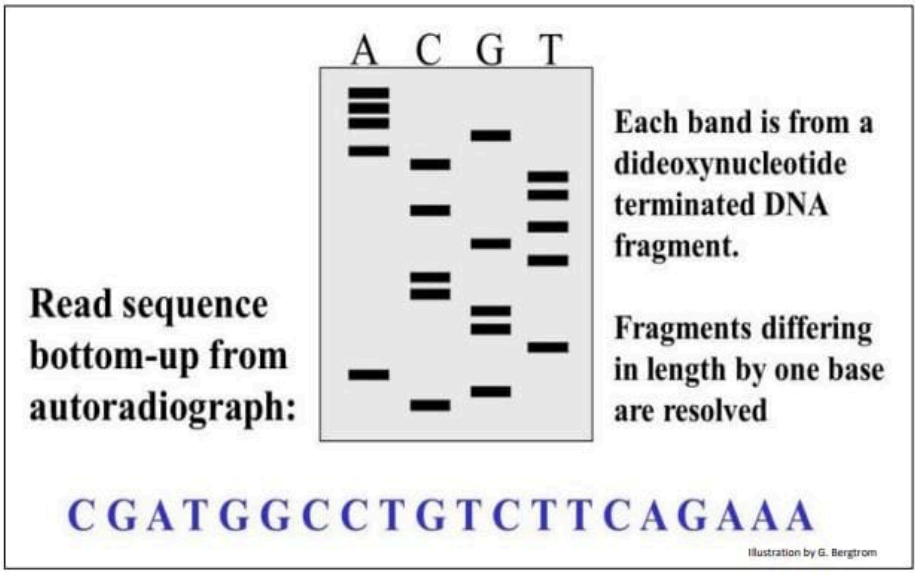


A different ddNTP, (ddATP, ddCTP, ddGTP or ddTTP) is added to each of the four tubes. Finally, DNA polymerase is added to each tube to start the DNA synthesis reaction. During DNA synthesis, different length fragments of new DNA accumulate as the ddNTPs incorporate randomly, opposite complementary bases in the template DNA being sequenced. The expectations of the didieoxy sequencing reactions in the four tubes are illustrated below.



A short time after adding the DNA polymerase to begin the reactions, the mixture is heated to separate the DNA strands and fresh DNA polymerase is added to repeat the synthesis reactions. These sequencing reactions are repeated as many as 30 times in order to produce enough radioactive DNA fragments to be detected. When the heat-stable **Taq DNA polymerase** from the thermophilic bacterium *Thermus aquaticus* became available (more later!), it was no longer necessary to add fresh DNA polymerase after each replication cycle. The many heating and cooling cycles required for what became known as *chain-termination DNA sequencing* were soon automated using inexpensive *programmable thermocyclers*.

Since a small amount of a radioactive deoxynucleotide (usually 32P-labeled ATP) was present in each reaction tube, the newly made DNA fragments are radioactive. After electrophoresis to separate the new DNA fragments in each tube, autoradiography of the electrophoretic gel reveals the position of each terminated fragment. The DNA sequence can then be read from the gel as illustrated in the simulated autoradiograph below.

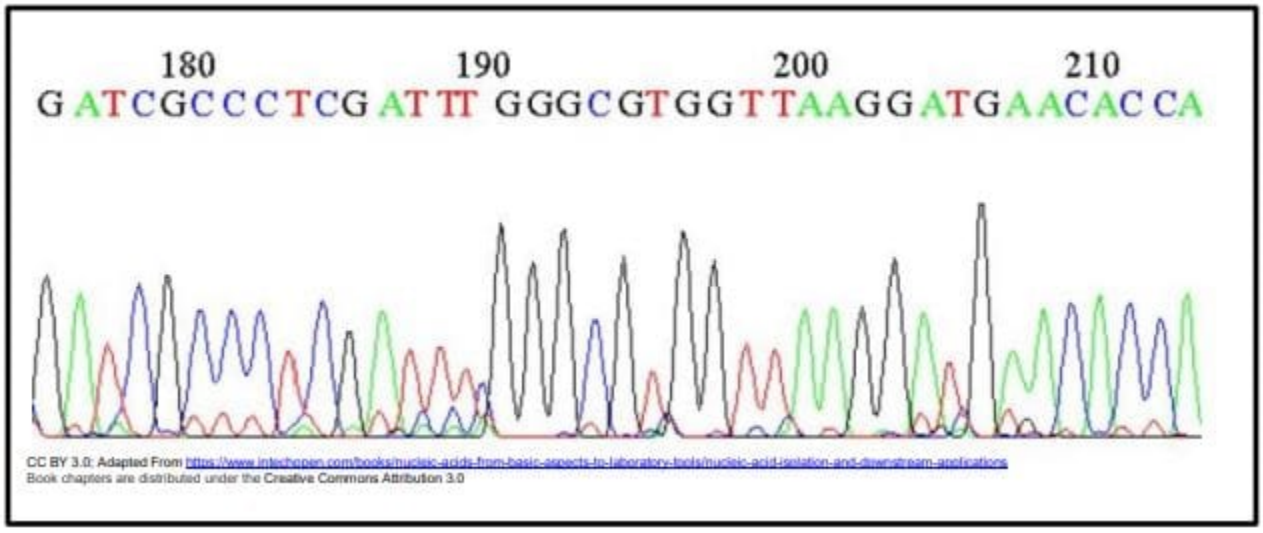


As shown in the cartoon, the DNA sequence can be read by reading the bases from the bottom of the gel, starting with the C at the bottom of the C lane. Try reading the sequence yourself!

267 Manual Dideoxy Sequencing

The first semi-automated DNA sequencing method was invented in Leroy Hood's California lab in 1986. Though still Sanger sequencing, the four dideoxynucleotides in the sequencing reaction were tagged for detection with a fluorescent dyes instead

radioactive phosphate-tagged nucleotides. After the sequencing reactions, the reaction products are electrophoresed on an 'automated DNA sequencer'. UV light excites the migrating dye-terminated DNA fragments as they pass through a detector. The color of their fluorescence is detected, processed and sent to a computer, generating color-coded graph like the one below, showing the order (and therefore length) of fragments passing the detector and thus, the sequence of the strand.



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A most useful feature of this sequencing method is that a template DNA could be sequenced in a single tube, containing all the required components, including *all* four dideoxynucleotides! That's because the fluorescence detector in the sequencing machine separately sees all the short ddNTP-terminated fragments as they move through the electrophoretic gel.

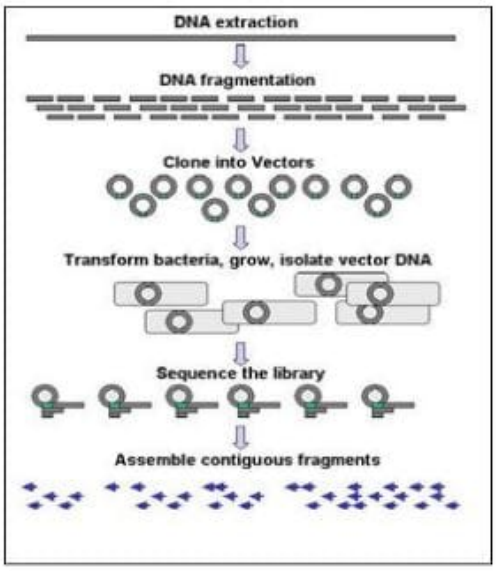
Hood's innovations were quickly commercialized making major sequencing projects possible, including whole genome sequencing. The rapidity of automated DNA sequencing led to the creation of large sequence databases in the U.S. and Europe.

The NCBI (National Center for Biological Information) maintains the U.S. database. Despite its location, the NCBI archives virtually all DNA sequences determined worldwide. New 'tiny' DNA sequencers have made sequencing DNA so portable that in 2016, one was even used in the *International Space Station*. Expanding databases and new tools and protocols (some are described below) to find, compare and analyze DNA sequences have also grown rapidly.

268 Automated Sequencing Leads to Large Genome Projects

C. Large Scale Sequencing

Large-scale sequencing targets entire prokaryotic, and typically much larger eukaryotic genomes. The latter require strategies that either sequence long DNA fragments and/or sequencing DNA fragments more quickly. We already noted the shotgun sequencing used by Venter to sequence smaller and larger genomes (including our own... or more accurately, his own!). In shotgun sequencing, cloned DNA fragments 1000 base pairs or longer are broken down at random into smaller, more easily sequenced fragments. The fragments are themselves cloned and sequenced and non-redundant sequences are assembled by aligning overlapping regions of sequence. Today's computer software is quite adept at rapid overlapping sequence alignment as well as connecting and displaying long contiguous DNA sequences. Shotgun sequencing is summarized below.



Sequence gaps that remain after shotgun sequencing can be filled in by primer walking, in which a known sequence near the gap is the basis of creating a sequencing primer to “walk” into the gap region on an intact DNA that has not been fragmented. Another ‘gap-filling’ technique involves **PCR** (the Polymerase Chain Reaction, to be described shortly). Briefly, two oligonucleotides are synthesized based on sequence information on either side of a gap. Then PCR is used to synthesize the missing fragment, and the fragment is sequenced to fill in the gap.

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15.4: Genomic Libraries

A **genomic library** might be a tube full of **recombinant bacteriophage**. Each phage DNA molecule contains a fragmentary insert of cellular DNA from a foreign organism. The library is made to contain a representation of all of possible fragments of that genome. Bacteriophage are often used to clone genomic DNA fragments because:

- phage genomes are bigger than plasmids and can be engineered to remove a large amount of DNA that is not needed for infection and replication in bacterial host cells.
- the missing DNA can thus be replaced by foreign insert DNA fragments as long as 18- 20kbp (kilobase pairs), nearly 20X as long as typical cDNA inserts in plasmids.
- purified phage coat proteins can be mixed with the recombined phage DNA to make infectious phage particles that would infect host bacteria, replicate lots of new recombinant phage, and then lyse the cells to release the phage.

The need for vectors like bacteriophage that can accommodate long inserts becomes obvious from the following bit of math. A typical mammalian genome consists of more than *2 billion base pairs*. Inserts in plasmids are very short, rarely exceeding 1000 base pairs. Dividing *2,000,000,000* by *1000*, *you get 2 million*, a minimum number of phage clones that must be screened to find a sequence of interest. In fact, you would need many more than this number of clones to find a gene (or parts of one!). Of course, part of the solution to this “needle in a haystack” dilemma is to clone larger DNA inserts in more accommodating vectors.

From this brief description, you may recognize the common strategy for genetically engineering a cloning vector: determine the minimum properties that your vector must have and remove non-essential DNA sequences. Consider the **Yeast Artificial Chromosome** (**YAC**), hosted by (replicated in) yeast cells. YACs can accept humongous foreign DNA inserts! This is because to be a chromosome that will replicate in a yeast cell requires **one centromere and two telomeres**... and little else!

Recall that telomeres are needed in replication to keep the chromosome from shortening during replication of the DNA. The centromere is needed to *attach chromatids to spindle fibers* so that they can separate during *anaphase* in *mitosis* (and *meiosis*). So along with a centromere and two telomeres, just include restriction sites to enable recombination with inserts as long as 2000 Kbp. That’s a YAC! The tough part of course is keeping a 2000Kbp long DNA fragment intact long enough to get it into the YAC.

However a vector is engineered and chosen, sequencing its insert can tell us many things. They can show us how a gene is regulated by revealing known and uncovering new regulatory DNA sequences. They can tell us what other genes are nearby, and where genes are on chromosomes. Genomic DNA sequences from one species can probe for similar sequences in other species and comparative sequence analysis can then tell us a great deal about gene evolution and the evolution of species.

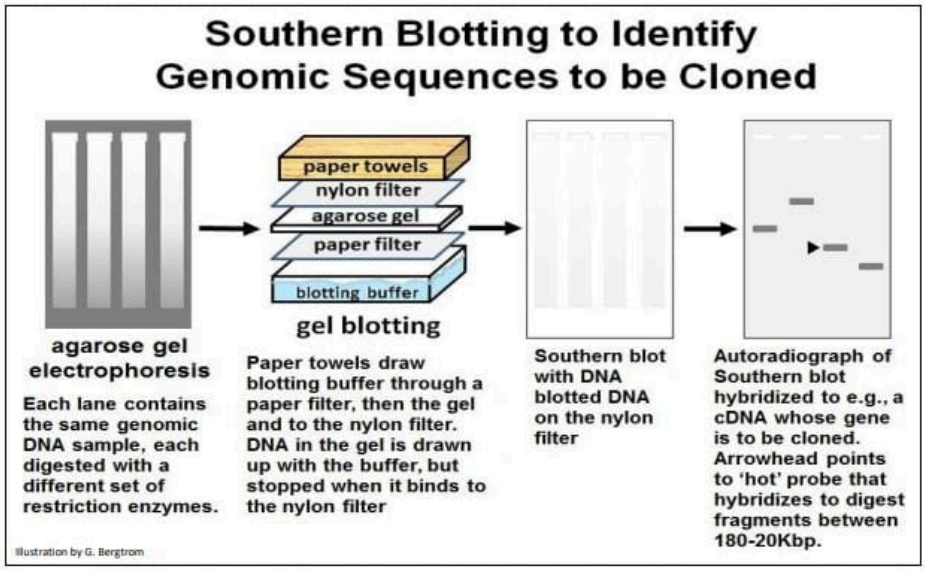
One early surprise from gene sequencing studies was that we share many common genes and DNA sequences with other species, from yeast to worms to flies... and of course vertebrates and our more closely related mammal friends. You may already know that the chimpanzee’s and our genomes are 99% similar. Moreover, we have already seen comparative sequence analysis showing how proteins with different functions nevertheless share structural domains.

Let’s look at cloning a genomic library in phage. As you will see, the principles are similar to cloning a foreign DNA into a plasmid, or in fact any other vector, but the numbers and details used here exemplify cloning in phage.

A. Preparing Genomic DNA of a Specific Length for Cloning

To begin with, high molecular weight (i.e., long molecules of) the desired genomic DNA are isolated, purified and then digested with a restriction enzyme. Usually, the digest is partial, aiming to generate overlapping DNA fragments of random length. When the digest is electrophoresed on agarose gels, the DNA (stained with ethidium bromide, a fluorescent dye that binds to DNA) looks like a bright smear on the gel. All of the DNA could be recombined with suitably digested vector DNA. But, to further reduce the number of clones to be screened for a sequence of interest, early cloners would generate a **Southern blot** (named after Edward Southern, the inventor of the technique) to determine the size of genomic DNA fragments most likely to contain a desired gene.

Beginning with a gel of genomic DNA restriction digests, the Southern blot protocol is illustrated below



To summarize the steps:

a) Digest genomic DNA with one or more restriction endonucleases.

b) Run the digest products on an agarose gel to separate fragments by size (length). The DNA appears as a smear when stained with a fluorescent dye.

c) Place a filter on the gel. The DNA transfers (blots) to the filter for e.g., 24 hours

d) Remove the blotted filter and place it in a bag containing a solution that can denature the DNA.

e) Add radioactive probe (e.g., cDNA) containing the gene or sequence of interest. The probe hybridizes (bind) to complementary genomic sequences on the filter

f) Prepare an autoradiograph of the filter and see a 'band' representing the size of genomic fragments of DNA that include the sequence of interest.

Once you know the size (or size range) of restriction digest fragments that contain the DNA you want to study, you are ready to:

a) run another gel of digested genomic DNA.

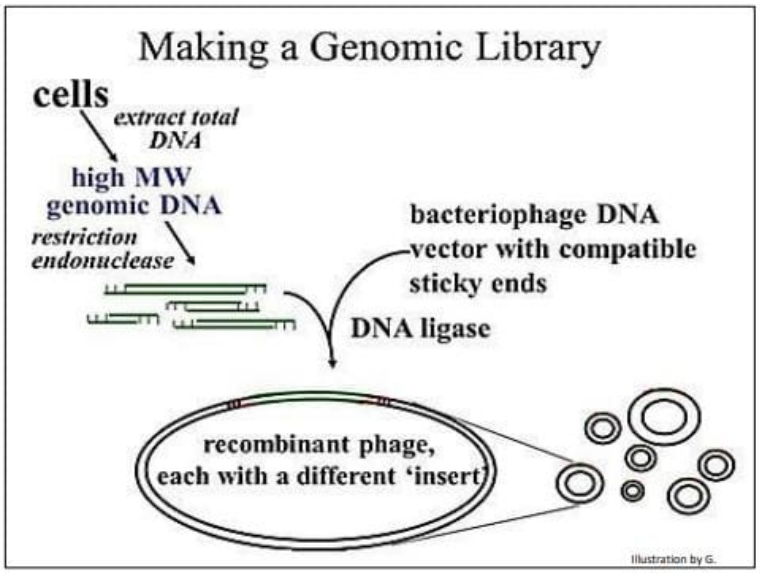
b) cut out the piece of gel containing the fragments that 'lit up' with your probe in the autoradiograph.

c) remove (elute) the DNA from the gel piece into a suitable buffer

d) prepare the DNA for insertion into (recombination with) a genomic cloning vector

B. Recombining Size-Restricted Genomic DNA with Phage DNA

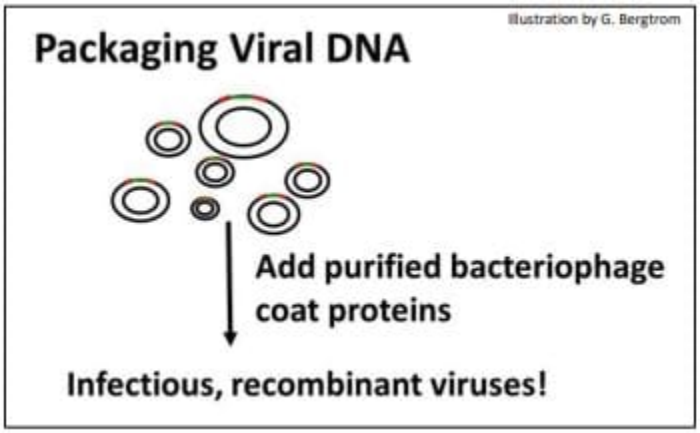
After elution of restriction digested DNA fragments of the right size range from the gels, the DNA is mixed with compatibly digested phage DNA at concentrations that favor the formation of H-bonds between the ends of the phage DNA and the genomic fragments. Addition of DNA ligase covalently links the recombined DNA molecules. These steps are abbreviated in the illustration below.



The recombinant phage that are made next will contain sequences that become the genomic library.

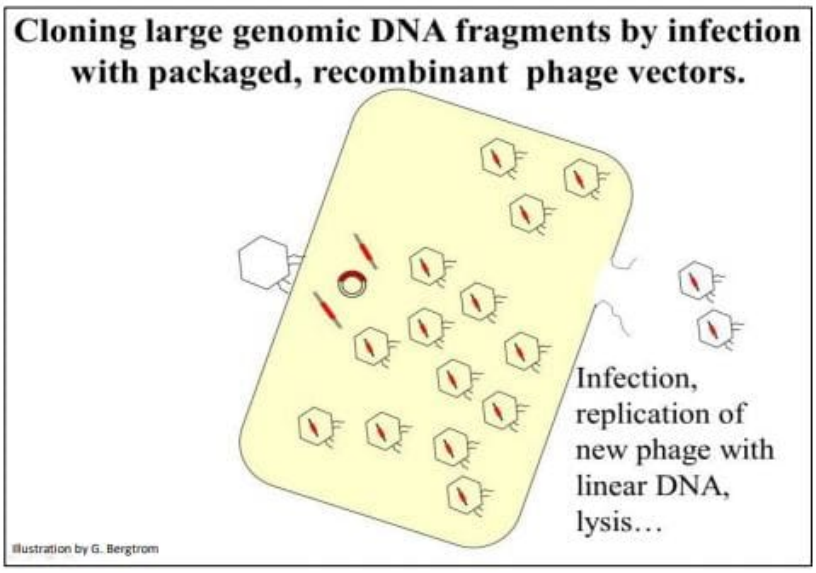
C. Creating Infectious Viral Particles with Recombinant Phage DNA

The next step is to **package** the recombined phage DNA with added purified viral coat proteins to make infectious phage *particles* (below)



269 Genomic Libraries: Make and Package Recombinant Phage DNA

Packaged phage are added to a culture tube full of host bacteria (typically *E. coli*). After infection, the recombinant DNA enters the cells where it replicates and directs the production of new phage that eventually lyse the host cell (illustrated below).



The recombined vector can also be introduced directly into the host cells by **transduction** (which is to phage DNA what transformation is to plasmid DNA). Whether by infection or *transduction*, the recombinant phage DNA ends up in host cells which produce new phage that eventually lyse the host cell. The released phages go on to infect more host cells until all cells have lysed. What remains is a tube full of **lysate** containing cell debris and lots of recombinant phage particles.

270 Infect Host with Recombinant Phage to Make a Genomic Library

D. A Note About Some Other Vectors

We've seen that phage vectors accommodate larger foreign DNA inserts than plasmid vectors, and YACs even more..., and that for larger genomes, the goal is to choose a vector able to house larger fragments of 'foreign' DNA so that you end up screening fewer clones. Given a large enough eukaryotic genome, it may be necessary to screen more than a hundred thousand clones in a phage-based genomic library. Apart from size-selection of genomic fragments before inserting them into a vector, selecting the appropriate vector is just as important. The table below lists commonly used vectors and the sizes of inserts they will accept.

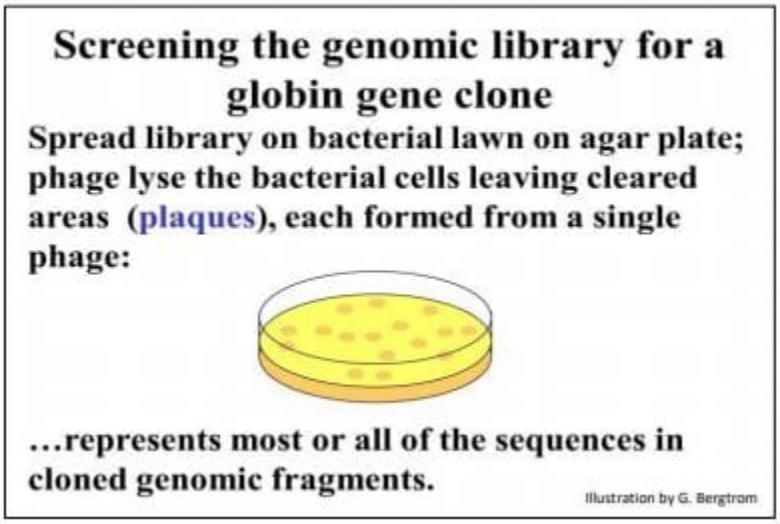
Vector Type	Insert Size (thousands of bases)
Plasmids	up to 15
Phage Lambda (λ)	up to 25
Cosmids	up to 45
Bacteriophage P1	70 to 100
P1 artificial chromosomes (PACs)	130 to 150
Bacterial artificial chromosomes (BACs)	120 to 300
Yeast artificial chromosomes (YACs)	250 to 2000

Click on the links to these vectors to learn more about them. We will continue this example by screening a phage lysate genomic library for a recombinant phage with a genomic sequence of interest.

E. Screening a Genomic Library; Titering Recombinant Phage Clones

A phage lysate is **titered** on a **bacterial lawn** to determine how many virus particles are present. A bacterial lawn is made by plating so many bacteria on the agar plate that they simply grow together rather than as separate colonies. In a typical **titration**, a lysate might be diluted 10-fold with a suitable medium and this dilution is further diluted 10-fold... and so on. Such **serial 10X dilutions** are then spread over bacterial (e.g., E. coli) lawns. What happens on such a culture plate?

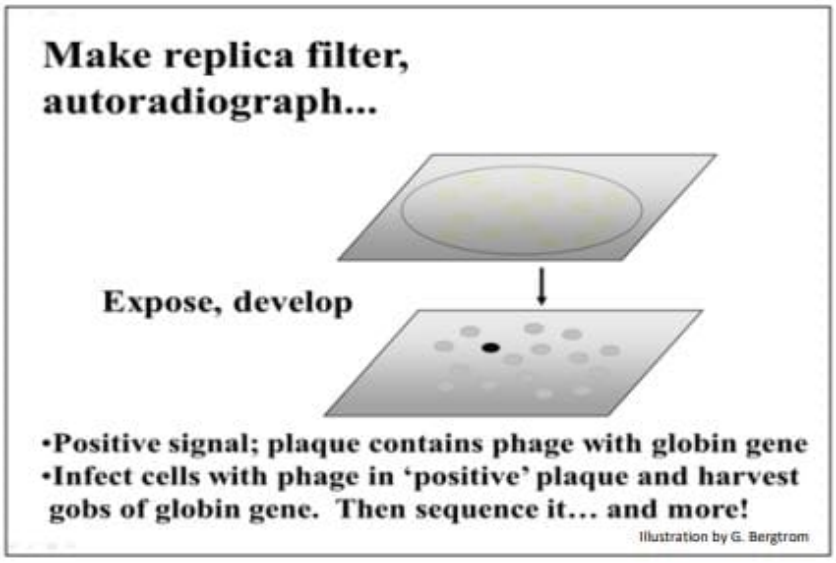
Let's say that when 10 µl of one of the dilutions are spread on the bacterial lawn, they infect 500 E. coli cells on the bacterial lawn. After a day or so, there will be small clearings in the lawn called **plaques**..., 500 of them in this example. These are 500 tiny clear spaces on the bacterial lawn created by the lysis of first one infected cell, and then progressively more and more cells neighboring the original infected cell. Each plaque is thus a clone of a single virus, and each virus particle in a plaque contains a copy of the same recombinant phage DNA molecule (below).



If you actually counted 500 plaques on the agar plate, then there must have been 500 virus particles in the 10 µl seeded onto the lawn. And, if this plate was the fourth dilution in a 10-fold serial dilution protocol, there must have been 2000 (4 X 500) phage particles in 10 µl of the original undiluted lysate.

F. Screening a Genomic Library; Probing the Genomic Library

In order to represent a *complete genomic library*, it is likely that many plates of such a dilution (~500 plaques per plate) will have to be created and then screened for a plaque containing a gene of interest. But, if only size-selected fragments were inserted into the phage vectors in the first place, the plaques represent only a partial genomic library, requiring screening fewer clones to find the sequence of interest. For either kind of library, the next step is to make replica filters of the plaques. Replica plating of plaques is similar to making a replica filter bacterial colonies. While much of the phage DNA in a plaque is encased in viral proteins, there will also be DNA on the plaque replicas that were never packaged into viral particles. The filters can be treated to denature the latter DNA and then directly hybridized to a probe with a known sequence. In the early days of cloning, probes for screening a genomic library were usually an already isolated and sequenced cDNA clone, either from the same species as the genomic library, or from a cDNA library of a related species. After soaking the filters in a radioactively labeled probe, X-Ray film is placed over the filter, exposed and developed. Black spots will form where the film lay over a plaque containing genomic DNA complementary to the radioactive probe. In the example illustrated below, a globin cDNA might have been used to probe the genomic library (globin genes were among the first to be cloned!).



G. Isolating a Gene for Further Study

Cloned genomic DNA fragments are much longer than any gene of interest, and always longer than any cDNA from a cDNA library. They are also embedded in a genome that is thousands of times as long as the gene itself, making the selection of an appropriate vector necessary. If the genome can be screened among a reasonable number of cloned phage (~100,000 plaques for instance), the one plaque producing a positive signal on the autoradiograph would be further studied.

This plaque should contain the gene of interest. The next step is to find the gene within a genomic clone that can be as much a 20kbp long. The traditional strategy is to purify the cloned DNA, subject it to restriction endonuclease digestion, and separate of the digest particles by *agarose gel electrophoresis*. Using *Southern Blotting*, the separated DNA fragments are denatured and blotted to a nylon filter. The filter is then probed with the same tagged probe used to find the positive clone (plaque). The smallest DNA fragment containing the gene of interest can itself be *subcloned* in a suitable vector, and grown to provide enough DNA for further study of the gene.

271 Screen a Genomic Library, Pick and Grow a Phage Clone

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15.5: The Polymerase Chain Reaction (PCR)

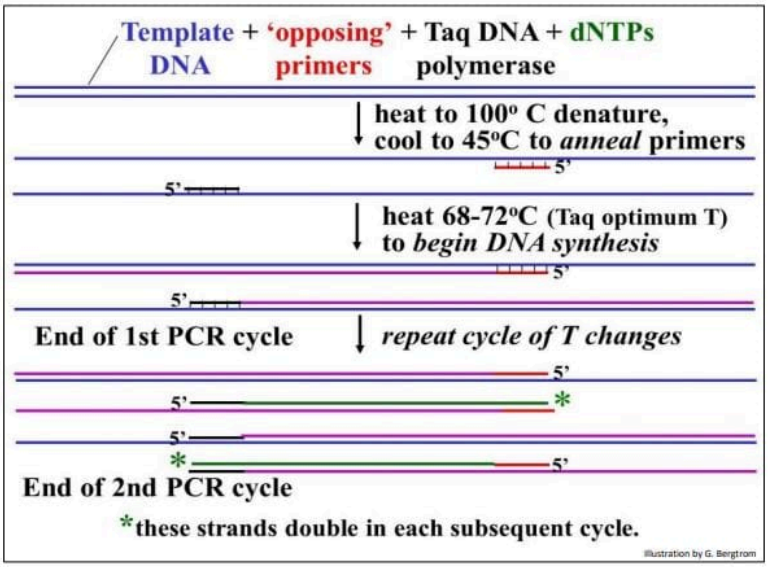
The polymerase chain reaction (PCR) can *amplify* a region of DNA from any source, even from a single cell's worth of DNA or from fragments of DNA obtained from a fossil. This amplification usually takes just a few hours, generating millions of copies of the desired *target* DNA sequence. The effect is to purify the DNA from surrounding sequences in a single reaction! Kary B. Mullis was awarded a Nobel Prize in 1993 for his development of PCR, which is now the basis of innumerable research studies of gene structure, function and evolution as well as applications in criminal forensics, medical diagnostics and other commercial uses. PCR is described in detail below.

A. PCR - the Basic Process

Typical PCR relies on knowing two bits of DNA sequence that will be used to design and synthesize short oligonucleotide sequences (*oligomers*) in the laboratory. The two oligomers are chosen to be complementary to sequences opposite strands of double-stranded DNA containing the gene to be studied. We say that the two oligomers face, or **oppose** each other. That just means that the 3' end of one oligomer faces the 3' end of the opposing oligomer. This way the two oligomers can serve as *primers* for the elongation replication of both strands of a double stranded target DNA sequence. Check out the link below for further explanation.

272 PCR: Design and Synthesize Opposing Oligonucleotide Primers

The first step in PCR is to add oligomer primers to the target DNA from which a gene (or other genomic sequence) is to be amplified. The mixture is then heated to denature the target DNA. The mixture is cooled to allow the primers to H-bond to complementary target DNA strands. Next, the four deoxynucleotide precursors to DNA (dATP, dCTP, dTTP and dGTP) are added along with a small amount of a DNA polymerase. New DNA strands will now lengthen from the oligonucleotide primers on the template DNAs. To make lots of the PCR product, this reaction cycle must be repeated many times. Therefore, after allowing elongation, the mixture is heated to denature (separate) all the DNA strands. When the mixture is again cooled, the oligomers again find complementary sequences with which to H-bond. Early versions of PCR originally relied on an *E. coli* DNA polymerase, which is inactivated by heating, and so had to be re-added to the PCR mixture for each elongation cycle. Just as with DNA sequencing, researchers very quickly switched to the heat-stable **Taq polymerase**, of *Thermus aquaticus*. The enzymes of *T. aquaticus* remain active at the very high temperatures at which these organisms live. Since heating does not destroy the Taq polymerase *in vitro*, PCR, like DNA sequencing reactions, could be automated with programmable *thermocylers* that raised and lowered temperature required by the PCR reactions. There was no longer a need to replenish a DNA polymerase once the reaction cycles were begun. Thermocyling in a typical PCR amplification is illustrated below for the first two PCR cycles, the second of which, produces the first strands of DNA that will actually be amplified exponentially.

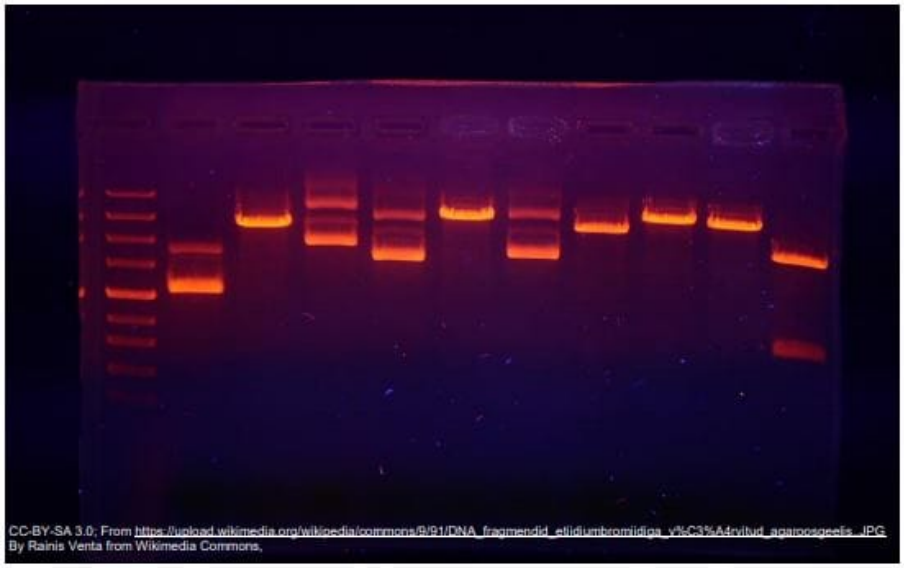


You can see from the illustration that the second cycle of PCR has generated the two DNA strands that will be templates for doubling and re-doubling the desired product after each subsequent cycle. A typical PCR reaction might involve 30 PCR cycles, resulting in a nearly exponential amplification of the desired sequence.

273 PCR: The Amplification Process

> Challenge:
>
> Starting with a pair of complementary target DNA molecules (after the 3rd PCR cycle), how many double stranded PCR products should you theoretically have at the end of all 30 PCR cycles?

The amplified products of PCR amplification products are in such abundance that they can easily be seen under fluorescent illumination on an ethidium bromide-stained agarose gel (below).



In this gel, the first lane (on the left) contains a *DNA ladder*, a mixture of DNAs of known lengths that can be used to estimate the size of the PCR fragments in the 3 rd and 4th lanes (the gel lane next to the ladder is empty). The two bright bands in lanes 3 and 4 are PCR products generated with two different oligomer primer pairs. PCRamplified DNAs can be sequenced and used in many subsequent studies.

B. The Many Uses of PCR

PCR-amplified products can be labeled with radioactive or fluorescent tags to serve as hybridization probes for

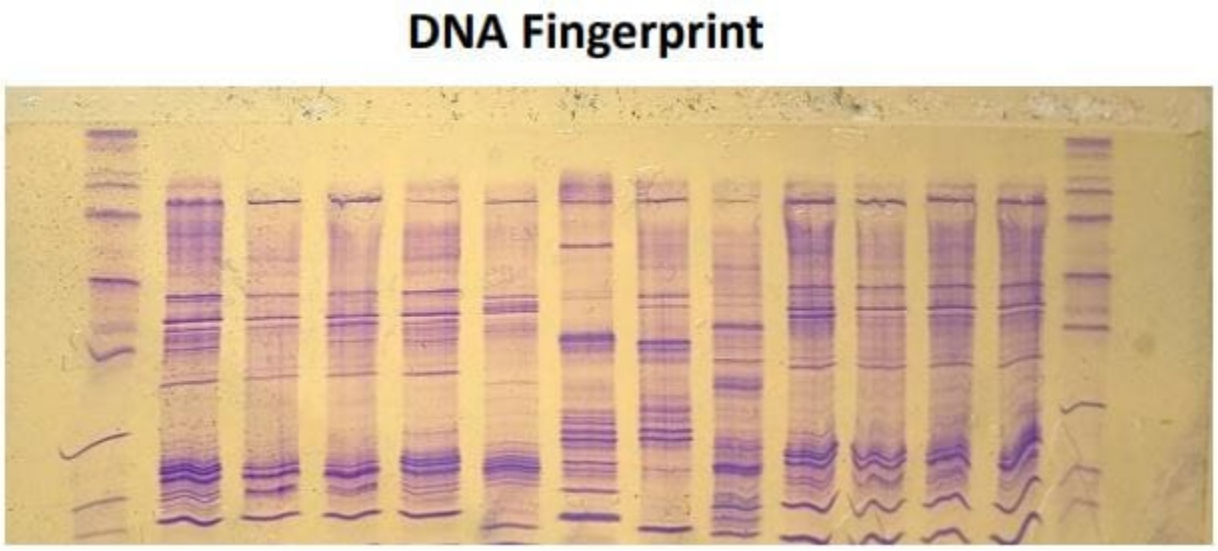
- screening cDNA or genomic libraries and isolation of clones.
- determining migration position on a Southern blot.
- determining migration position on a northern blot (a fanciful name for RNAs that are separated by size on gels and blotted to filter).
- and more!

1. Quantitative PCR

We noted above that PCR has wide applications to research, medicine and other practical applications. A major advance was *Quantitative PCR*, applied to studies of differential gene expression and gene regulation. In Quantitative PCR, initial cDNAs are amplified to detect not only the presence, but also the relative amounts of specific transcripts being made in cells.

2. Forensics

Another application of PCR is in forensic science, to identify a person or organism by comparing its DNA to some standard, or control DNA. An example of one of these acrylamide gel DNA *fingerprints* is shown below.



Using this technology, it is now possible to detect genetic relationships between near and distant relatives (as well as to exclude such relationships), determine paternity, demonstrate evolutionary relationships between organisms, and on many occasions, solve recent and even 'cold-case' crimes. Click Sir Alec Jeffries to learn about the origins of DNA fingerprinting in real life ...and on all those TV CSI programs! Check out here for a brief history of the birth of DNA fingerprinting, and to see how analysis of changes in gene activity that occur after death may even help ID criminals. For a video on DNA fingerprinting, click Alu and DNA fingerprinting. *Alu* is a highly repeated ~300bp DNA sequence found throughout the human genome. *Alu* sequences are *short interspersed elements*, or **SINES**, a retrotransposon we saw earlier. DNA fingerprinting is possible in part because each of us has a unique number and distribution of *Alu* SINEs in our genome. To read more about Alu sequences and human diversity, click Alu Sequences and Human Diversity.

Intriguing examples of the use of PCR for identification include establishing the identities of Egyptian mummies, the Russian Tsar deposed and killed during the Russian revolution (along with his family members), and the recently unearthed body of King Richard the 3rd of England. Variant PCR protocols and applications are manifold and often quite inventive! For a list, click Variations on Basic PCR.

274 The Power of PCR: Some Examples

3. Who are your Ancestors?

Tracing your ethnic, racial and regional ancestry is related to DNA fingerprinting, in that it relies on PCR amplification of genes and other DNA regions and comparison of these your sequences to distinguishing DNA markers in large sequence databases. The price of these services have come down, and as a result, their popularity has gone up in recent years. Typically, you provide spit or a salivary (buccal) swab to the service and they amplify and sequence the DNA in your samples. The analysis compares your DNA sequences to database sequences looking for patterns of ethnic and regional markers that you might share with the database(s). Based on these comparisons, you are provided with a (more..., or less) accurate map of your DNA-based ancestry. Folks who are spending around $100.00 (less when on sale!) often ask just how accurate are these analyses, and what do they actually mean. For example, what does it mean if your DNA says you are 5% native American? In fact, different services can sometimes give you different results! You can get some answers and explanations DNA Ancestry Testing.

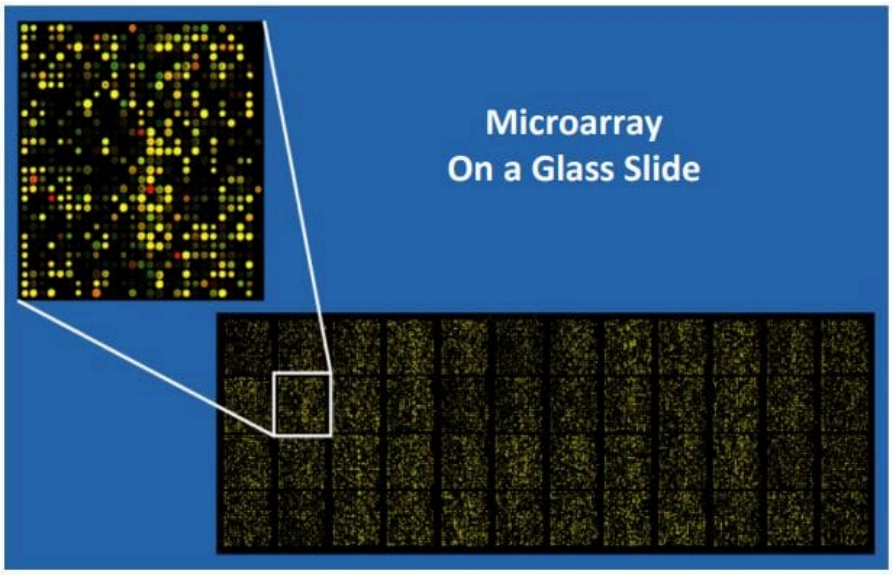
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15.6: Genomic Approaches- The DNA Microarray

Traditionally, when cellular levels of a protein were known to change in response to a chemical effector, molecular studies focused on control of the transcription of its gene. These studies often revealed that the control of gene expression was at the level of transcription, turning a gene on or off through interactions of transcription factors with DNA. However, protein levels are also controlled post-transcriptionally, by regulating the rate of mRNA translation or degradation. Studies of transcriptional and posttranscriptional regulation mechanisms are seminal to our understanding of how the correct protein is made in the right amounts at the right time.

We may have suspected, but now know that control of gene expression and cellular responses can be more complex than increasing or decreasing the transcription of a single gene or translation of a single protein. Whole genome sequences and new techniques make possible the study of the expression of virtually all genes in a cell at the same time, a field of investigation called **genomics**. Genomic studies reveal networks of regulated genes that must be understood to more fully explain the developmental and physiological changes in an organism. When you can 'see' all of the RNAs being transcribed from active genes in a cell, you are looking at a cell's **transcriptome**. By analogy to genomics, **transcriptomics** defines studies of 'webs' of interactive RNAs. Again, by analogy to genomics and transcriptomics, the broad study of active and inactive proteins in cells or tissues, how they are modified (processed) before use and how they interact is called **proteomics**. The technologies applied to proteomic studies include protein microarrays, immunochemical techniques and others uniquely suited to protein analysis (click Proteomics Techniques-Wikipedia for more information). *Protein Microarrays* are increasingly being used to identify protein-protein interactions, as well as the different states of proteins under different cellular conditions. Read even more about these exciting developments and their impact on basic and clinical research at Protein Microarrays from ncbi.

Finally think about this: creating a proteomic library analogous to a genomic library would seem a daunting prospect. But efforts are underway. Check out A stab at mapping the Human Proteome for original research leading to the sampling of a tissue-specific human proteome, and click Strategies for Approaching the Proteome for more general information. Let's look at some uses of DNA microarrays. This technology involves 'spotting' DNA (e.g., cloned DNA from a genomic or cDNA library, PCR products, oligonucleotides...) on a glass slide, or chip. In the language of microarray analysis, the slides are the probes. Spotting a chip is a robotic process. Because the DNA spots are microscopic, a cellspecific transcriptome (cDNA library) can fit on a single chip. A small genome microarray might also fit on a single chip, while larger genomes might need several slides. A primary use of DNA microarrays is **transcriptional profiling**. A genomic microarray can probe a mixture of fluorescently tagged target cDNAs made from mRNAs, in order to identify many (if not all) of the genes expressed in the cells at a given moment (i.e., its transcriptome). cDNA microarray probes can also probe quantitative differences in gene expression in cells or tissues during normal differentiation or in response to chemical signals. They are also valuable for genotyping, (i.e. characterizing the genes in an organism). Microarrays are so sensitive that they can even distinguish between two genes or regions of DNA that differ by a single nucleotide. Click Single Nucleotide Polymorphisms, or SNPs to learn more. In the microarray below, each colored spot (red, yellow, green) is a different fluorescently tagged molecule hybridizing to target sequences on the microarray. In the fluorescence microscope, the spots fluoresce different colors in response to UV light.



With quantitative microarray methods, the brightness (intensity) of the signal from each probe can be measured. In this way, we can compare the relative amounts of cDNA (and thus, different RNAs) in the *transcriptome* of different tissues or resulting from different tissue treatments. A table of different applications of microarrays (adapted from Wikipedia) is shown on the next page.

Application of Technology	Synopsis
Gene Expression Profiling	In a transcription (mRNA or gene expression) profiling experiment the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression.
Comparative genomic hybridization	Assessing genome content in different cells or closely related organisms, where one organism's genome is the probe for a target genome from a different species.
GeneID	Small microarrays to check IDs of organisms in food and feed for genetically modified organisms (GMOs), mycoplasmas in cell culture, or pathogens for disease detection. These detection protocols often combine PCR and microarray technology.
CHIP; Chromatin immunoprecipitation	DNA sequences bound to a particular protein can be isolated by immunoprecipitating the protein. The fragments can be hybridized to a microarray (such as a tiling array) allowing the determination of protein binding site occupancy throughout the genome.
DamID	Analogously to ChIP, genomic regions bound by a protein of interest can be isolated and used to probe a microarray to determine binding site occupancy. Unlike ChIP, DamID does not require antibodies but makes use of adenine methylation near the protein's binding sites to selectively amplify those regions, introduced by expressing minute amounts of protein of interest fused to bacterial DNA adenine methyltransferase.
SNP detection	Identifying single nucleotide polymorphism among alleles within or between populations. Some microarray applications make use of SNP detection, including Genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating **germline** mutations in individuals or **somatic** mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.
Alternative splicing protection	An exon junction array design uses probes specific to the expected or potential splice sites of predicted exons for a gene. It is of intermediate density, or coverage, to a typical gene expression array (with 1-3 probes per gene) and a genomic tiling array (with hundreds or thousands of probes per gene). It is used to assay the expression of alternative splice forms of a gene. Exon arrays have a different design, employing probes designed to detect each individual exon for known or predicted genes, and can be used for detecting different splicing isoforms

Application of Technology	Synopsis
Tiling array	Genome tiling arrays consist of overlapping probes designed to densely represent a genomic region of interest, sometimes as large as an entire human chromosome. The purpose is to empirically detect expression of transcripts or alternatively spliced forms which may not have been previously known or predicted.

YouTube Video

This interactive element is not available in this format. Visit the link or scan the QR code to access it online.

View this YouTube Video online

The Power of Microarrays. https://youtu.be/88rzbpclscM

If you like world records, check out the salamander with the largest genome, 10X bigger than our own: The HUGE Axolotl Genome. What do they do with all that DNA? And can our current technologies figure it out? For the original report, click on the following link: here.

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15.7: Ome-Sweet Ome

- **Genome -** total cell’s DNA content, identical in every cell of an organism
- A cell’s total coding DNA (excluding non-coding DNA)
- <="" strong="">A cell’s metabolic landscape (i.e., metabolic status)
- A cell’s total DNA-modification/chromatin topography
- <="" strong="">The pattern of methylation of DNA in the genome
- A cell's profile of protein content
- <="" strong="">A cell's RNA transcript profile and steady state at any given moment

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15.8: From Genetic Engineering and Genetic Modification

By enabling us to focus on how genes and their regulation have evolved, these genomic, transcriptomic and proteomic technologies have vastly increased our knowledge of how cells work at a molecular level. We continue to add to our knowledge of disease process and in at least a few cases, how we can treat disease. The use of technologies to genetically modify organisms is more controversial, despite the best of human intentions. Some genetically modified organisms (GMOs) aim to increase food productivity to better feed the world. The introduction 'beneficial' genes into some GMOs have made:

- drought-resistant crops to increase the range where major food crops can be grown.
- pest-resistant crops to reduce reliance on environmentally toxic chemical pesticides.
- herbicide-resistant crops that survive chemicals used to destroy harmful plants.

The quest for "improved" plant and animal varieties has been going on since before recorded history. Farmers have been cross-breeding cows, sheep, dogs, and crop varieties from corn to wheat, hoping to find faster growing, larger, hardier, (you name it) varieties. It is the manipulation of DNA (the essence of the genetic material itself) that is at the root of controversy. Controversy is reflected in opinions that GMO foods are potentially dangerous, and that their cultivation should be banned. However, the general consensus is that attempting to ban GMOs is too late! In fact, you are probably already partaking of some GMO foods without even knowing it. Perhaps the good news is that after many years of GMO crops already in our food stream, the emerging scientific consensus is that GMO foods are no more harmful than unmodified foods. The current debate is whether or not to label foods that are (or contain) GMO ingredients as genetically modified.

In an odd but perhaps amusing take on the discomfort some folks feel about GMOs, a startup company has genetically modified Petunias. When grown in water, their flowers are white, but when 'watered' with beer, they will produce pink flowers or purple flowers depending on how much beer they get (Check it out at Can Beautiful Flowers Change Face?). According to the company, they seek "to bring what it sees as the beauty of bioengineering to the general public" (and perhaps some profit as well?).

More recently, we have CRISPR and related tools that can precisely edit gene (in fact any DNA) sequences. And unlike the "quack medicines" of old, these tools have the real potential to cure disease, destroy disease-carrying vectors, cure cancer, improve crops and possibly alter the course of evolution. The speed with which one can accomplish such good (or evil) is truly awesome.

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15.9: Key Words and Terms

2',3' dideoxy CTP chemotherapy	genome	regulatory networks
alternative splicing	genome projects	restriction endonucleases
automated DNA sequencing	genomic library	reticulocyte
autoradiography	insert DNA	reverse transcriptase
BACs and YACs	library screening	RNA probes
bacterial artificial chromosome vectors	linkers	RNAse
blunt ends	Northern blot	shotgun sequencing
cDNA	oligo d(T) column	single nucleotide polymorphisms
cDNA hairpin loop	PCR	SNPs
cDNA library	PCR primers	Southern blot
cDNA probes	PCR steps	sticky ends
chemiluminescence	phage lambda vectors	systematics
cosmid vectors	plasmids	Taq polymerase
di-deoxy chain termination	poly(A) tail	thermophilic bacteria
di-deoxy sequencing method	polymerase chain reaction	thermophilic DNA polymerases
DNA ligase	primer	*Thermus aquaticus*
DNA sequencing	primer walking	transcriptome
elution	probe hybridization	transformation
ethidium bromide	proteome	vectors
fluorescence	recombinant vector	Western blot
forensics	recombination	yeast artificial chromosome vectors
Genetic (DNA) fingerprint		

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16: MEMBRANE STRUCTURE

16.1: Overview
16.2: Plasma Membrane Structure
16.3: Membrane Proteins
16.4: How Membrane Proteins are Held in Membranes
16.5: A Diversity of Membrane Protein Functions
16.6: Glycoproteins
16.7: Glycolipids
16.8: Glycoproteins and Human Health
16.9: Key Words and Terms

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16.1: Overview

The plasma membrane has the same **phospholipid bilayer** construction as all intracellular membranes. All membranes are a **fluid mosaic** of proteins attached to or embedded in the phospholipid bilayer. The different proteins and to some extent, different phospholipids, structurally and functionally differentiate one kind of cellular membrane from another. **Integral** (**trans-membrane**) **proteins** span the phospholipid lipid bilayer, with one **hydrophobic** domain and two **hydrophilic** domains. In the case of the plasma membrane, the *hydrophilic domains* interact with the aqueous extracellular fluid on one side and the cytoplasm on the other, while the *hydrophobic domain* keeps the proteins anchored in the membrane. Once embedded in the fatty acid interior of a membrane, integral membrane proteins cannot escape! In contrast, **peripheral membrane proteins** bind to membrane surfaces, typically held in place by hydrophilic interactions with charged features of the membrane surface (phospholipid heads, hydrophilic surface domains of integral proteins). Integral membrane proteins are often glycoproteins whose sugars face the outside of the cell. Cells thus present a sugar coating, or **glycocalyx**, to the outside world. As cells form tissues and organs, they become bound to extracellular proteins and glycoproteins that they, or other cells, secrete to form an **extracellular matrix**. We will spend much of this chapter looking at characteristic structures and biological activities of plasma membrane proteins and their functions.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. distinguish components of the membrane that can move (diffuse) laterally in the membrane from those that can *flip* (switch) from the outer to the inner surface of the phospholipid bilayer
2. compare the fluid mosaic membrane to earlier membrane models and cite the *evidence* for and against each (as appropriate).
3. describe how cells might make their plasma membranes and suggest an experiment that would demonstrate your hypothesis.
4. distinguish between *transmembrane* and *peripheral* membrane proteins, and provide specific examples of each.
5. decide whether a newly discovered protein might be a membrane protein.
6. predict the effect of *molecular* and *physical* influences on membrane fluidity
7. suggest how organisms living in *warm tropical waters* have adapted to the higher temperatures. Likewise, fish living under the *arctic ice*.
8. explain how salmon are able to spend part of their lives in the ocean and another part swimming upstream in freshwater, without their cells shriveling or exploding
9. list the diverse *functions* of membrane proteins.
10. speculate on why only eukaryotic cells have evolved *sugar coated* cell surfaces.
11. compare and contrast the *glycocalyx* and *extracellular matrix* of cells.

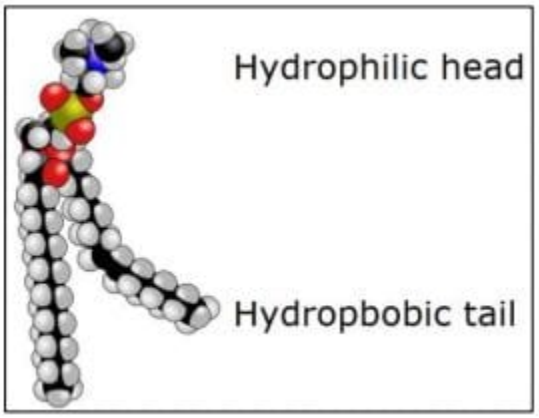
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16.2: Plasma Membrane Structure

In eukaryotic cells, the **plasma membrane** surrounds a cytoplasm filled with ribosomes and organelles. Organelles are structures that are themselves encased in membranes. Some organelles (nuclei, mitochondria, chloroplasts) are even surrounded by double membranes. All cellular membranes are composed of two layers of phospholipids embedded with proteins. All are selectively permeable (semi-permeable), allowing only certain substances to cross the membrane. The unique functions of cellular membranes are due to their different phospholipid and protein compositions. Decades of research have revealed these functions (see earlier discussions of mitochondrial and chloroplast function for instance). Here we'll describe general features of membranes, using the plasma membrane as our example.

A. The Phospholipid Bilayer

Gorter and Grendel predicted the bilayer membrane structure as early as 1925. They knew that red blood cells (erythrocytes) have no nucleus or other organelles, and thus have only a plasma membrane. They also knew that the major chemical component of these membranes were **phospholipids**. The space-filling molecular model below shows the basic structure of phospholipids, highlighting their **hydrophilic** (polar) heads and **hydrophobic** tails.



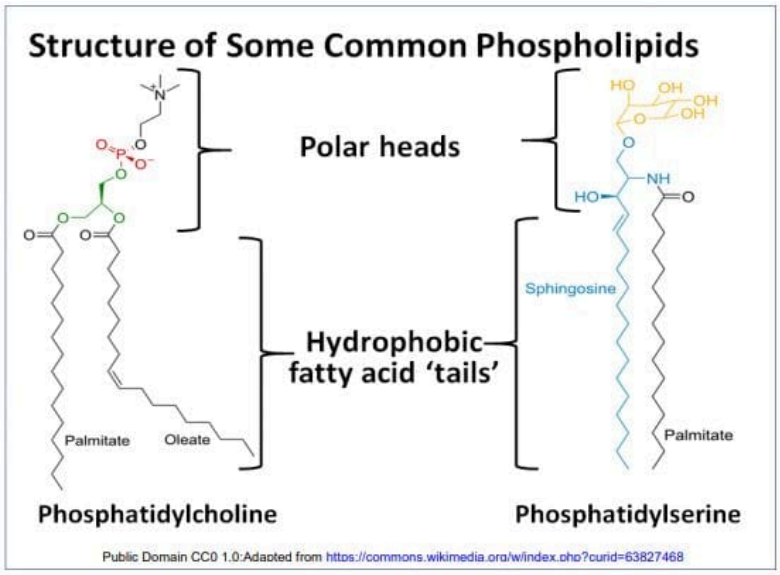
Molecules with hydrophilic and hydrophobic domains are **amphipathic** molecules. Gorter and Grendel had measured the surface area of red blood cells. They then did a 'blood count' and then disrupted a known number of red blood cells. They then measured the amount of phospholipids in the membrane extracts. From this, they calculated that there were enough lipid molecules per cell to wrap around each cell twice. From these observations, they predicted the **phospholipid bilayer** with fatty acids interacting within the bilayer. Curiously, Gorter and Grendel had made two calculation errors in determining the amount of phospholipid per cells. Nevertheless, their errors compensated each other so that, while not strictly speaking correct, their conclusion remained prophetic! Common membrane phospholipids are shown below.

Amphipathic molecules mixed with water spontaneously aggregate to 'hide' their hydrophobic regions from the water. In water, these formed actual structures called liposomes that sediment when centrifuged!

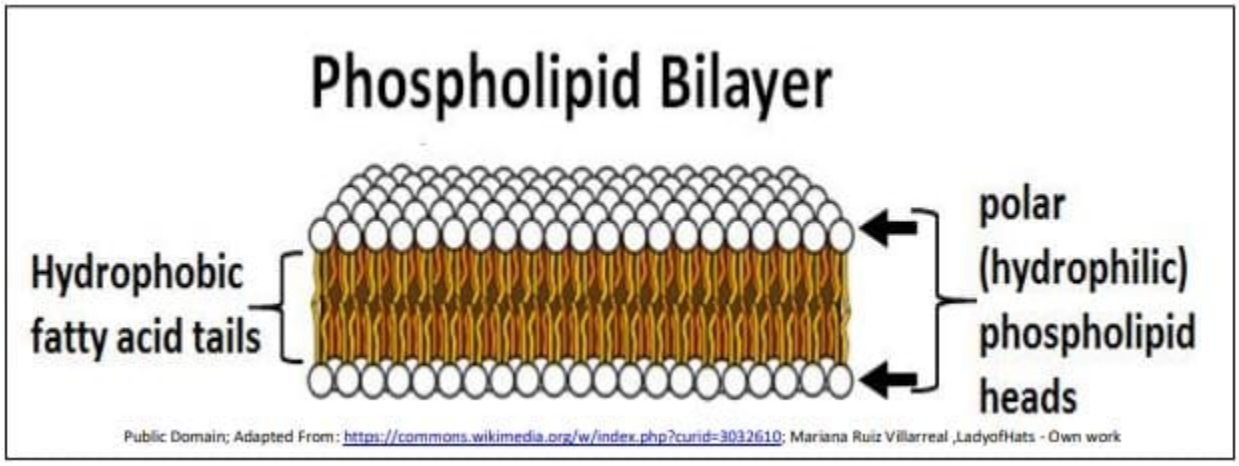
276 Membrane Lipids and the Phospholipid Bilayer

277 Experiments with and Uses of Liposomes

Liposome membrane structure is consistent with the predicted phospholipid bilayer, with the hydrophobic tails interacting with each other and the polar heads facing away from each other, forming a *phospholipid bilayer*. This led to a picture of membrane architecture based on phospholipid interactions. An iconic illustration of the phospholipid bilayer, with its hydrophobic fatty acid interior and hydrophilic external surfaces is drawn below.

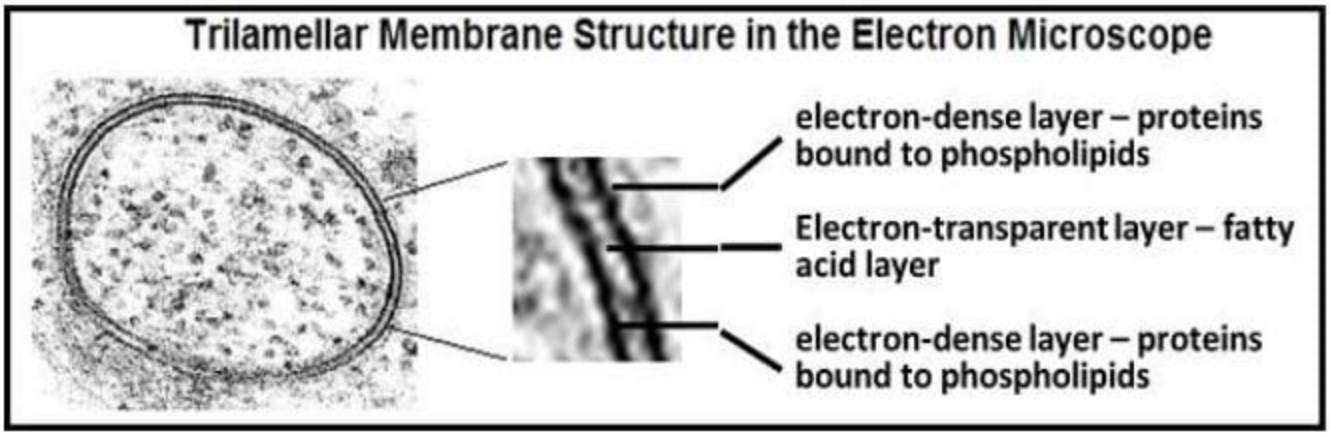


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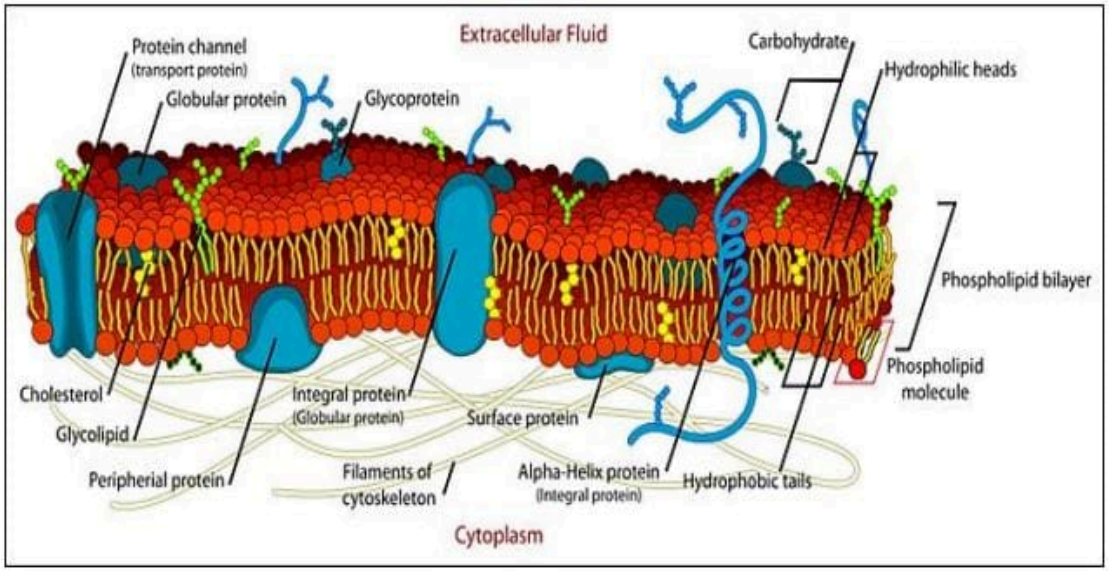
B. Models of Membrane Structure

In 1935, Davson and Danielli suggested that proteins might be bound to the polar heads of the phospholipids in the plasma membrane, creating a protein/lipid/protein sandwich. Decades later, J.D. Robertson observed membranes in the transmission electron microscope at high power, revealing that all cellular membranes had a **trilamellar** structure. The classic *trilamellar* appearance of a cellular membrane in the electron microscope is illustrated below



The **trilamellar** structure is consistent with the protein-coated hydrophilic surfaces of a phospholipid bilayer in Davson and Danielli's protein-lipid-protein sandwich. Observing that *all* cellular membranes had this trilamellar structure, Robertson he further proposed his **Unit Membrane** model: *all membrane*s consist of a clear phospholipid bilayer coated with electron-dense proteins.

The static view of the trilamellar models of membrane structure implied by the Davson-Danielli or Robertson models was replaced in 1972 by Singer and Nicolson's **Fluid Mosaic** model (see *The fluid mosaic model of membrane*s. Science 175:720- 731). They suggested that in addition to **peripheral proteins** that *do* bind to the surfaces of membranes, many integral membrane proteins actually span the membrane. *Integral membrane proteins* were imagined as a *mosaic* of protein 'tiles' embedded in a phospholipid medium. But unlike a mosaic of glazed tiles set in a firm, cement-like structure, the protein 'tiles' were predicted to be mobile (*fluid*) in a *phospholipid sea*. In this model, membrane proteins are anchored in membranes by one or more *hydrophobic* domains; their *hydrophilic* domains would face aqueous external and cytosolic environments. Thus, like phospholipids themselves, membrane proteins are *amphipathic*. We know that cells expose different surface structural (and functional) features to the aqueous environment on opposite sides of a membrane. Therefore, we also say that cellular membranes are **asymmetric**. A typical model of the plasma membrane of a cell is illustrated below.



In this model, peripheral proteins have a hydrophobic domain that does not span the membrane, but that anchors it to one side of the membrane. Other peripheral (or socalled "*surface*") proteins are bound to the membrane by interactions with the polar phosphate groups of phospholipids, or with the polar domains of integral membrane proteins.

Because of their own aqueous hydrophilic domains, membrane proteins are a natural barrier to the free passage of charged molecules across the membrane. On the other hand, membrane proteins are responsible for the **selective permeability** of membranes, facilitating the movement of specific molecules in and out of cells. Membrane proteins also account for specific and selective interactions with their extracellular environment. These interactions include the adhesion of cells to each other,

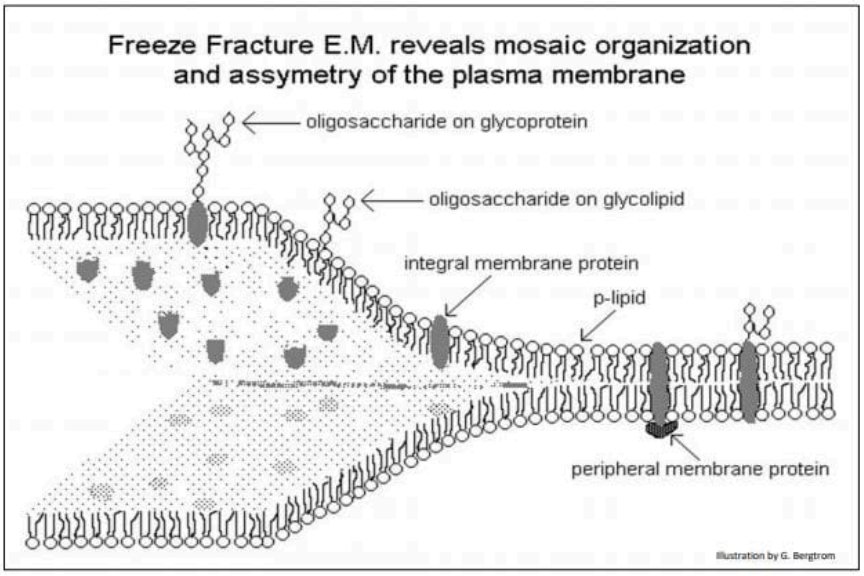
their attachment to surfaces, communication between cells (both direct and via hormones and neurons), etc. The 'sugar coating' of the extracellular surfaces of plasma membranes comes from **oligosaccharides** covalently linked to membrane proteins (as **glycoproteins**) or to phospholipids (as **glycolipids**). Carbohydrate components of **glycosylated** membrane proteins inform their function. Thus, glycoproteins enable specific interactions of cells with each other to form tissues. They also allow interaction with extracellular surfaces to which they must adhere. In addition, they figure prominently as part of receptors for many hormones and other chemical communication biomolecules. Protein domains exposed to the cytoplasm, while not glycosylated, often articulate to components of the cytoskeleton, giving cells their shape and allowing cells to change shape when necessary. Many membrane proteins have essential enzymatic features, as we will see. Given the crucial role of proteins and glycoproteins in membrane function, it should come as no surprise that proteins constitute an average of 40-50% of the mass of a membrane. In some cases, proteins are as much as 70% of membrane mass (think cristal membranes in mitochondria!).

278 Properties of Proteins Embedded in a Phospholipid Bilayer

279 Different Membrane Compositions

C. Evidence for Membrane Structure

Membrane asymmetry refers to the different membrane features facing opposite sides of the membrane. This was directly demonstrated by the scanning electron microscope technique of **freeze-fracture**. The technique involves freezing of isolated membranes in water and then chipping the ice. When the ice cracks, the encased membranes split along a *line of least resistance*... that turns out to be between the hydrophobic fatty acid opposing tails in the interior of the membrane. Scanning electron microscopy then reveals features of the interior and exterior membrane surfaces. Among the prominent features in a scanning micrograph of freeze-fractured plasma membranes are the pits and opposing *mounds* facing each other on opposite flaps of the membrane, as illustrated below.



Other features shown here are consistent with phospholipid membrane structure.

280 Freeze Fracture Electron Microscopy of Cell Membranes

Cytochemistry confirmed the asymmetry of the plasma membrane, showing that only the external surfaces of plasma membranes are sugar coated, Check the link below for more detailed descriptions of the experiments.

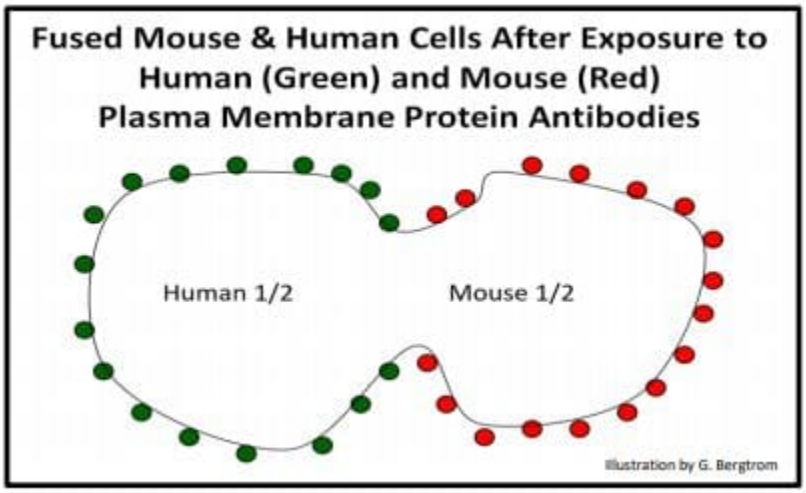
281 EM Cytochemical Demonstration of Membrane Asymmetry

Finally, the asymmetry of membranes was also demonstrated biochemically. In one experiment, whole cells treated with proteolytic enzymes, followed by extraction of the membranes and then isolation of membrane proteins. In a second experiment, plasma membranes were isolated from untreated cells first, and *then* treated with the enzymes. In a third experiment, proteins were extracted from plasma membranes isolated from untreated cells. Electrophoretic separation of the three protein extracts

by size demonstrated that different components of integral membrane proteins were present in the two digest experiments, confirming the asymmetry of the plasma membrane. Again, for more details, check the link below.

282 Electrophoretic Demonstration of Membrane Asymmetry

The idea that membranes are *fluid* was also tested. In yet another elegant experiment, antibodies were made to mouse and human cell membrane proteins. Membranes were isolated and injected into a third animal (a rabbit most likely). The rabbit saw the membranes and their associated proteins as foreign and responded by making specific anti-membrane antibody molecules. The antibodies against each membrane source were isolated and separately tagged with different colored fluorescent labels so that they would glow a different color when subjected to ultraviolet light. After mouse and human cells were mixed under conditions that caused them to fuse, making human-mouse hybrid cells. When added to fused cells, the tagged antibodies bound to the cell surface proteins. After a short time, the different fluorescent antibodies were seen to mix under a fluorescence microscope under UV light. The fluorescent tags seemed to moving from their original location in the fused membranes. Clearly, proteins embedded in the membrane are not static, but are able to move laterally in the membrane, in effect floating and diffusing in a "sea of phospholipids". The mouse antibodies as seen in the hybrid cell right after fusion are cartooned below.

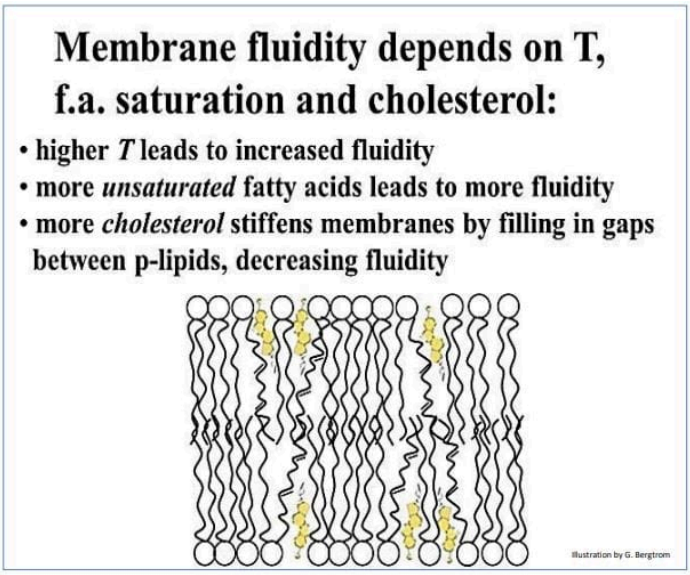


283 Two Demonstrations of Membrane Fluidity: The Fluid Mosaic

D. Membrane Fluidity is Regulated

1. Chemical Factors Affecting Membrane Fluidity

As you might imagine, the fluidity of a membrane depends on its chemical composition and physical conditions surrounding the cell, for example the outside temperature. Factors that affect membrane fluidity are summarized below.



Just as heating a solution causes dissolved molecules and particulates to move faster, phospholipid and protein components of membranes are also more fluid at higher temperatures. If the fatty acids of the phospholipids have more *unsaturated* (C=C) carbon bonds, these hydrophobic tails will have more kinks, or bends. The kinks tend to push apart the phospholipid tails. With more space between the fatty acid tails, membrane components can move more freely. Thus, more *polyunsaturated* fatty acids in a membrane make it more fluid. On the other hand, cholesterol molecules tend to fill the space between fatty acids in the hydrophobic interior of the membrane. This reduces the lateral mobility of phospholipid and protein components in the membrane. By reducing fluidity, cholesterol reduces membrane permeability to some ions.

2. Functional Factors Affecting Membrane Fluidity

Evolution has adapted cell membranes to different and changing environments to maintain the fluidity necessary for proper cell function. **Poikilothermic**, or coldblooded organisms, from prokaryotes to fish and reptiles, do not regulate their body temperatures. Thus, when exposed to lower temperatures, *poikilotherms* respond by increasing the *unsaturated* fatty acid content of their cell membranes. At higher temperatures, they increase membrane *saturated* fatty acid content. Thus, the cell membranes of fish living under the arctic ice maintain fluidity by having high levels of both monounsaturated and polyunsaturated fatty acids. What about fish species that *range* across warmer and colder environments (or that live in climates with changing seasons). For these fish, membrane composition can change to adjust fluidity to environment.

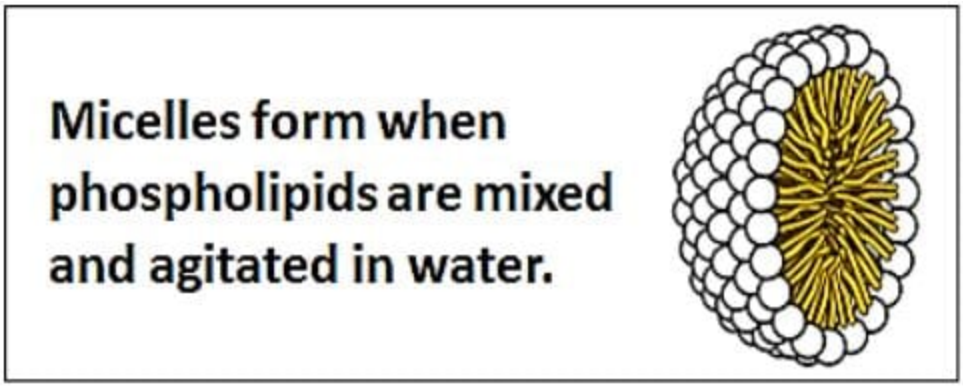
The warm-blooded (**homeothermic**) mammals and birds maintain a more or less constant body temperature. As a result, their membrane composition is also relatively constant. But there is a paradox! Their cell membranes are very fluid, with a higher ratio of *polyunsaturated* fat to *monounsaturated* fats than say, reptiles. The apparent paradox is resolved however, when we understand that this greater fluidity supports the *higher metabolic rate* of the warm-blooded species compared to poikilotherms. Just compare the life styles of almost any mammal to a lazy floating alligator, or a snake basking in the shade of a rock!

284 Factors Influencing Membrane Fluidity

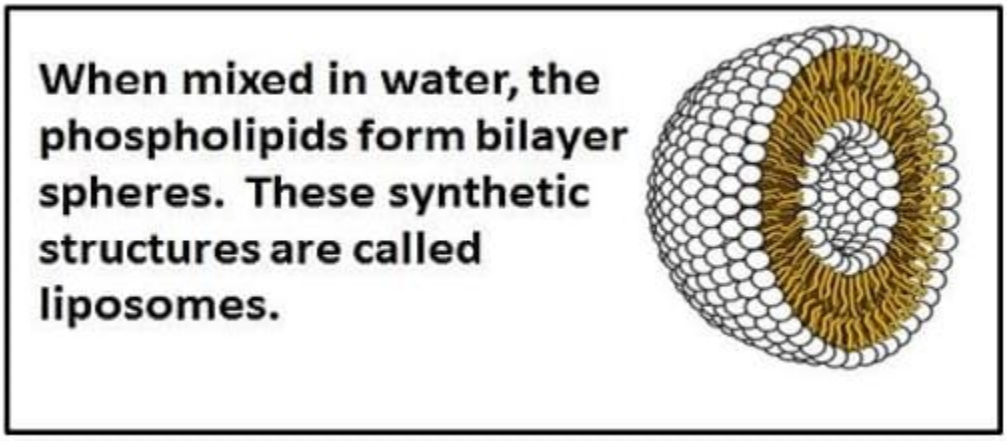
E. Making and Experimenting with Artificial Membranes

Membrane-like structures can form spontaneously. When phospholipids interact in an aqueous environment, they aggregate to exclude their hydrophobic fatty tails from water, forming micelles. Micelles are spherical phospholipid monolayer vesicles that self-assemble, a natural aggregation of the hydrophobic fatty acid domains of these amphipathic molecules.

A micelle is drawn below.



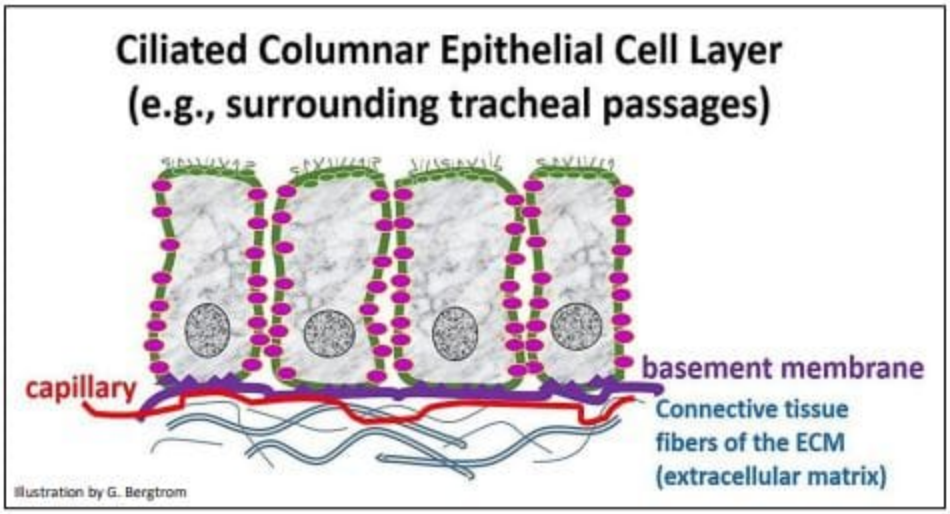
Micelles can further self-assemble into spherical phospholipid bilayers called **liposomes** (below).



When formed in the laboratory, these structures behave somewhat like cells, for example, forming a pellet at the bottom of a tube when centrifuged. Liposomes can be custom designed from different kinds of phospholipids and amphipathic proteins that become integral to the liposome membranes. When liposomes can be prepared in the presence of specific proteins or other molecules that can't cross the membrane. The trapped molecules cannot get out of this synthetic 'organelle'. Such were the studies that allowed the identification of the mitochondrial respiratory chain complexes. The ability to manipulate liposome content and membrane composition also make them candidates for the drug delivery to specific cells and tissues (google liposome for more information).

F. The Plasma Membrane is Segragated into Regions with Different Properties of Fluidity and Selective Permeability

As we will see shortly, fluidity *does not* result in an equal diffusion of all membrane components around the cell membrane surface. Instead, extracellular connections between cells as well as intracellular connections of the membrane to differentiated regions of the cytoskeleton, effectively compartmentalize the membrane into subregions. To understand this, imagine a sheet of epithelial like those in the cartoon below.



The sheet of cells exposes one surface with unique functions to the inside of the organ they line. It exposes the opposite surface, one with a quite different function, to the other side of the sheet. The lateral surfaces of the cells are yet another membrane compartment, one that functions to connect and communicate between the cells in the sheet. Components, i.e., membrane proteins illustrated with different symbolic shapes and colors, may remain fluid within a compartment. Of course, this *macrodifferentiation* of cell membranes to permit cell-cell and cell-environmental interactions makes intuitive sense.

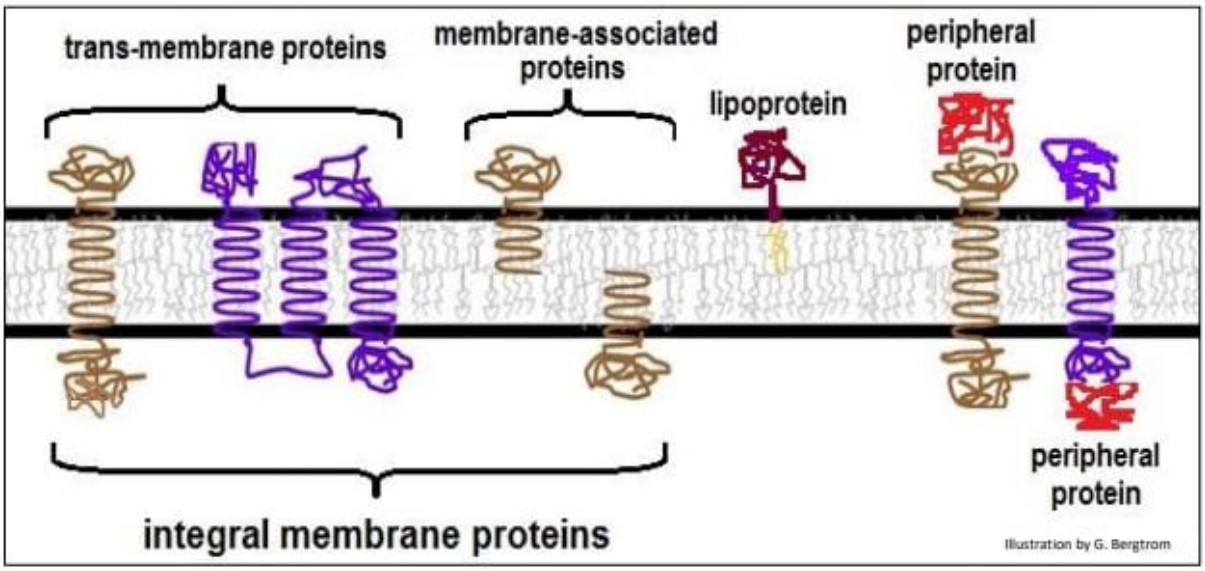
The recent observation that cellular membranes are even more compartmentalized was perhaps less anticipated. In fact, membranes are further divided into microcompartments. Within these compartments, components are fluid but seldom move between compartments. Studies indicate that cytoskeletal elements create and maintain these micro-discontinuities. For example, integral membrane proteins are immobilized in membranes if they are attached to cytoskeletal fibers (e.g., actin) in the cytoplasm. Furthermore, when aggregates of these proteins line up due to similar interactions, they form kind of *fence*, inhibiting other membrane components from crossing. By analogy, this mechanism of micro-compartmentalization is called the *Fences and Pickets* model; proteins attached to the cytoskeleton serve as the pickets. The movement across the fences (i.e., from one membrane compartment to another) is infrequent. Extra kinetic energy is presumably needed for a molecule to 'jump' a fence between compartments. Hence, this kind of motion, or *hop diffusion* distinguishes it from the Brownian motion implied by the original fluid mosaic model.

285 Membrane Domains: Regional Differentiation of a Plasma Membrane

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16.3: Membrane Proteins

Clearly, membrane proteins themselves have domains that keep membranes in or attached to the membrane, provide catalytic surfaces and allow interactions inside, across and outside of cells and organelles. Membranes anchor proteins in several ways. As noted, membrane proteins, like phospholipids, are amphipathic, with hydrophobic domains that non-covalently interact strongly with the fatty acid interior of membranes. Some integral membrane proteins span the entire membrane, with hydrophilic domains facing the cytosol or cell exterior. Peripheral proteins bind to a membrane surface through non-covalent interactions. Examples of integral and peripheral membrane proteins, glycoproteins and lipoproteins are illustrated below.

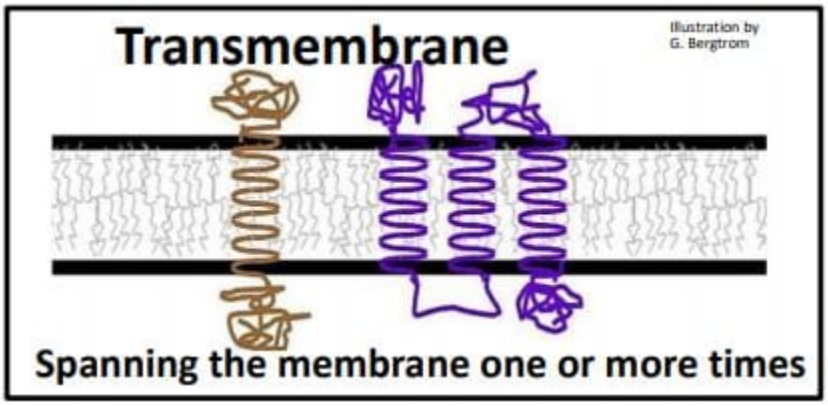


286 Domains of Membrane Proteins

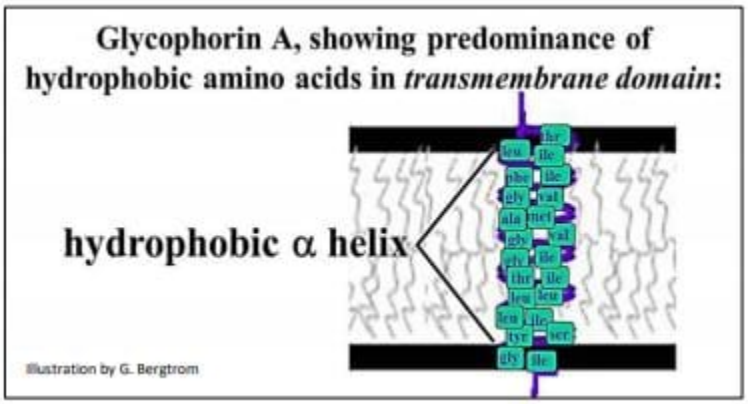
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16.4: How Membrane Proteins are Held in Membranes

The hydrophobic domain of integral membrane proteins consists of one or more alphahelical regions that interact with the hydrophobic interior of the membranes. Hydrophilic domains tend to have more tertiary structure with hydrophilic surfaces, and so face the aqueous cytosol and cell exterior. Two trans-membrane proteins are cartooned below.



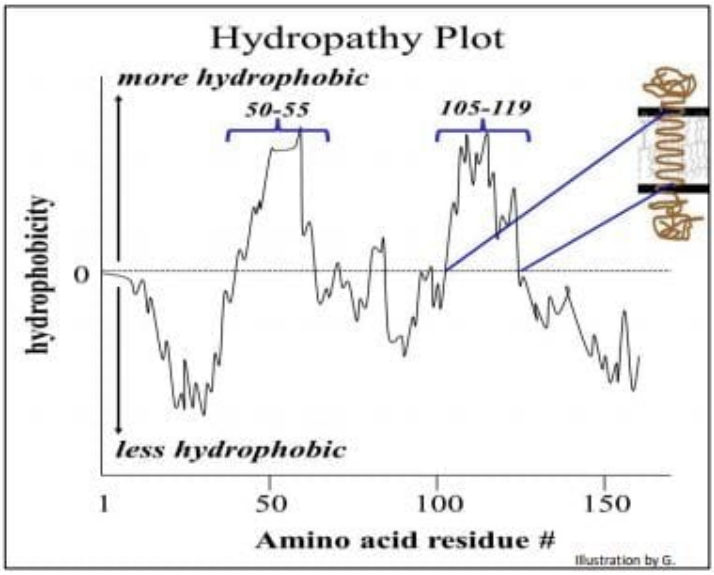
The protein on the left crosses the membrane once, while the one on the right crosses the membrane three times. How a transmembrane protein inserts into the membrane during synthesis dictates the locations of its N- and C-terminus. Transmembrane proteins can in fact cross a membrane more than once, which also determines the location of its N- and C-termini. N-terminal end of a plasma membrane polypeptide always ends up exposed to the outside of the cell. The alpha helical domains that anchor proteins in membranes are mostly non-polar and hydrophobic themselves. As an example, consider the amino acids in the alpha-helical domain of the red blood cell protein *glycophorin A*, a membrane protein that prevents red blood cells from aggregating, or clumping in the circulation. One glycophorin A polypeptide with its hydrophobic trans-membrane alpha helix is cartooned below. Glycophorin A monomers pair to form dimers in the plasma membrane.



Proteins that span membranes multiple times may include amino acids with charged, polar side chains, provided that these side chains interact between helices so that they are shielded from the fatty acid environment in the membrane. Because of these hydrophilic interactions, such proteins can create **pores** for the **transport** of polar molecules and ions; we will see some of these proteins later. Integral membrane proteins that do not span the membrane also have a hydrophobic helical domain that anchors them in the membrane, while their hydrophilic domains typically interact with intracellular or extracellular molecules to e.g., hold cells in place give cells and tissues their structure, etc.

The very presence of the hydrophobic alpha-helical domains in trans-membrane proteins makes them difficult if not impossible to isolate from membranes in a biologically active form. By contrast, the peripheral polypeptide *cytochrome c* readily dissociates from the cristal membrane, making it easy to purify. For many years, an inability to purify other cristal membrane electron carriers in biologically active form limited our understanding of the structure and function of the mitochondrial electron transport system.

Hydrophobic alpha-helical domains are in fact, a *hallmark* of membrane-spanning proteins. It is even possible to determine the primary structure of a polypeptide encoded by a gene before the protein itself has been isolated. For example, knowing the DNA sequence of a gene, we can infer the amino acid sequence of the protein encoded by the gene. A *hydrophobicity* analysis of the inferred amino acid sequence can tell us if a protein is likely to be a membrane protein. Let's look at a **hydropathy** (*hydrophobicity*) plot (below).



To see how an hydropathy plot can predict whether a protein is a membrane protein, check out the link below.

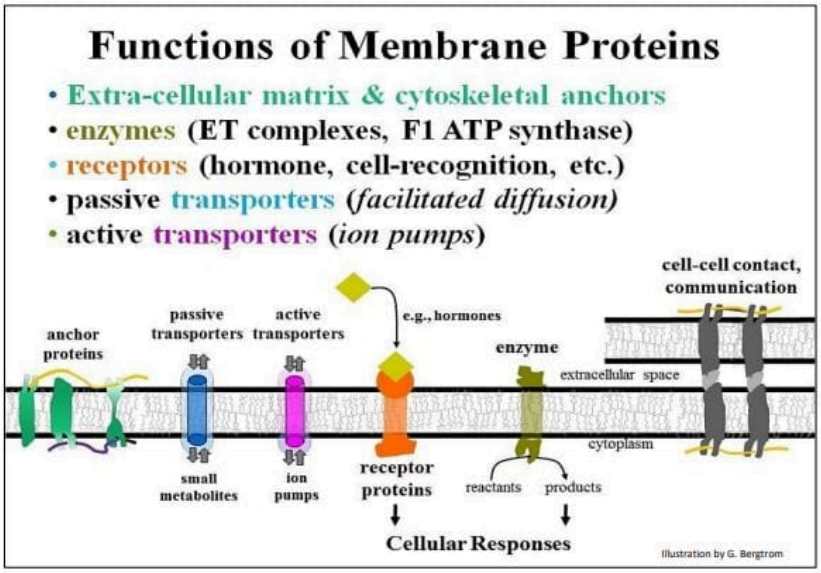
287 Hydropathy Predicts Hydrophobic Domains and Membrane Proteins

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16.5: A Diversity of Membrane Protein Functions

Example Membrane Protein Functions

- receptors for hormones or neurotransmitters
- antibodies of the immune system that recognize foreign substances (antigens)
- cell-recognition molecules that bind cells together
- cell membrane structures that directly pass chemical information between cells
- anchoring cells to extracellular surfaces like connective tissue
- molecular transport (entry into or exit of substances from cells)
- enzymes that catalyze crucial reactions in cells.



Transmembrane proteins perform most of the functions illustrated here. However, peripheral membrane proteins also play vital roles in membrane function. Remember that *Cytochrome c* in the electron transport system on the mitochondrial cristal membrane is a peripheral protein. Other peripheral membrane proteins may serve to regulate the transport or signaling activities of transmembrane protein complexes or may mediate connections between the membrane and cytoskeletal elements. The peripheral membrane proteins by shown here do not penetrate membranes. They bind reversibly to the internal or external surfaces of the biological membrane with which they are associated. We will be looking more closely at what holds membrane proteins in place and how they perform their unique functions. Check out major membrane protein functions, actions and cellular locations below

Basic Function	Specific Actions	Examples
Facilitated Transport	Regulate diffusion of substances across membranes along a concentration gradient	Ca^{2+} & other ion channels, glucose transporters
Active Transport	Use energy to move ions from low to high concentrations across membranes	Mitochondrial protein pumps, the Na^{+}/K^{+} ion pump in neurons
Signal Transduction	For e.g., hormones that can't enter cells, these convey information from molecular signals to cytoplasm, leading to a cellular response	Protein hormone and growth factor signaling, antibody/antigen interactions, cytokine mediation of inflammatory responses etc.

Cell-cell interactions	Cell-cell recognition and binding to form tissues	Formation of desmosomes gap junctions and tight junctions.
Anchors to cytoskeleton	Link membrane proteins to cytoskeleton	Give cells their shape, cell movement and response to molecular signals
Enzymatic	Usually multifunctional proteins with enzymatic activities	The F1 ATP synthase that uses proton gradient to make ATP; adenylyl cyclase that makes cAMP during signal transduction; note that some receptor proteins are linked to enzymatic domains in the cytoplasm

288 Diversity of Membrane Protein Structure and Function

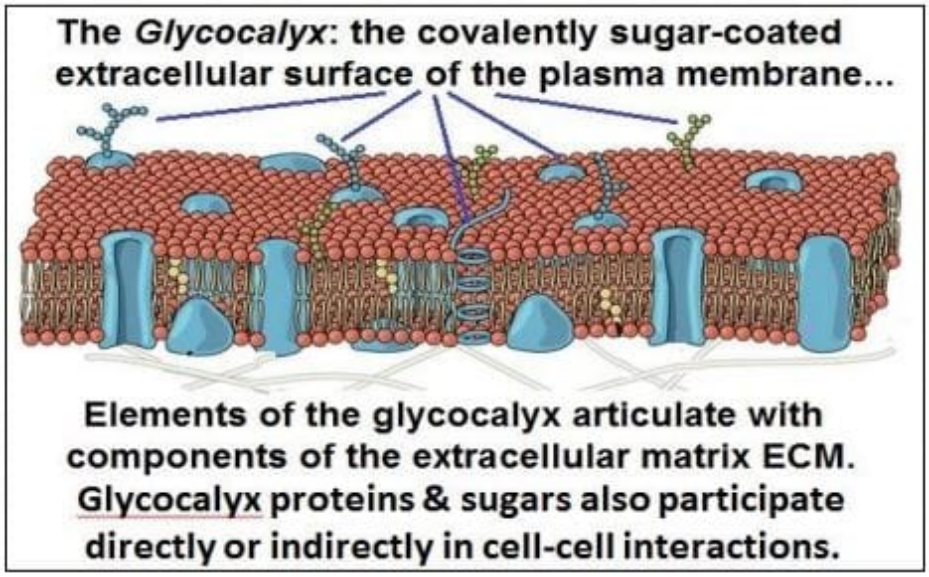
289 Pore Proteins May Cross the Membrane Many Times

290 Red Blood Cell (Erythrocyte) Membrane Protein Functions

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16.6: Glycoproteins

Membrane proteins are often covalently linked to **oligosaccharides**, which are branched *glycoside-linked* sugars (averaging around 15 sugar residues). As **glycans**, they are the sugars linked to **glycoproteins**. Glycoproteins are rare in the cytosol, but common on secreted and membrane proteins. Oligosaccharides are typically linked to proteins via the hydroxyl group on *serine* or *threonine*. Occasional linkages are to modified amino acids like **hydroxylysine** or **hydroxyproline** (*O-glycosylation*), and to the amide nitrogen on asparagine (*N-glycosylation*). The oligosaccharide domains of glycoproteins often play a major role in membrane protein function. For example, the glycoproteins, along with the polar domains of integral and peripheral proteins and glycolipids, are a major feature of the **glycocalyx**. A cell membrane and its glycocalyx are illustrated below.



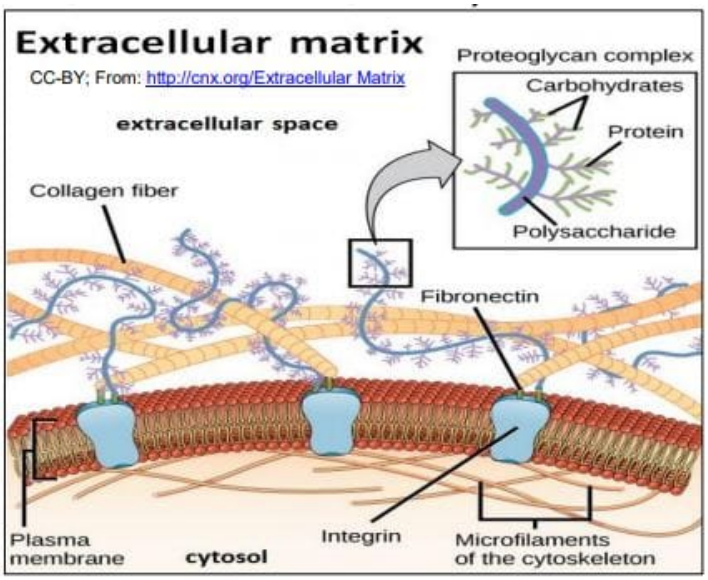
Oligosaccharides begin their synthesis in the rough endoplasmic reticulum (RER), with the creation of a **core glycoside**. Partial *glycans* are enzymatically linked to compatible amino acids of a membrane protein. As these proteins travel through the *Golgi vesicles* of the *endomembrane system*, **terminal glycosylation** attaches more sugars to the core glycoside to complete glycoprotein synthesis. When vesicles budding from the transGolgi vesicles fuse with the plasma membrane, the sugars on the glycoproteins end up on the exterior cell surface. This is illustrated in the link below.

291 The Path to Sugar Coated Cells

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16.7: Glycolipids

Glycolipids are phospholipids attached to oligosaccharides, and as noted, are part of the glycocalyx. Both are only found on the extracellular surface. Glycolipids are synthesized in much the same way as glycoproteins. Specific enzymes catalyze initial glycosylation of either phospholipids or polypeptides, followed by the addition of more sugars. Along with glycoproteins, glycolipids play roles in cell-cell recognition and the formation of tissues. The glycans on the surfaces of one cell will recognize and bind to carbohydrate receptors (**lectins**) on adjacent cells, leading to cell-cell attachment as well as intracellular responses in the interacting cells. Glycoproteins and glycolipids also mediate the interaction of cells with extracellular molecular signals and with chemicals of the **extracellular matrix** (**ECM**). The ECM includes components of connective tissue, basement membranes, in fact any surface to which cells attach.



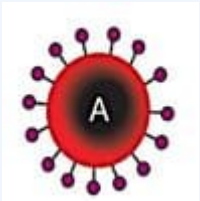
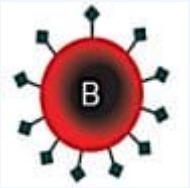
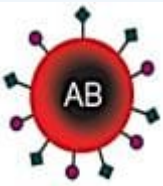
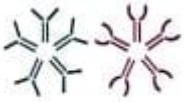
292 The Extracellular Matrix

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16.8: Glycoproteins and Human Health

We'll close this chapter with a few examples of glycoproteins that play crucial roles in human physiology. Let's look first at the major human blood groups. The major A, B, AB, O and Rh blood groups result from the presence or absence of glycoprotein **antigens** embedded in red blood cell membranes and the presence or absence in the blood, of **antibodies** against the antigens. Typically, exposure to *antigens* (foreign substances like bacteria, viruses, toxins...) generates **immunoglobulins**, the *antibody* molecules of our immune system; *immunoglobulins* are glycoproteins. The situation with blood groups is something of a paradox. The blood group antibodies already in the blood of a healthy person are *not* a response to foreign antigen invasion

You probably know that these blood groups must be compatible for a successful blood transfusion. A mismatch between donor and recipient can be devastating. The interaction of the red cell antigens of one blood group with antibodies in another blood group will cause the red cells to clump, restricting blood flow and ultimately killing the transfusion recipient. The table below summarizes why transfusions with mismatched A, B, AB, O blood groups must be avoided.

	Group A	Group B	Group AB	Group O
Cell-surface antigens				
Antibodies inthe blood			**None**	
Acceptable donor-recipient matches	Group A or Group O donors	Group B or Group O donors	Universal Recipient (Group AB, A, B, O donors)	Only Group O donors
Why red cells clump in mismatched blood	Anti-A from Group B donor binds, aggregates recipient red cells; recipient Anti B binds, aggregated donor red cells	Anti-B from Group A donor binds, aggregates recipient red cells; recipient Anti A binds, aggregates donor red cells		Antibodies in Group O blood will bind any donor red cell antigens and cause the cells to clump

Another red blood cell antigen is the Rh factor. People have either it (Rh+) or not (Rh-). In contrast, when an Rhrecipient receives blood from an Rh+ donor, the recipient's immune system makes defensive anti-Rh antibodies in the usual way. This too can cause blood cell clumping with bad consequences. A word to the wise: it's a good idea to know your own blood group!

Check the Red Cross website (here) or Wikipedia for more detail about blood groups.

The last example here involves the cell surface major histocompatibility complex (MHC) glycoproteins that distinguish self from non-self in body tissues and organs. Major organ Transplantation (liver, kidneys, heart) from donors into patients with failing organs has become, if not routine, then at least increasingly common. Before a transplant, MHC tissue typing determines donor and recipient compatibility, reducing the chances of the rejection of the transplanted organ. Since available donors are few, and good matches even fewer, patients wait on prioritized lists for a matched organ. Even when MHC typing is a match for a patient, the immune systems of transplant recipients are suppressed with hormones to reduce further the chance of rejection. Unlike the limited number of blood groups, many MHC proteins are analyzed to determine a match. Thus, it is not practical (or routinely necessary) to 'know' your MHC type!
In the next chapter, we look at membrane functions intrinsic to cellular existence itself.

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16.9: Key Words and Terms

amphipathic molecules	glycolipids	preipheral membrane proteins
asparagine	glycosylation	phospholipid bilayer
cell membrane	Golgi vesicles	plasma membrane
cell-cell attachment	Hyropathy plot	poikilothermic organisms
cytoskeleton	hydrophilic phosphate heads	RER
Davson-Danielli membrane model	hydrophobic fatty acid tails	Rough endoplasmic reticulum
endomembrane system	hydrophobicity plot	saturated fatty acids
exocytosis	hydroxyproline	serine
extracellular matrix (ECM)	hydroxylysine	temperature effects on membranes
fluid mosiac	integral membrane proteins	threonine
freeze facture method	membrane asymmetry	transmembrane proteins
membrane evolution	membrane proteins	unsaturated fatty acids
glycan	N-glycoslyation	
glycocalyx	O-glycoslyation	

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17: MEMBRANE FUNCTION

17.1: Introduction
17.2: Membrane Transport
17.3: Ligand and Voltage Gated Channels in Neurotransmission
17.4: Endocytosis and Exocytosis
17.5: Directing the Traffic of Proteins in Cells
17.6: How Cells are Held Together and How they Communicate
17.7: 17.7 Signal Transduction
17.8: Key Words and Terms

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17.1: Introduction

Small molecules like O2 or CO2 can cross cellular membranes unassisted; neither the hydrophilic surfaces nor the hydrophobic interior of the phospholipid bilayer are barriers to their transit. On the other hand, most molecules (even water!) need the help of *membrane transport proteins* to get in or out of cells and organelles. Transport proteins can act as *gates* that might be open or closed. When open, they permit diffusion of molecules into or out of cells along a concentration gradient so that their concentrations equalize across the membrane. Like the **passive diffusion** of small gasses, **facilitated diffusion** by membrane proteins does not require an input of energy. In contrast, some transport proteins are actually *pumps*, using chemical energy to move molecules across membranes against a concentration gradient. The result of this **active transport** is to concentrate solutes on one side of a membrane. For example, pumps that create sodium and potassium **ion gradients** are responsible for the *excitability of cells*. Recall that this is one of the fundamental properties of life: the ability of cells and organisms to respond to stimuli.

As you read this chapter, look for how allosteric change can regulate membrane function, where we consider how:

- membrane **gates** and **pumps** work
- membrane protein interactions allow cells to **self-assemble** into tissues and organs.
- cells direct protein **traffic** to the cytoplasm, into membrane themselves, into organelles, or out of the cell
- membrane proteins participate in **direct communication** between adjacent cells.
- membrane proteins are receptors for more **long-distance communications**
- membrane proteins are receptors for more **long-distance communications,** responding to neurotransmitters, hormones, and other external chemical signals.

Learning Objectives

1. Explain how/why one cell's plasma membrane differs from that of another cell type.
2. Explain how/why the plasma membrane differs from other membranes with in the same cell.
3. Determine if a solute crosses a plasma membrane by passive or facilitated diffusion.
4. Explain how *salmon* can spend part of their lives in the ocean and part swimming upstream in freshwater to spawn, without their cells shriveling or bursting.
5. Explain how *active transport* stores *chemical energy* (recall electron transport).
6. Explain the role of active transport in maintaining/restoring a cell's *resting potential*.
7. Compare and contrast different kinds of *gated channels*.
8. Describe the order of ion movements that generate an *action potential*.
9. Define and compare *exocytosis, pinocytosis, phagocytosis and receptor-mediated endocytosis*
10. Distinguish between signal molecules that enter cells to deliver their chemical message and those deliver their message only as far as the plasma membrane.
11. Trace an intracellular response to a *steroid hormone* to a likely *cellular effect*.
12. Trace a liver cell response to *adrenalin* from plasma membrane to glycogenolysis (*glycogen breakdown*).
13. Compare the *signal transduction* activities of different *G-protein receptors* leading to the first active kinase enzyme.
14. Explain how a liver cell can respond the same way to two different hormones (e.g., adrenalin and glucagon)..., and why this should be possible
15. Describe/explain how a phosphorylation cascade *amplifies* the cellular response to a small amount of an *effector* (signal) molecule.
16. Discuss the differences and interactions between the *glycocalyx*, *basement membrane* and *extracellular matrix* (*ECM*).
17. Explain ECM functions and identify components involved in those functions
18. Describe how the molecular structure of *fibronectin* supports its different functions.
19. Describe some structural relationships between cell surfaces and the *cytoskeleton*.
20. Compare and contrast the structures and functions of the different cell junctions.
21. Distinguish between the structures and functions of *cadherins, clathrin, COPs, adaptin, selectins, SNAREs* and *CAMs*.
22. State an hypothesis to explain why some cancer cells divide without forming a tumor

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17.2: Membrane Transport

The first control on the passage of molecules across membranes is the semi-permeable character of the membrane itself. Molecules move in and out of cells in one of three ways: **passive diffusion**, **facilitated transport** and **active transport**.

Only a few small, relatively uncharged molecules can cross a membrane unassisted (i.e., by passive diffusion). Hydrophilic molecules that must enter or leave cells do so with help, i.e., by *facilitated transport*. Passive and facilitated transport release the free energy inherent in concentration gradients as molecules diffuse across a membrane. In contrast, active transport consumes energy to create concentration gradients of specific solutes. The specificity of *facilitated* and *active transport* lies in integral membrane proteins that recognize and bind specific solutes for transport. As you may predict, allosteric regulation of these proteins controls the movement of their target molecules into or out of cells.

Despite its polarity, many believed that the small water molecules crossed membranes without help. Indeed, it does to a limited extent. However, others suspected that given its highly charged *polar covalent* bonds relative to its small size, water molecules require an assist to get across membranes efficiently. Let's begin with a closer look at passive diffusion and facilitated diffusion, followed by osmosis (a special case of facilitated diffusion), and finally, at active transport.

A. Passive Diffusion

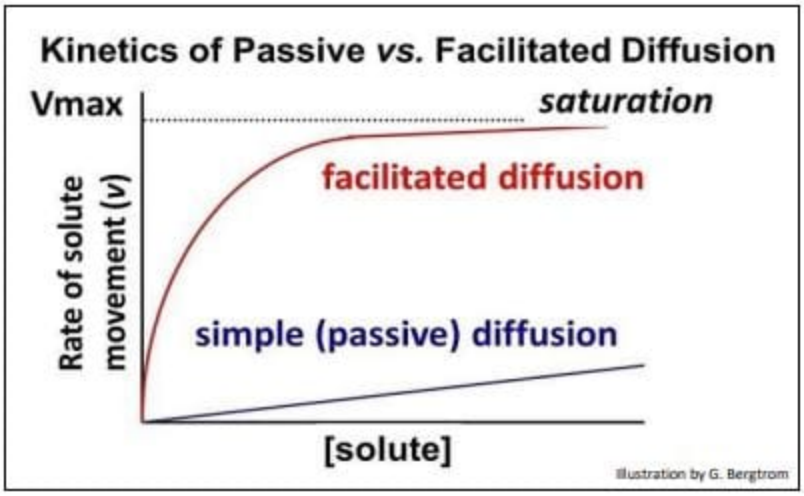
Passive diffusion is the movement of molecules over time by random motion (also called *Brownian motion*) from regions of higher concentration to regions of lower concentration. Significant passive diffusion across cellular membranes is limited to a few molecules, mostly gasses like O2, CO2, and N2, that can freely cross the hydrophobic phospholipid barrier. The rapid diffusion of gasses is essential for O2 and CO2 exchange between the alveolar capillaries and cells of the lungs during physiological respiration. O2 and CO2 exchange also occurs in mitochondria during cellular respiration. Diffusion across membranes does not require energy. In fact, diffusion releases energy - recall the movement of protons through the F1 ATPase proton gate that synthesizes ATP during mitochondrial oxidative phosphorylation.

The rate of diffusion of a molecule is dependent only on its own concentration. It is unaffected by the concentration of other molecules. Over time, random motion of solutes within and across compartments results in a **dynamic equilibrium** for each different solute over time. At equilibrium, solute molecules continue to diffuse across the membrane, but for each molecule moving across in one direction, another molecule of the same solute crosses in the other direction.

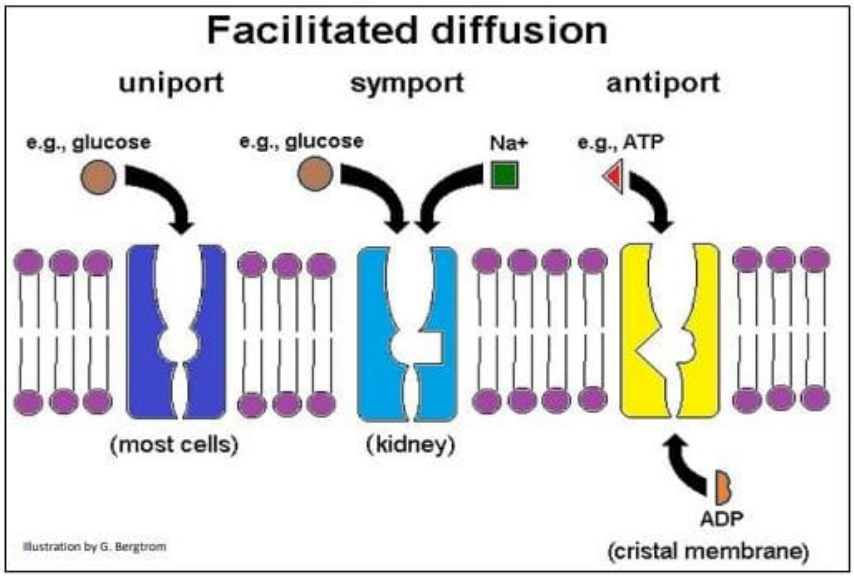
B. Facilitated Diffusion of Solutes and Ions

Like passive diffusion, *facilitated diffusion* is the spontaneous (downhill) passage of molecules or ions across membranes through specific transmembrane proteins. The kinetics of passive and facilitated diffusion reveals the differences between the two processes. To understand the latter, recall that the rate of enzyme catalysis is *saturable*. That is, as the concentration of substrate is increased, the rate of the catalyzed reaction approaches a maximum (V_{max}), when all enzyme molecules in solution are bound to substrate molecules. The same saturation phenomenon applies to facilitated transport – the rate of solute movement across a membrane is directly proportional to the number of transport proteins in the membrane.

The kinetics of passive and facilitated diffusion are illustrated by the graph shown below



Perhaps you see another similarity between facilitated diffusion and enzyme catalysis in this graph! Relative rates of facilitated diffusion are typically rapid, compared to those of passive diffusion. This is because the allosteric changes that accompany facilitated transport are rapid, just as they are during enzyme catalysis. There are three kinds of **facilitated transpor**t of solutes (below).



The GLUT protein (glucose transporter) protein shown above (left) allows glucose **uniport**, the specific transport of a single substance in or out of cells. Kidney cells have glucose transporters that **symport** (couple) the simultaneous movement of glucose and sodium ions; **SGLT** (**S**odium-**GL**ucose **T**ransporter) serves a similar function in small intestine cells, enabling absorption of dietary glucose and sodium. **Antiport** (above, right) allows the specific exchange of molecules across a membrane. In the example shown, ATP leaves the mitochondrial matrix, crossing the cristal membrane at the same time as ADP enters the matrix.

Whether by uniport, symport or antiport, each solute will independently cross a membrane down its concentration gradient, moving from higher concentration to where it is at a lower concentration. Recall that diffusion along a gradient releases free energy that depends on relative concentrations of the solutes.

Proteins mediating facilitated diffusion are of two kinds: **carrier proteins** and **channel proteins**. Carrier proteins allow solute transport. Ions, with their high charge-to-mass ratio, need help to cross the hydrophobic membrane barrier; this is the job of channel proteins that essentially serve as ion pores.

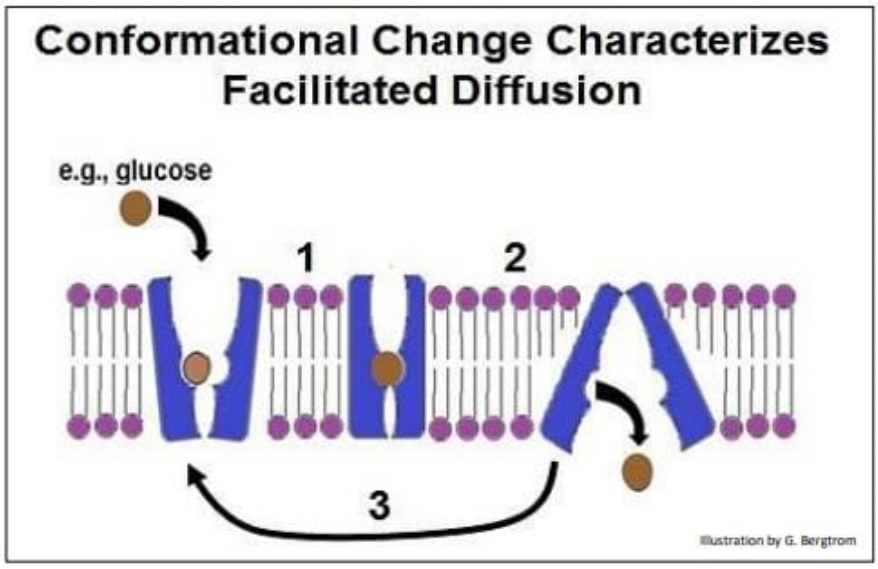
Like all transporter proteins, both carrier and channel proteins undergo allosteric change during transport. They are also typically subject to allosteric regulation, rather than being in a constant 'open' state. Examples of facilitated diffusion are considered in

more detail below.

1. Carrier Proteins

When a carrier protein binds a solute that must cross the membrane, it undergoes an allosteric change (illustrated below). During transport, the carrier protein undergoes another change in shape. When the solute reaches the other side of the membrane, it no longer has a high affinity for the carrier protein. After release of the solute, a final allosteric change restores the original conformation of the transport protein. These sequential conformational changes are illustrated on the next page.

Any given carrier protein is specific for a single solute, or at most a single family of closely related solutes. We just saw the GLUT1 transporter carrier protein that allows glucose (but not fructose or ribose!) to cross cell membranes. Different specific carrier proteins facilitate the transport of amino acids and other charged solutes across cell membranes. Once again, molecules that indicate cell status (i.e., a need to import or export solute) are *allosteric effectors* that regulate carrier proteins. The regulation of glucose transport into cells by insulin is a perfect



example. One consequence of insulin released during a meal (or just in anticipation of a meal) is the stimulation of glucose transporters to take up glucose. An inability of those transporters to respond to insulin accounts in part for Type II (adult onset) diabetes.

Water gets across membranes by osmosis (we'll look more closely at how osmosis affects cells in a moment). We noted that small amounts of water could cross the phospholipid bilayer unassisted. Water can also cross a membrane incidentally, when ions flow through their channel proteins. But most osmosis involves facilitated diffusion mediated by **aquaporins**. Some aquaporins only transport water. Others have evolved to co-facilitate the transport of glucose (see above), glycerol, urea, ammonia, carbon dioxide and even ions (protons) along with water. Like other carrier proteins, aquaporins are allosterically regulated to allow cells to meet their specific water balance requirements. So fundamental was the understanding of water balance that the discovery of aquaporins earned Peter Agre a Nobel Prize in Chemistry in 2003. Since Agre's discovery (in 1992), several genetic diseases have been linked to aquaporin gene mutations.

Kidney cells are critically involved in vertebrate water balance and have many aquaporins in their membranes. In a rare form of diabetes, abnormal aquaporins cause the kidneys to excrete unusually large volumes of water. In another example, aquaporin gene mutations lead to the development of cataracts in both eyes. Since their initial discovery, aquaporins have been described in bacteria and plants. To learn more, click Aquaporins.

2. Ion Channels

Allosteric regulation of ion channel proteins controls ion *homeostasis* in blood and extracellular fluids within narrow limits. Often, multiple integral proteins contribute to the formation of an ion channel. When stimulated, channel proteins rearrange to open a pore allowing specific ion transport. Some ion channels, like the glucosesodium ion symport system noted above, mobilize the

energy of diffusion of one solute (an ion in this case) to rapidly transport another solute through the same channel (acting like an ion channel and a carrier protein). Finally, ion channels are responsible for the excitability of cells, where Na+ , K+ and Ca++ channels collaborate in ion movements into and out of cells leading to neuronal or muscle cell responses (more shortly!)

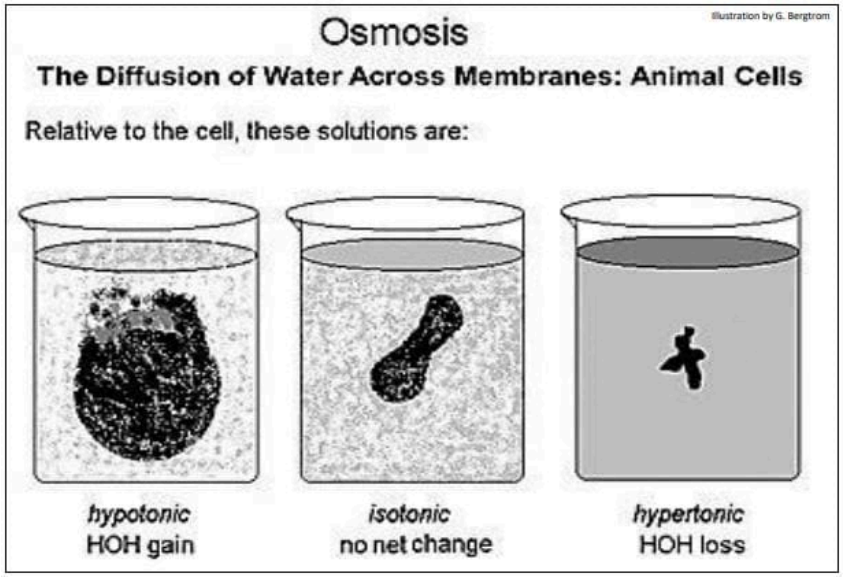
293 Passive and Facilitated Diffusion

C. Osmosis

Osmosis, the diffusion of water across membranes from lower to higher solute concentrations, is an essential activity. It allows cells to use water to maintain cellular integrity or to adapt to changes in the solute composition in the extracellular environment. Osmosis across cellular membranes relies on the facilitated transport of water by aquaporins. The passive diffusion of water molecules, can be demonstrated with an artificial (e.g., dialysis) membrane. Water will cross such a membrane if solute concentrations are higher on one side of the membrane. Water crosses the membrane "trying" to equalize the solute concentrations on both sides of the membrane. In effect, water movement is from the side of a membrane where the free water molecule concentration is higher (i.e., where the concentration of solute is lower) to the side where the concentration of free water is lower (i.e., where the concentration of solute is higher).

1. Osmosis in Plant and Animal Cells

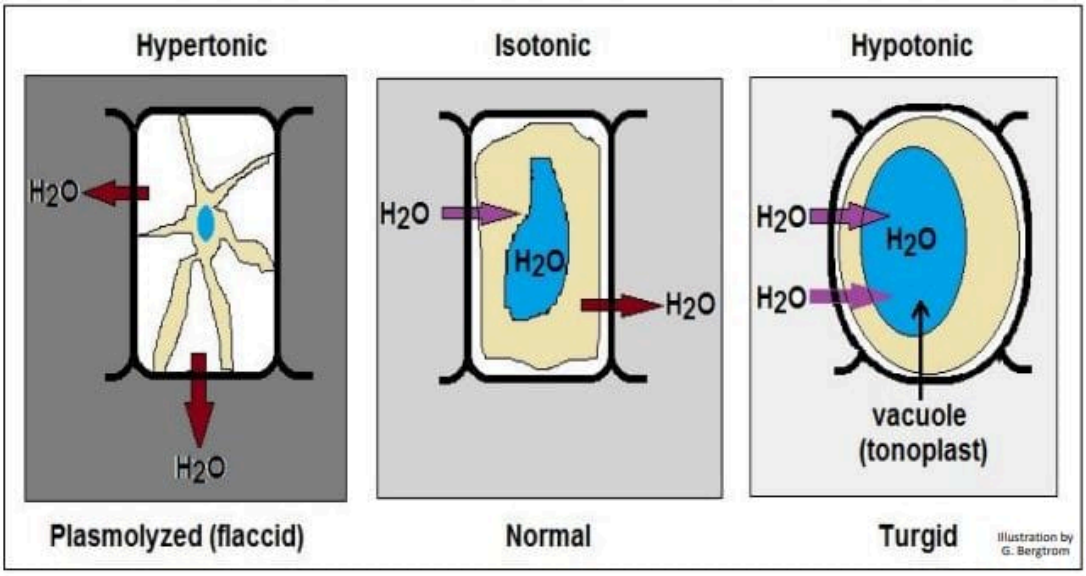
We could present this section in the context of free water concentrations, but we will do so in the more familiar terms of solute concentrations. Osmosis affects plant and animal cells according to the same principles, but with different effects. First, let us consider the effect of different experimental solute concentrations on animal cells, illustrated on the next page.



If the solute concentration inside and outside the cell is the same, there is no net movement into or out of the cells. The extracellular medium and cytosol are said to be **isotonic** to each other. When water diffuses into the cells from a low solute medium, the medium is said to by **hypotonic** to (less concentrated than) the cytosol. In this case, movement of water into a cell lowers the cytosol solute concentration. Animal cells swell and burst in a hypotonic solution. In **hypertonic** solutions (with a higher solute concentrations than the cytosol), animal cells shrivel up as water leaves the cell. From this brief description, you should conclude that *water crosses from the hypotonic to the hypertonic side of a membrane*.

As with animal cells, exposure of plant cells to hypotonic or hypertonic solutions causes the same directional water movements, but with some key differences due to their cell walls. In *hypotonic* solutions, water enters plant cells, moving into the cytosol and then into water vacuoles called **tonoplasts**. This results in higher **osmotic pressure** (water pressure) in the tonoplasts. The expanding tonoplast creates **turgor pressure**, compressing the cytosol against the cell wall. Rather than bursting, the cells and plant tissues stiffen and become *turgid*. Since water cannot enter plant cells indefinitely, water stops entering the cells when the *osmotic pressure* outside the cells and the *turgor pressure* inside the cells are at equilibrium. You encountered this phenomenon if you have ever over-watered houseplants. The stiffened leaves and stems become brittle and are easily snapped or broken.

In hypertonic medium, plant cells (like animal cells) lose water, resulting in **plasmolysis**. This is the effect of shrinkage of the plasma membrane. However, the plasma membrane remains tightly attached to the plant cell wall at several points. You may have seen under-watered plants with floppy or droopy stems and leaves. These have become **flaccid** due to loss of water and thus the loss of turgor pressure needed to keep leaves and stems upright. The effects of different solutions on plant cells are illustrated below

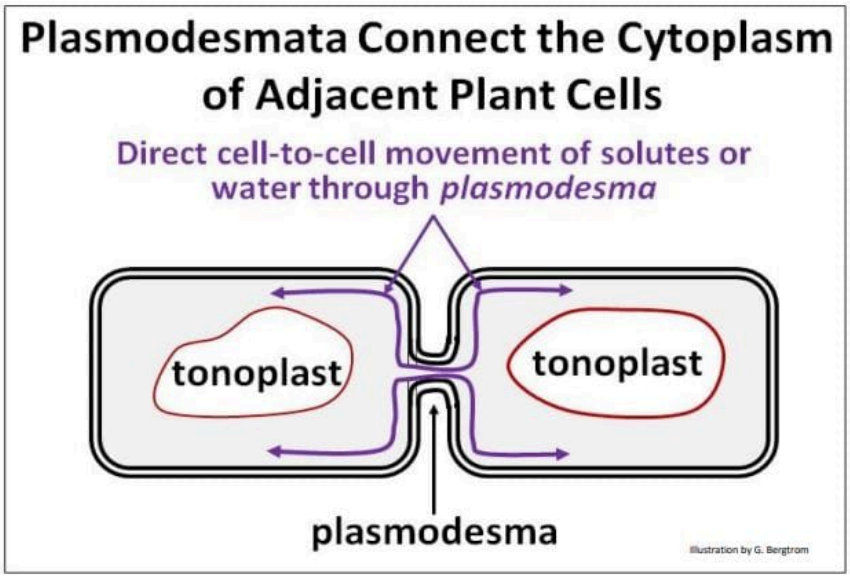


Formally, osmotic or turgor pressure is defined as the force per unit area (i.e., *pressure*) required to prevent the passage of water across a semipermeable membrane from a hypotonic to a hypertonic solution

2. Osmosis in Plant Life

While individual plant cells respond to changes in solute concentrations, these changes are rapidly communicated to adjacent cells through **plasmodesmata**. These structures connect the plasma membranes of adjacent cells through their cell walls, allowing rapid, direct sharing of physical and chemical information.

A **plasmodesma** is illustrated below.



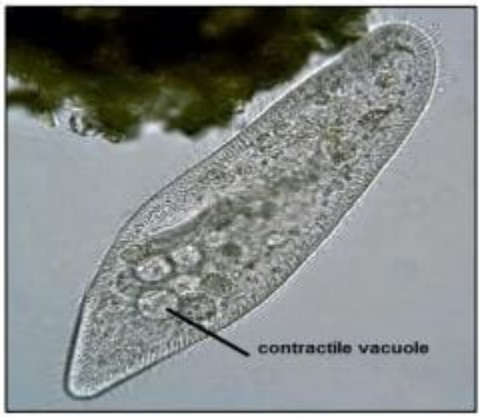
In this way, effects on osmotic pressure in a few cells created by changes in water availability are transmitted to adjacent cells,

affecting turgor pressure in those cells and, ultimately, in plant tissues.

Finally, plant life depends on water! Recall that plant cells require a continual supply of water for use in photosynthesis, to provide hydrogen to reduce CO2 to glucose. Photosynthesis as well as the loss of excess water from plant tissues (especially leaves) by transpiration lowers cellular osmotic pressure. As water moves up from the roots to replace water used and lost by leaf cells, the osmotic pressure drops in the *fine root hair cells* (with their high surface area). This draws water into the cells and roots by osmosis. Thus, osmotic pressure is the main force driving water into plants and, defying gravity, moving it up from the roots to the rest of the plant

3. Osmosis in Animal Life

Changes in osmotic environment can stress or kill an organism. For example, freshwater organisms (protozoa or fish) placed in sea water will die. Likewise, saltwater fish placed in freshwater. But organisms can **osmoregulate** (control the osmotic pressure in their cells), at least to a point. *Paramecium* for example, expels fresh water to prevent bursting as it takes on water. This is accomplished by a **contractile vacuole** (shown below).



Water constantly enters these freshwater protists because the solute concentration in the cytosol is always higher than the freshwater water they live in. To cope with a constant uptake of water, their *contractile vacuole*s collect excess water and then contract to expel the water. At a high-energy cost, *Paramecia* constantly pump water out of the cell to maintain water balance (i.e., correct osmotic pressure). Another protist strategy for coping with change in environmental solute concentrations (salinity) is to pump salts (or suitable salt solute substitutes in or out of the cell, as needed (For some details, see Protist Osmoregulation Genes Acquired by Eukaryotes from Bacteria by Horizontal Gene Transfer).

Larger organisms like freshwater fish cope with their hypotonic environment by urinating a lot! At the other end of the spectrum, salt-water fish cope with the high solute concentration of solutes (salts) in their environment by excreting excess salt. Salmon spend time in seawater growing to maturity and later swim upstream in fresh water to spawn. You can imagine how salmon and similar organisms have to **osmoregulate** to adapt to their changing, very different environments. In this case, **osmoregulation** begins when hormonal changes respond to changes in living circumstance and dictate a compensatory response.

4. Summing Up

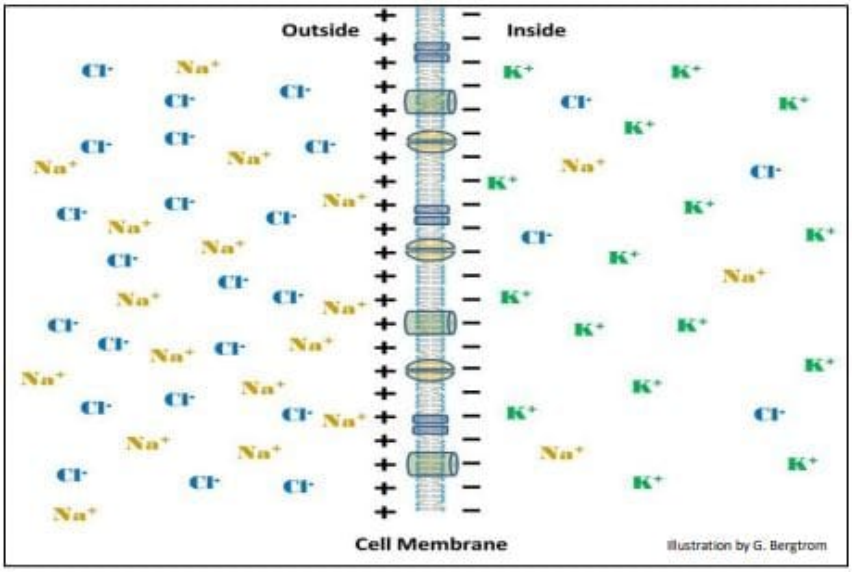
Osmosis is the movement of water across membranes to where solutes are at high concentration. At the same time, solutes that can diffuse across membranes move in or out of cells towards where they are at lower concentration, either passively, or by facilitated diffusion. We have evolved different facilitated transport proteins specific for different proteins. Finally, most water crosses membranes by facilitated diffusion through aquaporin proteins that serve as pores in cellular membranes.

294 Osmosis

D. Active Transport

Excitability (adaptation) is another of the defining properties of life. This property of all cells is based on chemical and electrical reactivity. Neurotransmitters released at a synapse cross the synaptic cleft from a “sending” neuron to a responding cell (another

neuron or a muscle cell). The neurotransmitter binds to receptors on the responding cell resulting in a **membrane depolarization**, a rapid change in the electrical potential difference across the cell membrane. While responses to neurotransmitters occur in fractions of a second, all cells are responsive, albeit not always as fast as neurons or muscle cells. Changes in membrane polarity of any cell depend on *unequal* concentrations of ions inside and outside cells. Cells at rest typically have a higher **[K^+]** in the cytosol and higher **[Cl^-]** and **[Na^+]** outside the cell (below).



These ionic differences across membranes are what enable such cells as neurons and muscle to respond to chemical and other (e.g., electrical) signals. Thus, cells have a **resting potential**, shown here with plus and minus signs on opposite sides of the membrane. The measured resting potential (difference in charge or **potential difference**) of most cells is typically between -50mv to -70mv. Disturbance of the resting potential (i.e., *membrane depolarization*), results from a flow of ions across membranes. Resting potentials sustained by ion gradients permit physiological response to chemical or other signals.

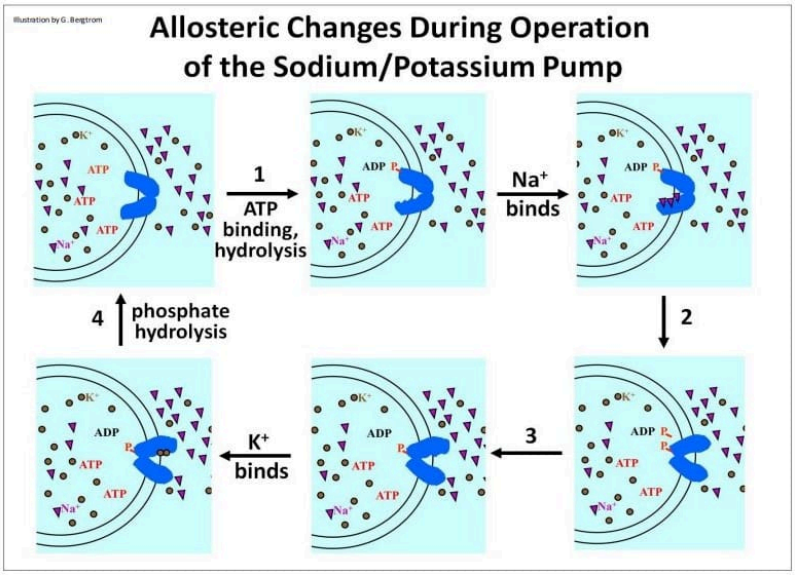
Resting potentials change when cells are excited, as well as by normal, but non-functional ion leakage. Whether incidental or intentional, the correct ion balance must be restored and maintained. This is accomplished by the **active transport** of ions across the membrane. This energetically unfavorable process requires an input of free energy, typically from ATP hydrolysis. The **Na^+/K^+** pump is an active transport protein complex linked to **ATPase** activity. Next, we consider ion flow during cell excitation and how ion pumps work.

Let's begin by looking at the allosteric changes that occur when the Na+ /K+ pump works to restore and maintain ion gradients (illustrated on the next page). In operation, the ATPase domain of the Na+ /K+ pump hydrolyzes ATP, leaving a phosphate attached to the pump and inducing the first of several allosteric changes in the pump proteins (**No. 1**, above). In its new conformation, the pump binds three Na+ ions, causing a second conformational change that in turn releases the Na+ ions into the extracellular fluid (**No. 2**). The release of Na+ ions outside the cell causes a third allosteric change (**No. 3**), after which two K+ ions from the extracellular fluid are able to bind to the pump protein. K+ binding causes the hydrolysis of the phosphate from the pump protein, returning the pump proteins to their original conformation (**No. 4**) and releasing the two K+ ions into the cytosol. The Na+ /K+ pump is ready for action again!

295 Potassium Leakage Helps to Maintain Cellular Resting Potentials

296 Active Transport by the Sodium/Potassium Pump

For his discovery of the ATPase-powered sodium/potassium pump and and his studies of how it works to maintain intracellular ion balance, Jens C. Skou earned a share of the Nobel Prize in Chemistry 1997. You can read more about Jens C. Skou at https://www.nobelprize.org/prizes/chemistry/1997/skou/auto-biography/

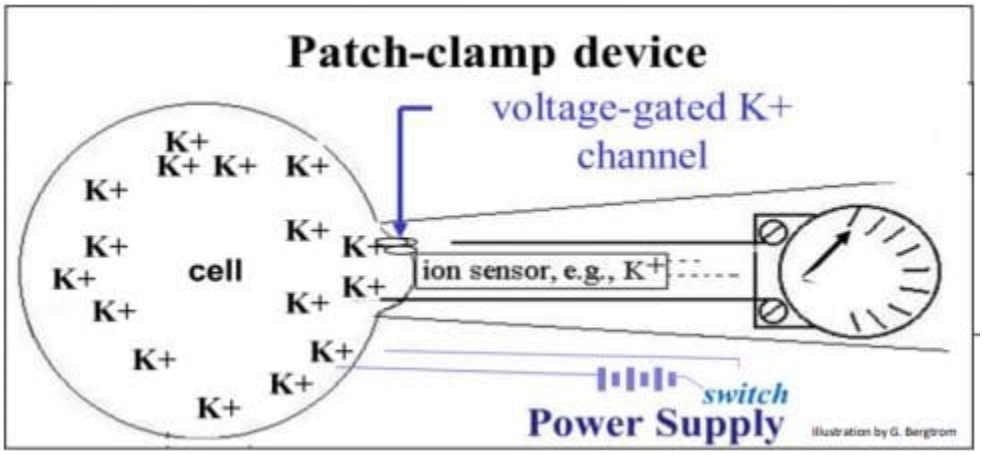


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17.3: Ligand and Voltage Gated Channels in Neurotransmission

A. Measuring Ion Flow and Membrane Potential

When neurotransmitters bind to their receptors, **ion channels** in responding neuron or muscle cells open. The resulting influx of Na^+ ions disrupts the **resting potential** of the target cell. The effect is only transient if the membrane potential remains negative. However, if enough Na+ ions enter the cell, the membrane becomes depolarized. If the cell experiences **hyperpolarization**, a localized *reversal of normal membrane polarity* (say from –70 mV to +65mV or more) will generate an **action potential**. This action potential will travel like a current along the neural or muscle cell membrane, eventually triggering a physiological response, e.g., the excitation of the next nerve cell in a neuronal pathway or contraction of the muscle cell. The **patch-clamp** device detects specific ion flow and any the resulting change in potential difference across the membrane. Principles of patch-clamp measurement are illustrated below.



In the example above, closing the switch on the power supply sends an electrical charge to the cell, opening up **voltage-gated ion channel**. In this case, a potassium sensor in the device detects the flow of K+ ions through the channel and out of the cell. At the same time, a volt meter registers the resulting change in membrane potential.

297 A Patch Clamp Device Can Record Membrane Potential and Ion Flow

298 Patch Clamp Measures Resting Potential and Depolarization

In addition to voltage-gated ion channels, the patch clamp device can measure ion flow through **ligand-gated ion channels** and **mechanically-gated ion channels**.

The former channels are *receptor-ion gates* that open when they bind an effector molecule. *Mechanically-gated ion channels* detect physical *pressure* or *stress* that result in a local membrane deformation, opening the channel.

299 Gated Ion Channels

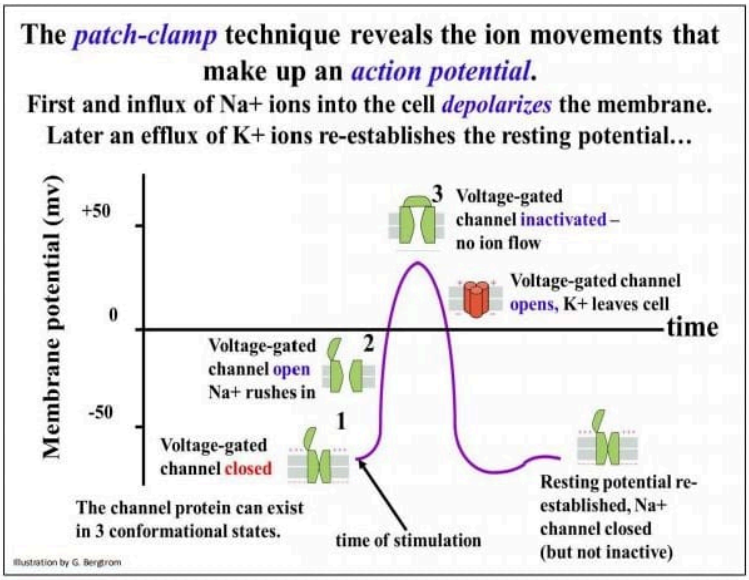
300 Types of Gated Ion Channels-Illustrated

Finally, cells maintain a high intracellular concentration of K^+ ions, causing K^+ ions to slowly leak from the cell, a phenomenon detectable by a patch-clamp. The presence of negative ions (Clions, organic ions) inside a cell limits the leakage. This creates the electronegative interior of a cell relative to outside the cell, i.e., the resting potential across its plasma membrane. The patch-clamp technique has been used to correlate the flow of ions and changes in membrane potential when a neuron fires, causing an action potential in a responding cell.

Such a correlation is described on the next page. In the illustration, follow the opening and closing of ion channels and the flow of ions. An action potential (in fact any shift from resting potential) results from facilitated diffusion of specific ions into or out of the cell through gated ion channels (green, above) that must open and close in sequence. The behavior of two different *voltage-gated ion channels* are illustrated in the graph. Electrical stimulation opens Na^+ channels. Na^+ ions rush into the cell, reducing the membrane potential from the resting state to zero, or even making the cytoplasm more positive than the extracellular fluid. If the reversal in polarity is high enough, a voltagegated K^+ opens and potassium ions rush into the cell, restoring the resting potential of the cell.

A cell can continue to respond to stimuli with action potentials for as long as there is sufficient Na+ outside the cell and K^+ inside the cell. While active transport of Na^+ and K^+ is not required to re-establish the resting potential, it will eventually be necessary to restore the balance of the two ions in the cell. If a nerve or muscle cell fires several times (or even if it just leaks ions), the [K^+] inside the cell and the [Na^+] outside the cell would drop to a point where the cell cannot generate an action potential when stimulated. Ultimately, it is the role of ATP-dependent Na^+/K^+ pumps to restore the appropriate Na^+:K^+ balance across the responding cell membrane. As we have seen, each cycle of pumping exchanges 3 Na^+ ions from the intracellular space for 2 K^+ ions from the extracellular space. The pump has two effects:

- It restores Na^+ concentrations in the extracellular space relative to the cytoplasm.
- It restores K^+ concentrations in the cytoplasm relative to the extracellular space.



301 Gated Ion Channels Open and Close in Order During an Action Potential

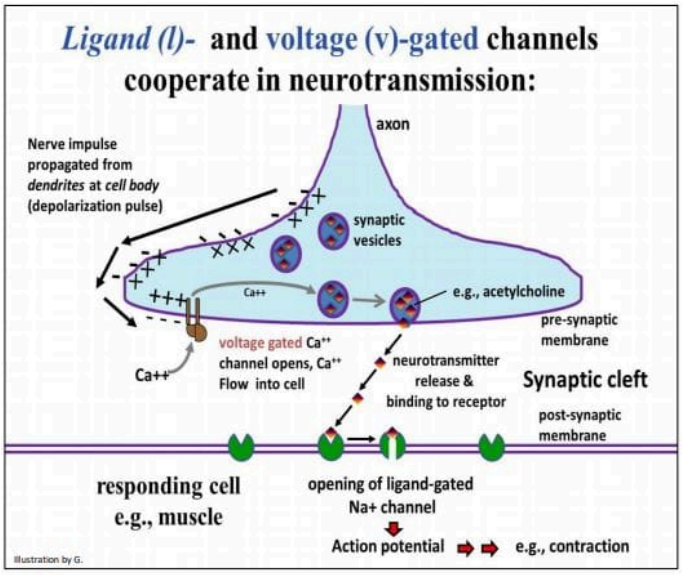
Together with the higher negative ion concentrations in the cytosol, the unequal exchange of Na+ for K+ ions maintains the resting potential of the cell over the long term and ensures that nerve and muscle cells remain excitable. Next, we will take a closer look at the role of both *ligand-gated* and *voltage-gated* ion channels in neurotransmission.

B. Ion Channels in Neurotransmission

Action potentials result in an orderly, sequential opening and closing of *voltage-* and *ligand-gated* channels along the neuronal axon. In the link below, you can see the sequential cycling of voltage-gated channels that propagates a localized action potential (membrane depolarization) along an axon towards a synapse.

302 Propogating an Action Potential Along an Axon

When a propagated depolarization reaches a synapse, gated ion channels either open or close in the neuron and the responding cell. The cooperation of voltage- and ligand-gated channels at a neuromuscular junction is illustrated below.



As you can see from the illustration, after a neuron fires, an electrical impulse (a moving region of *hyperpolarization*) travels down the axon to the nerve ending. At the nerve ending, the traveling charge difference (electrical potential) across the cell membrane stimulates a Ca++ -specific *voltage-gated channel* to open. Ca++ ions then flow into the cell because they are at higher concentrations in the *synaptic cleft* than in the cytoplasm.

The Ca^{2+} ions in the cell cause synaptic vesicles to fuse with the membrane at the nerve ending, releasing neurotransmitters into the synaptic cleft. Then, the neurotransmitters bind to a receptor on the responding cell plasma membrane. This receptor is a **ligand-gated channel** (also called a *chemically-gated channel*). Upon binding of the neurotransmitter ligand, the channel opens. The rapid diffusion of Na+ ions into the cell creates an action potential that leads to the cellular response, in this case, muscle contraction. We have already seen that K+ channels participate in restoring membrane potential after an action potential, and the role of the sodium/potassium pump in restoring the cellular Na^{+}/K^{+} balance.

303 The Role of Gated Ion Channels at Neuromuscular Junction

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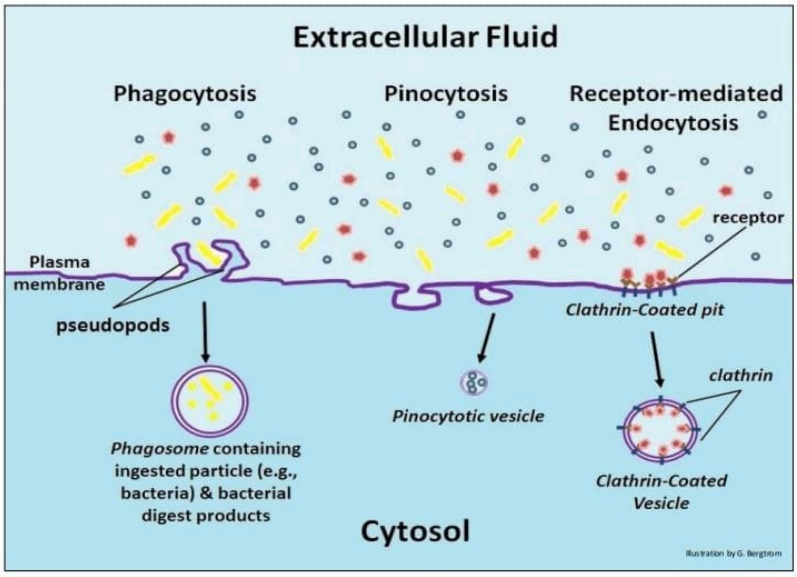
17.4: Endocytosis and Exocytosis

Endocytosis is a mechanism for internalizing large extracellular molecules (e.g., proteins), insoluble particles, or even microorganisms. The three main types of exocytosis are **phagocytosis**, **pinocytosis** and **receptor-mediated endocytosis**. Pinocytosis is non-specific. Phagocytosis targets large structures (e.g., bacteria, food particles...) and is not particularly specific. As its name suggests, receptor-mediated endocytosis is specific for substances recognized by a cell-surface receptor. **Exocytosis** is typically the secretion of large molecules. These could be proteins and glycoproteins like digestive enzymes and many peptide/polypeptide hormones, each of which must exit the cell to either the extracellular fluid or circulation. Exocytotic pathways also deliver membrane proteins made in cells to the cell surface.

A. Endocytosis

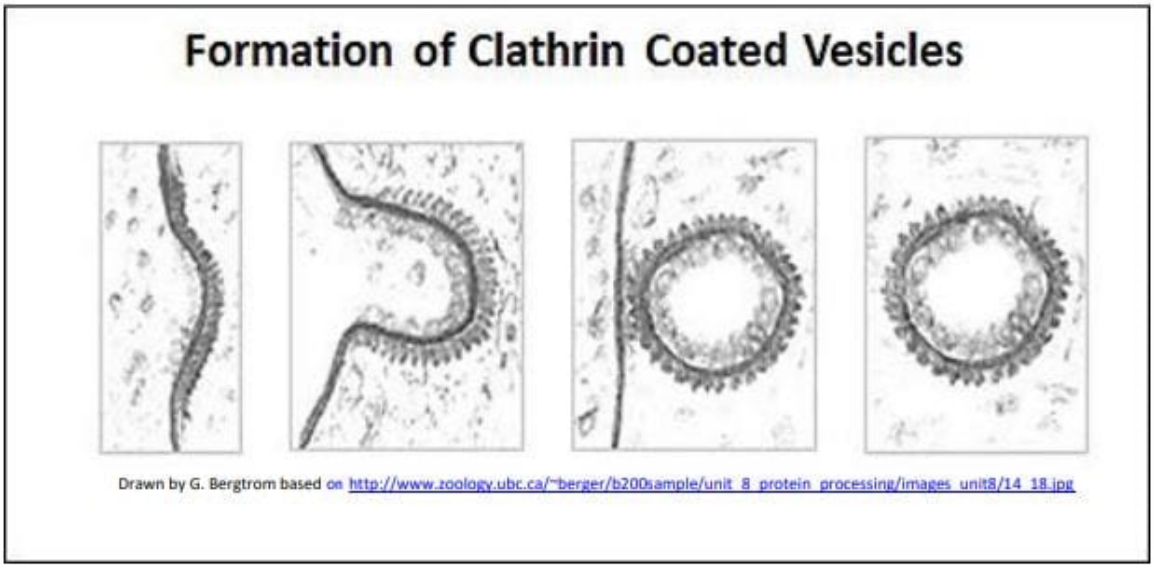
Different forms of endocytosis are illustrated on the next page.

1. **Phagocytosis** (above left): phagocytes extend *pseudopodia* by membrane **evagination**. The pseudopodia of amoeba (and amoeboid cells generally) engulf particles of food that end up in digestive vesicles (**phagosomes**) inside the cytosol. Phagocytes are a class of white blood cells that are part of our immune system. They engulf foreign particles that must be eliminated from the body. A *lysosome* fuses with the phagosome, after which stored hydrolytic enzyme are activated. The result is the digestion of the engulfed particles. Phagocytosis begins upon contact between the outer cell surface and those particles. The main kinds of endocytosis are phagocytosis, pinocytosis and receptor-mediated endocytosis, shown below.

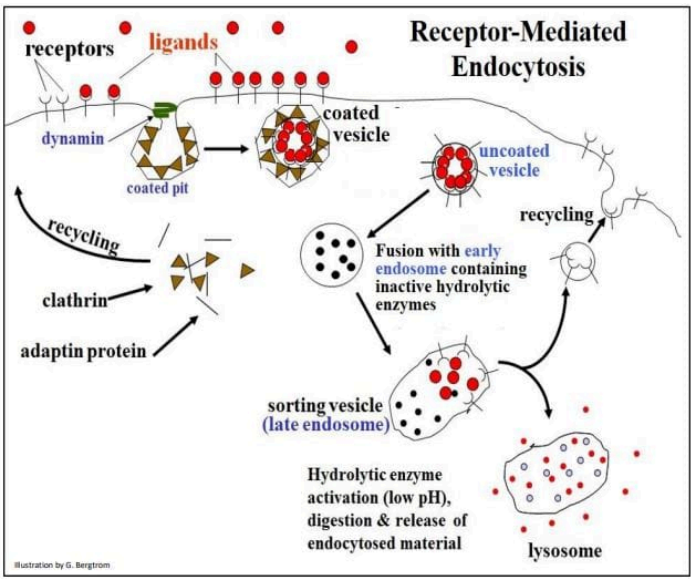


2. **Pinocytosis** (above center): pinocytosis is a non-specific, more or less constant pinching off of small vesicles that engulf extracellular fluid containing solutes; they are too small to include significant particulates.

3. **Receptor-mediated endocytosis** (above right): this kind of endocytosis relies on the affinity of **receptors** for specific extracellular substances. Upon binding their ligands, the receptors aggregate in differentiated regions of cell membrane called **coated pits**. The coated pits then invaginate and pinch off, forming a **coated vesicle**, thereby bringing their extracellular contents into the cell. After the coated vesicles deliver their contents to their cellular destinations, the vesicle membranes are recycled to the plasma membrane. Receptor-mediated endocytosis is perhaps the best understood mechanism for bringing larger substances into cells. The drawings below are taken from a series of electron micrographs that illustrates the invagination of coated pits to form *clathrin-coated vesicles*. The receptor and coat proteins are clearly visible as larger structures on the inner surfaces of the pits and on the outer surfaces of the clathrin-coated vesicles.



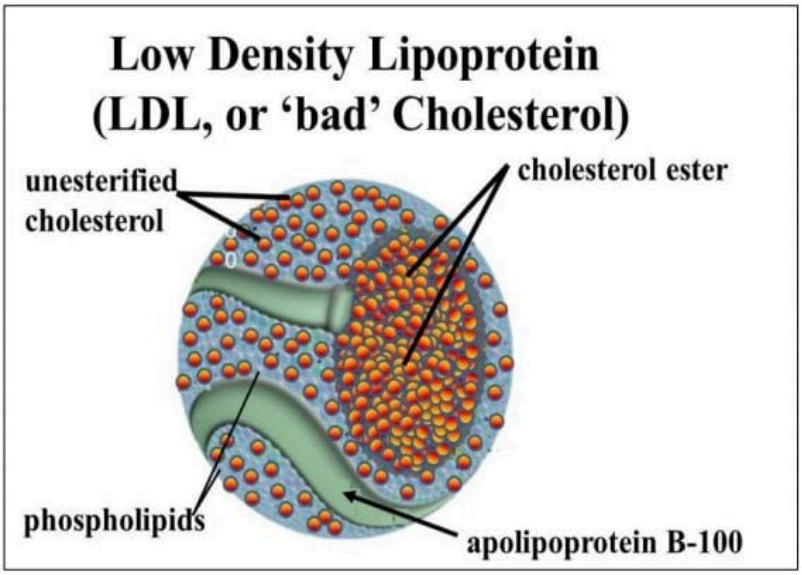
Watch fluorescently labeled proteins enter cells by receptor-mediated endocytosis live by following the bright spots in the video loop at Receptor-mediated endocytosis. **Clathrin**, a large protein, is the principal protein on the surface of the invaginated coated pit. Clathrin is linked to specific integral membrane proteins via **adaptor protein 1** (**AP1**). *AP1* recruits specific *cargo proteins* to bring into the cell when the coated pits invaginate. Some details of receptor-mediated endocytosis are illustrated below.



In the illustration, substances to be internalized have bound to their cell membrane receptors. The receptors then cluster to form a **coated pit**. Assisted by the protein **dynamin** (a GTPase), the coated pits invaginate. The final pinch-off of a **coated vesicle** requires GTP hydrolysis (not shown).

Once internalized, the coated vesicles lose their clathrin and associated adaptor protein coat. The uncoated vesicle fuses with an **early endosome** to form a **sorting vesicle** (i.e., *late endosome*). Sorting vesicles separate imported content from the receptors that are recycled to the membrane. In the vesicle that remains, now a **lysosome**, digestive enzymes catalyze hydrolysis of the vesicle contents. The digest products are then released for cellular use.

A well-known example of receptor-mediated endocytosis is the uptake of cholesterol bound to **low density lipoprotein** (**LDL**), a complex of phospholipid, protein and cholesterol illustrated below.

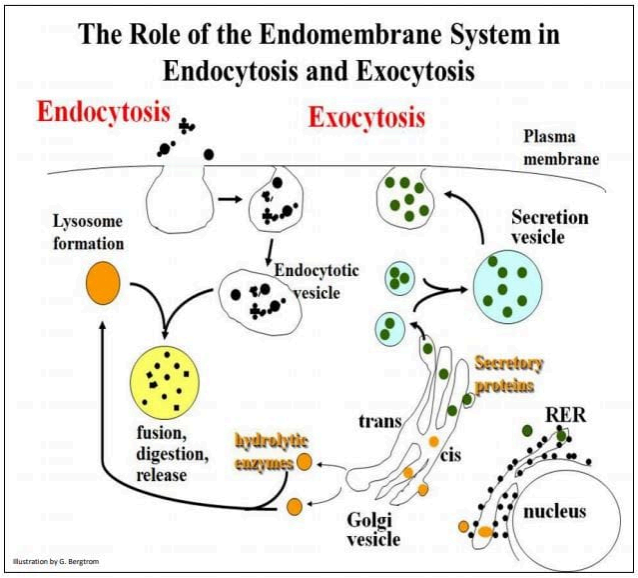


A single LDL complex carries as many as 15,000 molecules of cholesterol. LDL, sometimes called "bad cholesterol", is not good for you at high levels. On the other hand, high-density lipoprotein (HDL) is "good cholesterol". As one gets older, it is important to monitor one's HDL/LDL ratio; the higher it is the better!

B. Exocytosis

Maintaining cell size or volume seems to be a built-in component of the machinery of receptor-mediated endocytosis that balances endocytosis with membrane recycling. However, exocytosis is also necessary to restore plasma membrane internalized by pinocytosis and phagocytosis, and for eliminating cellular waste products. Exocytosis is also the end-point of a complex process of packaging proteins destined for secretion or for insertion into the membrane themselves. The pathways of exocytosis and endocytosis share common features, as illustrated on the next page.

Note that the formation of both lysosomes and secretion vesicles begins in the rough endoplasmic reticulum, followed by passage and maturation through Golgi vesicles. While *endocytotic vesicles* and *secretion vesicles* form in 'opposite directions', they both share common structural features with the plasma membrane, from which they are derived and with which they fuse (*respectively*).



The table on the next page lists some representative proteins packaged for secretion or destined to live in cell membranes.

Hormones	Immune System Proteins	Neurotransmitters	Other
Insulin	IgG (immunoglobulin G, a class of circulating antibodies)	acetylcholine	EGF (Epidermal growth factor)
Growth Hormone	IgM and other cell membrane antibodies		NGF (neural growth factor)
FSH (follicle stimulating hormone)	MHC (major histocompatability complex) proteins on cell surfaces	dopamine, adrenaline, noradrenaline & other monoamines	Fibrinogen (& other blood clotting factors)
Oxytocin		serotonin	Fibronectin (and other extracellular matrix proteins)
Prolactin		some amino acids (glutamate, aspartate, glycine)	Plant cell wall components
ACTH (adrenocorticotropic hormone)			Trypsin, pepsin, et al. (digestive enzymes of the gut)

As we have seen, many secretory and membrane proteins are glycoproteins, to which sugars are covalently attached starting in the rough endoplasmic reticulum. As we have also seen, their glycosylation begins in the RER. Check the link below to see the process again.

291 The Path to Sugar Coated Cells

Individual cells often produce more than a few packaged proteins at the same time, requiring the sorting of each protein to its correct destination – extracellular fluids, lysosomes, peroxisomes and other 'microbodies', and of course, membranes themselves. Next we consider how cells target proteins to their different intracellular and extracellular destinations.

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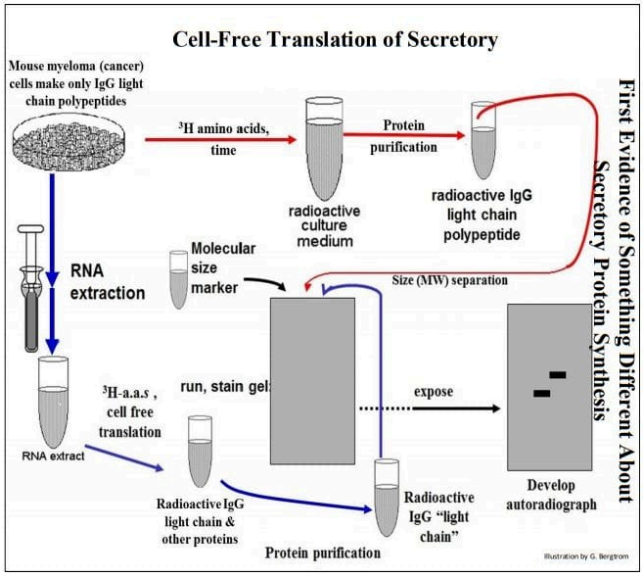
17.5: Directing the Traffic of Proteins in Cells

Each polypeptide protein translated by ribosomes from a sequence of bases in an mRNA has a specific functional location, either in the cytoplasm, on cellular membranes, inside organelles or in extracellular fluids. In this section we consider the movement and sorting of proteins in the endomembrane system as well as the transport of proteins into and out of organelles

A. Packaging Proteins in the RER

All protein synthesis begins in the same way, with the formation of an initiation complex and subsequent elongation cycles peptide bond formation and carboxylterminal amino acid addition. However, secretory proteins and those destined for lysosomes, peroxisomes or other microbodies, complete elongation directly into cisternae, or spaces enclosed by the **rough endoplasmic reticulum** (**RER**). It is possible to isolate and purify proteins secreted by cultured cells. A good model system for studying secretory protein synthesis turn out to be mouse myeloma cells.

Mouse myeloma cells were isolated and grown in culture, where they synthesize an *IgG light chain*, a polypeptide that is part of a mouse **immunoglobulin** molecule. Immunoglobulins are assembled from light and heavy chain polypeptides and secreted into the circulation. There they serve as circulating **antibodies** of the vertebrate immune system. Mouse myeloma cells are cancer cells that have lost the ability to make the heavy chain polypeptides. Instead, they secrete mostly the IgG light chain, making it easy to purify it from the cell culture medium. An early experiment revealed that secreted polypeptides made in an *in vitro* translation system are larger (longer) than the same polypeptides isolated from secretion fluids. This experiment is summarized on the next page.

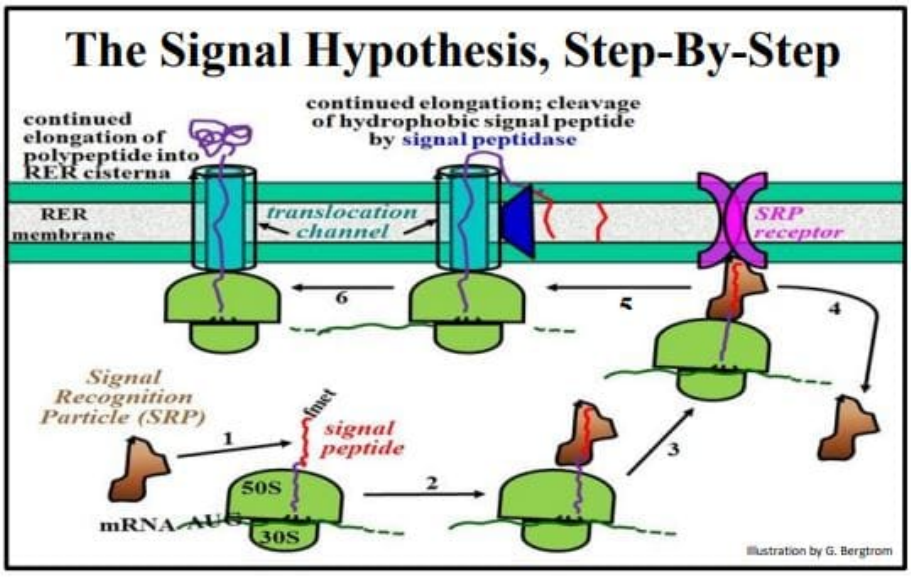


In one part of the experiment described above, myeloma cells were grown in the presence of radioactive amino acids. The resulting radioactive IgG light chain polypeptides were isolated (follow the red arrows). mRNA separately extracted from another batch of the myeloma cells was added to a cell-free translation system containing radioactive amino acids. The radioactive polypeptide synthesized *in vivo* and *in vitro* were separated on electrophoretic gels and autoradiographed (follow the blue arrows, above).

From the autoradiograph, the mature, secreted polypeptides made *in vivo* had migrated faster on the gel than had those translated *in vitro*. The cell-free translation product was indeed, larger than the *mature* secreted polypeptide. To explain these results, Gunther Blobel and colleagues suggested the **Signal Hypothesis**, according to which secretory protein genes encode extra amino acids as a short amino-terminal **signal peptide** that directs a growing secretory polypeptide to the RER. To explain the smaller (i.e., shorter) length of the mature, secreted polypeptide, they further proposed that the *signal peptide* is only a temporary 'traffic' signal, removed by an RER-associated enzyme as the polypeptide crossed the RER membrane into the cisternal space.

304 Formulating the Signal Hypothesis: Early Experiments

In the test of the *Signal Hypothesis* (which won Blobel the 1999 Nobel Prize in Physiology or Medicine), isolated RER membranes were included with mouse myeloma cell mRNA in cell-free protein synthesis systems. Electrophoretic autoradiographs this time showed that the polypeptides made *in vitro* in the presence of RER were the same size as the mature, secreted polypeptides. The RER must therefore contain *processing activity*, i.e., a **signal peptidase** that removes the signal peptide! The steps of the signal hypothesis that emerged from the experiments of Blobel and his colleagues are illustrated below.



Recall that the synthesis of any protein starts with assembly of a *translation initiation complex*, followed by polypeptide elongation. During elongation, the growing polypeptide moves through and emerges from a channel, or groove in the large subunit. As the N-terminal **signal sequence** (i.e., the signal peptide) of a secretory polypeptide emerges from this groove, it interacts with the RER membrane. Beginning at the lower left of the illustration above, the steps of the process are:

1. An **SRP** (**signal recognition particle**) binds to the hydrophobic *signal peptide*.
2. Elongation stops until the SRP-ribosome complex finds the RER membrane.
3. The ribosome-SRP complex binds to an **SRP receptor** on the RER membrane.
4. The SRP detaches from the growing polypeptide chain, to be recycled.
5. Translation elongation resumes through a **translocation channel**; a **signal peptidase** in the RER membrane catalyzes **co-translational** hydrolysis of the signal peptide, which remains embedded in the RER membrane.
6. Elongation continues and the growing polypeptide begins to fold in the RER.

305 Testing the Signal Hypothesis

306 Details of the Signal Hypothesis

Step 2 above requires that the SRP find and bind to the signal peptide before the nascent polypeptide gets too long and starts to fold into a 3D (tertiary) conformation. It turns out the ribosome itself may keep the signal peptide available by destabilizing electrostatic interactions that would otherwise lead to folding and an undoubtedly incorrect conformation. For more on ribosome involvement in protein folding, check out the link at Protein Folding-Destabilizing One Protein Strand at a Time.

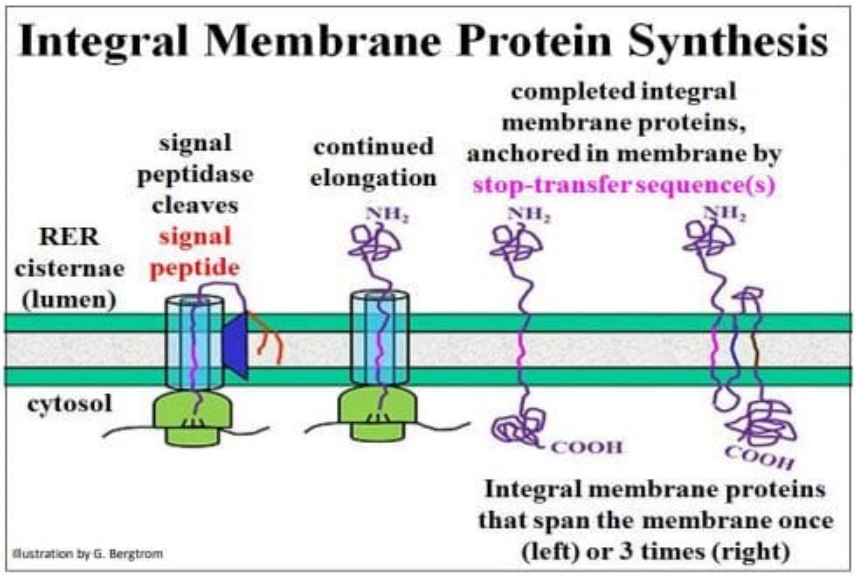
The secretory mechanism just described for eukaryotes has its counterpart in bacteria, which secrete proteins that assist in nutrient scavenging as well as cell wall synthesis. Partially elongated signal peptides guide mRNA-bound ribosomes to the cytoplasmic side of the plasma membrane, where the ribosomes bind and then pass elongating proteins through the plasma membrane into the space between the cell membrane and wall. As the protein exits the cell, a bacterial signal peptidase (**SPase**) cleaves the signal peptide. Apparently, the mechanism for the secretion of proteins evolved early and since been conserved. As we will see, this mechanism has been further coopted by eukaryotes for packaging proteins into some organelles and into membranes themselves. Some interesting speculations on the evolution of the protein packaging pathway are discussed in the link below.

307 Destinations of Protein Traffic and Evolution of Pathways

Early on, we discovered that antibiotics stop bacterial growth either by disrupting the cell wall or otherwise killing the cells outright. We now know that some antibiotics (e.g., *arylomycins*) disrupt plasma membrane *SPase* function, preventing proteins required in the space between the cell wall and membrane from ever making it out of the cell. Once used against **Staphylococcus aurease**, arylomycins are no longer effective because many strains have become resistant to these antibiotics (click Bacterial Signal Peptidase and Antibiotic Resistance to read about the mechanism of *arylomycin* resistance). As you may already know, *S. aurease* is now resistant to many antibiotics, and illness from untreatable infections has its own name, **MRSA** (Methicillin-Resistant Staph Aurease - dig on your own to see more about methicillin resistance). While named for *methicillin* resistance, MRSA now describes *nearly* untreatable *S. aurease* infections.

B. Synthesis of Membrane-Spanning (Integral) Proteins

N-terminal signal sequences also guide ribosomes translating *integral membrane proteins* to the RER. However, before such a protein can pass completely through the membrane, a **stop-transfer** sequence (a hydrophobic domain within the polypeptide chain) traps the protein in the fatty acid interior of the membrane. Multiple stoptransfer sequences account for transmembrane proteins that span a membrane more than once (below).



308 Integral Membrane Proteins Hae Stop Transfer Sequences

C. Moving and Sorting Packaged Proteins to Their Final Destination

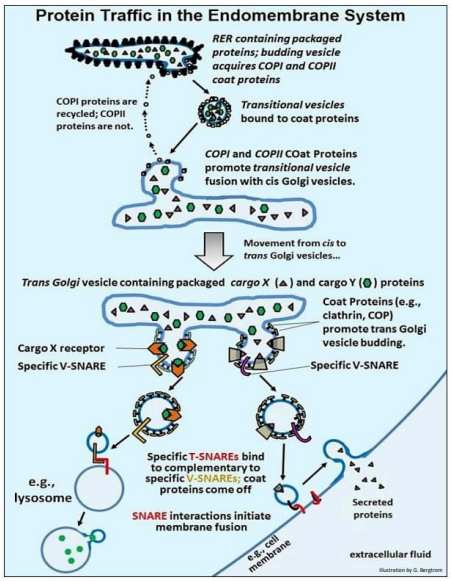
Like proteins packaged in RER, those made in the cytoplasm go to different destinations before they become functional. Let's look at the sorting mechanisms for proteins sequestered by the endomembrane system and those made in the cytoplasm.

1. Traffic on the Endomembrane Highway

We have already seen that, once packaged in the RER cisternae, proteins begin post-translational modification (by e.g., 'core glycosylation'). Transport vesicles that bud off from the RER carry packaged and membrane proteins to the cis vesicles of the Golgi apparatus. There, vesicle fusion is mediated by the recognition of complementary integral membrane proteins embedded in the two membranes. Later, such packaged proteins are sorted to different organelles or to the plasma membrane. Sorting starts as proteins move from the cis to the trans face of the Golgi vesicles, where specific sorting proteins associate with different packaged proteins in the trans Golgi vesicles. The packaged proteins then sort to vesicles that bud off from trans Golgi stacks. These vesicles move to their final destinations, recognizing and fusing with appropriate membranes. Some events of protein trafficking are animated at Events in Protein Trafficking and summarized in the illustration on the next page.

James E. Rothman, Randy W. Schekman and Thomas C. Südhof won the 2013 Nobel Prize in Physiology or Medicine for their studies of the regulation of vesicle traffic (click 2013 Nobel Prize in Physiology or Medicine for more information). Let's follow some proteins in and on RER membranes through the cell:

- **Transition vesicles** carrying their mix of packaged proteins bud off from the RER with the help of **COPI** and **COPII** coat proteins, and dissociate from the ribosomes originally attached to them. Transition vesicles however, remain associated with the COP proteins.
- These vesicles fuse with the *cis* Golgi vesicles, a process also mediated by COP proteins. COPI proteins detach during or after fusion, to be recycled back to the RER .
- Packaged proteins and membrane proteins are further processed as the pass through the Golgi vesicle stack, for example undergoing terminal glycosylation.



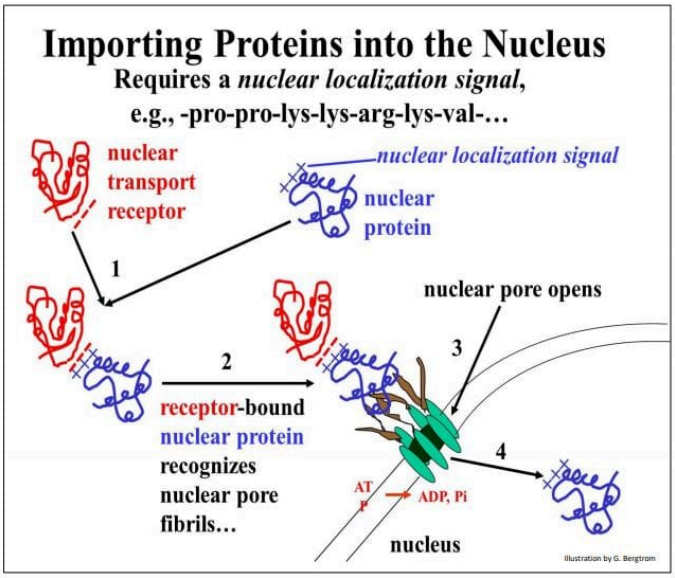
- At the *trans* face of the Golgi vesicles, **cargo receptor** proteins in the membranes to bind specific packaged proteins (now called **cargo proteins**). With the help of **clathrin** and other *COP proteins*, cargo protein-bound receptor proteins bud off from the trans Golgi stack. However this time, specific cargo proteins sort to separate vesicles with different cellular or extracellular destinations. These budding vesicles also acquire membrane **V-SNARE** (for vesicle-SNARE) proteins.
- When **V-SNARE** proteins on their vesicles bind to complementary **T-SNARE** (for target-SNARE) proteins on receiving membranes, the membranes fuse.
  - Some vesicles follow this pathway, fusing with **lysosomes** or similar vesicles to stock them with appropriate enzymes and other protein content. Coat proteins come off the fusing vesicle and are recycled, while vesicle contents are transferred into the next vesicle.
  - Vesicles containing secretory proteins typically fuse to form larger **secretory vesicles**. Secretory vesicles can be stored until the cells are signaled to release their contents from the cell. At that point, secretion vesicles fuse with the plasma membrane, releasing their contents to the extracellular fluid. Once again, coat proteins and clathrin dissociate from the secretory vesicle during fusion.

Other players have been left out of this discussion, notably those that hydrolyze nucleotide triphosphates to provide the energy for this protein trafficking. In addition, you might recognize other molecular players such as clathrin that play a role receptor-mediated endocytosis. Maybe that's not a surprise! After all, endocytosis is, at least partly, molecular traffic in the opposite direction of vesicle formation and secretion.

2. Nuclear Protein Traffic

Almost all proteins are encoded in the nucleus and translated in the cytosol. These include most of those found in nucleus itself, as well as in mitochondria and chloroplasts (see the *Endosymbiotic Hypothesis* for a description of intraorganelle gene expression). Proteins synthesized in the cytosol destined for these organelles contain oligopeptide traffic signals that direct them to their appropriate destinations. We saw earlier that large molecules (mRNAs, tRNAs) and even whole particles (i.e., ribosomal subunits) cross the nuclear envelope through nuclear pores.

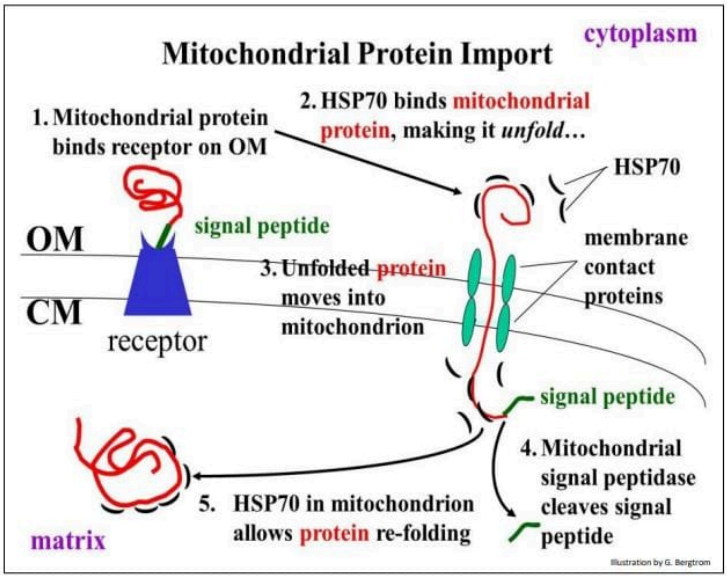
As for proteins headed for the nucleus, **nuclear localization signals** rich in positively charged amino acids (lysine, proline) enable binding to the negatively charged domain of a **nuclear transport receptor** protein in the cytosol. This process is illustrated below.



As the complex of the two proteins approach a nuclear pore, it interacts with **nuclear pore fibrils**, causing the pore to open. The two bound proteins then cross the double membrane of the nuclear envelope where they accumulate against a concentration gradient. This active transport comes from ATP hydrolysis as the nuclear proteins enter the nucleus.

3. Mitochondrial Protein Traffic

Recall that mitochondria contain their own genome and translational machinery. Thus, they transcribe RNAs and translating proteins of their own. However, genes in the nucleus encode many of the proteins found in mitochondria. Import of these proteins into mitochondria is illustrated below.



Unlike the co-translational packaging of proteins by the RER, mitochondrial protein transfer is post-translational. This means that mitochondrial proteins formed in the cytoplasm have already folded, assuming a tertiary structure. However, the folded protein exposes an N-terminal **signal peptide** on its surface that recognizes and binds to a **receptor protein** at the outer mitochondrial membrane. The *receptor protein* spans both the mitochondrial *outer membrane* (OM) and *cristal membrane* (CM).

The receptor protein delivers the protein to **membrane contact proteins** that also span both mitochondrial membranes. The membrane contact proteins acts as a channel, or pore, through which the mitochondrial protein will cross into the mitochondrial matrix.

But there is a problem: the folded protein *cannot* cross the membrane by itself! The entry of a completed mitochondrial protein in the cytoplasm requires a so-called **chaperone** protein, in this case the **HSP70** (*heat-shock 70*) protein. *HSP70* controls *unfolding* of the mitochondrial protein as it passes into the matrix. Upon removal of the signal peptide by a mitochondrial **signal peptidase**, another HSP70 molecule resident in the mitochondrion facilitates *refolding* of the protein into a biologically active shape. Recall that HSPs were initially discovered in heat

309 Protein Traffic to Nuclei and How They Communicate

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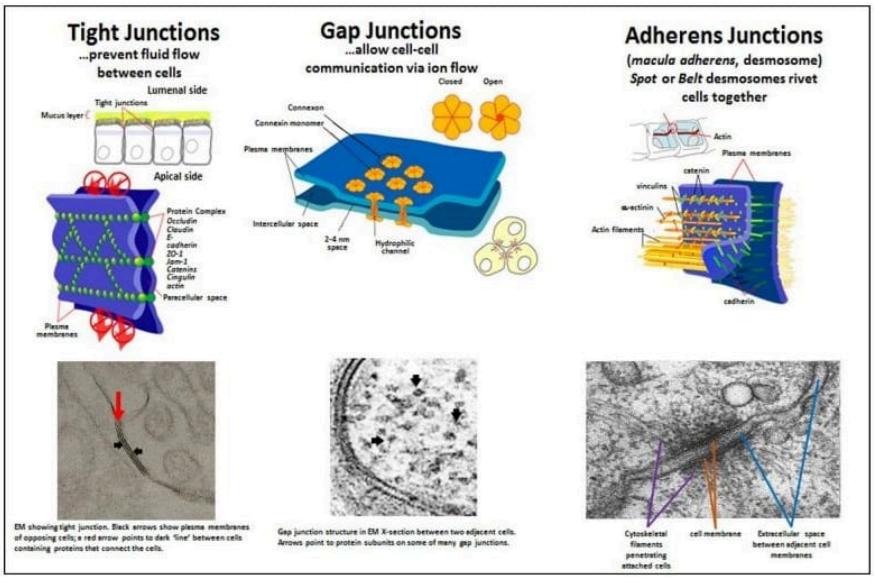
17.6: How Cells are Held Together and How they Communicate

Proteins and glycoproteins on cell surfaces play a major role in how cells interact with their surroundings and with other cells. Some of the proteins in the **glycocalyx** of adjacent cells interact to form cell-cell junctions, while others interact with extracellular proteins and carbohydrates to form the **extracellular matrix** (**ECM**). Still others are part of receptor systems that bind hormones and other signaling molecules at the cell surface, conveying information into the cell by **signal transduction**.

A. Cell Junctions

Cell junctions serve different functions in cells and tissues. Cell junctions in healthy cells serve to bind cells tightly, to give tissues structural integrity and to allow cells in contact with one another to pass chemical information directly between them. Electron micrographs and illustrations different cell junctions are shown on the next page.

- **Tight Junctions** (*zonula occludens*) are typical in sheets of epithelial cells that line the *lumens* of organs (e.g., intestines, lungs, etc.). *Zonula* refers to the fact that these structures form a band encircling an entire cell, attaching it to all surrounding cells. *Occludens* refers to 'water-tight' seal or *occluding barrier* of tight junctions, that stops extracellular fluids from crossing to the other side of a sheet of cells by passing between cells. **Tight junction membrane proteins** (**TJMPs**) create the waterproof barrier between cells.



- **Desmosomes** (*adherens junctions*) essentially glue (adhere) cells together, giving tissues their strength. **Belt desmosomes** (*zonula adherens*) surround entire cells, strongly binding them to adjacent cells. Spot desmosomes (*macula adherens*) act like rivets, attaching cells at 'spots'. In both cases, **cadherins** cross cell membranes from intracellular **plaque** proteins, spanning the intercellular space to link adjacent cell membranes together. Plaques are in turn, connected to intermediate filaments (keratin) of the cytoskeleton, further strengthening intercellular attachments and thus, the tissue cell layer.
- **Gap junctions**, the third cell junction, do not so much physically bind cells together as enable chemical communication between cells. **Connexon** structures made of **connexin** proteins act as pores that open to allow direct movement of ions and small molecules between cells. This communication by ion or molecular movement is quite rapid, ensuring that all cells in a sheet or other tissue in one metabolic state can respond to each other and switch to another state simultaneously. In plants, we have seen the plasmodesmata that perform functions similar to the gap junctions of animal cells.

310 Cell Junction Structure and Function

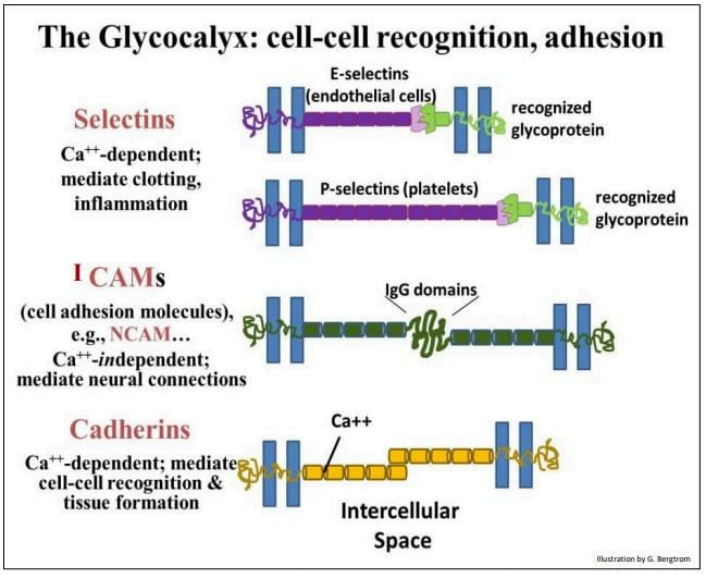
Many glycocalyx proteins that interact to form junctions between cells are glycoproteins. Generally, proteins that interact to bind

cells together are called **I**ntercellular **C**ell **A**dhesion **M**olecules (**ICAM**s). These include **selectins**. During blood clotting, selectins on one platelet recognize and bind specific receptors on other platelets, contributing to the clot. **NCAMs** are another kind of ICAM, ones with sugary immunoglobulin domains that interact specifically to enable neural connections. We've already seen the calcium-dependent **cadherins** involved in forming adherens junctions (desmosomes). These are essentially the 'glue' that binds cells together to form strong cohesive tissues and sheets of cells.

Some examples of membrane proteins that enable cell-cell recognition and adhesion are illustrated on the next page.

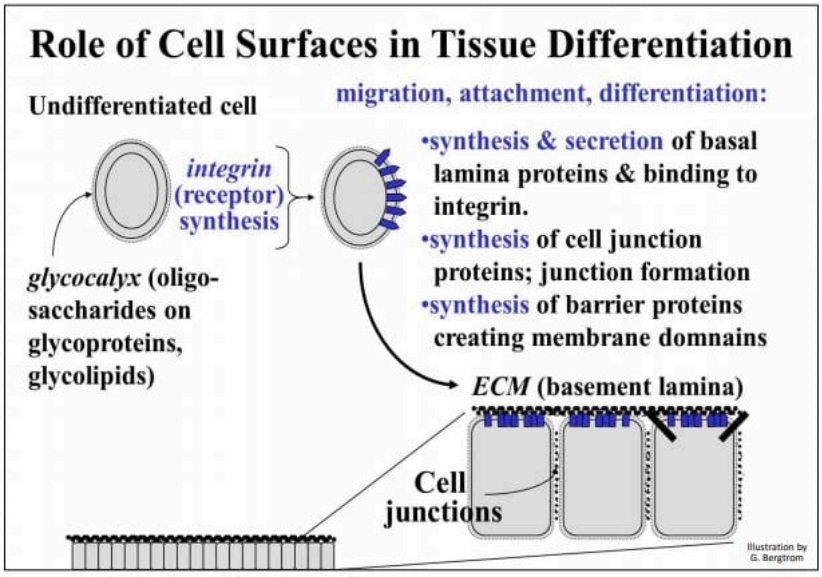
311 Glycocalyx: Sugars Covalently Linked to Plasma Membrane Proteins

312 Cell Adhesion Molecule Functions in the Glycocalyx



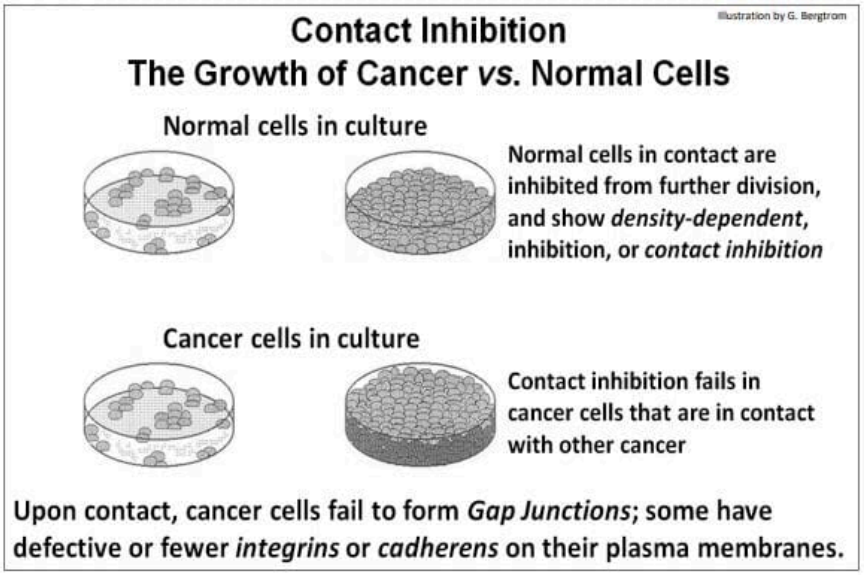
B. Cancer and Cell Junctions

During embryogenesis, cells migrate from a point of origin by attaching to and moving along an **extracellular matrix** (**ECM**), which acts as a path to the cell's final destination. This ECM (or basal lamina) is made up of secretions from other cells..., or from the migrating cells themselves! One major secretion is **fibronectin**. One of its functions is to bind to integral membrane proteins called **integrins**, attaching the cells to the ECM. During development, integrins respond to fibronectin by signaling cell and tissue differentiation, complete with the formation of appropriate cell junctions. An orderly sequence of gene expression and membrane protein syntheses enable developing cells to recognize each other as different or the same. The influences of cell surfaces on tissue differentiation are summarized below.



An early difference between eukaryotic normal and cancer cells is how they grow in culture. Normal cells settle to the bottom of a culture dish when placed in growth medium. Then they grow and divide, increasing in number until they reach **confluence**, when a single layer of cells completely covers the bottom of the dish. The cells in this monolayer seem to 'know' to stop dividing, as if they had completed formation of a tissue, e.g., a cell layer of epithelial cells. This phenomenon, originally called **contact inhibition**, implies that cells let each other know when they have finished forming a tissue and can stop cycling and dividing. In contrast, cancer cells do not stop dividing at confluence. Instead, they continue to grow and divide, piling up in multiple layers.

Among other deficiencies, cancer cells do not form *gap junctions* and typically have fewer *cadherens* and *integrins* in their membranes. Thus, cancer cells cannot inform each other of when they reach confluence. Neither can they form firm *adherens junctions*. *In vivo*, a paucity of integrins would inhibit cancer cells from binding and responding to *fibronectin*. Therefore they also have difficulty attaching firmly to an extracellular matrix, which may explain why many cancers metastasize, or spread from their original site of formation. These differences in growth in culture between normal and cancer cells are shown below.



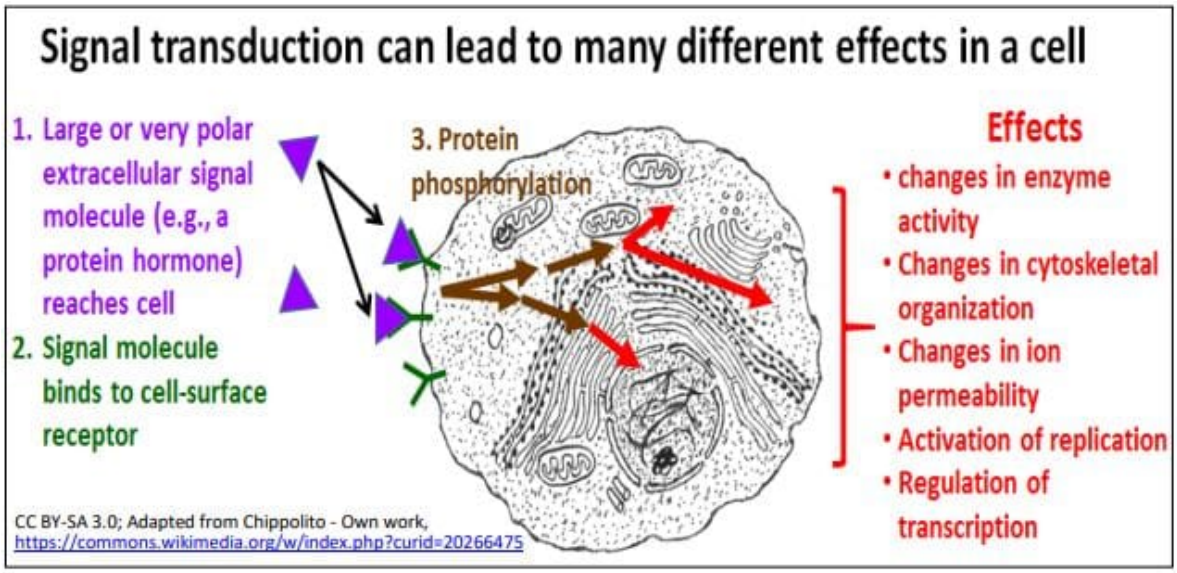
313 Formation of a Glycocalyx, Normal Development and Cancer

314 Role of the Extracellular Matrix in Cell Migration and Development

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17.7: 17.7 Signal Transduction

When hydrophobic chemical effector molecules such as steroid hormones reach a target cell they can cross the hydrophobic membrane and bind to an intracellular receptor to initiate a response. When large **effector** molecules (e.g., protein hormones) or highly polar hormones (e.g., adrenalin) reach a target cell, they can't cross the cell membrane. Instead, they bind to transmembrane protein receptors on cell surfaces. A conformational change initiated on the extracellular domain of the receptor induces further allosteric change on the cytoplasmic domain of the receptor. A sequential series of molecular events then converts information delivered by the external effector into intracellular information, a process called **signal transduction**. A general outline of signal transduction events is illustrated below.



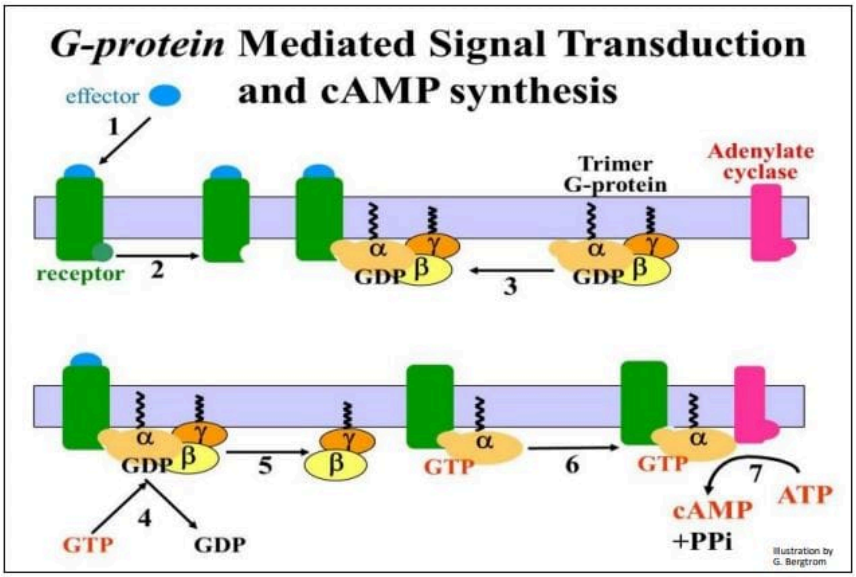
Many effects of signal transduction are mediated by a sequence, or *cascade* of protein phosphorylations catalyzed by *protein kinases* inside the cell. Here we will consider **G Protein-linked** and **enzyme-linked receptors**.

315 Introduction to Signal Transduction

A. G-Protein Mediated Signal Transduction by PKA (Protein Kinase A)

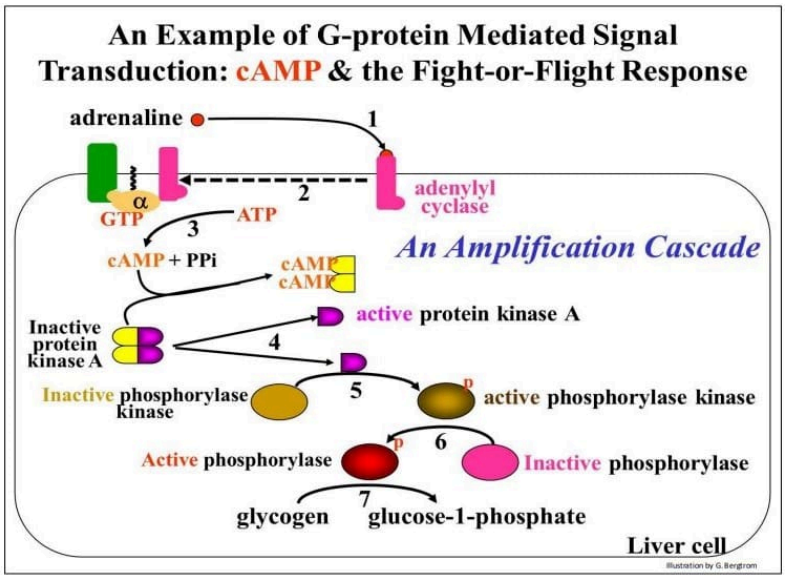
GTP-binding proteins (**G-Proteins**) transduce extracellular signals by inducing production of **second messenger** molecules in the cells. When hormones or other effector (signal) molecules bind to their membrane receptors, an allosteric change on the *cytoplasmic* domain of the receptor increases the affinity of the cytoplasmic domain the receptor for G proteins on the inner plasma membrane surface. G proteins are *trimers* consisting of α, β and γ subunits, embedded in the cytoplasmic surface of responsive cell membranes. G-protein-mediated signal transduction is illustrated in the seven steps shown on the next page.

The receptor changes shape upon binding its effector signal molecule (steps 1, 2). In this conformation, the receptor recognizes and binds to the G-protein trimer on the cytoplasmic surface of the plasma membrane (step 3). Upon binding of the trimer to the receptor, GTP displaces GDP on the α **subunit** of the G-protein (step 4).



After a conformational change, the α **subunit** dissociates from the β and γ subunits (step 5). In this illustration, the GTP-α subunit can now bind to a transmembrane enzyme, **adenylate cyclase** (step 6). Finally, the initial extracellular chemical signal is **transduced** to an intracellular response involving second messenger molecules (step 7). In this case, the second messenger is **cAMP**. The well-known **fight-or-flight** response to adrenaline in liver cells of higher animals is a good example of a cAMPmediated cellular response. After adrenalin binds to its receptors, G-proteins in turn bind to the cytoplasmic side of the receptor, which then binds to adenylate cyclase. cAMP binds to and activates **protein kinase A** (**PKA**), setting off the **amplification cascade** response. Some details of a G-protein mediated signal amplification cascade are detailed in the illustration on the next page.

After activation of adenylate cyclase (steps 1 and 2 in the drawing), cAMP is synthesized and binds to two of the four subunits of an **inactive PKA** (step 3). A conformational change dissociates the tetramer into two cAMP-bound inert subunits and two **active PKA** subunits (step 4). Each *active PKA* enzyme catalyzes phosphorylation and activation of an enzyme called **phosphorylase kinase** (step 5).



In step 6, phosphorylase kinase catalyzes **glycogen phosphorylase** phosphorylation. Finally, at the end of the *phosphorylation cascade*, the now active *glycogen phosphorylase* catalyzes the hydrolysis glycogen to glucose-1-phosphate (step 7). This results in a rapid retrieval free glucose from liver cells into the circulation. Remind yourself of how this works by reviewing the conversion of glucose-1 phosphate (G-1-P) to G-6-P in glycolysis and its fate in gluconeogenesis. Of course, the increase in circulating glucose

provides the energy for the *fight-or-flight* decision.

317 G-Protein Activation of Protein Kinase A and a *Fight-or-Flight* Response

In addition to activating enzymes that break down glycogen, cAMP-activated PKA mediates cellular responses to different effectors resulting in a phosphorylation cascade leading to

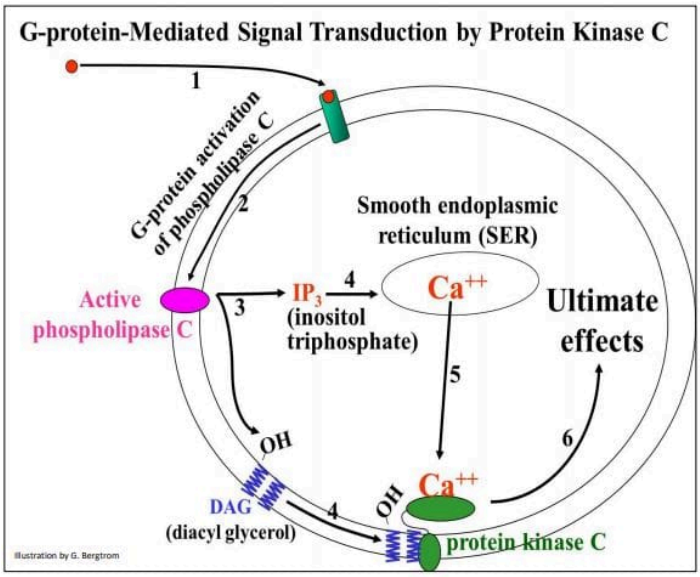
- Activation of enzymes catalyzing glycogen synthesis.
- Activation of *lipases* that hydrolyze fatty acids from triglycerides.
- Microtubule assembly.
- Microtubule disassembly.
- Mitogenic effects (activation of enzymes of replication).
- Activation of transcription factors increasing/decreasing gene expression.

Of course, when the cellular response is no longer needed by the organism, it must stop producing the signal molecules (hormone or other effector). As their levels drop, effector molecules dissociate from their receptors and the response stops. This is all possible because binding of signals to their receptors is freely reversible! This is animated for G-protein based signal transduction in the link below.

316 G-protein Signal Transduction

B. Signal Transduction using PKC

Many responses involving G-proteins begin by activating the integral membrane *adenylate cyclase*. A different G-protein-mediated signaling pathway generates other second messengers. **Protein kinase C** (**PKC**) plays a major roles in the activating these other second messengers and subsequent **phosphorylation cascades** in which the activation of just a few enzyme molecules in the cell results in the activation of many more enzymes. Like *PKA*, *PKC*-mediated signal transduction also **amplifies** the cell's first molecular response to the effector. The role of G-proteins is similar for PKA and **PKC signal transduction**. Responses can include diverse effects in different cells..., or even in the same cells using different effector signals. But, PKC and PKA signal transduction differ in that PKC activation requires an additional step, as well as the generation of two intracellular messenger molecules. The events leading to the activation of **PKC** are illustrated below.



Here are details of the steps leading to **PKC** activation. An effector signal molecule binds to its receptor, activating an integral membrane **phospholipase C** enzyme. *Phospholipase C* catalyzes formation of cytosolic **inositol triphosphate** (**IP_3**) and membrane bound **diacyl glycerol** (**DAG**), two of those other intracellular second messenger molecules. IP3 interacts with receptors on smooth endoplasmic reticulum, causing the release of sequestered Ca++ ions into the cytoplasm. Finally, **Ca^{2+}** ions and DAG activate **Protein Kinase C** (**PKC**) that then initiates a phosphorylation amplification cascade leading cell-specific responses.

318 G-Protein Activation of Protein Kinase C and Phospholipase C

Protein Kinase C mediated effects include:

- Neurotransmitter release.
- Hormone (growth hormone, leutinizing hormone, testosterone) secretion leading to cell growth, division and differentiation.
- Glycogen hydrolysis, fat synthesis.

Additionaly independent *phospholipase C* effects include:

- Liver glycogen breakdown.
- Pancreatic amylase secretion.
- Platelet aggregation.

PKA and PKC are **serine-threonine kinases** that they place phosphates on serine or threonine in target polypeptides. Let's consider tyrosine kinases next.

C. Receptor Tyrosine Kinase-Mediated Signal Transduction

The intracellular activity of these receptors is in the cytoplasmic domain of the receptor itself. When bound to its effector, receptor-kinase catalyzes phosphorylation of specific tyrosine amino acids in target proteins. While studying the action of **nerve growth factor** (**NGF**) and **epidermal growth factor** (**EGF**) in stimulating growth and differentiation of nerve and skin, Stanley Cohen and Rita Levi-Montalcini discovered the **EGF receptor**, the first *enzyme-linked tyrosine kinase*..., and won the 1986 Nobel Prize in Physiology or Medicine! Watch the animation of receptor kinase signal transduction at the link below (a description is provided in the next few paragraphs).

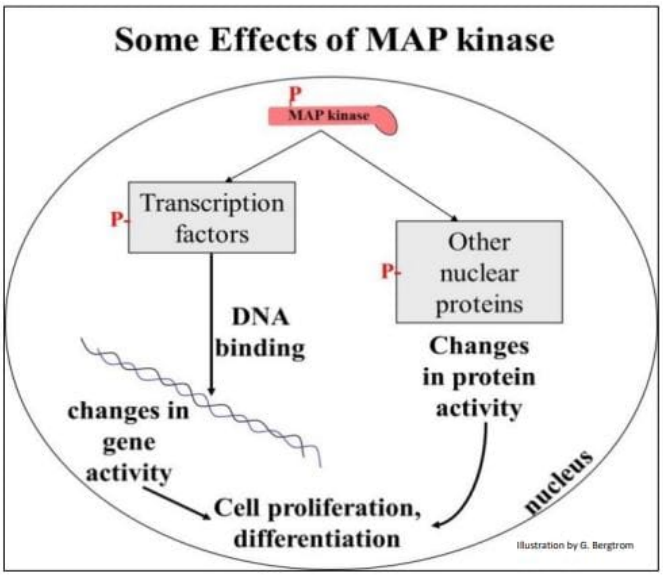
319 Receptor Kinase Signal Transduction

Monomer membrane receptor kinases dimerize when they bind effector ligands, at which point sulfhydryl group-containing **SH_2 proteins** bind to each monomer. This activates the kinase domain of the receptor. After multiple cross-phosphorylations of the receptor monomers, the SH_2 proteins fall away allowing the receptors to interact with other cytoplasmic proteins to continue the response pathway. The characteristic response to EGF and NGF signaling is cellular proliferation. Not surprisingly, mutations correlated with cancer cells often lie in signaling pathways leading to cell proliferation (growth and division). Cancer-causing genes, or **oncogenes**, were actually first discovered in viruses, but J. Michael Bishop and Harold Varmus won the 1964 Nobel Prize in Physiology or Medicine for showing that cells were actually the origin of a chicken retrovirus (the Rous Sarcoma Virus). *Oncogene*s turn out to be mutations of genes for proteins in mitogenic signal transduction pathways. Under normal circumstances, mitogenic chemical signals (like *EGF*) bind to their receptors and induce target cells to begin dividing.

The **Ras** protein-mediated activation of a phosphorylation cascade leading to the **MAP** (**mitogen-activated protein**) **kinase** is an example of such a signal transduction pathway, one with a central role in many receptor kinase signaling pathways. The Ras gene was one of those originally discovered as an oncogene whose mutation leads to uncontrolled cell division, i.e., cancer. Ras gene/protein activity may in fact be responsible for up to 30% of all cancers!

320 The RAS Oncogene, its Normal Mitogenic Effects and Cancer

MAP kinase phosphorylates *transcription factor*s and other nuclear proteins that affect gene activity leading to cell proliferation and differentiation, as shown below.



D. Signal Transduction in Evolution

We saw that signal transduction typically takes a few signal molecules interacting with a few cell surface receptors to amplify a response in a cascade of enzymatic reactions, typically phosphorylations, to activate (or inactivate) target proteins. Amplification cascades can take a single effector-receptor interaction and magnify its effect in the cell by orders of magnitude, making the signaling systems rapid and highly efficient. The range of cellular and systemic (organismic) responses to the same chemical signal is broad and complex. Different cell types can have receptors for the same effector, but respond differently. For example, adrenalin targets cells of the liver and blood vessels among others, with different effects in each. As it happens, adrenaline is also a neurotransmitter. Apparently, as organisms evolved, they became more complex in response to environmental imperatives, adapting by coopting already existing signaling systems in the service of new pathways. Just as the same signal transduction event can lead to different pathways of response in different cells, evolution has allowed different signal transduction pathways to engage in **crosstalk**. This is when two different signal transduction pathways intersect in the same cells. In one example, the cAMP produced at the front end of the PKA signaling pathway can activate (or under the right circumstances, inhibit) enzymes in the MAP kinase pathway. These effects result in changes in the levels of active or inactive transcription factors and can therefore modulate the expression of a gene using two (or more) signals. We are only beginning to understand what looks less like a linear.

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17.8: Key Words and Terms

action potential	fight-or-flight	peroxisomes
active transport	flaccid	phagocytosis
adaptin	free energy	phospholipase C
adenylate cyclase	G protein subunits	phosphorylase kinase
adherens junctions	gap junctions	pinocytosis
adrenaline	gluconeogenesis	PKA
allosteric change regulates transport	GLUT1	PKC
antiport	glycolysis	plasmodesmata
aquaporins	good cholesterol	plamolysis
bad cholesterol	G-protein-linked receptors	poikilothermic organisms
basal lamina	Heat shock protein	potential difference
belt desmosomes	HSP70 protein	protein kinase A
Ca^{2+}	hydrophilic corridor	protein kinase C
cadherin	hypertonic	protein packaging
cargo receptor	hypotonic	protein phosphorylation
carrier proteins	IgG light chain	proton gate
cell adhesion molecules	inositol triphosphate	proton pump
cell-cell attachment	integrin	receptor-mediated endocytosis
cell-cell recognition	ion channels	RER membrane
cell-free translation	ion flow	resting potential
channel proteins	ion pumps	secondary active transporters
chaperone proteins	IP_3	serine-threonine kinases
cholesterol effects in membranes	isotonic	signal peptide
clathrin	LDL (low density lipoprotein)	signal recognition particle
coated pits	ligand (chemically) gated channels	signal sequence
coasted vesicle	lysosome	signal transduction
connexins	MAP kinase	smooth endoplasmic reticulum
contact inhibition	mechanically gated channels	sodium-potassium pump
contractile vacuole	membrane depolarization	solute concentration gradients
COP	membrane hyperpolarization	solute transport
cotransport	membrane invagination	sorting vesicle
coupled transport	membrane potential	spot desmosomes
cytoskeleton	microbodies	stop-transfer sequence
DAG	mitochondrial membrane contact proteins	symport
diffusion kinetics	mitogenic effects	tight junction membrane proteins

early endosome	nerve growth factor	tight junctions
ECM	neurotransmitters	TJMPs
effector molecules	NGF	tonoplast
EGF	nuclear envelope	T-SNARE
endocytosis	nuclear pore fibrils	turgid
endomembrane system	nuclear transport receptor	turgor pressure

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18: THE CYTOSKELETON AND CELL MOTILITY

18.1: Introduction
18.2: Cytoskeletal Components
18.3: The Molecular Structure and Sub-Cellular Organization of Cytoskeletal Components
18.4: Key Words and Terms

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18.1: Introduction

The cell as it appears in a microscope was long thought to be a bag of liquid surrounded by a membrane. The electron microscope revealed a **cytoskeleton** composed of thin and thick rods, tubes and filaments. Other intracellular structures and organelles are enmeshed in these **microfilaments**, **intermediate filaments** and **microtubules**. We will compare the molecular compositions of these structures and their subunit proteins. In aggregate, they account for **organelle location** in cells, the **shapes** of cells, and cell **motility**. Cell motility includes the movement of cells and organisms, as well as the internal movements of organelles (e.g., vesicles) and other structures in the cell. Of course, these movements are not random..., and they require chemical energy! A long and well-studied system of cell motility is the interaction of **actin** and **myosin** during **skeletal muscle contraction**. We will first consider a **paradox** suggesting that ATP was required for contraction BUT ALSO for relaxation of *muscle fibers*. Then we look at experiments that resolve the paradox. Animals control skeletal muscle contraction, but some muscles contract rhythmically or with little or no control on the part of the animal - think cardiac muscles of the heart, or smooth muscles like those in digestive and circulatory systems. We will also look at the role of calcium ions and regulatory proteins in controlling the response of skeletal muscles our commands, and finally, at the elasticity of skeletal muscles.

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Compare and contrast roles of cytoskeletal structures in different kinds of *cell motility*.

2. Distinguish the roles of microfilaments, microtubules and intermediate filaments in the *maintenance and alteration of cell shape* and structure.

3. Suggest how ciliary and spindle fiber microtubules can maintain their length.

4. Explain how spindle fiber microtubules can change their length.

5. Propose an experiment to show which part of a *motor protein* has *ATPase activity*.

6. Define the actin-myosin *contraction paradox*.

7. Outline the steps of the *contraction cycle* involving myosin and actin.

8. Compare and contrast mucle and flagellar structure and function.

9. Explain why *smooth muscle*s do not show striations in the light microscope.

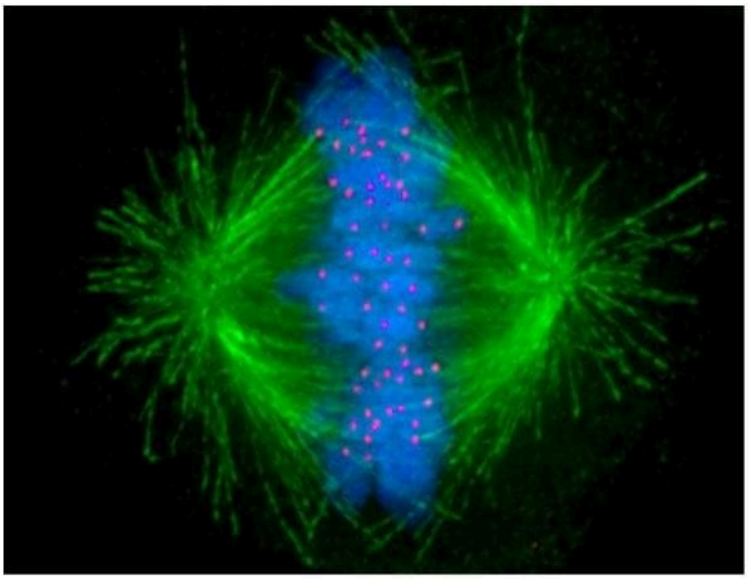
10. Outline the structure of a skeletal muscle, from a whole muscle down to a sarcomere.

11. Propose alternate hypothesis to explain *hereditary muscle weakness* involving specific proteins/genes, and suggest how you might test one of them.

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18.2: Cytoskeletal Components

Most eukaryotic cells look like a membrane-bound sac of cytoplasm containing a nucleus and assorted organelles in a light microscope. In the late 19th century, microscopists described a dramatic structural re-organization of dividing cells. In **mitosis**, duplicated chromosomes (i.e., **chromatids**) condense in the nucleus just as the nuclear membrane dissolves. **Spindle fibers** emerge and then seem to pull the chromatids apart to opposite poles of the cell. Spindle fibers turn out to be bundles of **microtubules**, each of which is a polymer of **tubulin** proteins. Let's look below at that fluorescence micrograph of a mitosing **metaphase** cell again; most of the cell other than what is fluorescing is not visible in the micrograph.



To get this image, *antibodies* were made against purified microtubule, kinetochore and chromosomal proteins (or DNA), and then linked to different **fluorophores** (organic molecular fluorescent tags). When the fluorophores were added to dividing cells in metaphase, they bound to their respective fibers. Upon UV light irradiation, the *fluorophores* emit different colors of visible light, visible in a fluorescence microscope. Microtubules are green, metaphase chromosomes are blue and kinetochores are red in the micrograph.

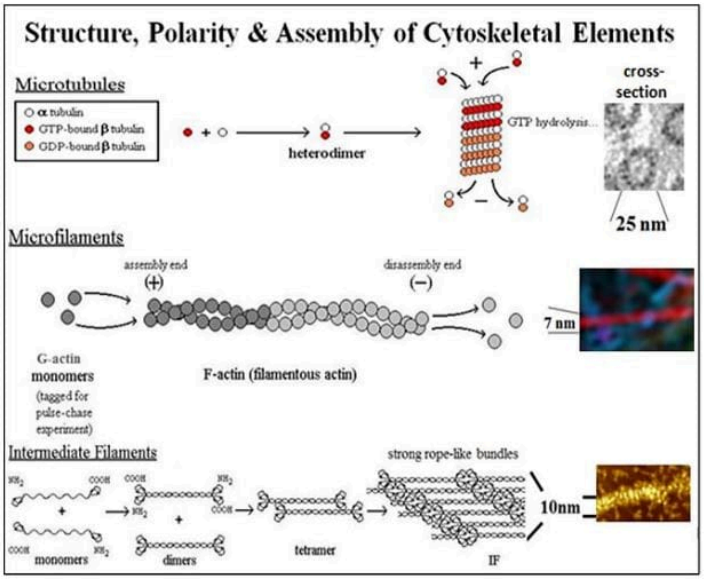
Both mitosis and meiosis are very visible examples of movements *within* cells, both already described by the late 19th century. As for movement in whole organisms, mid20th century studies focused on what the striations (or stripes) seen in skeletal muscle in the light microscope might have to do with muscle contraction. The striations turned out to be composed of a protein complex originally named **actomyosin** (*acto* for active; *myosin* for muscle). Electron microscopy later revealed that actomyosin (or **actinomyosin**) is composed of thin filaments (**actin**) and thick filaments (**myosin**) that slide past one another during muscle contraction.

Electron microscopy also hinted at a more complex cytoplasmic structure of cells in general. The **cytoskeleton** consists of fine rods and tubes in more or less organized states that permeate the cell. The most abundant of these cytoskeletal components are **microfilaments**, **microtubules** and **intermediate filaments**. But, even myosin is present in non-muscle cells, albeit at relatively low concentrations. Microtubules account for chromosome movements of mitosis and meiosis, while together with microfilaments (i.e., actin), they enable organelle movement inside cells (you may have seen **cytoplasmic streaming** of *Elodea* chloroplasts in a biology lab exercise). Microtubules also underlie **cilia-** and **flagella-**based motility of whole cells such as paramecium, amoeba, phagocytes, etc., while actin microfilaments and myosin enable muscle and thus higher animal movement! Finally, the cytoskeleton is a dynamic structure. Its fibers not only account for the movements of cell division, but they also give cells mechanical strength and unique **shapes**. All of the fibers can disassemble, reassemble and rearrange, allowing cells to change shape, for example, creating *pseudopods* in amoeboid cells and spindle fibers of mitosis and meiosis. In this chapter we look at the molecular basis of cell structure and different forms of cell motility.

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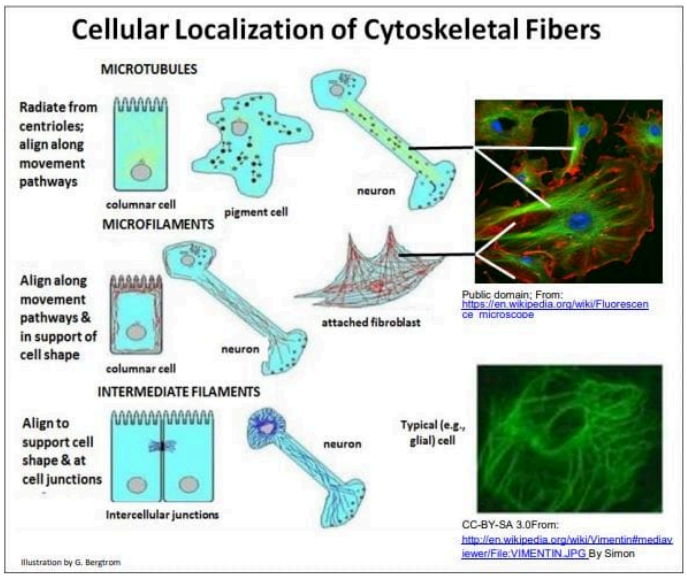
18.3: The Molecular Structure and Sub-Cellular Organization of Cytoskeletal Components

Of the three main cytoskeletal fibers, intermediate filaments serve a mainly structural role in cells. Microtubules and microfilaments have dual functions, dynamically maintaining cell shape and enabling cell motility. For example, when attached to the plasma membrane, microfilaments maintain cell shape. However, by interacting with **motor proteins** (e.g., *myosin*), they can pull or push against a muscle cell membrane, changing the shape of the cell. Likewise, motor proteins such as *dynein* and *kinesin* can move 'cargo' to and fro along microtubule tracks from one point to another in the cell. We will look at how motor proteins interact with microtubules and microfilaments shortly. At this point, let's take another look at the drawings and micrographs of the three main cytoskeletal filaments of eukaryotic cells (below) that we saw earlier in the text.



321 Introduction to the Cytoskeleton

The location and general functions of microtubules, microfilaments and intermediate filaments were demonstrated by immunofluorescence microscopy. After exposing cells to fluorophore-tagged antibodies against either microtubule, microfilament (actin) or intermediate filament proteins, fluorescence micrographs of the stained cells revealed the different locations of the fibers in cells. The typical localization of the different cytoskeletal fibers is shown below.



These localizations are consistent with known functions of the major cytoskeletal component filaments in cell structure and motility. Despite the small size of prokaryotic cells, they too were recently found have previously unsuspected cytoplasmic structures that could serve as a cytoskeleton (ncbi-A Prokaryotic Cytoskeleton?). So perhaps **all** (not just eukaryotic) cells are more than an unorganized bag of fluid sap! Next, we consider specific roles of microtubules, microfilaments, intermediate filaments and related proteins in the eukaryotic cytoskeleton.

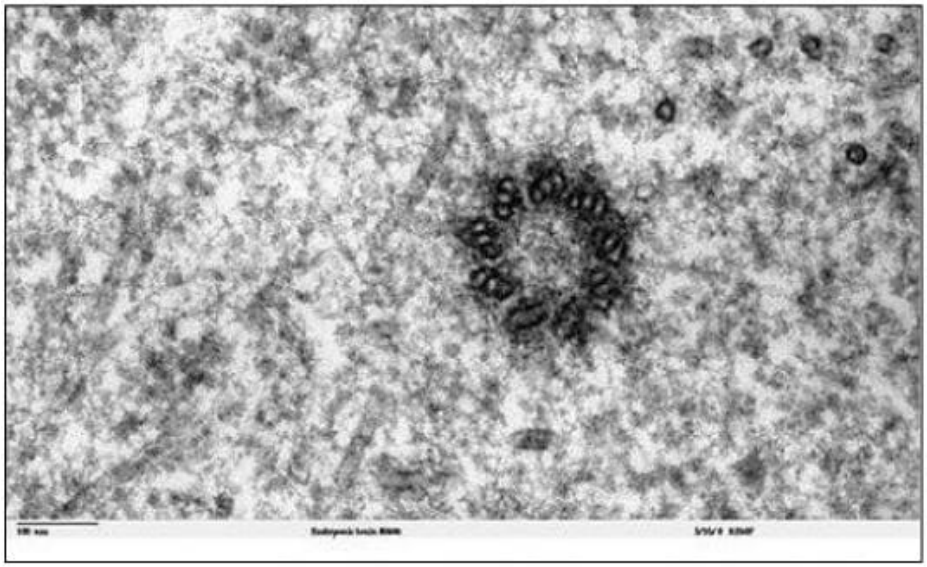
322 Microtubules, Microfilaments, and Intermediate Filaments in Cells

A. Microtubules - an Overview

Microtubules assemble from dimers of α**-tubulin** and β**-tubulin** monomers. After formation, α**/**β-tubulin dimers add to a growing, or plus end (**+end**), fueled by **GTP** hydrolysis. Disassembly at the **-end** of microtubules powers changing the shape of cells or the separation and movement of chromatids to opposite poles of cells during cell division (i.e., mitosis or meiosis). Isolated single microtubules were shown to grow by addition to one end and to disassemble at the opposite end, thus distinguishing the *+ends* and *-ends*. A summary of this experiment demonstrating microtubule **polarity** is in the link below.

323 Demonstration of the Polarity and Dynamics of Microtubules

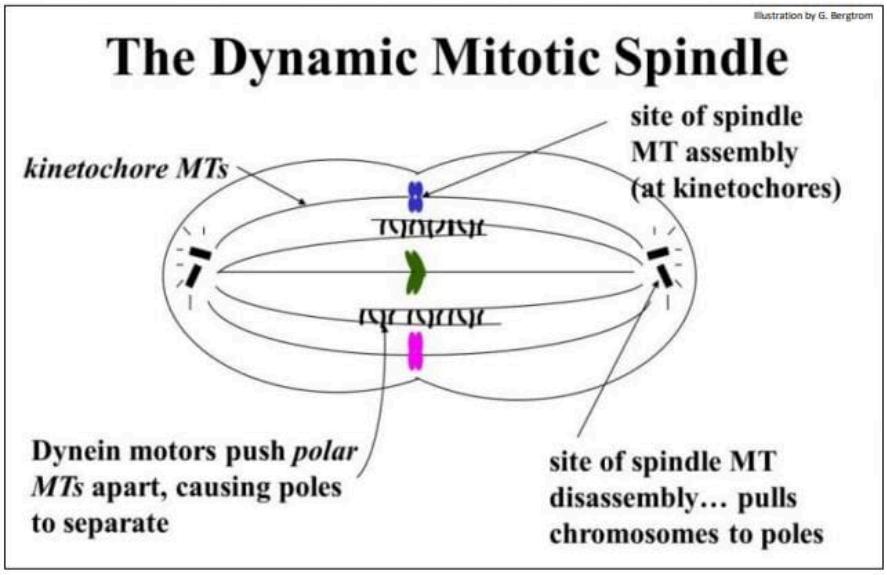
Microtubules in most cells can seem disordered. In interphase, they tend to radiate from centrioles in non-dividing animal cells, without forming discrete structures. However, in the run-up to cell division, microtubules reorganize to form spindle fibers. This reorganization is *nucleated* from **centrioles** in animal cells and from a more amorphous **microtubule organizing center** (**MTOC**) in plant cells. A typical centriole (or **basal body**) in has a '*9 triplet*' microtubule array as seen in the electron micrograph cross section (below).



1. The Two Kinds of Microtubules in Spindle Fibers

a) Kinetochore Microtubules

Duplicated chromosomes condense in prophase of mitosis and meiosis, forming visible paired **chromatids** attached at their **centromeres**. Specific proteins associate with centromeres to make a **kinetochore** during condensation. As the spindle apparatus forms, some spindle fibers attach to the kinetochore; these are the kinetochore microtubules. By **metaphase**, bundles of kinetochore microtubules stretch from the kinetochores at the cell center to the polar centrioles or *MTOCs* of the dividing cell, as drawn below.



We now know that the *+ends* of kinetochore microtubules are in fact at the kinetochores, where these fibers are assembled! At **anaphase**, forces generated when microtubules shorten at their *–ends* (disassembly ends) separate the chromatids. Microtubule disassembly at centrioles/MTOCs provides the force that draws daughter chromosomes to the opposite poles of the cell as cell division continues.

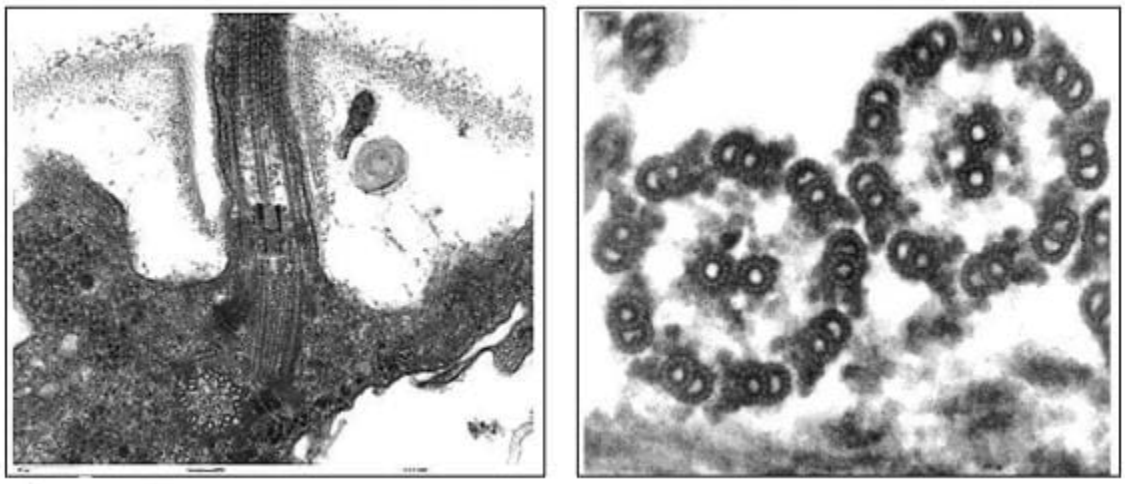
b) Polar Microtubules

The spindle fiber **polar microtubules** extend from centrioles/MTOCs at opposite poles of the cell. They do not bind to kinetochores of chromatids, but instead, overlap at the center of the dividing cells. As *kinetochore microtubule*s pull at chromatids in anaphase, *polar microtubule*s slide past one another in opposite directions, pushing apart the poles of the cell. In this case, **dynein** (a **motor protein** attached to microtubules) catalyzes ATP hydrolysis to power microtubule sliding. Dynein motors on the microtubules from one pole of the cell in effect, 'walk' along overlapping microtubules extending from the opposite pole. The role of microtubule disassembly at the centrioles (i.e., at the minus end) was demonstrated in a clever experiment in which a tiny laser beam was aimed into a cell at spindle fibers attached to the kinetochore of a pair of chromatids (see this animated at the link below).

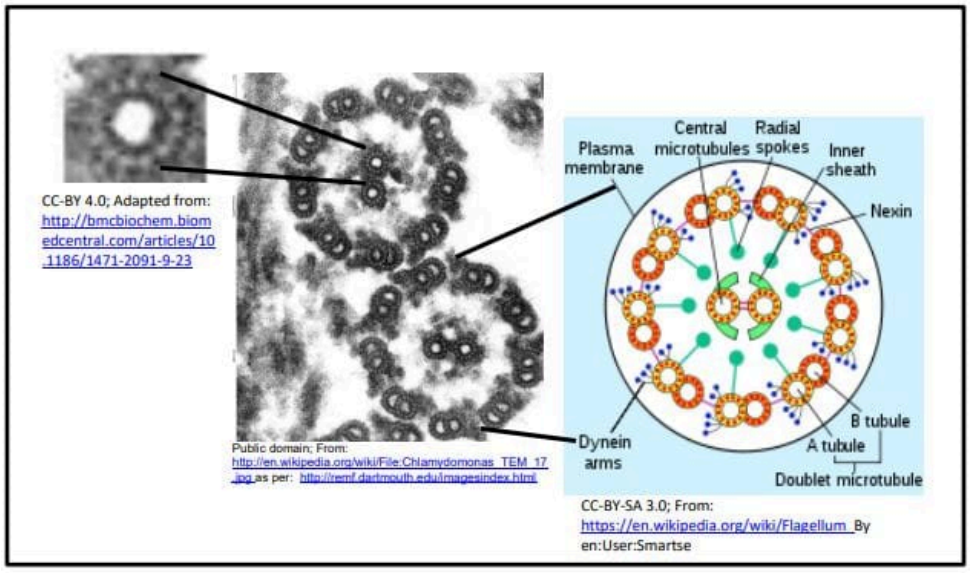
324 Spindle Fiber Microtubules Generate Force on Chromatids

2. Microtubules in Cilia and Flagella

The microtubules of cilia or flagella emerge from a **basal body**, shown at the left in the electron micrograph below



Basal bodies are structurally similar to centrioles, organized as a ring of nine microtubule triplets. Cilia and flagella formation begin at basal bodies but show a typical **9+2 arrangement** (9 outer doublet plus 2 central microtubules) in cross section (shown in the micrograph, above right). After detergent treatment to remove the membranes of isolated cilia or flagella, the remaining **axonemes** preserve the 9+2 microtubule arrangement. The structural relationship between the axonemes of a cilium or flagellum and an individual microtubule are shown in the cross sections below.

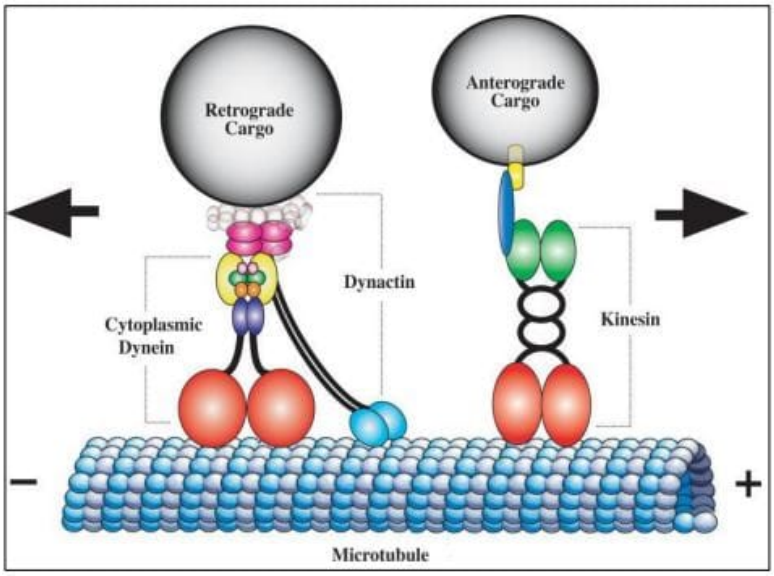


It is possible to see the tubulin subunits that make up a microtubule polymer in cross section. Each tubule is made up of a ring of 13 tubulin subunits. The microtubules in the 'doublets' share tubulins, but are also composed of 13 tubulins. When fully formed, the 25 nm diameter microtubules appear to be a hollow cylinder. When microtubules are isolated, they typically come along with dynein motor proteins and other **Microtubule-Associated Proteins** (**MAPs**), some of which hold microtubules together in an axoneme.

3. Microtubule Motor Proteins Move Cargo from Place to Place in Cells

Motor proteins such as dynein and kinesin, are *ATPases*; they use the free energy of ATP hydrolysis to power intracellular motility. Let's take a closer look at how these two major motor proteins carry **cargo** from place to place inside of cells. Organelles are a typical cargo. Examples include **vesicles** formed at the *trans Golgi face* containing secretory proteins, pigments or neurotransmitters. **Secretory vesicles** move along microtubule tracks to the plasma membrane for *exocytosis*. Vesicles containing *neurotransmitters* move from the cell body of neurons along microtubule tracks in the axons, reaching the nerve ending where they become **synaptic vesicles**. In a chameleon, **pigment vesicles** in skin cells disperse or aggregate along microtubule tracks to change skin color to match the background.

Motor proteins carry cargo vesicles in opposite directions. The transport of neurotransmitters is a well-understood example. Neurotransmitter vesicles arise from the endomembrane system in neuron **cell bodies**. Powered by ATP, **kinesin** powers **anterograde** vesicle movement from the *cell body* to nerve endings. In contrast, an ATP-dependent **dynein** motor, as part of a **dynactin** complex, powers **retrograde** movement of empty vesicles back to the cell body. Motor protein structure and action are below.



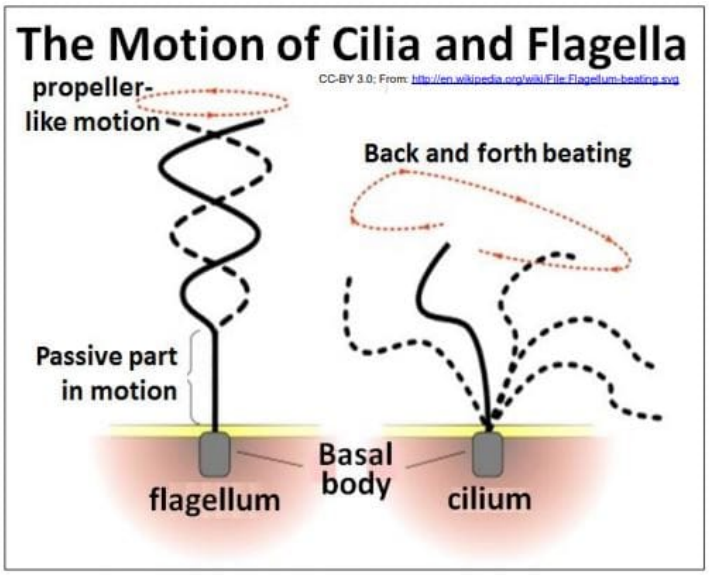
A fanciful (and not too inaccurate!) cartoon of a motor protein 'walking along an axonal microtubule is animated at this link: Kinesin 'walking' an organelle along a microtubule.

325 Microtubule Motor Proteins

At this point, we can look at several specific kinds of cell motility involving microtubules and microfilaments.

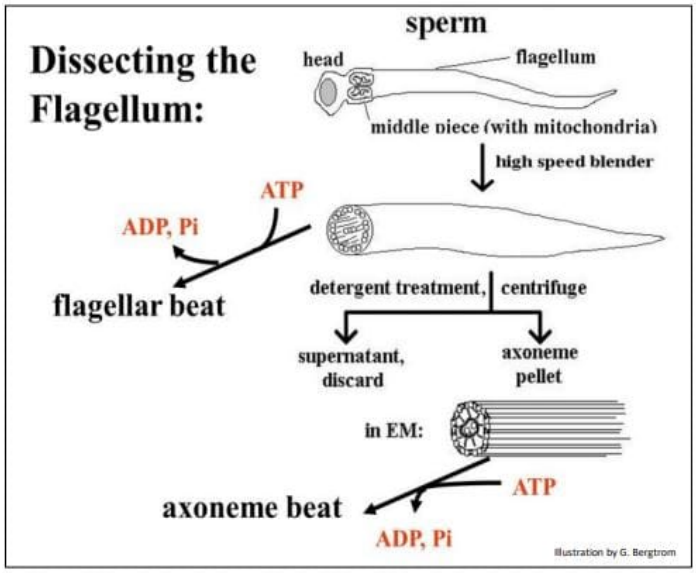
4. The Motor Protein Dynein Enables Axonemes to Bend

Take a look at the cross-section of axonemes a few illustrations ago. In the 9+2 axoneme of cilia and flagella, dynein arms attached to the **A tubules** of the outer doublets walk along the **B tubules** of the adjacent doublet. If only the doublets on one side of an axoneme take a walk while those on the other side hold still, the microtubules will slide past one another and the axoneme (and therefore a cilium or flagellum) will bend. However, microtubule sliding is constrained by flexible **nexin** and **radial spoke** attachments. The movements of cilia and flagella are illustrated below.



The differences in flagellar motion (wave-like propeller) and ciliary motion (single plane, back and forth beat) result in part from which microtubules are sliding at a given moment and the nature of their restraint by axoneme proteins. Let's look at some experiments that demonstrate these events. CC-BY 3.0; From: en.Wikipedia.org/wiki/File:Fl...um-beating.svg CMB3e 467.

Experiments on isolated *axonemes* demonstrate the sliding microtubule mechanism of ciliary and flagellar motility. In one experiment, isolated flagella and purified axonemes were both shown to 'beat' in the presence of added ATP (below).

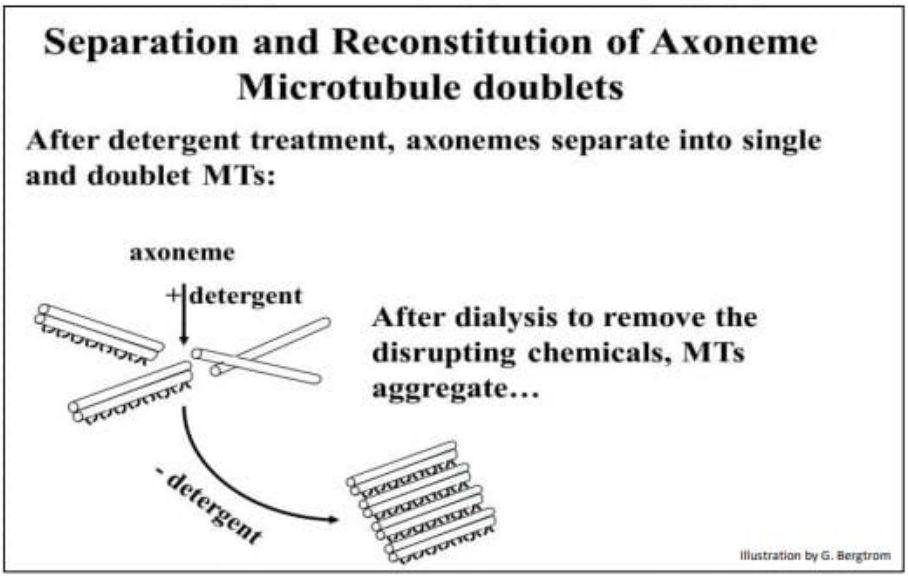


Agitating sperm or ciliated cells in a high-speed blender for a few seconds will shear and detach flagella or cilia from the rest of the cell. Adding ATP to detached cilia or flagella will cause them to beat, a phenomenon easily seen in a light microscope. Axonemes isolated from detached cilia or flagella by *detergent treatment* (to disrupt membranes) retain their characteristic 9+2 microtubule arrangement as well as other ultrastructural features..., and will even 'beat' in the presence of ATP!

326 9+2 Microtubule Array in Axonemes that Beat

Additional detergent treatment removes **radial spokes**, **nexin** and other proteins from the axoneme, causing the microtubules to separate. Dissociated microtubule doublets and central 'singlets' can then be observed in the electron microscope.

When separated microtubules are dialyzed to remove the detergents, the doublet microtubules re-associate, forming sheets, as shown in the cartoon below.



ATP added to these '*reconstituted*' microtubule doublets causes the microtubules to separate as the ATP is hydrolyzed. When such preparations are fixed for electron microscopy *immediately after* adding the ATP, they are caught in the act of sliding. See this animated in the first link below.

327 Proof of Sliding Microtubules During the Bending of Flagella and Cilia

328 Bacterial Flagella are Powered by a Proton Gradient

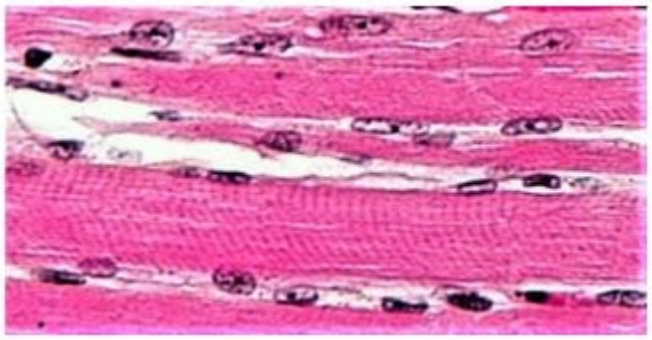
329 The Effects of Different Drugs on Microtubules and Cancer

C. Microfilaments - Structure and Role in Muscle Contraction

At 7 nm in diameter, **microfilaments** (actin filaments) are the thinnest cytoskeletal component. Globular actin (**G-actin**) monomers polymerize to form linear **F-actin** polymers. Two polymers then combine to form the twin-helical actin microfilament. As with microtubules, microfilaments have a **+end** to which new actin monomers are added to assemble *F-actin*, and a **–end** at which they disassemble when they are in a dynamic state, such as when a cell is changing shape. When one end of a microfilament is anchored to a cellular structure, for example to **plaques** in the cell membrane, motor proteins like myosin can use ATP to generate a force that deforms the plasma membrane and thus, the shape of the cell. One of the best-studied examples of myosin/actin interaction is in skeletal muscle where the sliding of highly organized thick myosin rods and the thin actin microfilaments results in muscle contraction.

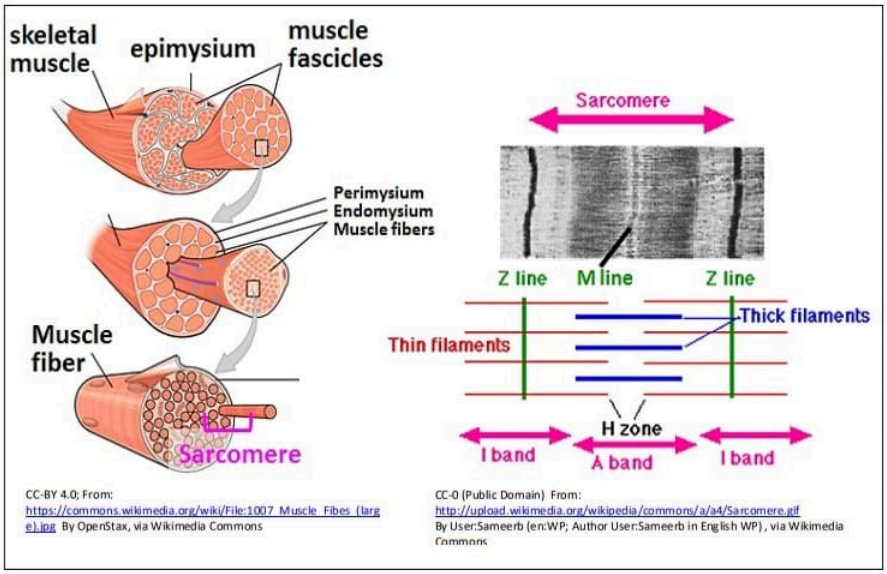
1. Thick and Thin Filaments of Skeletal Muscle Contraction

Bundles of parallel muscle cells make up a skeletal muscle. Light microscopy of skeletal muscle thin sections show **striated** muscle cells (**myocytes**, below).



The dark purplish structures surrounding the myocyte are mitochondria, which will provide the ATP to fuel contraction. Skeletal muscle is made up of 'aligned', bundled myocytes. The bundled myocytes (also called *myofibers*) are further organized into fascicles that are finally bundled into a muscle. The blowout illustration on the next page shows this anatomical organization and fine structure of a muscle (left panel).

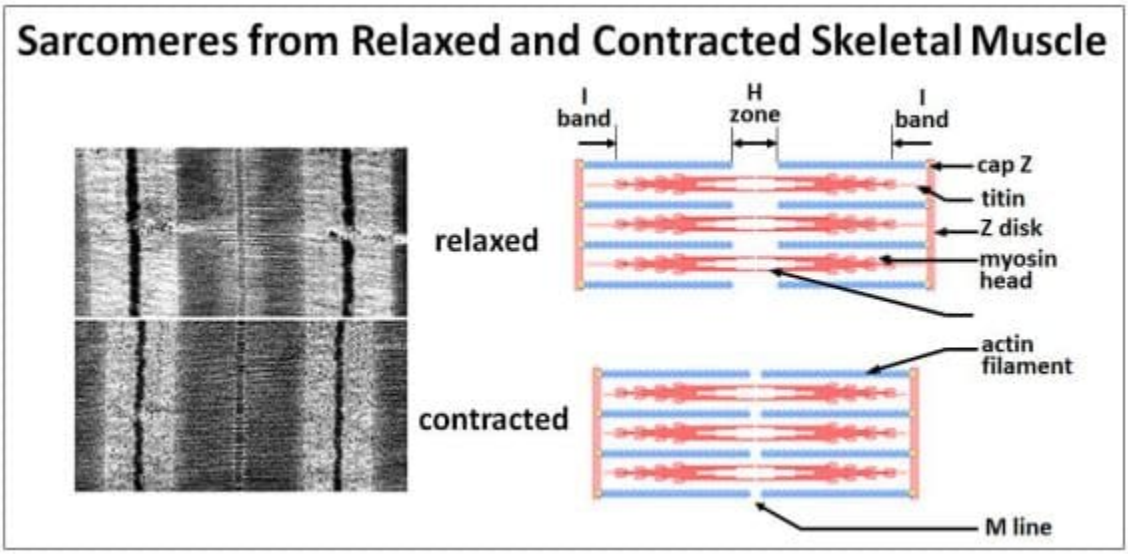
High-resolution electron microscopy from the 1940s revealed the fine structure of skeletal muscle (right panel of the illustration), allowing characterization of the **sarcomere**. The dark bands of the striations in the light micrograph of myocytes are regions of aligned, adjacent sarcomeres. A pair of **Z lines** demarcate a *sarcomere* (Z for *zwischen*, German for *between*). The **I band** is a relatively clear region of the sarcomere, largely made up of thin (actin) microfilaments. The **A band** at the center of the sarcomere consists of overlapping thin and thick (actin and myosin) filaments, while the **H zone** is a region where myosin does not overlap actin filaments. An **M line** lies at the center of the H zone. Multiple repeating sarcomeres of myocytes *aligned in register* in the fascicles give the appearance of striations in whole muscles.



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CC-0 (Public Domain) From: http://upload.wikimedia.org/wikipedia/commons/a/a4/Sarcomere.gif By User:Sameerb (en:WP; Author User:Sameerb in English WP), via Wikimedia Commons

2. The Sliding Filament Model of Skeletal Muscle Contraction

Electron microscopy of relaxed and contracted muscle shown below is consistent with the sliding of thick and thin filaments during contraction. Additional key structures of the sarcomere can be seen in the drawing at the right.



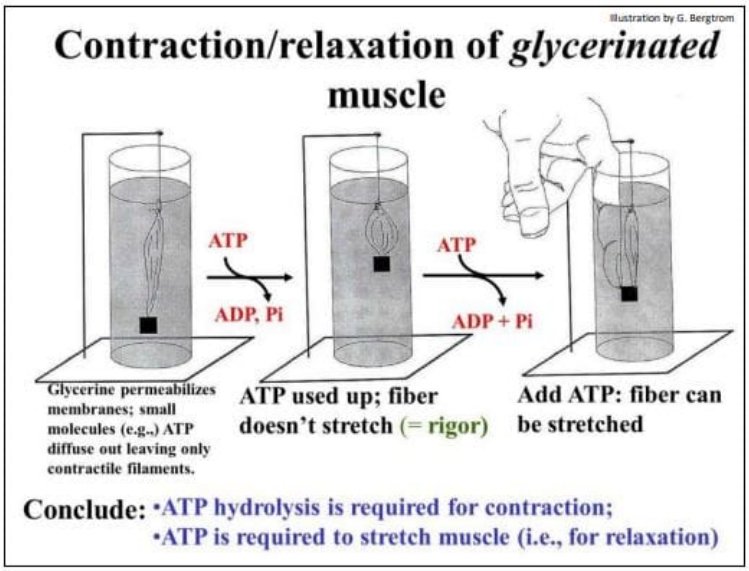
Note that in the sarcomeres of a contracted muscle cell, the H zone has almost disappeared. While the width of the A band has not changed after contraction, the width of the I bands has decreased and the Z-lines are closer in the contracted sarcomere. The best explanation here was the **Sliding Filament Hypothesis** (**model**) of skeletal muscle contraction.

330 The Sliding Filament Model of Skeletal Muscle Contraction

3. The Contraction Paradox: Contraction and Relaxation Require ATP

The role of ATP in fueling the movement of sliding filaments during skeletal muscle contraction was based in part on experiments with **glycerinated fibers** (muscle fibers soaked in glycerin to permeabilize the plasma membrane). The soluble cytoplasmic components leak out of glycerinated fibers, but leave the sarcomere structures intact, as visualized by electron microscopy. Investigators found that, if ATP and calcium were added back to glycerinated fibers, the ATP was hydrolyzed and the fiber could still contract... and even lift a weight!

The contraction of a glycerinated muscle fiber in the presence of ATP is illustrated below.



When assays showed that all of the added ATP had been hydrolyzed, the muscle remained contracted. It would not relax, even with the weight it had lifted still attached! Attempting to manually force the muscle back to its relaxed position didn't work. But the fiber could be stretched when fresh ATP was added to the preparation! Moreover, if the experimenter let go immediately after stretching the fiber, it would again contract and lift the weight! A cycle of forced stretching and contraction could be repeated until all of the added ATP was hydrolyzed. At that point, the fiber would again no longer contract..., or if contracted, could no longer be stretched.

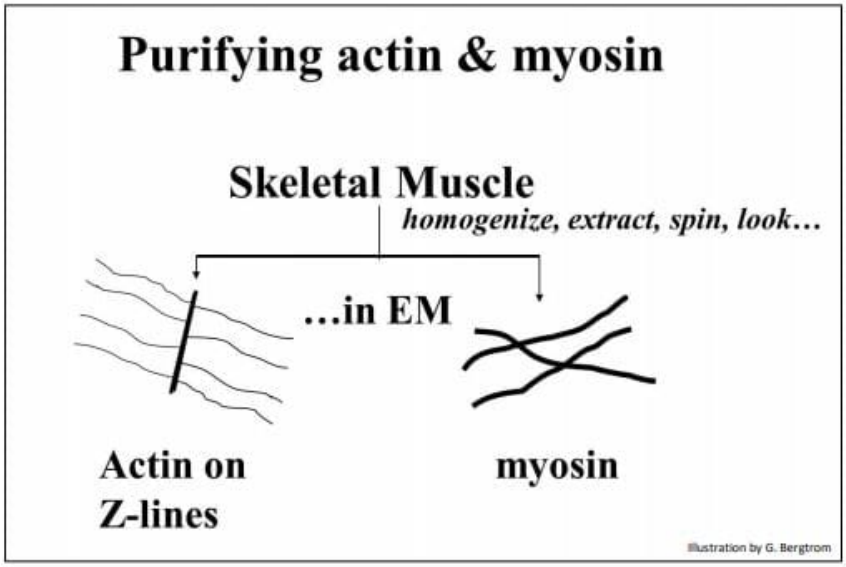
The contraction paradox then, was that ATP hydrolysis was required for muscle contraction *as well as* for relaxation (stretching). The paradox was resolved when the functions of the molecular actors in contraction were finally understood. Here we review some of the classic experiments that led to this understanding

331 The Contraction Paradox

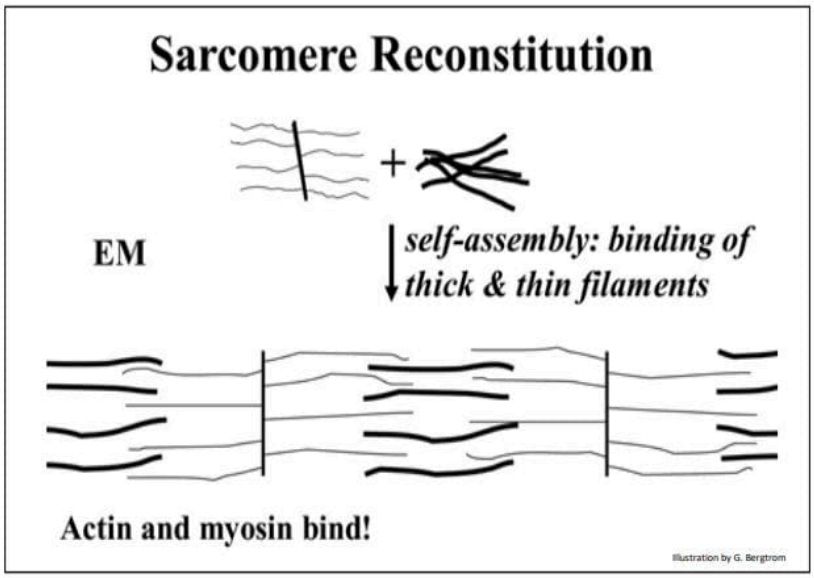
4. Actin-Myosin Interactions In Vitro: Dissections and Reconstitutions

An early experiment hinted at the interaction of actin and myosin in contraction. Homogenates of skeletal muscle were viscous. The viscous component was isolated and shown to contain a substance that was called **actomyosin** (*acto*, active; *myosin*, muscle substance). Under appropriate conditions, adding ATP to *actomyosin* preparations caused a decrease in viscosity. However, after the added ATP was hydrolyzed, the mixture became viscous again. Extraction of the nonviscous preparation (before it re-congealed and before the ATP was consumed) led to the biochemical separation of two the main substances we now recognize as the **actin** and **myosin** filaments of contraction. What's more, adding these components back together reconstituted the viscous actomyosin extract (now referred to as **actinomyosin** to reflect its composition). And..., adding ATP to the reconstituted solution eliminated its viscosity. The ATP-dependent viscosity changes of actinomyosin solutions were consistent with an ATP-dependent separation of thick and thin filaments. Perhaps actin and myosin also separate in glycerinated muscles exposed to ATP, allowing them to stretch and relax.

The advent of electron microscopy provided further evidence of a role for ATP in both contraction and relaxation of skeletal muscle The purification of skeletal muscle *actin* (still attached to Z Lines) from *myosin* is cartooned below, showing what the separated components looked like in the electron microscope.



Next, when actin (still attached to Z-Lines) and myosin were mixed, electron microscopy of the resulting viscous material revealed thin filaments interdigitating with thick filaments. The result of this reconstitution experiment is shown below.

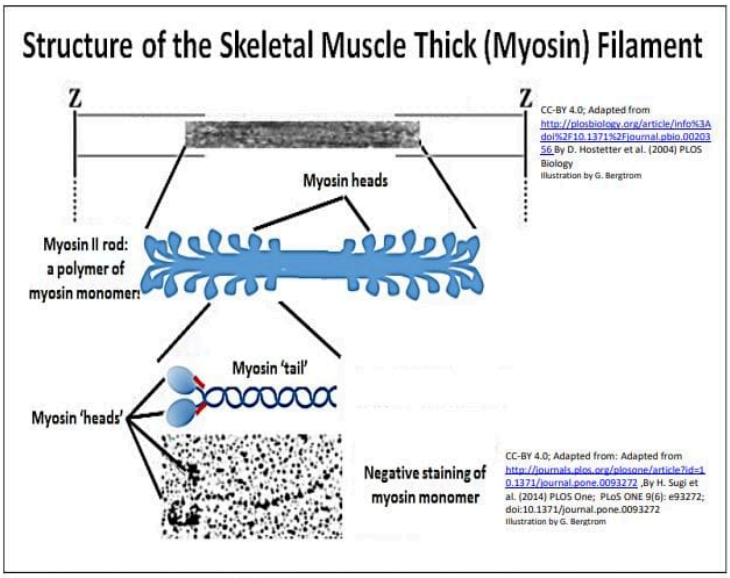


As expected, when ATP was added to these extracts, the solution viscosity dropped, and electron microscopy that the revealed thick (myosin) and thin (actin) filaments had again separated. The two components could again be isolated and separated by centrifugation.

In yet further experiments, actinomyosin preparations could be spread on over an aqueous surface, forming a film on the surface of the water. When ATP was added to the water, the film visibly “contracted”, pulling away from the edges of the vessel, reducing its surface area! Electron microscopy of the film revealed shortened sarcomere-like structures with closely spaced Z lines and short I bands..., further confirming the sliding filament model of muscle contraction.

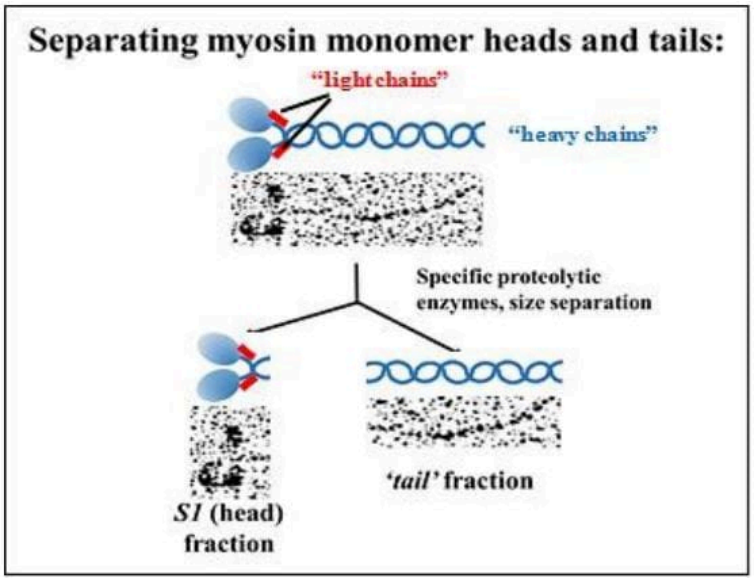
332 *In Vitro* & Electron Microscope Evidence for a Sliding Filament Model

When actin and myosin were further purified from isolated actinomyosin, the thick myosin rods could be dissociated into large ~599Kd myosin monomers. Thus, thick filaments are massive polymers of myosin monomers! The molecular structure of myosin thick filaments is shown below.



An early observation of isolated actin filaments was that they had no ATPase activity. On the other hand, while isolated myosin preparations did have an ATPase activity, they would only catalyze ATP hydrolysis very slowly compared to intact muscle fibers. Faster ATP hydrolysis occurred only if myosin filaments were mixed with microfilaments (either on, or detached from Z-lines). In the electron microscope, isolated myosin protein monomers appeared to have a double-head and single tail regions. Biochemical analysis showed that the myosin monomers themselves were composed of the two heavy chain and two pairs of light chain polypeptides shown in the illustration above

High magnification, high resolution electron micrographs and the corresponding illustration below show the component structures of myosin monomers.

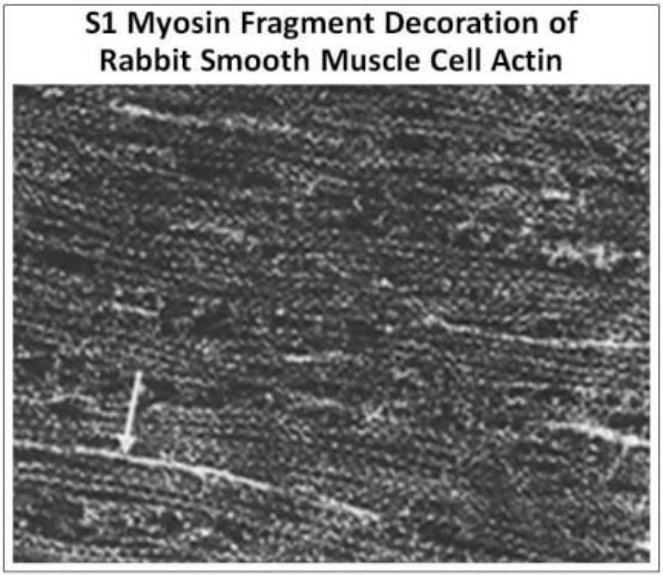


Proteolytic enzymes that hydrolyze peptide linkages only between specific amino acids, can 'cut' the heavy chains of myosin monomers into **S1** (head) and **tail** fragments. Electron micrographs of these two fragments after separation by ultracentrifugation are shown above. S1 fragments were shown to have a slow ATPase activity, while the tails had none. The slow activity was not an artifact of isolation; mixing the S1 fraction with isolated actin filaments resulted in a higher rate of ATP hydrolysis. Clearly, myosin heads are ATPases that interact with actin microfilaments.

333 Thick Filament & Myosin Monomer Structure

The direct demonstration of an association of S1 myosin head fragments with rabbit smooth muscle actin microfilaments is shown

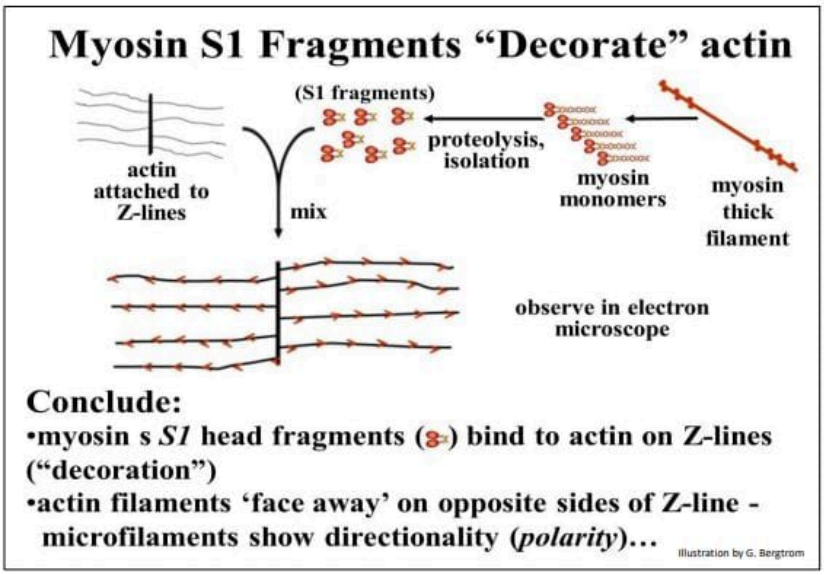
below.



Just as for skeletal muscle, smooth muscle contraction is due to actin-myosin sliding, though smooth muscle is not striated and lacks sarcomere morphology; a white arrow in the micrograph points to one of several myosin (thick) filaments visible in the micrograph. The interaction of the S1 myosin heads with actin filaments dramatically alters their morphology. In this image, the diagonal stripes, or **arrowhead**-like appearance of the S1-actin binding all along actin filaments indicates that F-actin filaments are *polar*, with a plus (+) and a minus (–) end, as was expected.

The same "decoration" of microfilaments with arrowheads is seen when S1 heads (or even intact myosin monomers) bind to thin sections of skeletal muscle sarcomeres, preparations of actin still attached to the Z lines, and with isolated Factin preparations. These images are consistent with the requirement that myosin must bind to actin to achieve a maximum rate of ATPase activity during contraction.

The arrowheads on *decorated actin* still attached to Z lines always face in opposite directions, as shown below.

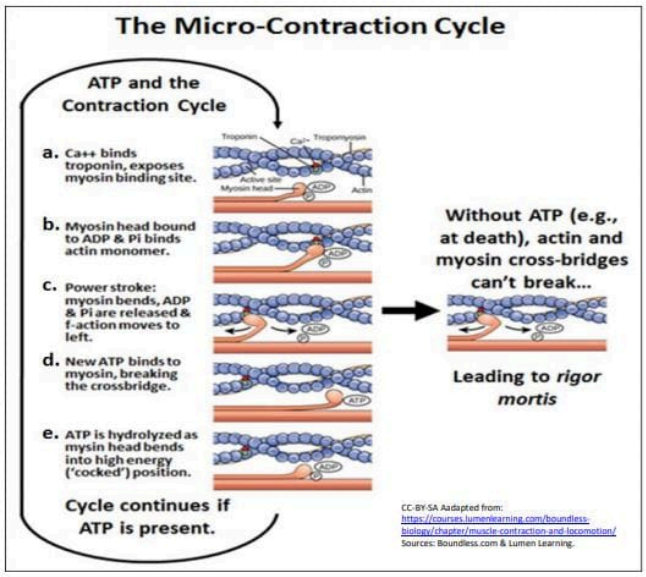


These opposing arrowheads, consistent with the sliding filament model of contraction in which *bipolar* thick filament pull actin filaments towards each other from opposite sides of the myosin rods, drawing the Z-lines closer together and shortening sarcomeres.

5. Allosteric Change and the Micro-Contraction Cycle

Whereas dynein and kinesin are *motor proteins* that 'walk' along microtubules, the myosin monomer is a motor protein that walks along microfilaments. In each case, these motor proteins are ATPases that use free energy of ATP hydrolysis to effect conformational changes that result in the walking, i.e., motility. In skeletal muscle, allosteric changes in myosin heads enable the myosin rods to do the walking along F-actin filaments

When placed in sequence such different myosin head conformations are likely the same as would occur during a **micro-contraction cycle** (illustrated below).



To help you follow the sequence, follow the small red dot on a single monomer in the actin filament. Here are the steps:

a. In the presence of Ca^{2+} ions, myosin binding sites on actin are open (Ca^{2+} - regulation of muscle contraction is discussed in more detail below).

b. Myosin heads with attached ADP and Pi bind to open sites on actin filaments.

c. The result of actin-myosin binding is an allosteric change in the myosin head, a bending of the hinge region, that pulls the attached microfilament (follow the red dot - it has moved from right to left!). This bit of **micro-sliding** of actin along myosin is the **power stroke**.

d. In its 'bent' conformation, the myosin head, still bound to an actin monomer in the F-actin, binds ATP, causing ADP and Pi to come off the myosin head and dissociating it from the actin.

e. Once dissociated from actin, myosin heads catalyze ATP hydrolysis, resulting in another conformational change. The head, still bound to ADP and Pi, has bent at its hinge, taking on a high-energy conformation that stores the energy of ATP hydrolysis.

f. The stored free energy is released during the power stroke. If Ca^{2+} has been removed, the myosins remain in the high-energy conformation of step e, until a release of Ca^{2+} again signals contraction.

Micro-contraction cycles of actin sliding along myosin continue as long as ATP is available. During repetitive *micro-contraction cycles*, myosin heads on the thick filaments pull actin filaments attached to Z-lines, bringing the Z lines closer to each other. The result is shortening of the sarcomere and ultimately, of muscle cells and the entire muscle. In the *absence* of ATP (as after the death of an organism), the *micro-contraction cycle* is interrupted. All myosin heads will remain bound to the actin filaments in the state of muscle contraction or relaxation (stretch) at the time of death. This is **rigor mortis** at the molecular level (see the illustration above). At the level of whole muscle, **rigor mortis** results in the inability to stretch or otherwise move body parts when

ATP is, once and for all, depleted.

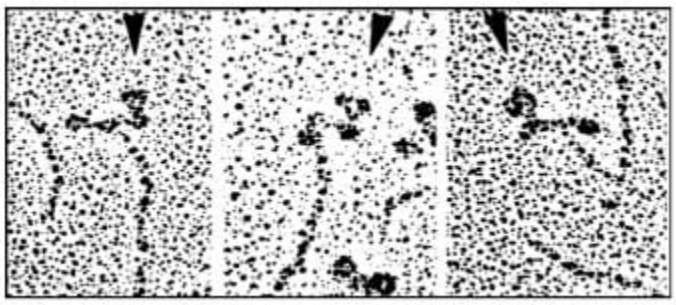
6. Resolving the Contraction Paradox

The myosin head micro-contraction cycle resolves the contraction paradox:

- **ATP is necessary for muscle contraction**: In step **e** in the illustration above, as ATP on myosin heads is hydrolyzed, the heads change from a low-energy to a high-energy conformation. The myosin heads can now bind to actin monomers (step **b** in the micro-contraction cycle). This results in of the **power stroke** (step **c**), where free energy released by an allosteric change in myosin, pulls the actin along the myosin, in effect causing a micro-shortening of the sarcomere, in other words, contraction!
- **ATP is necessary for muscle relaxation**: At the end of step **c**, myosin remains bound to actin until ATP can again bind to the myosin head. Binding of ATP in step **d** displaces ADP and inorganic phosphate (Pi) and breaks actinmyosin cross-bridges. A removal of Ca^{2+} from sarcomeres at the end of a contraction event blocks myosin binding sites on actin, while the rapid breakage of actin-myosin cross-bridges by ATP-myosin binding allows muscle relaxation and the sliding apart of the actin and myosin filaments (i.e., stretching). This leaves the myosin heads in the 'cocked' (high-energy) conformation, ready for the next round of contraction.

To summarize, ATP-myosin binding breaks actin-myosin cross-bridges. The muscle can then relax and stretch. Free energy of ATP hydrolysis, now stored in a high-energy myosin conformation, is released during the microcontraction power stroke

Electron microscopic examination of myosin monomer heads at different ionic strengths or when bound to antibodies (as shown below), provides visual evidence that myosin heads are flexible and can take on alternate stable conformations, as would be expected during the micro-contraction cycle.



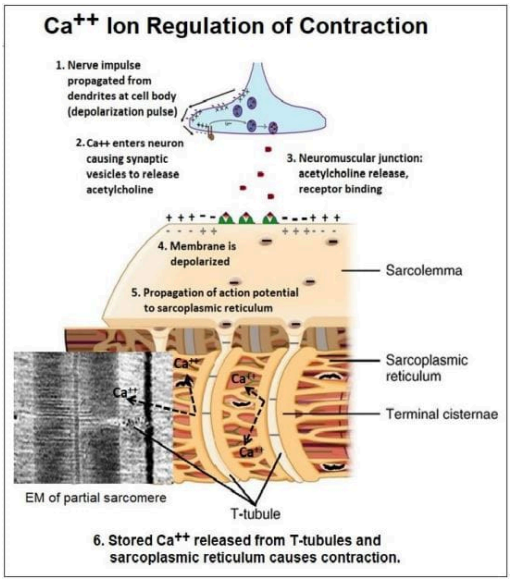
The arrow heads point to bound antibody molecules (immunoglobulins). For a video of conformational change in myosin monomers at Myosin heads in Action.

335 An Actin-Myosin Contraction Cycle Resolves the Contraction Paradox

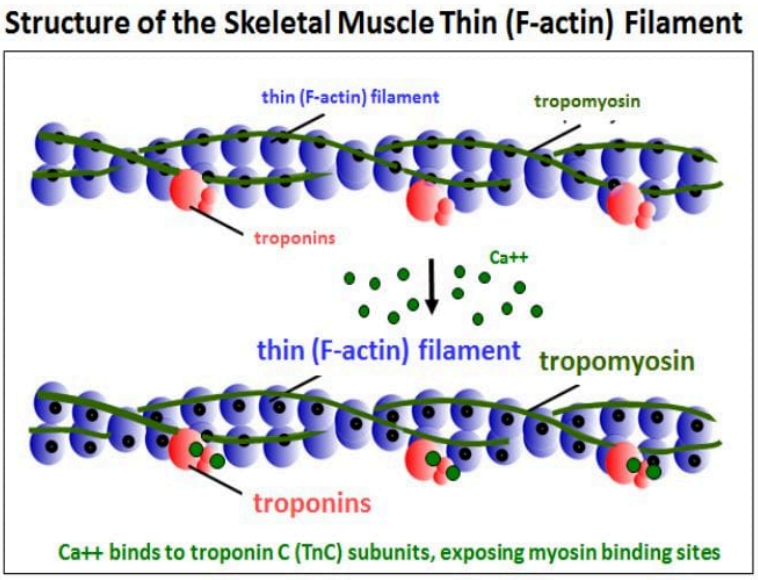
336 Binding and Hydrolysis of ATP Changes Myosin Head Conformation

7. Ca^{2+} Ions Regulate Skeletal Muscle Contraction

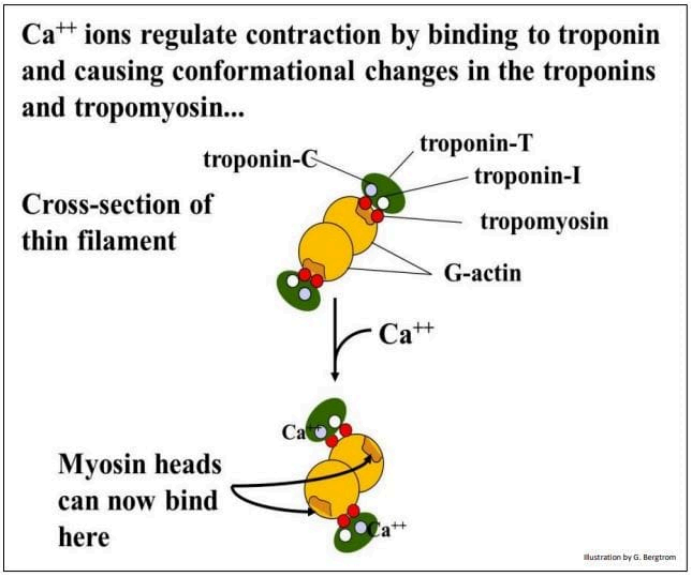
Typically, the neurotransmitter acetylcholine released by a motor neuron binds to receptors on muscle cells to initiate contraction. In early experiments, Ca^{2+} was required, along with ATP, to get glycerinated skeletal muscle to contract. It was later demonstrated that Ca^{2+} ions were stored in the **sarcoplasmic reticulum**, the smooth endoplasmic reticulum of muscle cells. As we have seen, an action potential generated in the cell body of a neuron propagates along an axon to the nerve terminal, or synapse. In a similar fashion, an action potential generated at a neuromuscular junction travels along the **sarcolemma** (the muscle plasma membrane) to points where it is continuous with **transverse tubules** (**T-tubules**). The action potential then moves along the T-tubules and then along the membranes of the sarcoplasmic reticulum. This propagation of an action potential opens Ca^{2+} channels in the sarcoplasmic reticulum. The Ca^{2+} released bathes the sarcomeres of the myofibrils, allowing filaments to slide (i.e., contraction). The action potential at a neuromuscular junction that initiates contraction is summarized in the illustration below.



Ca^{2+} ions released from the sarcoplasmic reticulum bathe the myofibrils, where they bind to one of three **troponin** molecules to regulate skeletal muscle contraction. The **three troponins** and a **tropomyosin** molecule are bound to actin filaments.. Experiments using *anti-troponin* and *anti-tropomyosin* antibodies localize these proteins on thin filaments spaced at regular intervals in electron micrographs. The drawing below models this association of the **troponin** subunits and **tropomyosin** with the thin filaments.



In resting muscle, tropomyosin (a fibrous protein) lies along the actin filament where it covers up the myosin binding sites of seven G-actin subunits in the microfilament. In this conformation, **troponin T** (*tropomyosin-binding troponin*) and **troponin I** (*inhibitory troponin*) hold the tropomyosin in place. The 'cross-section illustration below illustrates the role conformational changes in troponin C upon binding Ca++ in regulating contraction.



337 Regulation of Skeletal Muscle Contraction by Calcium

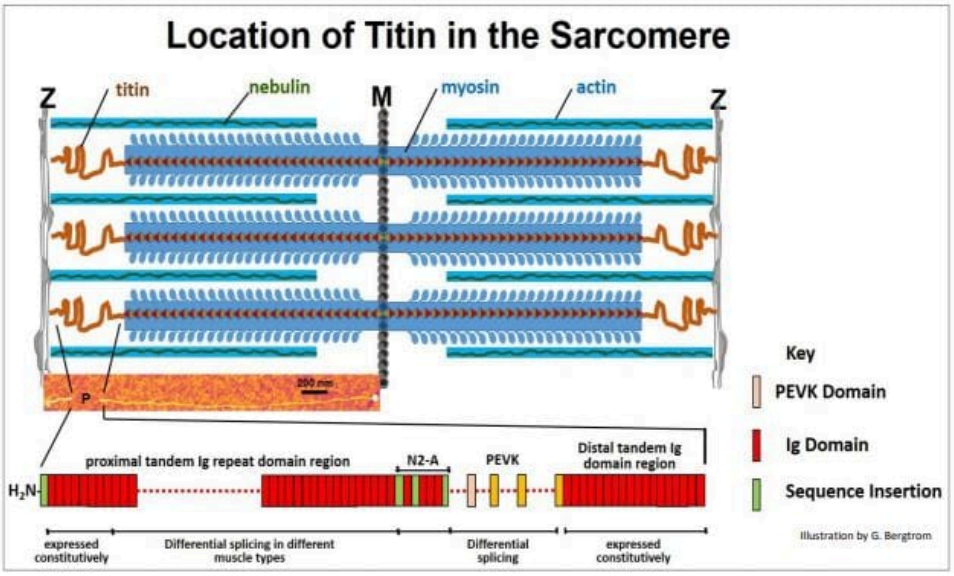
8. Muscle Contraction Generates Force

Contraction by ATP-powered sliding of thin along thick filaments generates force on the *Z-lines*. In three dimensions, the Z-lines are actually **Z-disks**) to which the actin thin filaments are attached. The protein α**-actinin** in the Z-disks anchors the ends of the actin filaments to the disks so that when the filaments slide, the Z-disks are drawn closer, shortening the sarcomeres. Another Z-disk protein, **desmin**, is an intermediate filament organized around the periphery of Z-disks. Desmin connects multiple Z-disks in a myofibril. By keeping the Z-Disks in register, muscle cell, and ultimately, muscle contraction is coordinated. Finally, actin filaments at the ends of the cell must be connected to the cell membrane for a muscle cell to shorten during myofibril contraction. Several proteins, including **syntrophins** and **dystrophin** (another intermediate filament protein) anchor the free ends of microfilaments coming from Z-disks to the cell membrane. Still other proteins anchor the cell membrane in this region to the extracellular matrix (tendons) that are in turn, attached to bones! Force generated by myosin hydrolysis of ATP and the sliding of filaments in individual sarcomeres are thus transmitted to the ends of muscles to effect movement. If the name *dystrophin* sounds familiar, it should! The gene and its protein were named for a mutation that causes muscular dystrophy, resulting in a progressive muscle weakening.

338 Contraction Generates Force Against Z Disks and Cell Membranes

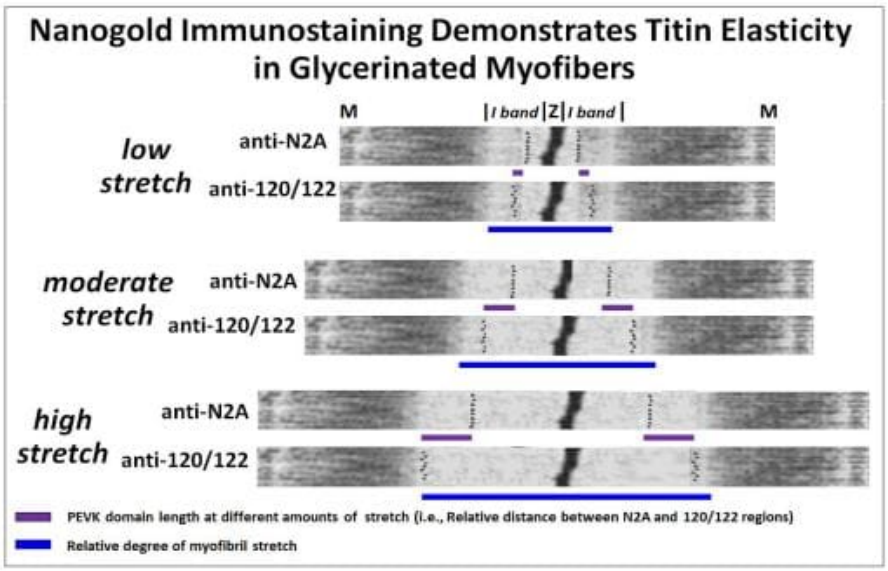
9. The Elastic Sarcomere: Do Myosin Rods Just Float in the Sarcomere?

In fact, myosin rods are anchored to proteins in the **Z discs** and **M-lines**. In 1954, R. Natori realized that when contracted muscle relaxes, it lengthens beyond its resting state, then shortening again to its resting length. He proposed that this elasticity must be due to a fiber in the sarcomere. Twenty-five years later, the elastic structure was identified as **titin**, a protein that holds several molecular records! The gene for titin contains the largest number of *exons* (363) of known proteins. After actin and myosin, titin is also the most abundant protein in muscle cells. At almost 4 x 10⁶ Da, the aptly named *titin* is the also the largest known polypeptide. Extending from the Z discs to the M line of sarcomeres, titin coils around thick filaments along the way. *Titin* is anchored at Z-disks by α**-actinin** and **telethonin** proteins. At the *M-line*, titin binds to **myosin-binding protein C** (**MYBPC3**) and **calmodulin**, among others (e.g., *myomesin*, *obscurin* and *skelamin*). Some if not all of these proteins must participate in keeping the myosin thick filaments positioned and in register in the sarcomere. This is similar to how Z-disks bind the ends of actin filaments to keep sarcomeres in register. The location of titin and several other sarcomere proteins is illustrated below.



Coiled titin molecules (in red in the illustration) extend from the Z to M lines. The colorized electron micrograph of one extended titin molecule in the middle of the illustration above should convince you of the length (35,213 amino acids!) of this huge polypeptide! Titin's elastic features are largely in the region labeled P in the micrograph, between Z discs and the myosin rods. The many domains of this P region are shown at the bottom of the illustration. With all the binding (and other) functions, you might expect that titin has many domains. It does! They include **Ig** (immunoglobulin) domains, **fibronectin** domains (not shown here), **PEVK** and **N2A** domains (that helps bind titin to α-actinin in Z-disks). Which and how many Ig and/or PEVK domains are present in a particular muscle depends on which alternative splicing pathway is used to form a titin mRNA. Over a micron long, *Titin* functions as a molecular spring, as Natori predicted. Its coiled domains compress during contraction, passively storing some of the energy of contraction. When skeletal muscle relaxes, Ca^{2+} is withdrawn from the sarcomere, ATP displaces ADP from myosin heads and actin and myosin dissociate. The muscle then stretches, typically under the influence of gravity or an opposing set of muscles. However, during contraction, 244 individually folded protein domains of titin were compressed, and during relaxation, these domains de-compress; the stored energy of compression also helps to power relaxation. At the same time, titin connections limit the stretch so that a potentially overstretched muscle can 'bounce' back to its normal relaxed length.

In a particularly elegant experiment, R. Linke et al. provided a visual demonstration of myofiber **elasticity** consistent with the coiled spring model of titin structure. They made antibodies to peptide domains on either side of the *PEVK* domain of *titin* (*N2A* and *I20-I22*) and attached them to **nanogold particles** (which will appear as electron dense granules in transmission electron microscopy). Then individual *myofibers* were stretched to different lengths, fixed for electron microscopy and treated with the *nanogold*-linked antibodies. The *antibodies* localize to and define the boundaries of the titin *PEVK* domains. The image below does not show original immune-stained electron micrographs but show alternate sarcomere micrographs with simulated localization of nanogold particles, reflecting actual results.

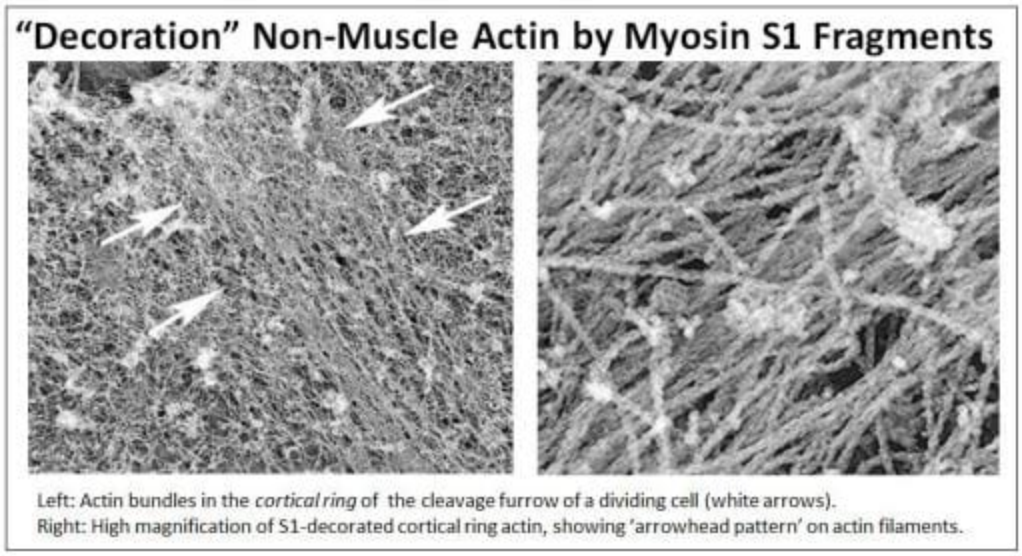


In the experiment, increased stretch lengthened the I bands on either side of Z lines of sarcomeres (blue bars). Likewise, the titin PEVK domains also lengthened as is evident from the increased distance between the nanogold-linked N2A and 120/122 antibodies that bind on either side PEVK domains. This demonstration of titin (and therefore sarcomere) elasticity) is consistent with the storage of some of the free energy of contraction when the molecule is compressed, and the passive release of that energy during relaxation. Since titin tethers thick filaments to Zdisks and M-lines, it also limits the amount of sarcomere stretch during relaxation. An animation from Linke's lab is at http://www.titin.info/.

D. Non-muscle Microfilaments

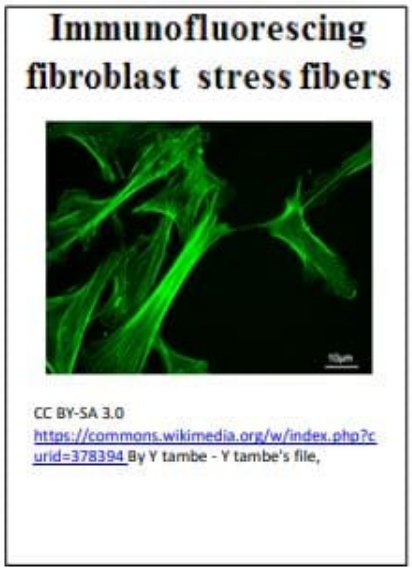
Electron microscopy revealed that thin (~10 nm) filaments permeated the cytoskeleton of eukaryotic cells. These were suspected to be actin microfilaments. Microfilaments typically lie in the cortex of cells, just under the plasma membrane, where they support cell shape. These same microfilaments can also re-organize dynamically, allowing cells to change shape. A dramatic example of this occurs in dividing cells, during cytokinesis when the dividing cell forms a *cleavage furrow* in the middle of the cell. The cortical microfilaments slide past each other with the help of *non-muscle myosin*, progressively pinching the cell until it divides into two new cells.

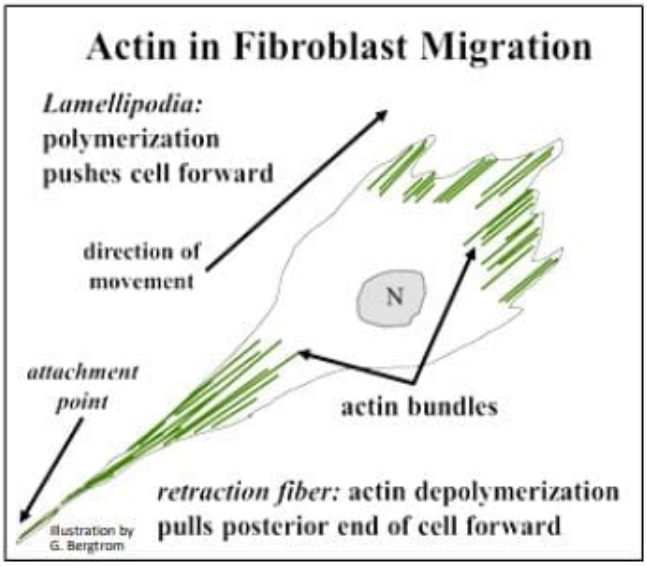
To test whether these 10 nm 'microfilaments' were in fact actin, intact myosin monomers or S1 myosin head fragments were placed atop electron micrographs of many different cell types. When viewed in the electron microscope, such preparations always revealed that the 10 nm microfilaments were decorated with *arrowheads*, just like S1 fragment decorated muscle cell actin or Z line-bound actin! Clearly, these cytoplasmic microfilaments are a form of F-actin. In the example shown below, cells in cytokinesis were treated with S1 myosin head fragments.



See the role of cortical filaments in cytokinesis at Cortical Actin Filament Action in Cytokinesis. Of course, actin microfilaments are involved in all manner of cell motility in addition to their role in cell division. They enable cell movement and cytoplasmic streaming inside cells. And while they give intestinal microvilli strength, they even enable microvilli to move independent of the passive pressures of peristalsis.

Other examples of microfilaments in cell motility include the ability of **amoeba** and other *phagocytic cells* to extend **pseudopodia** to engulf food or foreign particles (e.g., bacteria), respectively. Similarly, when fibroblast cells move along surfaces, they extend thin **filipodia** into the direction of movement by assembling actin bundles along the axis of cell movement. Actin **stress fibers** that help to maintain cell shape fluorescence green in the immunofluorescence micrograph below (left panel). The dual roles of actin in fibroblast movement are also illustrated (below right).

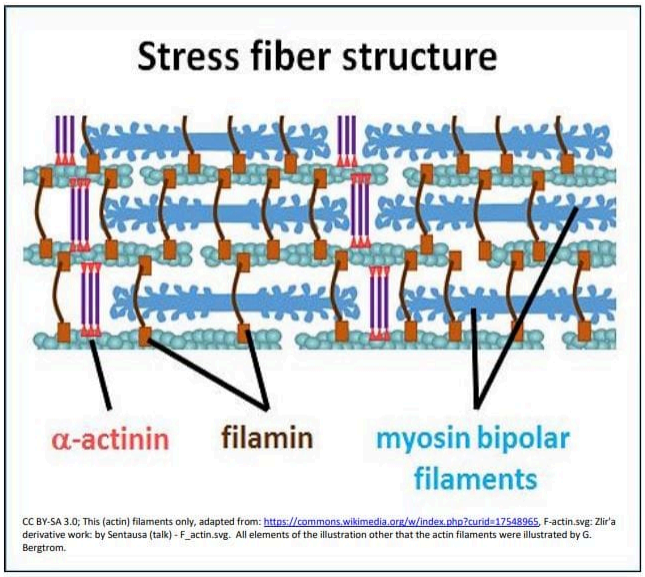




As we saw for microtubule-mediated cell motility, some actin-mediated motility may be primarily based on actin assembly and disassembly, as in the extension of filipodia at the moving front of a fibroblast. As the fibroblast moves forward, a **retraction fiber** at the hind-end of the cell remains attached to the surface (*substratum*) along which it is migrating. Eventually however, actin-myosin interactions (in fact, sliding) causes retraction of most of this 'fiber' back into the body of the cell.

Movements mediated by stress fibers may also explain the cytoplasmic streaming that distributes cellular components and nutrients throughout a cell. The movements of both involve actin-myosin interactions. Studies of non-muscle cell motility suggest the structure and interacting molecular components of stress fibers. They reveal overlapping myosin and actin filaments that slide

during movement, as illustrated below.



Filamin in this drawing is shown holding actin filaments together at an angle, while α**- actinin** also helps to bundle the actin (thin) filaments. **Titin** (not shown) also seems to be associated with stress fibers. However, unlike highly organized skeletal muscle sarcomeres, the proteins and filaments in stress fibers are not part of Z- or M-line superstructures. Could such less-organized non-muscle stress fiber filament bundles be the evolutionary predecessor to sarcomeres in muscle cells?

E. Actins and Myosins are Encoded by Large Gene Families

Actins may be the most abundant protein in cells! At least six different actin **isoforms** encoded by a large actin **gene family** have nearly identical amino acid sequences, all of which are involved in cytoskeletal function. The β-actin isoform predominates. Genes for some isoforms are expressed in a cell-specific manner.

Are all actin isoforms functionally significant? Myosin monomers (or S1 heads) decorate virtually all actins. This makes one wonder if any one actin is an adaptation, however subtle, such that the absence of one isoform would pose a significant threat to the survival of an organism? Since amino acid sequence differences between actins would not predict dramatically different protein function, could they underlie some as yet unknown physiological advantage to different cells? In mice, the loss of a γ-actin gene has little effect on the organism, while loss of the β-actin gene in mice is lethal at embryonic stages. In contrast, studies show that a mutated β-actin gene in humans correlate with delayed development and later neurological problems (e.g., epilepsy), kidney and heart abnormalities, but is not lethal. In fact, people with such mutations can lead nearly normal, healthy lives (Beta-Actin Gene Mutations and Disease).

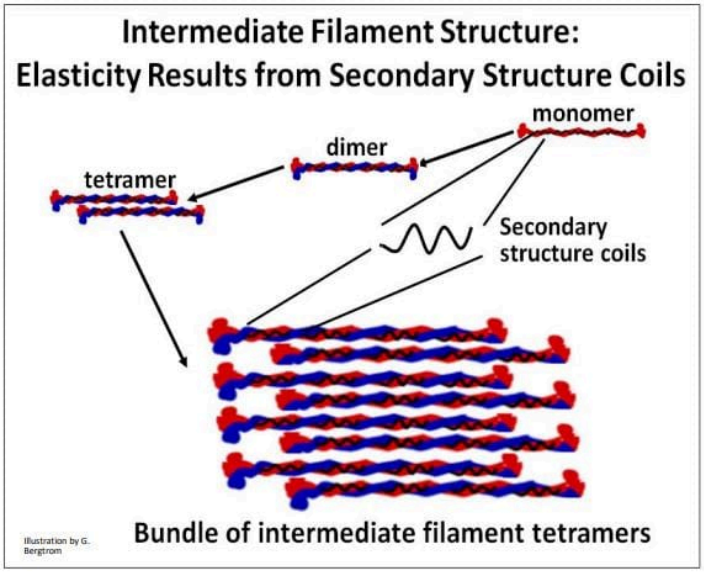
Like the actins, myosin genes encoding variant isoforms comprise a large eukaryotic gene family. All isoforms have ATPase activity and some are clearly involved in cell motility. Unique functions are not yet known for other isoforms, but different myosin monomers can decorate actin, and myosins from one species can decorate actin filaments of other species, even across wide phylogenetic distances.

F. Intermediate Filaments - an Overview

These 10 nm filaments are proteins with extended secondary structure that in fact, do not readily fold into tertiary structures, and they have no enzymatic activity. Recall their intercellular location in desmosomes where they firmly bind cells together to confer tensile strength to tissues. Within cells, intermediate filaments permeate the cells where they participate in regulating and maintaining cell shape. Recall their role in anchoring actin to either Z-disks or plasma membrane plaques in muscle cells, transmitting the forces of contraction to the shortening of the sarcomeres and then to the actual shortening of a muscle. The extracellular **keratins** that make up fur, hair, fingernails and toenails, are proteins related to intermediate filaments. Unlike intracellular intermediate filaments, keratins are bundles of rigid, insoluble extracellular proteins that combine to align to form stable, unchanging secondary structures. Finally, **lamins** are intermediate filaments that make up structural elements of the

nuclear lamina, a kind of **nucleoskeleton**.

CMB3e 492 As we saw earlier, intermediate filament subunits have a common structure consisting of a pair of monomers, each with globular domains at their C- and N-terminal ends, separated by coiled rod regions. Monomers are non-polar; i.e., unlike microtubules and actin filaments, they do not have 'plus' and 'minus' ends. The basic unit of intermediate filament structure is a dimer of monomers. Dimers further aggregate to form tetramers and larger filament bundles. Like microtubules and actin filaments, intermediate filament bundles can disassemble and reassemble as needed when cells change shape. Unlike microtubules and actin, intermediate filaments can stretch, a property conferred by the coiled rod regions of the filaments. This should be reminiscent of titin molecules! The structural features and elasticity of intermediate filaments is illustrated in the cartoon below.



In the bundled intermediate filaments that permeate the cytoplasm of cells, the ability to stretch contributes to the viscosity of cytoplasm, and is even called **viscoelasticity**. This elastic property is thought to allow actins and microtubules a degree of freedom of movement of cells, and within the cytoplasm of cells.

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18.4: Key Words and Terms

"9+2"	F-actin	myosin ATPase
α tubulin	F-actin polarity	myosin
A-band	flagella	myosin "heads"
acetylcholine	fluorescence microscopy	neuromuscular junction
acidic keratin	force transduction	nuclear lamina
actin	G-actin	plus and minus ends
actin-binding proteins	hair, horn	protofilaments
actin-myosin interactions	I-band	psuedopodia
actin-myosin paradox	intermediate filaments	sarcomere
action potential	intestinal microvilli	sarcoplasmic reticulum
aoeboid movement	keratin	sarcolemma
ATPase	keratin isoforms	scales, feathers, fingernails
axoneme	lamins	secretion vesicle transport
β tubulin	membrane depolarization	skeletal muscle contraction
basal body	microfilaments	skeletal muscle relaxation
basic keratin	microtubule assembly end	sliding filament model
Ca^{2+} regulation of contraction	microtubule disassembly end	syncytium
Ca^{2+} release v. active transport	microtubule doublets	thick and thin filaments
cell motility	microtubule organizing center	titin
centriole	microtubule polarity	transverse (T) tubules
cilia	microtubule-associated proteins	tread-milling
contraction regulation	microtubules	tropomyosin
cortical cellular microfilaments	mitotic, meiotic spindle fibers	troponin I
creatine phosphate	M-line	troponin T
cross-bridges	motor proteins	troponins
cytoplasmic streaming	MTOC	troponin C
cytoskeleton	muscle cell	tubulin heterodimer
desmosomes	muscle fiber	tubulins
dynein	myocyte	viscoelasticity
evolution of actin genes	myofiber	Z-disks
evolution of myosin genes	myofibril	Z-line

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19: CELL DIVISION AND THE CELL CYCLE

19.1: Introduction
19.2: Bacterial Cell Division and the Eukaryotic Cell Cycle
19.3: Regulation of the Cell Cycle
19.4: When Cells Die
19.5: Disruption of the Cell Cycle Checkpoints Can Cause Cancer
19.6: Key Words and Terms

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19.1: Introduction

Mitosis is the condensation of chromosomes from chromatin and their separation into dividing cells. **Cytokinesis** is the process that divides a cell into two new cells after duplicated chromosomes are safely on opposite sides of the cell. Mitosis and Cytokinesis together are a relatively short time in the **cell cycle**. While cell cycle times vary, imagine a cell that divides every 20 hours. Mitosis and cytokinesis would last about 1-1.5 hours in the life of this cell. Mitosis is divided into 4-5 phases (depending on whose text you are reading!), the last of which overlaps cytokinesis. Mitosis takes about an hour and cytokinesis about 30 minutes in this example.

The rest of a 20-hour cell cycle is spent in **interphase**, so-called because 19th century microscopists saw nothing happening in cells when they were not in mitosis or actually dividing. However, by the 1970s, experiments had revealed that interphase itself could be divided into discrete phases of cellular activity, called **G_1**, **S** and **G_2**, occurring in that order. It turns out that **kinases** regulate progress through the cell cycle, catalyzing timely *protein phosphorylations*. The early experiments led to the discovery of *mitosis-promoting factor* (**MPF**), one of these kinases.

Kinase-regulated events are **checkpoints** that cells must pass through in order to enter the next step in the cell cycle. As you might guess, the failure of a checkpoint can have serious consequences. **Carcinogenesis**, the runaway proliferation of cancer cells, is one such consequence that we will consider in this chapter. We will also look at the fate of differentiating cells and at details of cellular end-of-life events, including **apoptosis**, or **programed cell death**).

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Describe the *phases of the cell cycle* and what occurs in each.

2. Interpret experiments leading to our understanding of the s*eparation* of chromosomal events from duplication of the DNA contained in those chromosomes.

3. Describe the role of *cyclin* and *cdk* (cyclin-dependent kinases) in MPF.

4. Compare the roles of different cyclins and cdks in regulating the cell cycle.

5. Define cell-cycle checkpoints that monitor cell cycle activities.

6. Explain the molecular interactions between DNA damage, cell cycle checkpoints (arrest of the cell cycle if vital activities are blocked) and apoptosis.

7. State an hypotheses for how cell cycling errors can *transform* normal cells into *cancer* cells.

8. List some examples of apoptosis in humans and other organisms.

9. Compare and contrast examples of apoptosis and *necrosis*.

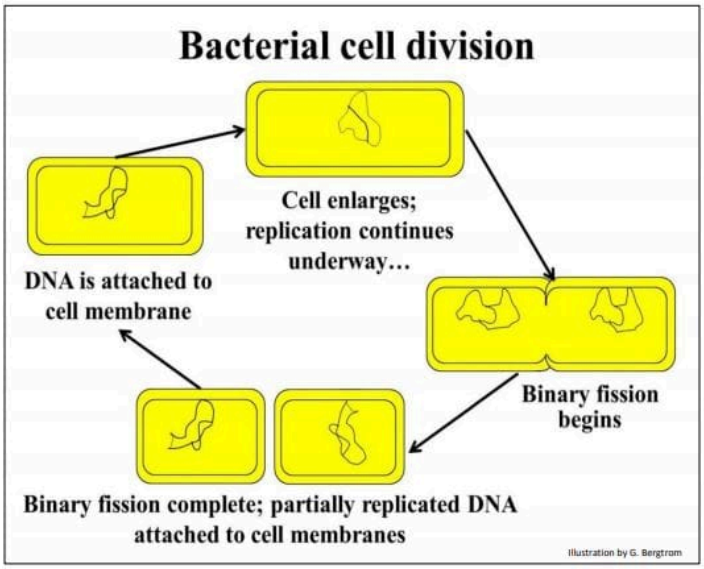
10. Formulate an hypothesis to account for the degradation of cyclin after mitosis.

11. Research and explain how different chemotherapeutic agents work and the biochemical or molecular basis of their side effects.

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19.2: Bacterial Cell Division and the Eukaryotic Cell Cycle

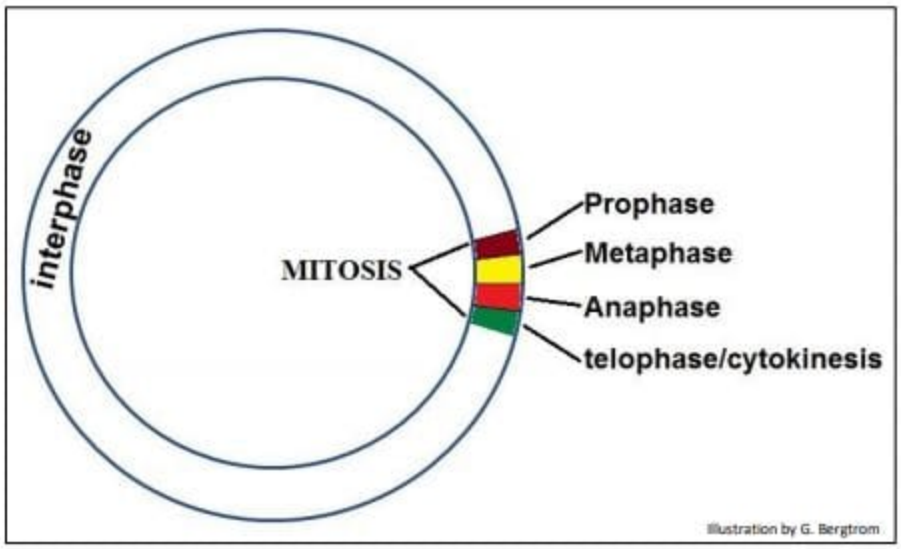
The life of actively growing bacteria is not separated into a time for duplicating genes (i.e., DNA synthesis) and one for **binary fission** (dividing and partitioning the duplicated DNA into new cells). Instead, the single circular chromosmome of a typical bacterium is replicating even before fission is complete, so that the new daughter cells already contained partially duplicated chromosomes. Cell growth, replication and fission are illustrated below



339 Binary Fission

The roughly 30-60 minute life cycle of an actively growing bacterium is not divided into discrete phases. On the other hand, typical eukaryotic cells have a roughly 16-24 hour cell cycle (depending on cell type) that is divided into four separate phases. In the late 1800s, light microscopy revealed that some cells lost their nuclei while forming **chromosomes** (from *chroma*, colored; *soma*, bodies). In **mitosis**, paired, attached chromosomes (**chromatids**) were seen to separate and to be drawn along **spindle fibers** to opposite poles of dividing cells. Thus homologous chromosomes were equally partioned to the daughter cells at the end of cell division. Because of the same chromosomal behavior was observed in **mitosis** in diverse organisms, chromosomes were soon recognized as the stuff of inheritance, the carrier of genes!

The short period of intense mitotic activity was in stark contrast to the much longer '*quiet*' time in the life of the cell, called **interphase**. The events of mitosis itself were described as occurring in 4 phases occupyiing a short time as shown below



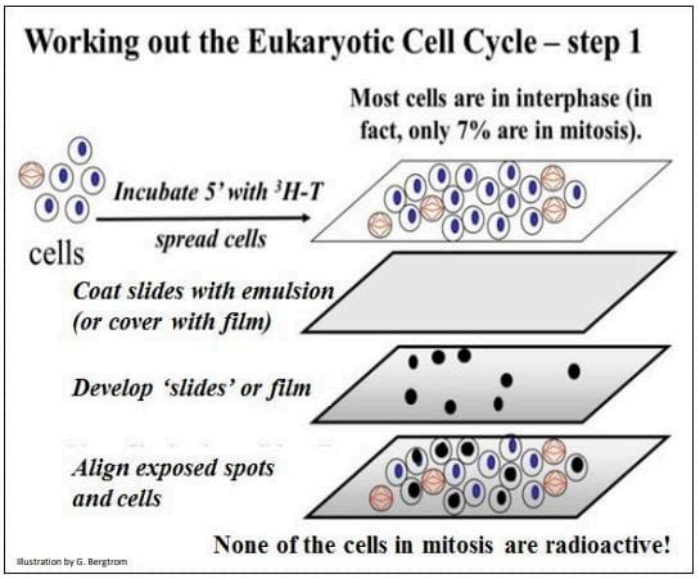
Depending on whom you ask, **cytokinesis** (the cell movements of actually dividing a cell in two) is *not* part of mitosis. In that sense, we can think of three stages in the life of a cell: interphase, mitosis and cytokinesis. Of course, it turned out that interphase is not cellular 'quiet time' at all!

A. Defining the Phases of the Cell Cycle

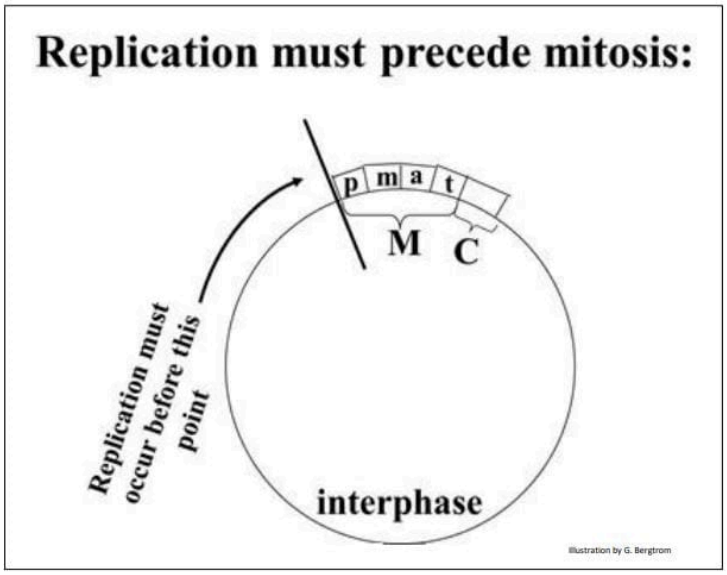
Correlation of the inheritance of specific traits with that of chromosomes was demonstrated early in the early 20th century, most elegantly in genetic studies of the fruit fly, *Drosophila melanogaster*. At that time, chromosomes were assumed to contain the genetic material and that both were duplicated during mitosis. The first clue that this was not so came only after the discovery that DNA was in fact the chemical *stuff of genes*. The experiment distinguishing the time of chromosome formation from the time of DNA duplication is summarized below.

1. Cultured cells were incubated with ^{3}**H-thymine**, the radioactive base that cells will incorporate into thymidine triphosphate (dTTP), and then into DNA.
2. Cultured cells were incubated with ^{3}H-thymine, the radioactive base that cells will incorporate into thymidine triphosphate (dTTP), and then into DNA.
3. Slides were dipped in a light-sensitive emulsion containing the same light sensitive chemicals as found in the emulsion-side of film.
4. After some time to allow the radioactivity on the slide to '*expose*' the emulsion, the slides were developed (in much the same way as developing film).
5. The resulting **autoradiographs** in the microscope revealed images in the form of dark spots created by exposure to **hot** (i.e., radioactive DNA.

If DNA replicates in chromosomes undergoing mitosis, then when the developed film is placed back over the slide, any dark spots should lie over the cells in mitosis, and not over cells that are not actively dividing. The experimental is illustrated below



Observation of the autoradiographs show that none of the cells in mitosis is radioactively labeled. But some of the cells in interphase were! Therefore, DNA synthesis must take place sometime in interphase, before mitosis and cytokinesis (illustrated below).



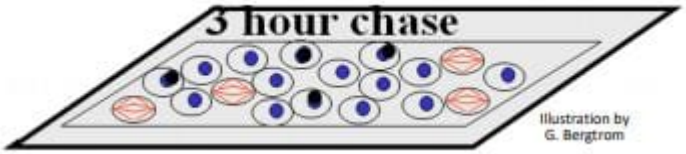
340 Experiments that Reveal Replication in Interphase of the Cell Cycle

Next a series of **pulse-chase** experiments were done to determine when in the cell cycle DNA synthesis actually takes place. Cultured cells given a short *pulse* (exposure) to ^{3}H-thymine and then allowed to grow in non-radioactive medium for different times (the *chase*). At the end of each chase time, cells were spread on a glass slide and again prepared for autoradiography. Analysis of the autoradiographs identified distinct periods of activity within interphase: Gap1 (**G_1**), a time of DNA *synthesis* (**S**) and Gap 2 (**G_2**). Here are the details of these very creative experiments, performed before it became possible to synchronize cells in culture so that they would all be growing and dividing at the same time.

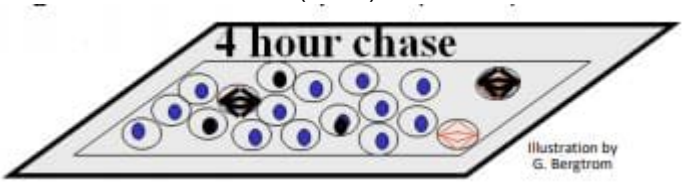
- Cells were exposed to 3H-thymine for just 5 minutes (the **pulse**) and then centrifuged. The radioactive supernatant was then discarded.
- The cells were rinsed and centrifuged again to remove as much labeled precursor as possible.
- The cells were re-suspended in fresh medium containing unlabeled (i.e., nonradioactive) thymine and further incubated for different times (the chase periods).After dipping the slides in light-sensitive emulsion, exposing and developing the film, the

autoradiographs were examined, with the following results:

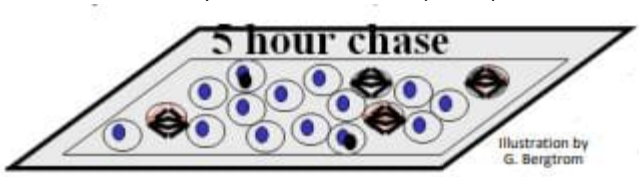
- After a 3-hour (or less) chase period, the slides looked just like they would immediately after the pulse. That is, none of the 7% of the cells that were in mitosis is radioactively labeled, but many interphase cells showed labeled nuclei, as shown below.



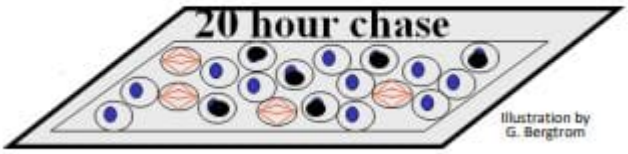
- After 4 hours of chase, a few of the 7% of the cells that were in mitosis were labeled, along with others in interphase (below).



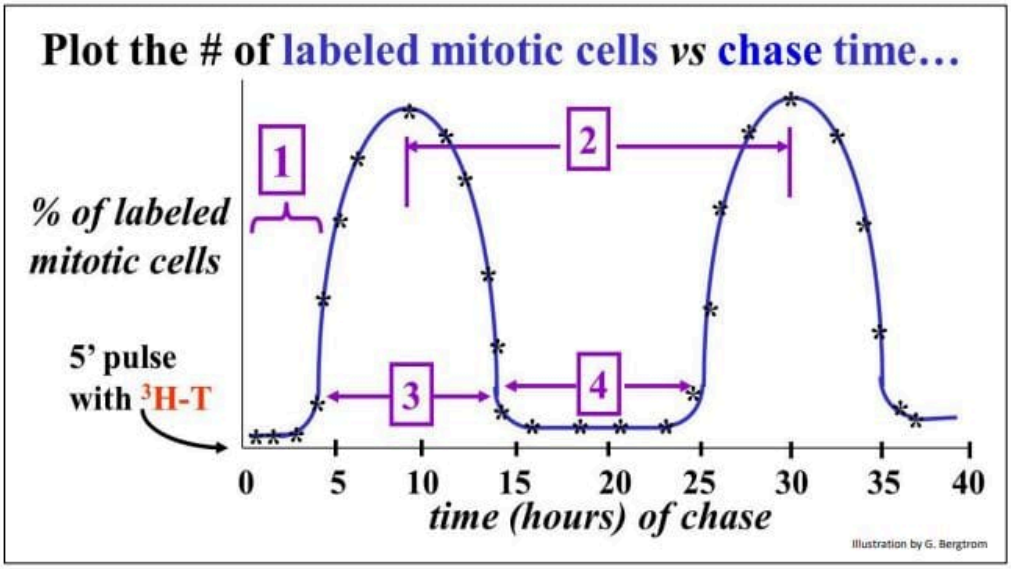
- After a 5 hour chase, most cells in mitosis (still about 7% of cells on the slide) were labeled; many fewer cells in interphase were labeled (below).



- After a 20 hour chase, none of the 7% of cells that were in mitosis is labeled. Instead, all of the labeled cells are in interphase (below).



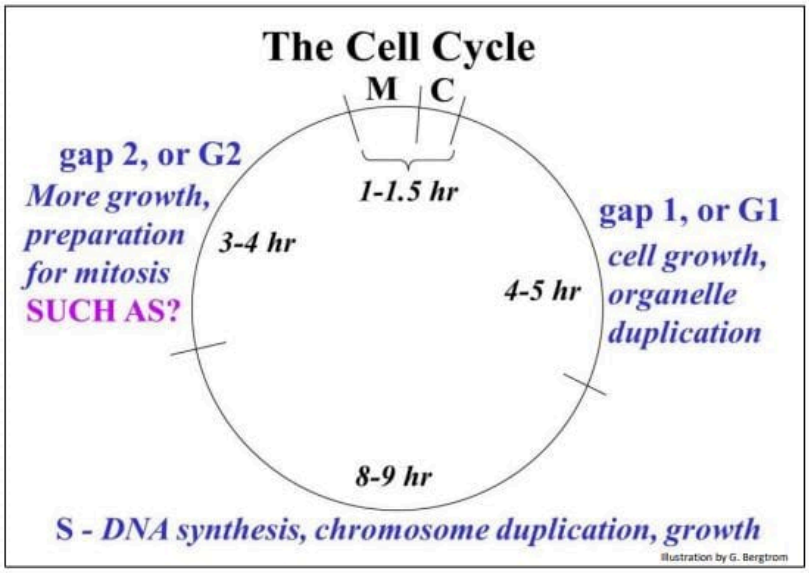
- The graph below plots a count of *radiolabeled mitotic cells* against *chase times*.



The plot defines the duration of events, or phases of the cell cycle as follows:

- The first phase (interval **#1** on the graph) must be the time between the end of DNA synthesis and the start of mitosis, defined as **Gap 2** (**G_2**).
- Cell doubling times are easily measured. Assume that the cells in this experiment doubled every 20 hours. This would be consistent with the time interval of 20 hours between successive peaks in the number of radiolabeled mitotic cells after the pulse (interval **#2**).
- Interval **#3** is easy enough to define. It is the time when DNA is synthesized, from start to finish; this is the **synthesis**, or **S** phase.
- One period of the cell cycle remains to be defined, but it is not on the graph! That would be the time between the end cell division (i.e., mitosis and cytokinesis) and the beginning of DNA synthesis (*replication*). That interval can be calculated from the graph as the time of the cell cycle (~20 hours) minus the sum of the other defined periods of the cycle. This phase is defined as the **Gap 1** (**G_1**) phase of the cycle.

So at last, here is our cell cycle with a summary of events occurring in each phase.



During all of interphase (**G_1, S and G_2**) , the cell grows in size, preparing for the next cell division. Growth in **G_1** includes the synthesis of enzymes and other proteins that will be needed for replication.

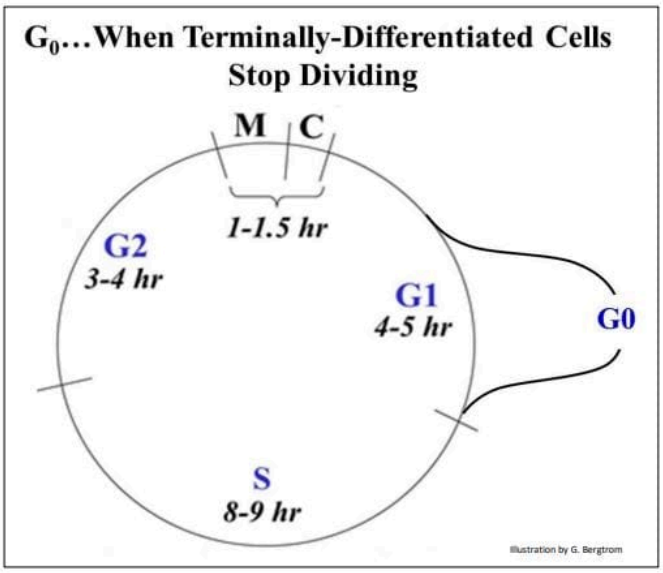
DNA is replicated during the S phase, along with the synthesis of new *histone* and other proteins that will be needed to assemble new *chromatin*. **G_2** is the shortest time of interphase and is largely devoted to preparing the cell for the next round of mitosis and cytokinesis. Among the proteins whose synthesis increases in this time are the *tubulins* and proteins responsible for condensing chromatin into the paired chromatids representing the duplicated chromosomes. **Cohesin** is a more recently discovered protein made in the run-up to mitosis. It holds centromeres of chromatids together until they are ready to separate.

341 Events in the Phases of the Cell Cycle

In a final note, typical dividing cells have generation times ranging from 16 to 24 hours. Atypical cells, like newly fertilized eggs, might divide every hour or so! In these cells, events that normally take many hours must be completed in just fractions of an hour.

B. When Cells Stop Dividing

Terminally differentiated cells are those that spend the rest of their lives performing a specific function. These cells no longer cycle. Instead, shortly after entering G_1 they are diverted into a phase called **G_0**, as shown below.



Referred to as **terminally differentiated**, these cells normally never divide again. With a few exceptions (e.g., many neurons), most terminally differentiated cells have a finite lifespan, and must be replaced by stem cells. Examples include red blood cells. With a half-life of about 60 days, they are regularly replaced by reticulocytes produced in bone marrow

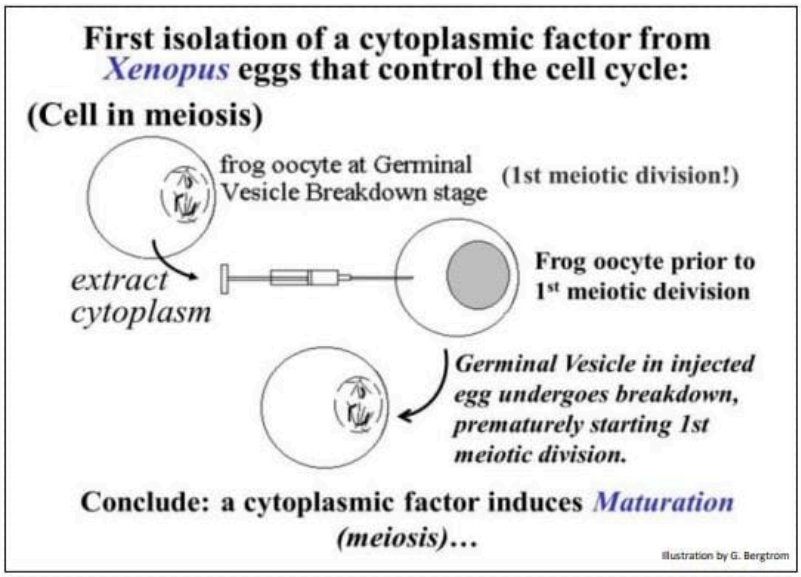
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19.3: Regulation of the Cell Cycle

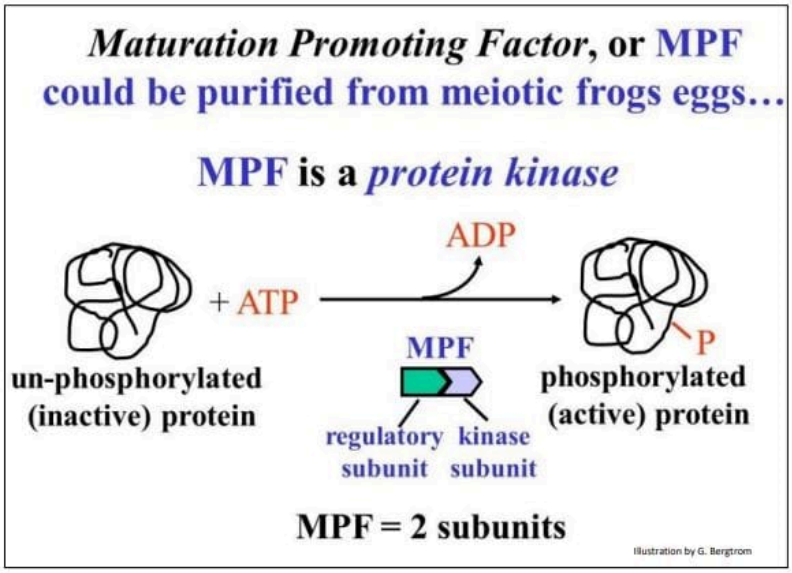
Progress through the cell cycle is regulated. The cycle can be controlled or put on 'pause' at any one of several phase transitions. Such **checkpoints** monitor whether the cell is on track to complete a successful cell division event. Superimposed on these controls are signals that promote cell differentiation. Embryonic cells *differentiate* as the embryo develops. Even after *terminal differentiation* of cells that form all adult tissues and organs, *adult stem cells* will divide and differentiate to replace worn out cells. Once differentiated, cells are typically signaled in $\mathbf{G_1}$ to enter $\mathbf{G_0}$ and stop cycling. In some circumstances cells in $\mathbf{G_0}$ are recruited to resume cycling. However, if this occurs to by mistake, the cells may be *transformed* to cancer cells. Here we consider how the normal transition between phases of the cell cycle is controlled.

A. Discovery and Characterization of *Maturation Promoting Factor (MPF)*

Growing, dividing cells monitor their progress through the phases. Cells produce internal chemical signals that tell them when it's time to begin replication or mitosis, or even when to enter into G_0 when they reach their terminally differentiated state. The experiment that first demonstrated a chemical regulator of the cell cycle involved fusing very large frog's eggs! The experiment is described below.



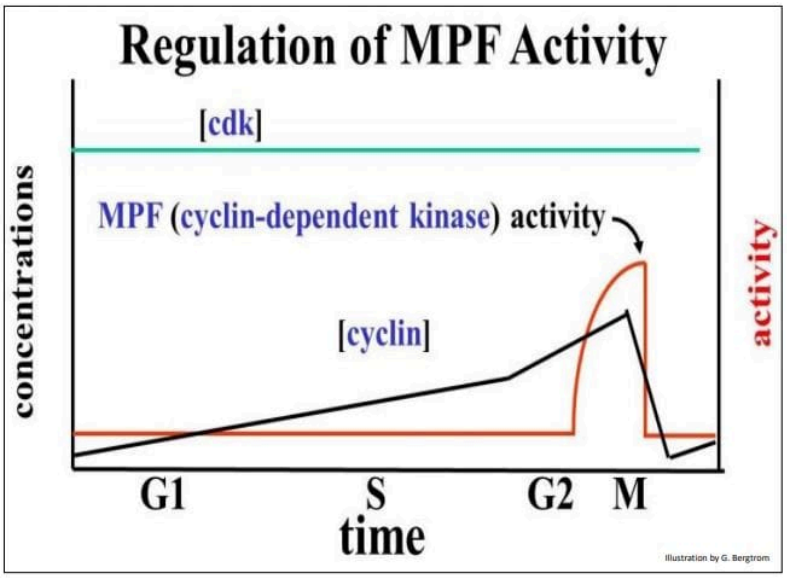
The hypothesis tested here was that frog oocyte cytoplasm from **germinal vesicle stage** oocytes (i.e., in mid-meiosis) contains a chemical that caused the cell to lose its nuclear membrane, condense its chromatin into chromosomes and enter meiosis. Cytoplasm was withdrawn from one of these mid-meiotic oocytes with a fine hypodermic needle, and then injected into a pre-meiotic oocyte. The mid-meiotic oocyte cytoplasm induced premature meiosis in the immature oocyte. A **maturation promoting factor** (**MPF**) could be isolated from the mid-meiotic cells and injected into pre-meiotic cells; it caused them to enter meiosis. **MPF** turns out to be a protein kinase made up of two polypeptide subunits as shown below.



MPF was then also shown to stimulate s*omatic cells* in $\mathbf{G_2}$ to enter premature mitosis. So conveniently, *MPF* can also be **Mitosis Promoting Factor**! Hereafter we will discuss the effects of *MPF* as being equivalent in mitosis and meiosis. When active, *MPF* targets many cellular proteins.

342 Discovery of MPF Kinase and Its Role in Meiosis and Mitosis

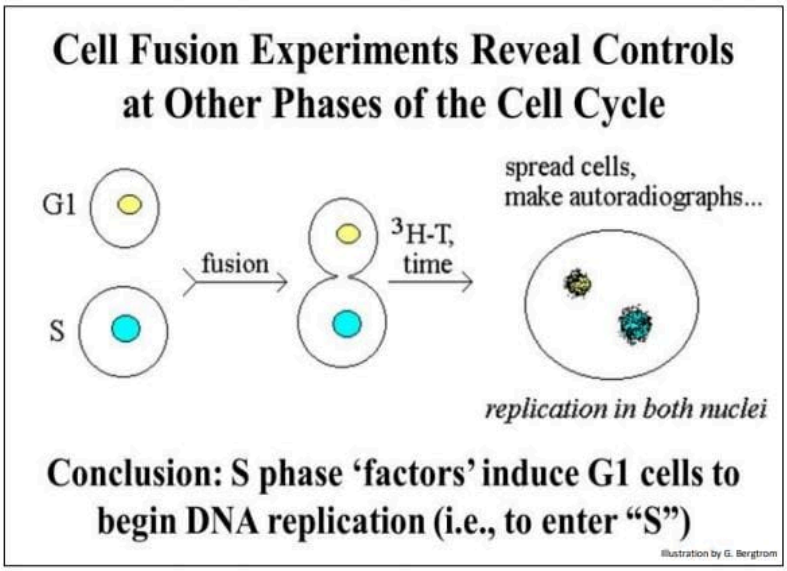
Assays of *MPF* activity as well as the actual levels of the two subunits over time during the cell cycle are graphed below.



One subunit of *MPF* is **cyclin**, a regulatory polypeptide. The other subunit, **cyclin-dependent kinase** (**cdk**), contains the kinase enzyme *active site*. Both subunits must be bound to make an active kinase. Cyclin was so-named because its levels rise gradually after cytokinesis, peak at the next mitosis, and then fall. Levels of the cdk subunit do not change significantly during the life of the cell. Because the kinase activity of MPF requires *cyclin*, it tracks the rise in cyclin near the end of the $\mathbf{G_2}$, and its fall after mitosis. Cyclin begins to accumulate in $\mathbf{G_1}$, rising gradually and binding to more and more *cdk* subunits. *MPF* reaches a threshold concentration in $\mathbf{G_2}$ that triggers entry into mitosis. For their discovery of these central molecules Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse won the 2001 Nobel Prize in Physiology or Medicine.

B. Other Cyclins, CDKs and Cell Cycle Checkpoints

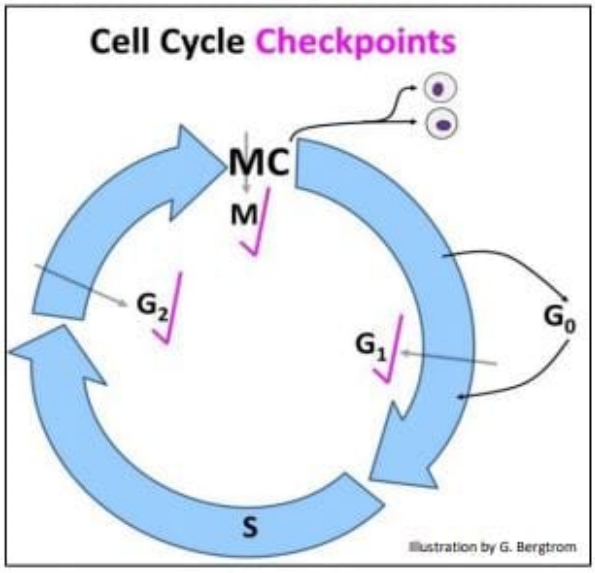
Other chemical signals accumulate at different points in the cell cycle. For example, when cells in S are fused with cells in **G_1**, the **G_1** cells begin synthesizing DNA (visualized as ^{3}H-thymine incorporation). An experiment showing control of progress to different phases of the cell cycle is illustrated below.



An **S** phase factor could be isolated from the **S** phase cells. This factor also turns out to be a two-subunit protein kinase, albeit a *different* one from MPF. Just as MPF signals cells in **G_2** to begin mitosis, the **S** phase kinase signals cells in **G_1** to enter the **S** phase of the cell cycle. *MPF* and the **S** phase kinase govern activities at two of several cell cycle **checkpoints**. In each case, the activity of the kinases is governed by prior progress through the cell cycle. In other words, if the cell is not ready to begin mitosis, active *MPF* production is delayed until it is. Likewise, the **S** phase kinase will not be activated until the cell is ready to begin DNA synthesis.

343 Cell Cycle Control at Check Points and the Go "Phase"

The sequence of signals that control progress through the cell cycle is probably more intricate and extensive than we currently know, but the best-described checkpoints are in **G_1**, **G_2** and **M** (below).



We generally envision checkpoints as monitoring and blocking progress until essential events of a current phase of the cell cycle phase are completed. These kinases are part of molecular sensing mechanisms that act by phosphorylating cytoplasmic and/or nuclear proteins required by upcoming phases of the cycle. Let's take a closer look at some events that are monitored at these *checkpoints* in more detail.

1. The G_1 Checkpoint

The G_1 checkpoint controls the transition from the G_1 to the S phase of the cell cycle. If actively dividing cells (e.g., stem cells) in G_1 fail to complete their preparation for replication, the S-phase kinase won't be produced and the cells won't proceed the S phase until the preparatory biochemistry catches up with the rest of the cycle. To enter S, a cell must be ready to make proteins of replication, like DNA polymerases, helicases, and primases among others. Only when these molecules have accumulated to (or become active at) appropriate levels, is it "safe" to enter S and begin replicating DNA. This description of **G_1 checkpoint** activity is consistent with the idea that all checkpoints delay cycling until a prior phase is complete.

What about cells that are fully differentiated? Such *terminally differentiated* cells stop producing the active **G_1** *checkpoint* kinase and stop dividing. These cells are arrested in **G_0** (see below). As an interesting side-note, recall that somatic cells are **diploid** and germ cells (sperm, egg) are **haploid**. So, are cells in **G_2** that have already doubled their DNA 'tetraploid', however briefly? Whether or not we can call **G_2** cells tetraploid (officially, probably not), it is clear that **G_1** cells and **G_0** cells are diploid!

2. The G_2 Checkpoint

Passage through the **G_2** checkpoint is only possible if DNA made in the prior **S** phase is not damaged. Or if it was, that the damage has been (or can be) repaired (review the proofreading functions of DNA polymerase and the various DNA repair pathways). Cells that do successfully complete replication and pass the **G_2** checkpoint must prepare to make the proteins necessary for the upcoming mitotic phase. These include nuclear proteins necessary to *condense* chromatin into chromosomes, *tubulins* for making *microtubules*, etc. Only when levels of these and other required proteins reach a threshold can the cell begin mitosis. Consider the following two tasks required of the **G_2** checkpoint (in fact, any checkpoint):

- sensing whether prior phase activities have been successfully completed.
- delaying transition to the next phase if those activities are unfinished.

But what if sensing is imperfect and a checkpoint is leaky? A recent study suggests that either the **G_2** checkpoint **is** leaky, or at least, that incomplete activities in the **S** phase are tolerated, and that some DNA repair is not resolved until mitosis is underway in M! Check it out at DNA repair and replication during mitosis.

3. M Checkpoint

The M checkpoint is monitored by the original *MPF* phosphorylation of proteins that: (a) bind to chromatin causing it to condense and form chromatids, (b) lead to the breakdown of the nuclear envelope, and (c) enable spindle fiber formation,. In addition, tension in the spindle apparatus at metaphase tugs at the kinetochores holding the duplicated chromatids together. When this tension reaches a threshold, MPF peaks and an activated **separase** enzyme causes the chromatids to separate at their centromeres. Beginning in *anaphase*, tension in the spindle apparatus draws the new chromosomes to opposite poles of the cell. Near the end of mitosis and cytokinesis, proteins phosphorylated by MPF initiate the breakdown of cyclin in the cell. Passing the **M** checkpoint means that the cell will complete mitosis and cytokinesis, and that each daughter cell will enter a new **G_1** phase.

Dividing yeast cells only seem to have the three checkpoints discussed here. More complex eukaryotes use more *cyclins* and *cdks* to control the cell cycle at additional checkpoints. Different *cyclins* show cyclic patterns of synthesis, while *cdks* remain at constant levels throughout the cell cycle (as in MPF). Different gene families encode evolutionarily conserved *cdks* or *cyclins*. But each *cyclin/cdk* pair has been coopted in evolution to monitor different cell cycle events and to catalyze phosphorylation of phase-specific proteins. To learn more, see Elledge SJ (1996) *Cell Cycle Checkpoints: Preventing an Identity Crisis*. Science 274:1664-1672.

344 Cyclin/cdk Checkpoint for Cell Cycle Phases

C. The G_0 State

This is not really a phase of the cell cycle, since cells in **G_0** have reached a terminally differentiated state and have stopped dividing. In development, terminally differentiated cells in tissues and organs no longer divide. Nevertheless, most cells have finite half-lives (recall our red blood cells that must be replaced every 60 days or so). Because cells in many tissues are in **G_0** and can't divide, they must be replaced by stem cells, which can divide and differentiate. Some cells live so long in **G_0** that they are nearly never replaced (muscle cells, neurons). Other cells live short lives in **G_0** (e.g., stem cells, some embryonic cells). For example, a **lymphocyte** is a differentiated *immune system* white blood cell type. However, exposure of lymphocytes to foreign chemicals or pathogens activates **mitogens** that cause them to re-enter the cell cycle from **G_0**. The newly divided cells then make the antibodies that neutralize the chemicals and fight off the pathogens. The **retinoblastoma** (**Rb**) protein is an example of a mitogen. Like other mitogens, the Rb protein is a transcription factor that turns on genes that lead to cell proliferation. What if cells continue cycling when they aren't supposed to? Or, what if they are inappropriately signaled to exit G_0? Such cells are in trouble! Having escaped normal controls on cell division, they can become a focal point of cancer cell growth. You can guess from its name that the retinoblastoma gene was discovered as a mutation that causes retinal cancer. For more about the normal function of the Rb protein and its interaction with a G_1 cdk, check out the link below.

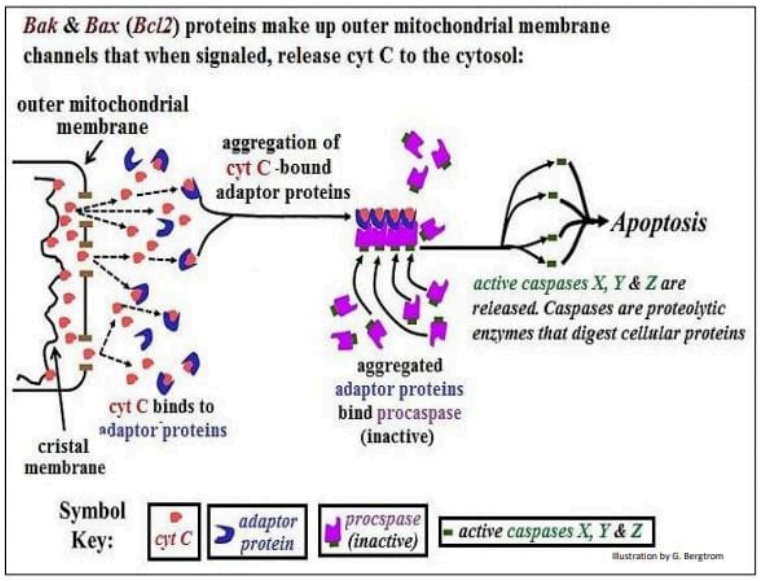
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345 Rb Gene Encodes Transcription Factor Regulatory Subunit

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19.4: When Cells Die

As noted, few cell types live forever; most live for a finite time. Most are destined to turn over (another euphemism for dying), mediated by **programmed cell death**, or **apoptosis**. This occurs in normal development when cells are only temporarily required for a maturation process (e.g., embryonic development, metamorphosis). When no longer necessary or when genetically or otherwise damaged, such cells are detected and signaled to undergo *apoptosis*. Programmed cell death starts with an external signal programmed to appear at a specific time in development. The signal molecule acts on target cells to induce transcription of **Bcl2** genes. *Bcl2* proteins **Bak** and **Bax** are outer mitochondrial membrane channel components that allow the release of *cytochrome C into the cytoplasm*. This sets off molecular events leading to *apoptosis*. The role of *cytochrome C* in apoptosis is illustrated below.



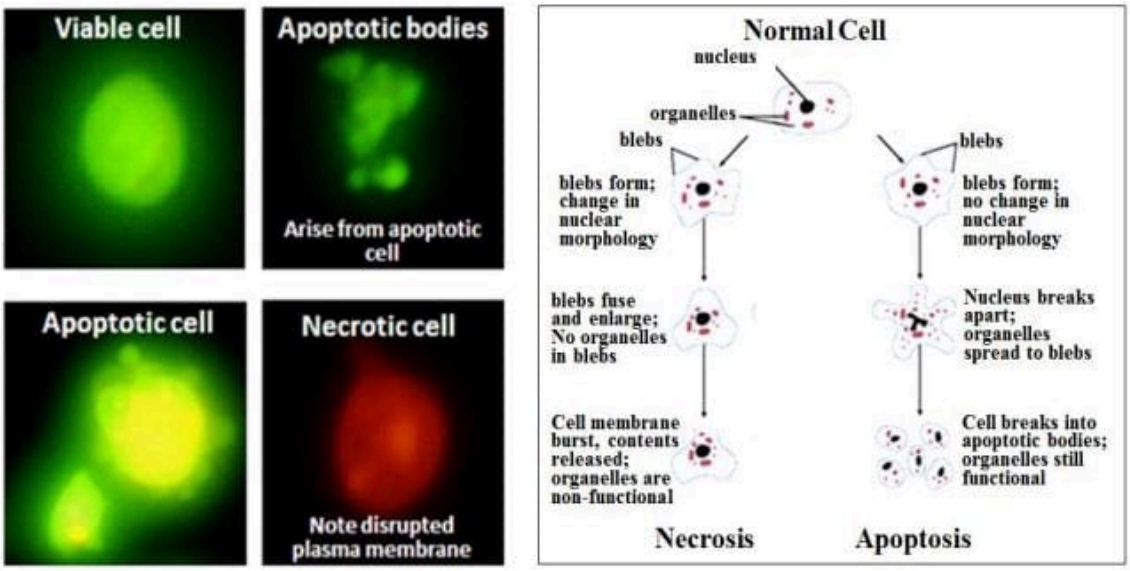
Mitochondrial exit of cytochrome C is possible because it is a *peripheral* membrane protein, only loosely bound to the cristal membrane. It exists in equilibrium between membrane-bound and unbound states. As some cytochrome C molecules exit the intermembrane space, others detach from the cristal membrane and follow. In the cytosol, cytochrome C binds to **adaptor** proteins that then aggregate. The cytochrome c-adaptor complex has a high affinity for a biologically **inactive procaspase**. Binding of *procaspase* to the *cytochrome C-adaptor* complex causes an allosteric change in the *procaspase*, releasing an active **caspase**. *Caspases* are *proteolytic enzymes* that start the auto-digestion of the cell.

One example of apoptosis is amphibian metamorphosis. *Thyroid hormone* signals tadpole metamorphosis. The hormone causes tadpoles to digest their own tail cells, allowing reabsorption and recycling of the digestion products. These in turn serve as nutrients to grow adult frog structures. For their work in identifying apoptosis genes, Sydney Brenner, H. Robert Horvitz and John E. Sulston shared the 2002 Nobel Prize in Physiology or Medicine.

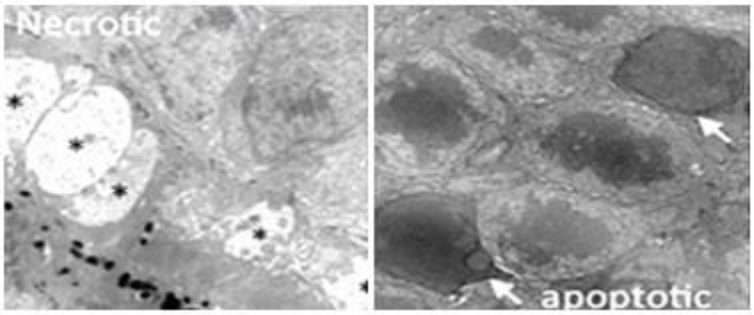
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19.5: Disruption of the Cell Cycle Checkpoints Can Cause Cancer

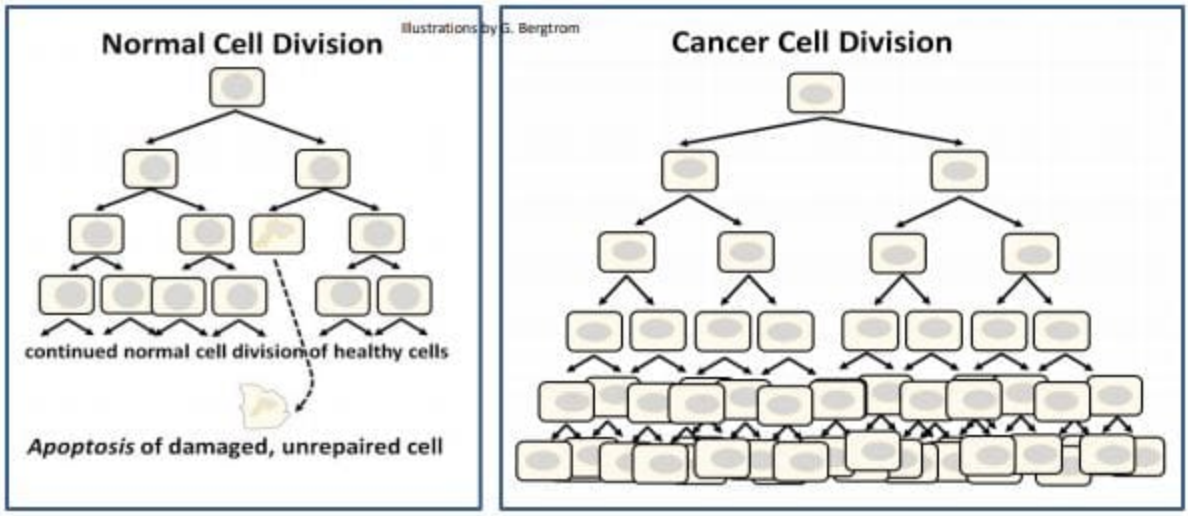
If a checkpoint fails or if a cell suffers physical damage to chromosomes during cell division, or if it suffers a debilitating somatic mutation in a prior **S** phase, it may selfdestruct in response to a consequent biochemical anomaly. This is another example of *apoptosis*. On the other hand, when cells die from external injury, they undergo **necrosis**, an *accidental* rather than a programmed death. In the cells shown below, apoptosis or necrosis were chemically induced, followed and identified as apoptotic or necrotic using fluorescent markers (propidium iodide, green; acridine orange, orange).



Only green-fluorescing (apoptotic) cells eventually formed apoptotic bodies. In contrast, necrotic (orange-fluorescing) cells lose their plasma membranes, do not form such 'bodies' and will eventually disintegrate. (400x magnification). Differences in ultrastructure between necrosis and apoptosis are also seen in electron micrographs of cone and rod cells (left and right, respectively) below. An asterisk indicates the cytoplasmic swelling characteristic of necrotic cone. White arrows point to nuclei characteristic of apoptosis of in rod cells.



As we've noted, cycling cells continue to divide until they attain $\mathbf{G_0}$ in the terminally differentiated state. Most terminally differentiated cells are cleared by *apoptosis* when they reach the end of their effective lives, to be replaced by stem cells. We also noted that accidental signaling can bring cells out of $\mathbf{G_0}$, leading to renewed cell proliferation. While these cells are obviously abnormal, they are not detected by apoptotic defense mechanisms. Thus, they undergo uncontrolled cell divisions, becoming cancer cells. Likewise, physically damaged or mutated cells may sometimes escape apoptotic clearance. When they do, they may also become cancer cells. Apoptotic clearance and uncontrolled cancer cell proliferation are compared below

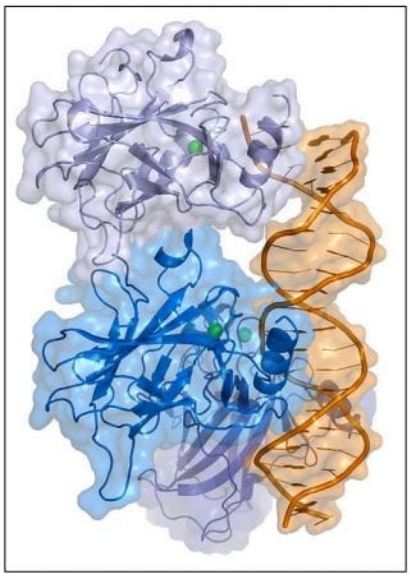


346 Apoptosis (Programmed Cell Death) vs. Necrosis

A. P53 Protein Mediates Normal Cell Cycle Control

Cancerous growth could result if a normal dividing cell should suffer a somatic mutation that disrupts normal cell cycle control. Think an over-expression of *cdk* for example. Alternatively, imagine *cyclin* levels in daughter cells that never drop; such cells would never stop cycling.

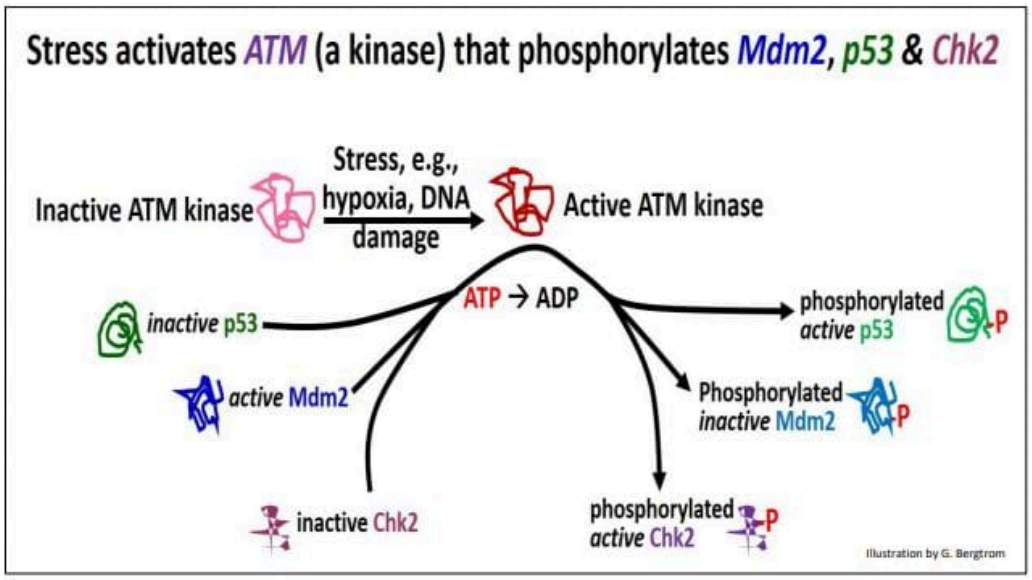
Other possibilities include a cell in $\mathbf{G_0}$ that is stimulated to begin cycling again by an inappropriate encounter with a hormone or other signal. If undetected, these anomalies can transform cells to cancer cells. The **p53 protein** (illustrated below) is a DNA-binding, gene-regulatory protein that detects some of these anomalies and enables dividing cells to repair the damage before proceeding through cell cycle check points..., or failing that, will lead to apoptosis of the cell.



Not surprisingly, mutations in the gene for the **P53 protein** (called **TP53** in humans) are associated with many human cancers (pancreatic, lung, renal cell, breast, etc.). As many as half of human cancers are associated with mutated *p53* genes. Thus, *p53* is one of a class of **tumor suppressor proteins**. Studies of humans with a condition known as **LFS** (*Li-Fraumeni syndrome*) have at least one mutated *p53* allele. The mutation leads to a ~100% lifetime risk of cancer, beginning in childhood. In cultured cells, mutagenized p53 genes exhibit key characteristics of cancer cells, including unregulated cell proliferation and suppression of apoptosis.

1. How p53 Works

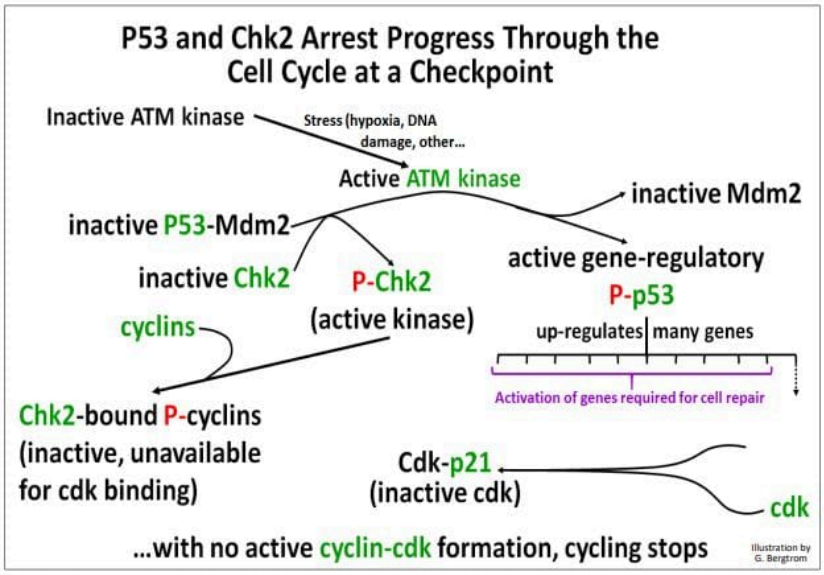
The *p53 protein* is normally bound to an active **Mdm2** protein. To enable cell cycle checkpoints, *p53-Mdm2* must separate and be kept separate to allow p53 time to act. In dividing cells, physical stress or chemical stress such as DNA damage during cell growth can activate an **ATM kinase**. ATM kinase in turn, phosphorylates *Mdm2*, causing it to dissociate from p53. The same kinase also phosphorylates another kinase, **Chk2**, as well as the now 'free' **p53**. ATM kinase-initiated events are further detailed below.



Each of the proteins and enzymes phosphorylated by the ATM kinase has a role in cell cycle checkpoint function and cell cycle arrest while errors are corrected:

- Now separated from Mdm2, **Phospho-p53** actively *up-regulate*s several genes, including the **p21** gene.
- The **P21** protein binds to **cdks**; **cyclins** can't bind **P21-cdks**.
- Active **Phospho-Chk2** catalyzes cyclin phosphorylation; **phospho-cyclins** can't bind to **p21-cdks**.
- The inability of *cyclins* to bind **cdks** specifically blocks the cell cycle between the **G_1** and **S**, and the **G_2**-to-**M** phases.

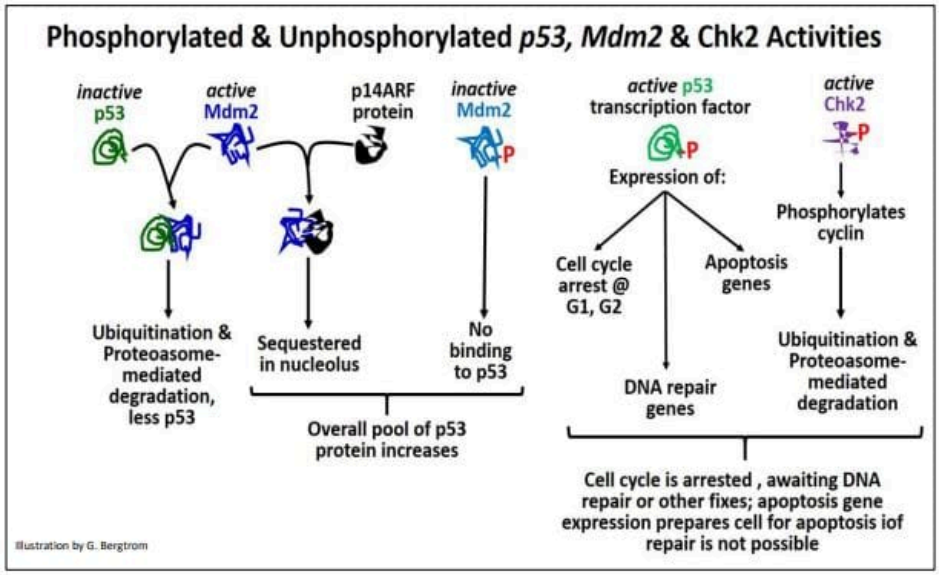
These kinase-mediated events at cell cycle checkpoints are illustrated below.



The cell cycle is remains arrested while the cell attempts to finish essential biochemical activities necessary to correct stress-induced or other physical or chemical aberrations before moving on to the next phase of the cycle. If DNA repairs or other corrections are successful, the cell can progress to the next phase.

If not, **proteasomes** target the **Chk2-cyclin** complex for degradation. Likewise, any **P53** remaining bound to unphosphorylated **Mdm2** is also targeted for proteasome destruction. The result is that any cell unable to correct effects of stress or chemical damage, or to repair DNA damage, is target for **apoptosis**.

The levels and activity of **p53** as well as the other proteins discussed above, control both the amount of **p53** protein available to respond to cell cycling anomalies, and the responses themselves. Phosphorylation (activation) of **p53** not only leads to a rapid arrest of the cell cycle, but also to the activation of genes encoding proteins required for DNA repair *and* of proteins required for apoptosis (in the event that repair efforts fail). The interactions of **p53** with different proteins leading to alternate cell fates are summarized below.



To sum up, **p53** suppresses malignant tumor growth either by

- allowing DNA or other cellular repair before resumption of normal cell cycling, preventing unregulated cell divisions; after repair, p53 and other proteins are inactivated and/or destroyed and the cell cycle can resume.
- The inability to repair/correct cell cycling problems sets in motion events leading to apoptosis, thereby also blocking tumorigenesis by killing off damaged cells.

It should be clear now why a mutant **p53** that reduces or eliminates **p21** protein production or blocks essential DNA repair protein production, will allow damaged cells to enter **S** and keep them replicating and dividing, transforming such them into cancer cells. In an interesting twist, it seems that compared to humans, few whales or elephants die from cancer, despite having thousands of times more cells than humans. The reason seems to be that, at least for elephants, they have as many as 20 copies (40 alleles) of their p53 genes! Thus a mutation in one allele of one of them may have little effect, while the tumor-repressing effects of the remaining p53 genes prevails. Read about this recent research at Whales and Elephants Don't Get Cancer!

2. The Centrality of p53 Action in Cell Cycle Regulation

Because of its multiple roles in regulating and promoting DNA repair, and in controlling cell cycle checkpoints, p53 has been called "*the Guardian of the Genome*"! Here is further evidence of this central role.

a) 'Oncogenic Viruses'

Cancer causing viruses include Human Papilloma Virus (**HPV**), Epstein Barr Virus (**EBV**), human immunodeficiency virus (**HIV**), Hepatitis B and C viruses (**HBV, HCV**), Human herpes virus 8 (**HHV-8**) and simian virus 40 (**SV40**).

There is a demonstrated link between SV40, **p53** and cancer. SV40 is a viral contaminant of polio vaccines that were used in the 1960s. The virus is tumorigenic in mammals, though an association of SV40 and cancer in humans is unproven. In infected cells, SV40 DNA enters the nucleus where it can integrate into the host cell genome. SV40 infections are usually latent, (i.e., they cause no harm). However, activation can lead to cellular transformation and the growth of malignant sarcomas in muscles as well as tumors in other organs. The RNA polymerase II in infected cells transcribes the SV40 genes, producing proteins that replicate and encapsulate the viral DNA in a membrane to make new viral particles. However, the relatively small SV40 genome does not encode all of the enzymes and factors need for viral DNA replication. The infected cells themselves provide these factors, producing them only during the **S** phase. At that time, the SV40 **large T antigen** (made soon after infection) enters the host cell nucleus where it regulates transcription of genes essential to viral replication and viral particle formation. The *large T antigen* also binds to **p53**, interfering with transcription of proteins whose genes are regulated by **p53**. Unable to exercise checkpoint functions, the host cell divides uncontrollably, forming cancerous tumors. Deregulation of the cell cycle by *large T antigen* ensures progress to the S phase and unregulated co-replication of viral and host cell DNA.

b) p53 and Signal Transduction

Stress can activate signal transduction pathways. For example, mutations affecting the **MAPK** (MAP kinase) signaling pathway can lead to tumorigenesis. This can be explained by the observation that when activated, the MAPK pathway leads to amplified production of a kinase that phosphorylates **p53**. Active **phospho-p53** in turn augments activation of the MAPK signal transduction pathway. You may recall that MAPK signal transduction typically ends with a mitogenic response.

Another example of p53 interaction is with **FAK** (**focal adhesion kinase**) proteins. *FAK* activity is increased by *integrin*-mediated signal transduction. Recall that membrane integrins bind *fibronectin*, contributing to formation of the extracellular matrix, or *ECM*. Elevated *FAK* activity participates in the regulation of cell-cell and cell-ECM adhesion at **focal adhesion points**. Another role for FAK is to bind directly to inactive p53 and increase p53-Mdm2 binding. As we have just seen, persistent p53-Mdm2 is targeted for ubiquitination... and ultimate destruction! In fact, abnormally high levels of FAK are associated with many different tumor cell lines (colon, breast, thyroid, ovarian, melanoma, sarcoma...). These result when p53 is unable properly to activate cell cycle checkpoints.

While the interactions implied here are complex and under active study, these **p53** activities certainly confirm its central role as both **guardian of the genome** and as **guardian of cell division**.

B. Growth and Behavior of Cancer Cells

Different cancer cell types have different growth and other behavioral properties. You may have heard of **slow growing** and **fast growing** cancers. *Colon* cancers are typically slow growing. Periodic *colonoscopies* that detect and remove colorectal tumors in middle-age or older people can prevent the disease (although the risks of disease and the procedure itself must be balanced). *Pancreatic* cancers are fast growing and usually go undetected until they reach an advanced stage. The twin goals of medical research are to detect the different cancers early enough for successful intervention, and of course, to find effective treatments.

A single mutated cell in a tissue can become the growth point of a **tumor**, essentially a mass of cells cloned from the original mutated one. **Benign tumors** or growths (for example breast and uterine *fibroid*s in women, or common moles in any of us) stop growing and are not life threatening. They are often surgically removed for the comfort of the patient (or because cells in some otherwise benign tumors may have a potential to become cancerous).

Malignant tumors (also called *malignant neoplasms*) are cancerous and can grow beyond the boundaries of the tumor itself. When tumor cells are shed they may enter the bloodstream and travel to other parts of the body, the phenomenon called **metastasis**. Cancer cells that metastasize can become the focal point of new tumor formation in many different tissues. Because cancer cells continue to cycle and replicate their DNA, they can undergo yet more somatic mutations. These further changes can facilitate metastasis and cancer cell growth in different locations in the body.

C. Cancer Treatment Strategies

There are many different kinds of cancers originating in different tissues of the body. They all share the property of uncontrolled cell division, albeit for different molecular and not always well-understood reasons. The two major cancer treatment strategies developed in the 20th century all aim at disrupting replication in some way.

- **Radiation therapy** relies on the fact that most cells in our bodies do not divide, aiming mutagenic radiation at tumors in the hope that replicating DNA will be mutated at so many sites (i.e., genes) that the tumor cells can no longer survive or replicate properly.
- **Chemotherapy** is used to attack tumors that do not respond well to radiation or that are not easily be reached by radiation technologies, and to fight cancers that do not even form focused tumors (such as lymphomas and leukemias involving lymph and blood cells). These *chemotherapies* also aim to derange replication or mitotic activities. For example, recall **cordycepin** (dideoxyadenosine triphosphate, or ddATP). When present during replication, ddATP is incorporated into a growing DNA chain, after which no additional nucleotides can be added to the DNA strand. That makes ddATP a potent chemotherapeutic disruptor of replication. **Taxol** is another chemo drug that acts in this case, not by inhibiting S phase replication, but by blocking spindle fiber microtubules from depolymerizing, thus blocking mitotic anaphase and telophase in the latter part of the M and C phases of the cycle. **Colchicine** (a plant alkaloid) attacks cancer (and other dividing) cells by blocking microtubule formation and therefore preventing spindle fiber formation in mitotic prophase.

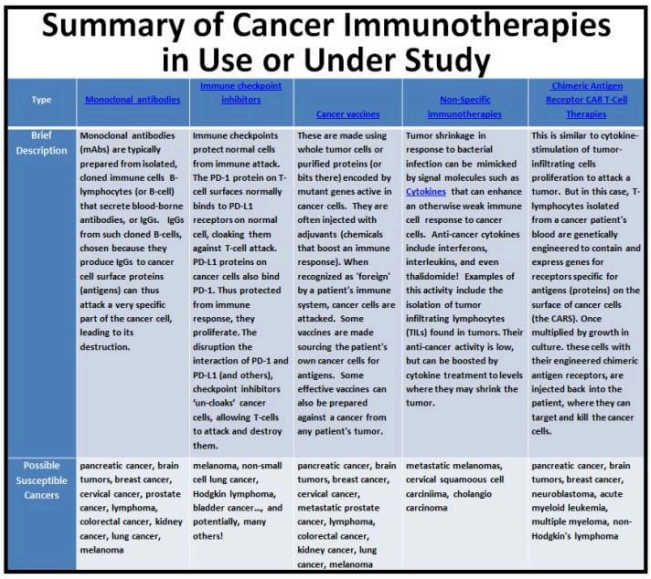
These therapies are not effective against all cancers, and of course, they don't target specific kinds of cancer cells. Their success relies simply on the fact that cancer cells proliferate rapidly and constantly while other cell types do not. Many if not all of the side effects of radiation and chemotherapies result from the damage done to normal dividing cells (e.g., hair follicle cells accounting for hair loss among many cancer patients, depletion of blood cells that fail to be replaced by stem cells in bone marrow).

Much research now is focused on mobilizing the body's own immune system to create more specific, targeted cancer treatments. In a fascinating bit of history, more than 100 years ago, Dr. William B. Coley injected a terminal cancer patient with streptococcal bacteria, who then emerged tumor-free upon his recovery from the infection (for details, check out The Earliest Cancer Immunotherapy Trials). The phenomenon of "Dr. Coley's Toxins" was initially thought to be an anti-tumor effect of the bacteria. But by 1948 it was widely attributed to the immune response activated by the infection. In the 1990s, scientists revisited the immune response to cancer, and by the turn of the 21st century, studies of cancer immunotherapy picked up steam (and more substantial research funding!).

Recent animal immunotherapy experiments and human clinical trials are promising. A few immunotherapies have already been approved by the U.S. FDA (Food and Drug Administration). Cancer immunotherapy strategies capitalize on the fact that your body sometimes recognizes cancer cell markers (e.g., cell surface molecules) as foreign, thus mounting an immune defense against those cells. But that response is sometimes not powerful enough to clear new, rapidly dividing cancer cells. Cancer apparently results when the immune response is weak. There are different, sometimes overlapping approaches to cancer immunotherapy. All are based on the fact that cancer cells that have mutated in some way and are producing aberrant proteins that the immune system can see as foreign enough to elicit an immune response, however slight. Some immunotherapies seek to boost that immune response. Others seek isolate or generate unique cancer cell antigens that will immunize a patient when injected with these cancer antigens. Some immunotherapies are summarized in the table on the next page. As you can see from the table, immuno-targeting cancer cells has already proven to be highly effective. In some cases the therapy is an example of *personalized medicine*, in which treatments are uniquely tailored to you as a patient. Issues with immunotherapies are that

- they are time and labor intensive, and costly to produce.
- while they may 'cure' you, they likely won't not work on someone else.
- like radiation and chemotherapy, immunotherapies come with their own unpleasant and sometimes severe side effects.

A more detailed discussion of cancer immunotherapies is on the *cancer.gov* website at Cancer Treatment Immunotherpay.



Summary of Cancer Immunotherapies in Use or Under Study

Type	Monoclonal antibodies	Immune checkpoint inhibitors	Cancer vaccines	Non-Specific immunotherapies	Chimeric Antigen Receptor CAR T-Cell Therapies
Brief Description	Monoclonal antibodies (mAbs) are typically prepared from isolated, cloned immune cells B-lymphocytes (or B-cell) that secrete blood-borne antibodies, or IgGs. IgGs from such cloned B-cells, chosen because they produce IgGs to cancer cell surface proteins (antigens) can thus attack a very specific part of the cancer cell, leading to its destruction.	Immune checkpoints protect normal cells from immune attack. The PD-1 protein on T-cell surfaces normally binds to PD-L1 receptors on normal cell, cloaking them against T-cell attack. PD-L1 proteins on cancer cells also bind PD-1. Thus protected from immune response, they proliferate. The disruption the interaction of PD-1 and PD-L1 (and others), checkpoint inhibitors 'un-cloaks' cancer cells, allowing T-cells to attack and destroy them.	These are made using whole tumor cells or purified proteins (or bits there) encoded by mutant genes active in cancer cells. They are often injected with adjuvants (chemicals that boost an immune response). When recognized as 'foreign' by a patient's immune system, cancer cells are attacked. Some vaccines are made sourcing the patient's own cancer cells for antigens. Some effective vaccines can also be prepared against a cancer from any patient's tumor.	Tumor shrinkage in response to bacterial infection can be mimicked by signal molecules such as Cytokines that can enhance an otherwise weak immune cell response to cancer cells. Anti-cancer cytokines include interferons, interleukins, and even thalidomide! Examples of this activity include the isolation of tumor infiltrating lymphocytes (TILs) found in tumors. Their anti-cancer activity is low, but can be boosted by cytokine treatment to levels where they may shrink the tumor.	This is similar to cytokine-stimulation of tumor-infiltrating cells proliferation to attack a tumor. But in this case, T-lymphocytes isolated from a cancer patient's blood are genetically engineered to contain and express genes for receptors specific for antigens (proteins) on the surface of cancer cells (the CARS). Once multiplied by growth in culture. these cells with their engineered chimeric antigen receptors, are injected back into the patient, where they can target and kill the cancer cells.
Possible Susceptible Cancers	pancreatic cancer, brain tumors, breast cancer, cervical cancer, prostate cancer, lymphoma, colorectal cancer, kidney cancer, lung cancer, melanoma	melanoma, non-small cell lung cancer, Hodgkin lymphoma, bladder cancer..., and potentially, many others!	pancreatic cancer, brain tumors, breast cancer, cervical cancer, metastatic prostate cancer, lymphoma, colorectal cancer, kidney cancer, lung cancer, melanoma	metastatic melanomas, cervical squamoous cell carciniima, cholangio carcinoma	pancreatic cancer, brain tumors, breast cancer, neuroblastoma, acute myeloid leukemia, multiple myeloma, non-Hodgkin's lymphoma

NOTE: The term *checkpoint inhibitor* in the context of immunotherapies is different than the term *checkpoints* describing portals to progress through the eukaryotic cell cycle.

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19.6: Key Words and Terms

anaphase	G_2 phase	mTOR signaling
apoptosis	*Guardian of the Genome*	necrosis
ATM kinase	immunotherapy	oncogenic viruses
benign tumors	intergrin	p14ARF
cancer cells	interphase	p21
CDKs	invasive tumors	p53
cell cycle	LFS	
cell cycle checkpoints	Li-Fraumeni Syndrome	PD-L1
chemotherapy	M checkpoint	programmed cell death
Chk2	M phase of the cell cycle	prophase
colchicine	malignant tumors	protein phosphorlyation
cyclin	MAPK	proteasome
cyclin level in cell cycle	maturation	radiation therapy
cytokinesis	maturation promoting	S phase
dideoxyNTP	Mdm2	signal transduction
elephant p53 genes	metaphase	SV40
FAK	metastasis	T antigens
G_0 of the cell cycle	mitosis	taxol
G_1 checkpoint	mitosis promoting factor	telophase
G_1 phase	mitotic phases	tumor suppressor protein
G_2 checkpoint	MPF	ubiquitination

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20: THE ORIGINS OF LIFE

20.1: Introduction
20.2: Thinking about Life's Origins- A Short Summary of a Long History
20.3: Formation of Organic Molecules in an Earthly Reducing Atmosphere
20.4: Origins of Organic Molecules in a Non-Reducing Atmosphere
20.5: Origins of Life Chemistries in an RNA World
20.6: Molecules Talk- Selecting Molecular Communication and Complexity
20.7: A Summary and Some Conclusions
20.8: Key Words and Terms

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20.1: Introduction

It is nearly universally accepted that there was a time, however brief or long, when the earth was a lifeless planet. Given that the cell is the basic unit of life, and that to be alive is to possess all of the **properties of life**, any cell biology textbook would be remiss without addressing the questions of **when** and **how** the first cells appeared on our planet. **Abiogenesis** is the origin of life from non-living matter. Of course describing abiogenesis is no longer possible by observation! Through experiment and educated guesswork, it has been possible to construct reasonable (if sometimes conflicting) scenarios to explain the **origins of life**, and hence our very existence.

In this chapter, we will see that different scenarios share at least one feature, namely a set of geologic, thermodynamic and chemical conditions that favored an accumulation of organic molecules and proto-structures that would eventually become a cell. Those permissive conditions would have been an ecological, climatological, and environmental **prebiotic laboratory** in which *many* experimental cells might have formed and competed. Hence the chapter title “Origins of Life”! Multiple origins were not only possible under these conditions, but also probable! According to Jeremy England, of MIT, the laws of thermodynamics dictate that "... when a group of atoms is driven by an external source of energy (like the sun or chemical fuel) and surrounded by a heat bath (like the ocean or atmosphere), matter inexorably acquires the key physical attribute(s) associated with life”. (Statistical Physics of Self Replication). Here is a reminder of those key attributes, or properties of life.

Properties of Life

1. **Evolution**: long term adaptation/speciation
2. **Cell-based**: Cells are the fundamental unit of life
3. **Complexity**: allows physical/biochemical changes (dynamic order)
4. **Homeostasis**: maintains balance between change and order
5. **Requires Energy**: needed to do *work* (cellular functions)
6. **Irritability**: immediate sensitivity and response to stimuli
7. **Reproduction**: the ability to propogate life
8. **Development**: programmed change, most obvious in multicellular organisms

Remember, to be alive is to possess not just some, but all of these properties! If entities with all of the properties of life (i.e., cells) did originate independently, they would have reproduced to form separate populations of cells. In this scenario, less successful populations go extinct while successful ones become dominant. Successful organisms would have spread, spawning populations and generating new species. The **take-home message** is that if conditions on a prebiotic earth favored the formation of the ‘first cell’, then why not the formation of two, or dozens or even hundreds of ‘first cells’? However, we will see that only one successful population of cells would survive to become the source of the common ancestor of all life on earth, while other populations became extinct.

As to the question of **when** life began, geological and geochemical evidence suggests the presence of life on earth as early as 4.1 billion years ago. As for **how** life began, this remains the subject of ongoing speculation. *All* of the scenarios described below attempt to understand the physical, chemical and energetic conditions that might have been the ideal laboratory for prebiotic “*chemistry experiments*”. What all the scenarios share are the following requirements.

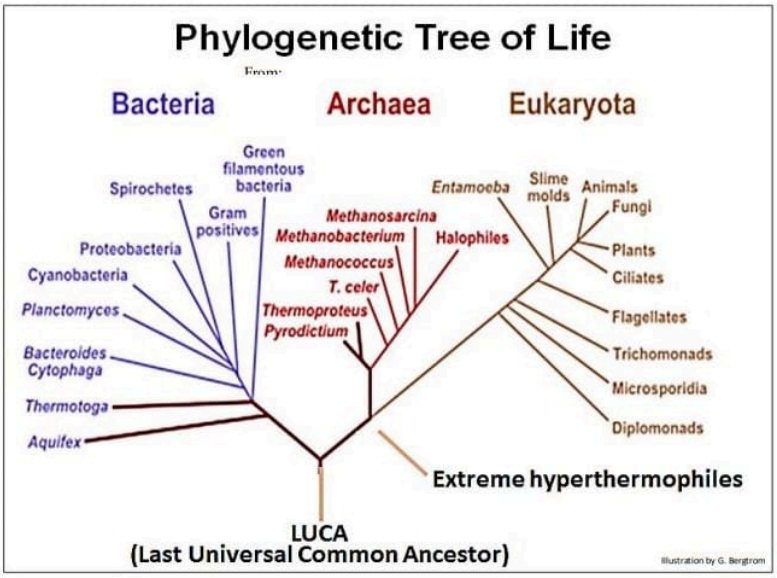
All Origins of Life Scenarios Must Explain:

- **Prebiotic Synthesis** of organic molecules and polymers
- the origins of **catalysis** and **replicative biochemistry**
- the **sources of free energy** to sustain prebiotic biochemistry
- the beginnings of **metabolism** sufficient for life
- the origins of **molecular information storage and retrieval**
- enclosure of life's chemistry by a semipermeable membrane

Let’s consider some tricky definitions. If one believes the origin of life was so unlikely that it could only have happened once (still a common view), then the very first cell (defined as the **progenote**, the progenitor of us all) is our common genetic ancestor.

On the other hand, what if there were many origins of life? Then there must have been more than one 'first cell', generating multiple populations of cells. Each such population, starting with its own 'progenote' would have evolved. In this scenario, only one cell population would survive; its evolved cells would have been the source of our **Last Universal Common Ancestor**, or **LUCA**. All populations of other first cells went extinct. The LUCA remains defined as the highly evolved cell(s) with genome, biochemistry and basic metabolic infrastructure that is shared among all things alive today.

Whatever the pathway to the first living cells on earth, molecular studies over the last several decades support the common ancestry of all life on earth, in the form of the LUCA. Look at the phylogenetic tree below showing the domains of life that we have seen before, with the *LUCA* at its root.



Regardless of the number of 'first cells', the LUCA's ancestors still descended from a progenote! So, how did we get to our own progenote, or first cell? Consider these common features of any life-origins scenario:

- reduction of inorganic molecules to form organic molecules
- a source of free energy to fuel the formation of organic molecules
- a scheme for catalytic acceleration of biochemical reactions
- separation of early biochemical 'experiments' by a semipermeable boundary

Next, consider some proposed scenarios for the creation of organic molecules:

- import of organic molecules (or even life itself) from **extraterrestrial** sources
- organic molecule synthesis on an earth with a **reducing atmosphere**
- organic molecule synthesis on an earth with a **non-reducing atmosphere**

Here we explore alternate free-energy sources and pathways to the essential chemistry of life dictated by these different beginnings. Then we look at possible scenarios of chemical evolution that must have occurred before life itself. Finally, we consider how primitive (read "simpler") biochemistries could have evolved into the present-day metabolisms shared by all existing life forms.

347 What any Life Origins Scenario Must Explain

Learning Objectives

When you have mastered the information in this chapter, you should be able to:

1. Explain how organic molecules would capture chemical energy on a *prebiotic earth*.
2. List the essential chemistries required for life and why they might have been *selected* during chemical evolution.

3. Discuss the different fates of prebiotically synthesized organic *monomers* and *polymers* and how these fates would influence the origins of the first cells on earth.
4. Compare and contrast two scenarios for *extraterrestrial origins* of organic molecules.
5. Summarize the arguments against Oparin's *primordial soup* hypothesis.
6. Summarize the evidence supporting origins of life in a *non-reducing* earth atmosphere.
7. Compare the *progenote* and the *LUCA*.
8. Discuss the evidence suggesting an origin of cellular life in the *late Hadean eon*.
9. Describe how life might have begun in deep ocean vents – compare the possibilities of life beginning in *black smokers* vs. *white smokers*.
10. Argue for and against an *autotroph-first* scenario for cellular origins.
11. Explain why some investigators place significance on the early origins of free energy storage in *inorganic proton gradients*.
12. Define *autocatalysis*, co-catalysis and co-catalytic sets; provide examples.
13. Define *coevolution*.
14. Describe the significance and necessity of *coevolution* before life. In what ways is coevolution a feature of living things? Explain.

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20.2: Thinking about Life's Origins- A Short Summary of a Long History

By all accounts, the earth must have been a very unpleasant place soon after its formation! For that reason, the period from 4.8 to 4.0 billion years ago is called the Hadean Eon, after Hades, the hell of the ancient Greeks! Until recently, geological, geochemical and fossil evidence suggested that life arose between 3.8 and 4.1 billion years ago. The 2017 discovery of 3.95 billion year-old sedimentary rocks in Labrador with evidence of life, points to an even earlier origin of life, (see From Canada Comes the Oldest Evidence of Life on Earth). In fact, questions about life's origins are probably "as old as the hills..." or at least as old as the ancient Greeks! We only have records of human notions of life's origins dating from biblical accounts and, just a bit later, from Aristotle's musings. While Aristotle did not suggest that life began in hell, he and other ancient Greeks did speculate about life's origins by *spontaneous generation*, in the sense of *abiogenesis* (*life* originating from *non-life*). He further speculated that the origins of life were gradual.

Later, the dominant theological accounts of creation in Europe in the middle ages muted any notions of origins and evolution. While a few mediaeval voices ran counter to strict biblical readings of the creation stories, it was not until the Renaissance in the 14th-17th century that an appreciation of ancient Greek *humanism* was reawakened, and with it, scientific curiosity and the ability to engage in rational questioning and research.

Many will recall that Louis Pasteur in the mid-19th century put to rest any lingering notions of life forming from dead (e.g., rotten, or fecal) matter. He showed that life would not form in sterilized nutrient solutions unless the broth was exposed to the air. Fewer know that much earlier, Anton Van Leeuwenhoek, the 17th century microscopist who first described bacteria and *animalcules*, mostly protozoa in pond water, had already tested the notion of spontaneous generation. By observing open and sealed containers of meat over time, he became convinced that 'large' animals like fleas and frogs do not arise *de novo* from putrid meat or slime. He also declared that insects come from other insects, and not from the flowers that they visited.

No lesser light than Charles Darwin suggested in 1859 that life might have begun in a "*warm little pond, with all sorts of ammonia and phosphoric salts, light, heat, electricity, &c., present, that a proteine compound was chemically formed ready to undergo still more complex changes*." He even realized that these chemical constituents would not have survived in the atmosphere and waters of his day, but must have done so in a prebiotic world. In *On the Origin of Species*, he referred to life having been 'created'. There, Darwin was not referring to a biblical basis of creation; he clearly meant that life originated "*by some wholly unknown process*" at a time before which there was no life. Finally, Pasteur's 1861 contribution was the irrefutable, definitive proof that 'invisible' *microbial life* likewise did not arise by spontaneous generation. Thus for creatures already on earth, they could only arise by **biogenesis** (*life-from-life*), the opposite of *abiogenesis*, a term that now applies to only the first origins of life!

Among Darwin's friends and contemporaries were Charles Lyell and Roderick Murchison, both geologists who understood much about the slow geological changes that shaped the earth. Darwin was therefore familiar with the concept of extended periods of geological time, amounts of time he believed was necessary for the natural selection of traits leading to species divergence.

Fast-forward to the 1920s when J.H.B.S. Haldane and A. Oparin offered an hypothesis about the life's origins based on notions of the chemistry and physical conditions that might have existed on a **prebiotic earth**. Their proposal assumed that the earth's atmosphere was hot, hellish and reducing (i.e., filled with inorganic molecules able to give up electrons and hydrogens). There are more than a few hypotheses for which chemicals were already present on earth, or that formed when the planet formed about 4.8 billion years ago. We'll start our exploration with Oparin and Haldane's *reducing atmosphere*. Then we look at the possibility that life began under *non-reducing conditions* (with passing reference to a few other ideas).

348 Early Ideas to Explain the Origins of Life

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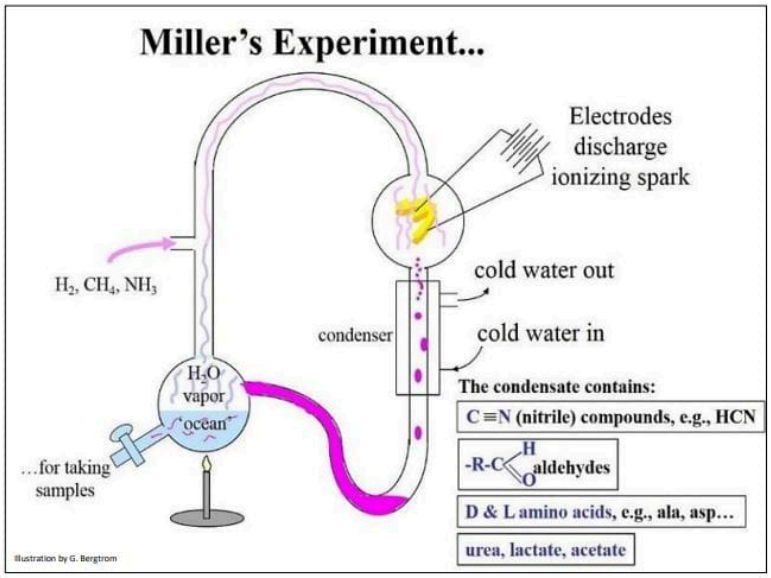
20.3: Formation of Organic Molecules in an Earthly Reducing Atmosphere

A prerequisite to the prebiotic chemical experimentation is a source of organic molecules. Just as life requires energy (to do anything and everything!), converting inorganic molecules into organic molecules requires an input of *free energy*. As we have seen, most living things today get free energy by oxidizing nutrients or directly from the sun by photosynthesis. Recall that in fact all the chemical energy sustaining life today ultimately comes from the sun. But, before there were cells, how did organic molecules form from inorganic precursors? Oparin and Haldane hypothesized a reducing atmosphere on the prebiotic earth, rich in inorganic molecules with *reducing power* (like H_2, NH_3, CH_4, and H_2S) as well as CO_2 to serve as a carbon source. The predicted physical conditions on this prebiotic earth were:

- lots of water (oceans).
- hot (no free O_2).
- lots ionizing (e.g., X, γ) radiation from space, (no protective ozone layer).
- frequent ionizing (electrical) storms generated in an unstable atmosphere.
- volcanic and thermal vent activity.

A. Origins of Organic Molecules and a Primordial Soup

Oparin suggested that abundant sources of free energy fueled the reductive synthesis of the first organic molecules to create what he called a “primeval soup”. No doubt, he called this primeval concoction a “soup” because it would have been rich in chemical (nutrient) free energy. The Oparin/Haldane proposal received strong support from the experiments of Stanley Miller and Harold Urey (Urey had already won the 1934 Nobel Prize in Chemistry for discovering deuterium). Miller and Urey tested the prediction that, under Haldane and Oparin’s prebiotic earth conditions, inorganic molecules could produce the organic molecules in what came to be known as the *primordial soup*. Their famous experiment, in which they provided energy to a mixture of inorganic molecules with reducing power, is illustrated below.



Miller’s earliest published data indicated the presence of several organic molecules in their *ocean* flask, including a few familiar metabolic organic acids (lactate, acetate, several amino acids...) as well as several highly reactive *aldehydes* and *nitriles*. The latter can interact in spontaneous chemical reactions to form organic compounds. Later analyses further revealed purines, carbohydrates and fatty acids in the flask. Later still, 50 years after Miller’s experiments (and a few years after his death), some un-analyzed sample collection tubes from those early experiments were discovered.

When the contents of these tubes were analyzed with newer, more sensitive detection techniques, they were shown to contain

additional organic molecules not originally reported, including 23 amino acids (to read more, click Surprise Goodies in the Soup!).

Clearly, the thermodynamic and chemical conditions proposed by Oparin and Haldane could support the *reductive synthesis* of organic molecules. At some point, Oparin and Haldane's evolving chemistries would have to have been internalized inside of semipermeable aggregates (or boundaries) destined to become cells. Examples of such structures are discussed below. A nutrient-rich primordial soup would likely have favored the genesis of *heterotrophic* cells that could use environmental nutrients for energy and growth, implying an early evolution of fermentative pathways similar to glycolysis. But, these first cells would quickly consume the nutrients in the soup, quickly ending the earth's new vitality!

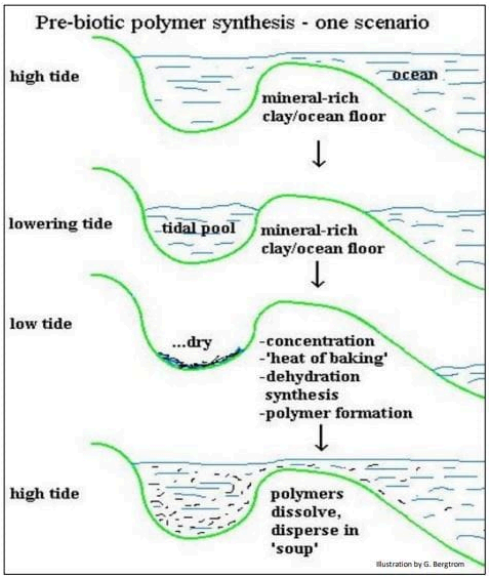
So, one must propose an early evolution of least small populations of cells that could capture free energy from inorganic molecules (**chemoautotrophs**) or even sunlight (**photoautotrophs**). As energy-rich organic nutrients in the 'soup' declined, autotrophs (notably photoautotrophs that could split water using solar energy) would be selected. Photoautotrophs would *fix* CO_2, reducing it with H^- ions from water. Photoautotrophy (**photosynthesis**) would thus replenish carbohydrates and other nutrients in the oceans and add O_2 to the atmosphere.

Oxygen would have been toxic to most cells, but a few already had the ability to survive oxygen. Presumably these spread, evolving into cells that could *respire*, i.e., use oxygen to *burn* environmental nutrients. Respiratory metabolism must have followed hard on the heels of the spread of photosynthesis.

Photosynthesis began between 3.5 and 2.5 billion years ago (the Archaean Eon). Eventually, photosynthetic and aerobic cells and organisms achieved a natural balance to become the dominant species in our oxygen-rich world.

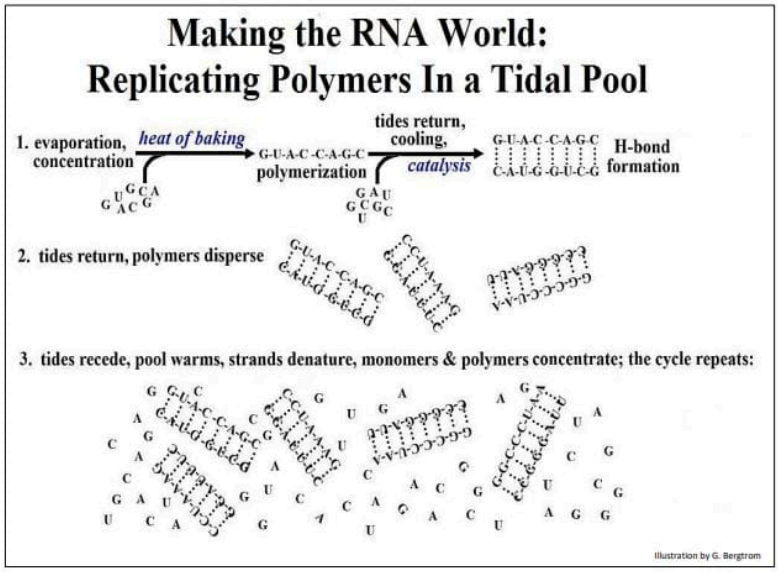
B. The Tidal Pool Scenario for an Origin of Polymers and Replicating Chemistries

In this scenario, prebiotic organic monomers would concentrate in tidal pools in the heat of a primordial day, followed by polymerization by dehydration synthesis. The formation of polymer linkages is an 'uphill' reaction requiring free energy. Very high temperatures (the **heat of baking**) can link monomers by dehydration synthesis in the laboratory, and may have done so in tidal pool sediments to form random polymers. This scenario further assumes the dispersal of these polymers from the tidal pools with the ebb and flow of high tides. The tidal pool scenario is illustrated below.



The concentration of putative organic monomers at the bottom of tidal pools may have offered opportunities to catalyze polymerization, even in the absence of very high heat. Many metals (nickel, platinum, silver, even hydrogen) are inorganic catalysts, able to speed up many chemical reactions. The heavier metals were likely to exist in the earth's crust as well as in the sediments of primordial oceans, as they do today. Such mineral aggregates in soils and clays have been shown to possess catalytic properties. Furthermore, metals (e.g., magnesium, manganese...) are now an integral part of many enzymes, consistent with an origin of biological catalysts in simpler aggregated mineral catalysts in ocean sediments.

Before life, the micro-surfaces of mineral-enriched sediment, if undisturbed, could have been able to catalyze the same or at least similar reactions repeatedly, leading to related sets of polymers. Consider the possibilities for RNA monomers and polymers, based on the assumption that life began in an RNA world. The possibilities are illustrated below.



The result predicted here is the formation not only of RNA polymers (perhaps only short ones at first), but of H-bonded double-stranded RNA molecules that might effectively replicate at each cycle of concentration, polymerization and dispersal. Heat and the free energy released by these same reactions could have supported polymerization, while catalysis would have enhanced the fidelity of RNA replication.

Of course, in the tidal pool scenario, repeated high heat or other physical or chemical attack might also degrade newly formed polymers. But what if some RNA double strands were more resistant to destruction. Such early RNA duplexes would accumulate at the expense of the weaker, more susceptible ones. Only the *fittest* replicated molecules would be selected and persist in the environment! The environmental accumulation of structurally related, replicable and stable polymers reflects a prebiotic chemical **homeostasis** (one of those properties of life!)

349 Life Origins in a Reducing Atmosphere

Overall, this scenario hangs together nicely, and has done so for many decades. However, there are now challenging questions about the premise of a prebiotic reducing environment. Newer evidence points to an earth atmosphere that was not at all reducing, casting doubt on the idea that the first cells on the planet were heterotrophs. Recent proposals posit alternative sources of prebiotic free energy and organic molecules that look quite different from those assumed by Oparin, Haldane, Urey and Miller.

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20.4: Origins of Organic Molecules in a Non-Reducing Atmosphere

A prebiotic non-reducing atmosphere is based on several assumptions: (1) The early earth would have had insufficient gravity to hold H_2 and other light gasses; thus "outgassing" would have resulted in a loss of H_2 and other reducing agents from the atmosphere. (2) Geological evidence suggests that the earth's oceans and crust formed early in the Hadean Eon, just a few hundred million years after formation of the planet. (3) Studies of 4.4 billion year old (early Hadean Eon) Australian *zircon* crystals suggest that their oxidation state is the same as modern day rocks, meaning that the early Hadean atmosphere was largely N_2 and CO_2, a distinctly *non-reducing* one! A colorized image of this Australian zircon is shown below.



So life might have begun in a non-reducing environment. Nevertheless, how far back can we date the appearance of the first actual cells on earth? Solid geological evidence of actual life dates to 3.5-3.95 billion years ago (i.e., the *Archaean Eon*). Softer evidence of microbial life exists in the form of graphite and other 'possible' remains as old as 4.1 billion years ago, near the end of the Hadean Eon. Thus, regardless of whether life began 3.5 or even 4.1 billion years ago, the evidence suggests that life's beginnings had to contend with a non-reducing environment.

Before we look more closely at other evidence of life origins under non-reducing conditions, let's consider the **Panspermia**, the possibility that life came to earth from **extraterrestrial** sources and a related hypothesis that prebiotic organic molecules came from extraterrestrial sources. Then we will examine how cells might have formed in localized, favorable **terrestrial** environments.

A. Panspermia - an Extraterrestrial Origin of Earthly Life

Panspermia posits that life itself arrived on our planet on comets or meteorites. Since these are unlikely to have sustained life in space, they must have been a kind of interstellar 'mailbox' into which dormant life forms were deposited. The cells in the mailboxes must have been *cryptobiotic*. Examples of cryptobiosis exist today (e.g., bacterial spores, brine shrimp!). Once delivered to earth's life-friendly environment, such organisms would emerge from dormancy, eventually populating the planet. There is however, no evidence of dormant or cryptobiotic life on comets or meteorites, and no hard evidence to support *Panspermia*. On the other hand, there is evidence at least consistent with an extraterrestrial source of organic molecules, and plenty to support more terrestrial origins of life. In any case, notions of *Panspermia* (and even extraterrestrial sources of organic molecules) simply beg the question of the conditions that would have led to the origin of life elsewhere!

While panspermia is not a favored scenario, it is nevertheless intriguing, in the sense that it is in line the likelihood that organic molecules formed soon after the *Big Bang*. Moreover, if ready-made organic molecules and water were available, we can expect (and many do!) that there is life on other planets. This expectation has stimulated serious discussion and funding of programs looking for signs of life on other planets. For example, NASA funded *Rover*'s search for (and discovery of) signs of water on Mars. It even supported the more earth-bound *Search for Extraterrestrial Intelligence* (the SETI program), based on the assumption that life not only exists elsewhere, but that it evolved high level communication skills (and why not?)! For a fascinating story about meteorites from Mars that contain water and that are worth more than gold, click Martian Obsession.

B. Extraterrestrial Origins of Organic Molecules

Even if life did not come to us ready-made, could organic molecules have arrived on earth from outer space? They are abundant, for example in *interstellar clouds*, and could have become part of the earth as the planet formed around 4.8 billion years ago. This suggests that there was no need to create them *de novo*. One hypothesis suggests meteorites, comets and asteroids, known to contain organic molecules, brought them here during fiery impacts on our planet. Comet and meteorite bombardments would have been common 3.8 or more billion years ago. In this scenario the question of how (*not on earth!*) free energy and inorganic molecular precursors reacted to form organic molecules is moot!

A related hypothesis suggests that those fiery hits themselves provided the free energy necessary to synthesize the organic molecules from inorganic ones- a *synthesis-on-arrival* scenario. With this hypothesis on the one hand, we are back to an organic oceanic primordial soup. On the other, some have suggested that organic molecules produced in this way (not to mention any primordial life forms) would likely have been destroyed by the same ongoing impacts by extraterrestrial bodies; witness the relatively recent dinosaur extinction by an asteroid impact off the coast of Mexico some 65.5 million years ago.

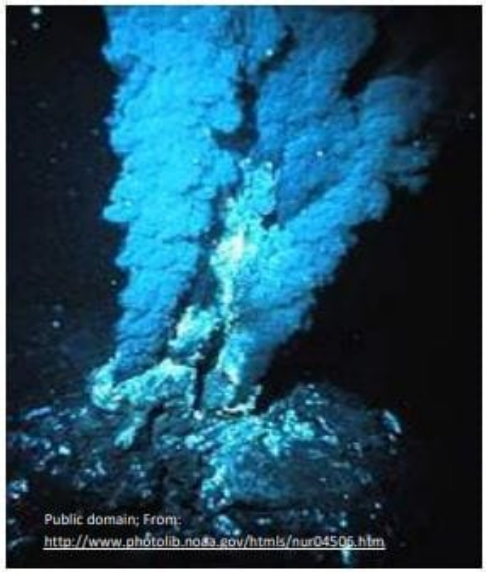
350 Life Origins in a Non-reducing Atmosphere?

C. Organic Molecular Origins Closer to Home

Deep in the oceans, far from the meteoric bombardments and the rampant free energy of an oxygen-free and ozone-less sky, deep-sea hydrothermal vents would have been spewing reducing molecules (e.g., H_2S, H_2, NH_4, CH_4), much as they do today. Some vents are also high in metals such as lead, iron, nickel, zinc copper, etc. When combined with their clay or crustal substrata, these minerals could have provided catalytic surfaces to enhance organic molecule synthesis. Could such localized conditions have been the focus of prebiotic chemical experimentation leading to the origins of life? Let's look at two kinds of deep-sea hydrothermal vents recognized today: **volcanic** and **alkaline**.

1. Origins in a High-Heat Hydrothermal Vent (Black Smoker)

The free energy available from a volcanic hydrothermal vent would come from the high heat (temperatures ranging to 350°C) and the minerals and chemicals expelled from the earth's mantle. A volcanic hydrothermal vent is illustrated below.



Conditions assumed for prebiotic volcanic hydrothermal vents could have supported catalytic syntheses of organic molecules from inorganic precursors (see Volcanic Vents and organic molecule formation). The catalysts would have been metallic (nickel, iron, etc.) minerals. Chemical reactions tested include some that are reminiscent of biochemical reactions in chemoautotrophic cells alive today. Günter Wächtershäuser proposed the Iron-sulfur world theory of life's origins in these vents, also called "black smokers". These vents now spew large amounts of CH_4 and NH_4 and experiments favor the idea that iron-sulfur aggregates in

and around *black smokers* could provide catalytic surfaces for the prebiotic formation of organic molecules like methanol and formic acid from dissolved CO_2 and the CH_4 and NH_4 coming from the vents. Wächtershäuser is also credited with the idea that prebiotic selection acted not so much on isolated chemical reactions, but on aggregates of *metabolic reactions*.

We might think of such metabolic *aggregates* as biochemical pathways or multiple integrated pathways. Wächtershäuser proposed the selection of cyclic chemical reactions that released free energy usable by other reactions. This prebiotic **metabolic evolution** of reaction chemistries (rather than a simpler chemical evolution) would have been essential to the origins of life. A variety of extremophiles (e.g., thermophilic archaea) now living in and around *black smokers* seems to be testimony to black smoker origins of life.

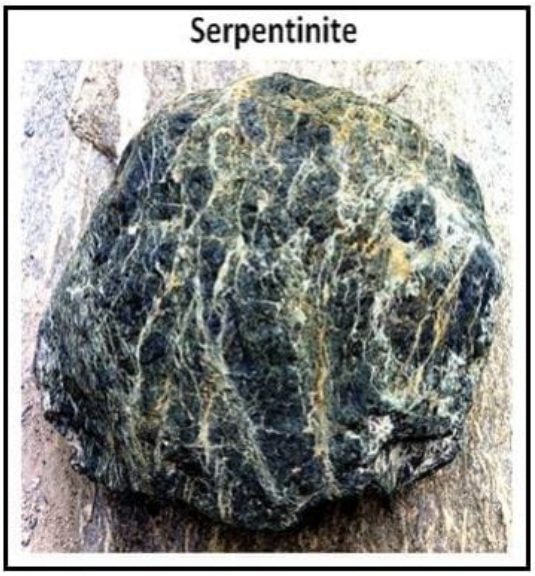
While the idea of selecting metabolic pathways has great merit, there are problems with a life-origins scenario in volcanic hydrothermal vents. For one thing, their high temperatures would have destroyed as many organic molecules as were created. Also, the extremophilic archaea now found around these volcanic vents cannot be the direct descendants of any cells that might have originated there. Woese's phylogeny clearly shows that archaea share a lineage with eukaryotes (not eubacteria - see above). Therefore, extremophilic cellular life originating in the vents must have first have given rise to a more moderate LUCA before then dying off themselves, after which extremophiles would once again evolve independently to re-colonize the vents! This mitigates against an extremophiles- first origins scenario. Given these concerns, recent proposals focus on life origins in less extreme **alkaline hydrothermal vents**.

2. Origins in an Alkaline Deep-Sea Vent (White Smoker)

Of the several scenarios discussed here, an origin of autotrophic life in *alkaline vents* is one of the more satisfying alternatives to a soupy origin of heterotrophic cells. For starters, at temperatures closer to 100°C-150°C, alkaline vents (**white smokers**) are not nearly as hot as are black smokers. An *alkaline vent* is shown below.



Other chemical and physical conditions of alkaline vents are also consistent with an origins-of-life scenario dependent on *metabolic evolution*. For one thing, the interface of alkaline vents with acidic ocean waters has the theoretic potential to generate many different organic molecules [Shock E, Canovas P. (2010) *The potential for abiotic organic synthesis and biosynthesis at seafloor hydrothermal systems*. Geofluids 10 (1-2):161-92)]. In laboratory simulations of alkaline vent conditions, the presence of dissolved CO_2 favors **serpentinization**, a reaction of water and heat with *serpentinite*, an iron-containing mineral found on land and in the oceanic crust. A sample of serpentinite is shown below.



Experimental serpentinization produces hydrocarbons and a warm aqueous oxidation of iron produces H_2 that could account for abundant H_2 in today's *white smoker* emissions. Also, during serpentinization, a mineral called *olivine* [$(Mg^{+2}, Fe^{+2})_2SiO_4$] reacts with dissolved CO_2 to form methane (CH_4). So, the first precondition of life, the energetically favorable creation of organic molecules, is possible in alkaline vents.

Proponents of cellular origins in a late-Hadean non-reducing ocean also realized that organic molecules formed in an alkaline (or *any*) vent would disperse and be rapidly neutralized in the wider acidic oceans waters. Somehow, origins on a nonreducing planet had to include some way to contain newly formed organic molecules from the start, and to power further biochemical evolution. What then, were the conditions in an alkaline vent that could have contained organic molecules and led to metabolic evolution and ultimately, life's origins? Let's consider an intriguing proposal that gets at an answer!

The porous rock structure of today's alkaline vents provides micro-spaces or micro-compartments that might have captured alkaline liquids emitted by *white smokers*. It turns out that conditions in today's alkaline vents also support the formation of hydrocarbon **biofilms**. Micro-compartments lined with such biofilms could have formed a primitive prebiotic membrane against a rocky "cell wall", within which alkaline waters would be trapped. The result would be a natural *proton gradient* between the alkaline solutions of organic molecules trapped in the microcompartments and the surrounding acidic ocean waters. Did all this happen?

Perhaps! Without a nutrient-rich environment, **heterotrophs-first** is not an option. That leaves only the alternate option: an **autotrophs-first** scenario for the origins of life. Nick Lane and his coworkers proposed that proton gradients were the selective force behind the evolution of early metabolic chemistries in the alkaline vent scenario (Prebiotic Proton Gradient Energy Fuels Origins of Life). Organized around biofilm compartments, prebiotic structures and chemistries would have harnessed the *free energy* of the *natural proton gradients*. In other words, the first protocells, and then cells, may have been **chemoautotrophs**.

Last but not least, how might chemoautotrophic chemistries on a non-reducing planet have supported polymer formation, as well as polymer replication? Today we see storage and replication of information in nucleic acids as separate from enzymatic catalysis of biochemical reactions. But are they all that s*eparate*? If replication is the faithful reproduction of the information needed for a cell, then enzymatic catalysis ensures the redundant production of all molecules essential to make the cell! Put another way, if catalyzed *polymer* synthesis is the replication of the workhorse molecules that accomplish cellular tasks, then what we call 'replication' is nothing more than the replication of nucleic acid *information* needed to faithfully reproduce these workhorse molecules. So, was there an early, coordinated, concurrent selection of mechanisms for the catalyzed metabolism as well as catalyzed polymer synthesis and replication? We'll return to these questions shortly, when we consider the origins of life in an RNA world.

Life-origins in a non-reducing (and oxygen-free) atmosphere raise additional questions. Would proton gradients provide enough free energy to fuel and organize life's origins? If so, how did cells arising from prebiotic chemiosmotic metabolism actually harness the energy of a proton gradient? Before life, were protocells already able to transduce gradient free energy into chemical free energy? And was ATP selected to hold chemical free energy from the start? Alternatively, was the relief of the gradient coupled at first to the synthesis of other high-energy intermediate compounds with e.g., thioester linkages? Later on, how did

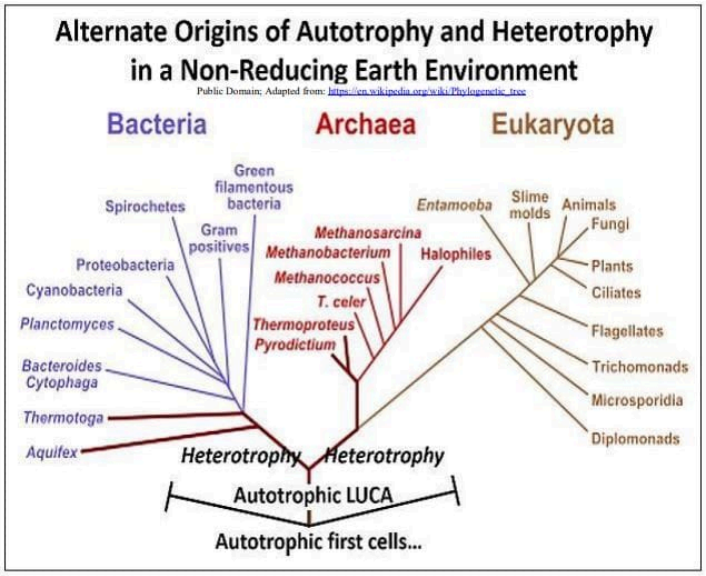
cells formed in alkaline vents escape the vents to colonize the rest of the planet?

Regardless of how proton gradient free energy was initially captured, the **chemoautotrophic LUCA** must have already have been using membrane-bound proton pumps and an ATPase to harness gradient free energy to make ATP, since all of its descendants do so. Finally, when did photoautotrophy (specifically **oxygenic photoautotrophy**) evolve? Was it a late evolutionary event? Is it possible that photosynthetic cells evolved quite early among some of the chemoautotrophic denizens of the white smokers, biding their time before exploding on the scene to create our oxygenic environment?

3. Heterotrophs-First vs. Autotrophs-First: Some Evolutionary Considerations

In the alkaline vent scenario, chemiosmotic metabolism predated life. Therefore, the first chemoautotrophic cells did not need the fermentative reactions required by cells in a heterotrophs-first origin scenario. Even though all cells alive today incorporate a form of glycolytic metabolism, *glycolysis may not be the oldest known biochemical pathway*, as we have thought for so long.

In support of a later evolution of glycolytic enzymes, those of the archaea show little structural resemblance to those of bacteria. If fermentative heterotrophy was a late evolutionary development, then LUCA and its early descendants would lack a well-developed glycolytic pathway. Instead, the LUCA must have been one of many 'experimental' autotrophic cells, most likely a chemoautotroph deriving free energy from inorganic chemicals in the environment. To account for heterotrophy in the three domains of life, it must have evolved separately in the two antecedent branches descending from the last universal common ancestor of bacterial, archaeal and eukaryotic organisms. The phylogeny shown below illustrates the *autotrophs-first* scenario.



4. Summing Up

Speculation about life's origins begins by trying to identify a source of free energy with which to make organic molecules. The first cells might have been heterotrophs formed in a reducing earth environment, from which autotrophs later evolved. On the other hand, the earliest cells may have been autotrophs formed under non-reducing conditions in the absence of a primordial soup. Then, only after these autotrophs had produced enough nutrient free energy to sustain them did heterotrophs belatedly emerge. Discoveries suggesting that the earth's atmosphere was a non-reducing one more that 4 billion years ago (soon after the formation of the planet), and that there was life on earth 3.95 billion years ago favor metabolic origins of autotrophic life in a thermal vent, likely an alkaline vent. Questions nevertheless remain about life-origins under non-reducing conditions. Even the composition of the prebiotic atmosphere is still in contention (see Non-reducing earth- Not so fast!).

For now, let us put these concerns aside for a moment and turn to events that get us from the LUCA and its early descendants to the elaborated chemistries common to all cells today. The descriptions that follow are educated guesses about pathways taken early on towards the familiar cellularity now on earth. They mainly address the selection of catalytic mechanisms, replicative metabolism, the web of intersecting biochemical pathways, and the even more intricate chemical communication that organized cell function and complexity.

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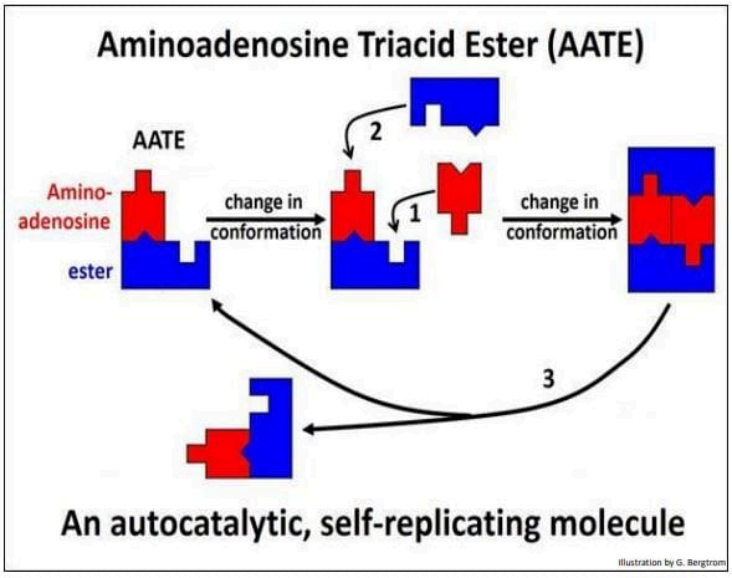
20.5: Origins of Life Chemistries in an RNA World

In the tidal pool scenario, with its feel of 'best-fit' with origins of life in a reducing environment, the energy for polymer formation from organic monomers came from an overheated earth environment. In that scenario, we considered the possibility that chains of nucleotides might have been synthesized, and then even replicated to form populations of nucleic acids. But if the prebiotic environment was non-reducing, where would the energy have come from to make any polymers, let alone ones that could replicate themselves? If you guessed that the energy was provided by a proton gradient between biofilm-enclosed acidic proto-cells and an alkaline ocean..., you would have been right! In this case, then polymers would have been synthesized in enclosed spaces, and not in tidal pools only to be dispersed and diluted in the wider oceans. And then, how would replicative, informational and catalytic chemistries have arisen from these organic monomers and polymers? Polypeptides would have formed, but they have no inherent chemical or structural basis for self-replication. Unlike polypeptides, we saw in describing the tidal pool scenario that polynucleotides (nucleic acids) do! In fact, evidence is accumulating to support the increasingly accepted hypothesis that life originated in a **RNA world**:

- Today's RNAs include ribozymes that catalyze their own replication (e.g., self-splicing introns).
- Some RNAs are part of **ribonucleoproteins** with at least **co-catalytic activity** (recall ribosomes, spliceosomes and the secretory signal recognition particle).
- **Retroviruses** (e.g., HIV) store their genetic information in *RNA genomes* that may have been integral to the emergence of cellular life.

Ribozymes, ribonucleoprotein structures and retroviruses may be legacies of a prebiotic RNA world. In fact, in an '*in vitro* evolution study', self-replicating ribozyme polymerases in a test tube become more efficient at replicating a variety of increasingly longer and more complex RNAs over time. For more about these autocatalysts, click Artificial Ribozyme Evolution Supports Early RNA World.

There are hypothetical *RNA world scenarios* for the origins of replicating, catalytic polymers, and even a real organic chemical **autocatalyst** that can catalyze its own synthesis. So, which may have come first? A self-replicating RNA or some other selfreplicating molecule, even a self-replicating organic molecule? Arguably, chemical evolution of an autocatalytic RNA is a stretch, but at least one organic molecule, **A**mino-**A**denosine **T**riacid-**E**ster (AATE), is a present-day self-replicating *autocatalyst*. Could an organic molecule like AATE have been a prebiotic prelude to the RNA world? The structure and replication of AATE are described below.



The replicative reaction proceeds in the following steps:

- The aminoadenosine triacid ester binds another molecule of aminoadenosine.
- The two aminoadenosines, now in opposite orientations, can attract and bind a second ester.

- After bond-rearrangements, the molecule separates into two molecules of AATE.

This reaction is catalytic because the stereochemistry of the reacting molecules creates an affinity of the aminoadenosine ester molecule first for an additional free aminoadenosine molecule, and then for a second free ester. The structure formed allows (i.e., catalyzes) linkage of the second aminoadenosine and ester followed by the separation of both AATE molecules. Subtle, sequential changes in the molecular conformation of the molecules result in the changes in affinities of the molecules for each other. In the replicative reaction, the AATE, free ester and free aminoadenosine concentrations would drive the reaction. Could AATE-like molecules have been progenitors of autocatalyzed polymer replication? *Could replication of a prebiotic AATElike molecule have led to an RNA world*? Could primitive RNAs have been stabilized by binding to short prebiotic peptides, becoming forerunners of ribozymes? The possibility of a prebiotic AATE-like molecule is intriguing because the 'triacid' includes a nucleotide base, the purine adenosine! On the other hand, the possibility of prebiotic replicating RNA-peptide complexes implies the origins of life in an **RNA-Protein world** (rather than exclusively RNA-world)! Whether life began in an RNA world or an RNA-protein world, catalyzed replication is of course another property of life.

353 AATE: An Autocatalytic, Self-Replicating Organic Molecule

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20.6: Molecules Talk- Selecting Molecular Communication and Complexity

In our complex human society, we define communication by its **specificity**. Without a careful choice of words, our speech would be at best, a source of magnificent misunderstanding, or just plain babel! What does this mean for prebiotic chemistries?

In terms of prebiotic chemical evolution, selection by definition would have favored protective accumulation of longer-lived molecular aggregates. Over time, the same selective imperatives would create webs of such aggregates, increasing the *range and specificity* of molecular interactions in a challenging environment. If this were to have occurred in an enclosed proto-cellular space, it should have resulted in a primitive *molecular communication* and a growing **complexity** (another property of life!). In fact, alof the properties of life must have accompanied the achievement of more and more complex intermolecular communication. Simply put, a prebiotic (or for that matter a cellular) genetic change that alters the rate of one catalytic reaction (if not destructive) will drive the selection of changes in components of other, interconnected metabolic chemistries. If *molecular communication* required the evolution of catalytic specificity, then the final elaboration of complexity and order as a property of life further requires the selection of mechanisms of **regulation** and **coordination**.

A. Intermolecular Communication Leads to an Early Establishment of Essential Interconnected Chemistries

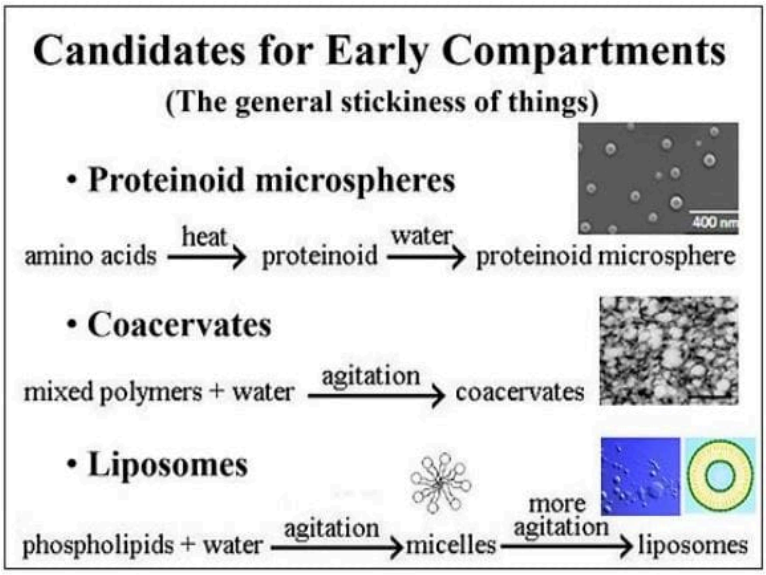
Earlier, we suggested that inorganic catalyst precursors to biological enzymes were probably minerals embedded in clay or other substrata, providing surfaces that would naturally aggregate organic molecules and catalyze repetitive reactions. Either the initial objects of prebiotic selection included external stable monomers and polymers, outside or as seems more likely, inside proto-cells. Later, selection would have favored polymers that enhanced growth and reproduction of successful aggregates. These polymers were likely those that catalyzed their own synthesis, perhaps collaborating with inorganic catalytic minerals. The result would be the elaboration of a web of *interconnected chemical reaction*s between molecules with high affinity for each other, thereby increasing the specificity of those reactions. In the context of life origins and evolution, **co-catalysis** describes the activities of these interconnected metabolic reactions.

As noted, high-affinity interactions are inherently **protective**. During prebiotic chemical/metabolic evolution, protected stable **molecular assemblies** would be targets of selection. Continuing co-evolution of catalysts, substrates and co-catalytic reaction sets would lead to more and more sophisticated *molecular communication*. Once established, efficient biochemical reaction sets would be constrained against significant evolutionary change. Any change (mutation) that threatened this efficiency would mean the end of a prebiotic chemical (or for that matter, cell) lineage! This explains why we find common pathways for energy-generation (e.g., autotrophic and fermentative), reproduction (replication), and information storage and retrieval (DNA, RNA, protein synthesis) in all of LUCA's descendants.

Sophisticated, effective communication requires coordination. In fact, effective communication is defined by **coordination**, the capacity to make *chemical decisions*. Selection of molecular aggregates that sequestered metabolic reactions behind a semipermeable membrane ensure that only certain molecules communicate with each other. This sequestration is likely to have occurred repeatedly during chemical evolution, beginning with the synthesis of larger, polymeric molecules and possibly, an aggregation of primitive lipoidal molecules. We can think of increasingly effective catalysis in an enclosed environment as a **conversation mediated by good speakers**! *Coordination* is a property that likely co-evolved with life itself!

B. Origins of Coordination

Let's look some possible structures churning around in the prebiotic chemistry set that might have self-assembled, or sequestered compatible chemistries of life. Along with the alkaline vent *biofilm compartment*, **coacervates**, **proteinoid microspheres** and **liposomes** have been considered as possible progenitors of biological membranes. Each can be made in the laboratory. They are demonstrably semipermeable, and in some cases can even replicate! Micrographs and the production of coacervates, proteinoid microspheres and liposomes are shown below



Oparin had proposed that the action of sunlight in the absence of oxygen could cause ionized, oppositely charged organic molecules (e,g, amino acids, carbohydrates, etc.) to form droplets from organic molecules in his primordial soup. These **coacervates** were actually produced in 1932, visualized by microscopy and demonstarted to be a semi-permeable compartment. They even behaved as if they were able to grow and reproduce (also as Oparin originally suggested they might).

In the 1950s, Sidney Fox produced **proteinoid microspheres** from short peptides that formed spontaneously from aqueous amino acid solutions heated to dryness (not unlike what happens in the tidal pool scenario of polymer formation from organic monomers). These can be seen by light and electron microscopy.

While **liposomes** are easily made in a laboratory, it isn't clear that they existed on a pre-biotic earth. Nevertheless, cell membranes must have had acquired their phospholipid bilayer structure by the time of LUCA since we all have them! Prior to LUCA, chemical rearrangenments must have occurred to enable incorporation of a phospholipid bilayer into whatever starting boundary life started with.

We have already considered the **biofilm** proposed for cellular origins in an alkaline vent. The formation of such biofilms would have separated acidic ocean protons from the interior of such protocells, creating a proton gradient. Such a gradient could have driven the early evolution of chemiososis as a means to create chemical energy, complete with the eventual selection of ATP synthases and the enzymes of proton transport, again because all cells descendent from LUCA's posess these biochemistries. Of course, proteinoid microspheres, coacervates, biofilm-based 'membranes and liposomes are not alive, and are therefore not cells. But one or another of them must have been where the enhanced **coordination of molecular communication** required for life began.

354 Protected Molecular Communication: Semipermeable Membranes

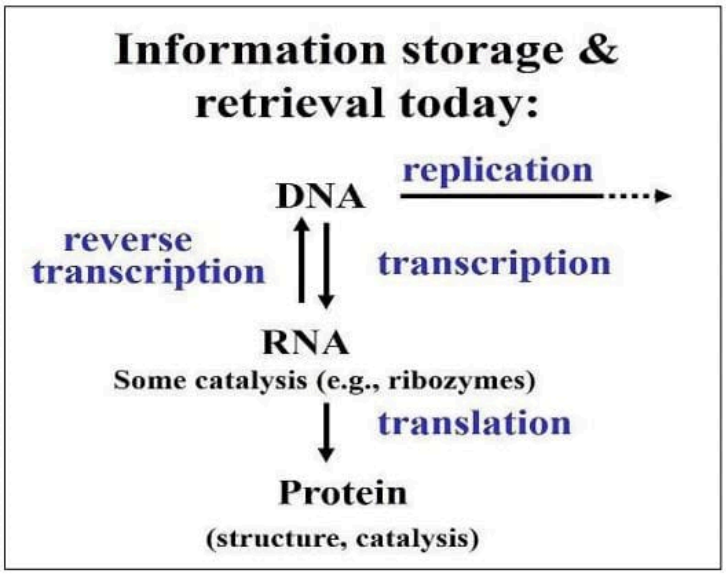
An important **take-home message** here is that whatever the original structure of the first cells, they arose soon after the organic chemical prerequisites of life began to acquire familiar metabolic functions. We need to see chemical and structural progress to cellularity as concurrent metabolic evolutionary events. At some point, selection of sequestered biochemistries led to **protocells**, then to the first cells, each with all of the properties of life.

Finally, selection of highly specific communication between cellular molecules allowed cells themselves to talk to one another, engage in group activities, and eventually join together to form multicellular organisms. Multicellularity is of course a characteristic of many if not most eukaryotes. But watch a great TED Talk on bacterial intercellular communication by Dr. Bonnie Bassler at Intercellular Communication in Bacteria.

C. Origins of Information Storage and Retrieval in an RNA World

Let us accept for now that molecular communication began concurrently with the packaging of interconnected co-catalytic sets into semipermeable structures. Then the most 'fit' of these structures were selected for efficient coordination of meaningful,

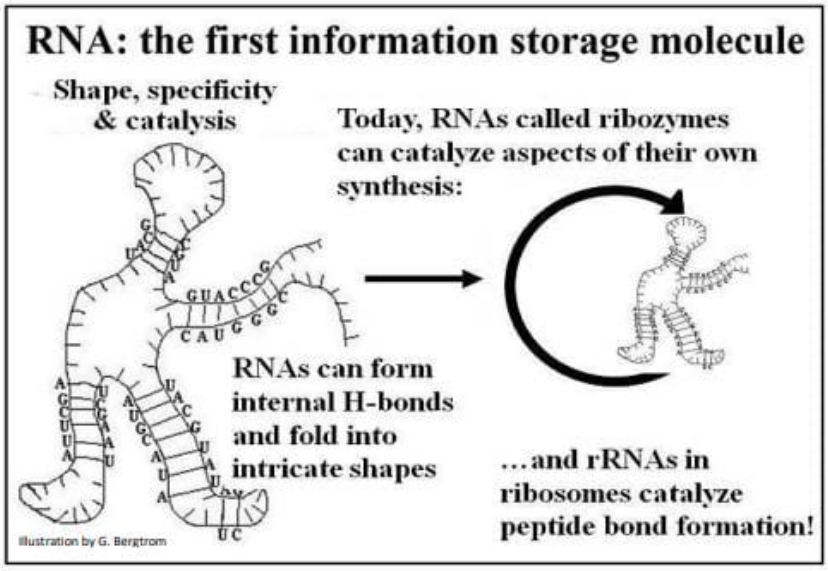
timely chemical messages. Ultimately, coordination requires **information processing**, **storage** and **retrieval**, something we recognize in Francis Crick's Central Dogma of information flow from DNA to RNA to protein. Cells and organisms do coordination quite well, but what do its beginnings look like? The answer may lie in the pre-biotic RNA world we discussed earlier. The Central Dogma, modified to account for reverse transcription and the behavior of retroviruses, is shown below.



We do not really know how cells came to rely on DNA to store, pass on and mobilize genetic information, but we have presented reasons to believe that the first replicating nucleic acid was RNA, creating an *RNA world*. Here is the evidence that leads us to this conclusion.

- Based on the stem-and-loop and other structures that form when RNA molecules undergo internal H-bonding, we know that RNAs can take on varied and intricate shapes.
- Diverse conformations are consistent with the evolution of specificity in the interaction of RNAs with themselves and/or with other molecules in the prebiotic environment.
- RNAs, either alone as *autocatalysts* (for example, self-splicing mRNAs) or in catalytic ribonucleoprotein complexes (e.g., ribosomes) exist in cells today.
- Some of these RNAs (specifically rRNAs), have a long phylogenetic heritage, shared by cells in all three domains of life.

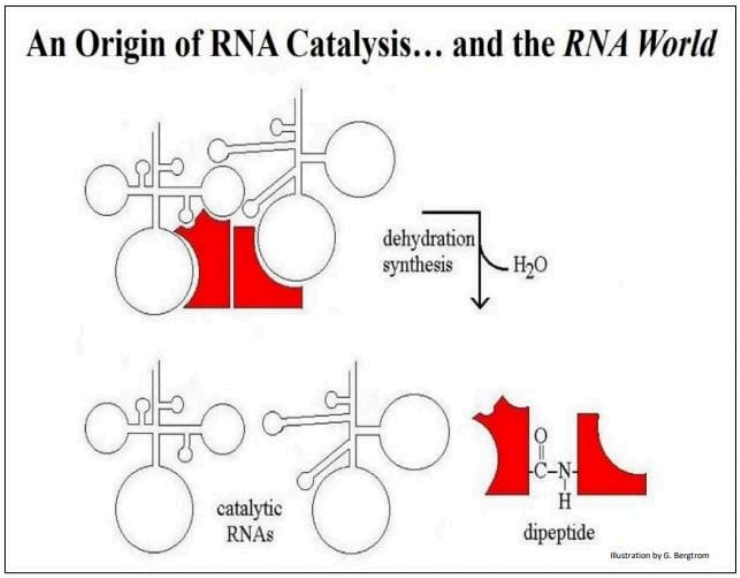
The propensity of single stranded RNA molecules to fold based on internal H-bonding can lead to those diverse three-dimensional shapes (tertiary structure). These structures could have interacted with other molecules in a environment. Because they could be replicated according to different prebiotic scenarios, the same RNAs could also pass on simple genetic information contained in their base sequences. The combination of informational and catalytic properties in a single molecule is illustrated below.



The capacity of RNAs as catalysts and warehouses of genetic information speaks to an efficient candidate for the first *dual* or *multi-purpose* polymer, a property that is not known and cannot be demonstrated for DNA. Read more about the proposed 'RNA worlds' in which life may have begun in Cech TR (2012) [*The RNA Worlds in Context*. In *Cold Spring Harbor Perspectives in Biology* (Cold Spring Harbor, NY: Cold Spring Harbor press) 4(7):a006742e].

355 Self-Replication: Information, Communication, and Coordination

What might RNA catalysis beyond self-replication have looked like in simpler times? Consider the interaction between a two hypothetical RNAs and different hypothetical amino acids bound to each, shown below.



The binding of each RNA to its amino acid would be a high affinity, specific interaction based on charge and shape complementarity. Likewise, the two RNAs seen in the illustration must have a high affinity for each other, also based on chemical and physical complementarities. One can even envision some strong H-bonding between bases in the two RNAs that might displace intra-strand H-bonding (not shown here). The result is that the two amino acids are brought together in a way that catalyzes peptide bond formation. This will require an input of free energy (recall that peptide bond is one of the most energy intensive reaction in cells). For now, assume a chemical energy source and let us focus on the specificities required for RNA catalytic activity.

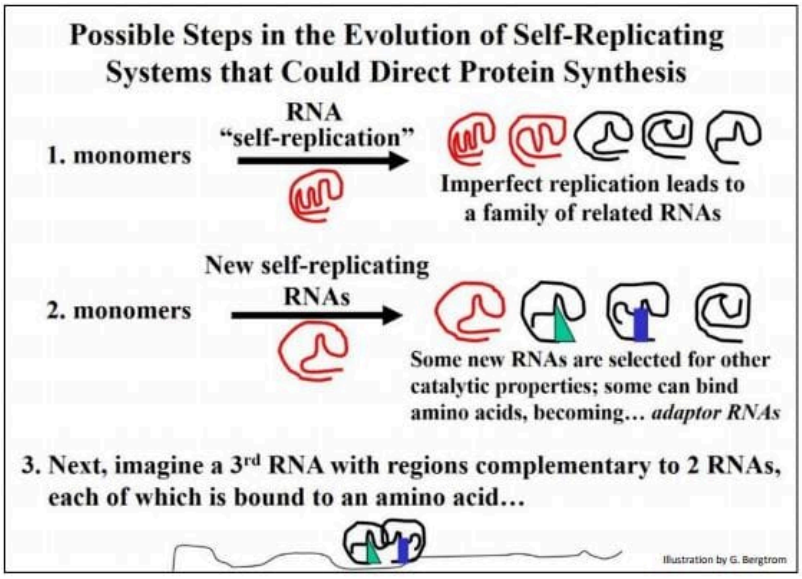
We know now that tRNAs the intermediaries between nucleic acids and polypeptide synthesis. So it's fair to ask how the kind of

activity illustrated above could have led to the tRNA-amino acid interactions we see today. There is no obvious binding chemistry between today's amino acids and RNAs, but there may be a less obvious legacy of the proposed bindings. This has to do with the fact that the genetic code is universal, which means that any structural relationship between RNA and amino acids must have been selected early (at the start!) of cellular life on earth. Here is the argument.

1. The code is indeed universal (or nearly so)
2. There is a correlation between the chemical properties of amino acids and their codons, for example
    - Charged (polar) amino acids are encoded by triplet codons with more G (guanine) bases.
    - Codons for uncharged amino acids more often contain a middle U (uracil) than any other base.

These correlations would mean that an early binding of amino acids to specifically folded RNAs was replaced in evolution by enzyme-catalyzed covalent attachment of an amino acid to a 'correct' tRNA, such as we see today.

What forces might have selected separation of the combined template/informational functions from most of the catalytic activities of RNAs? Perhaps it was the selection of the greater diversity of structure (i.e., shape) that folded polypeptides can achieve, compared to folded RNAs. After all, polypeptides are strings of 20 different amino acids compared to the four bases that make up nucleic acids. This potential for molecular diversity would in turn accelerate the pace of chemical (and ultimately cellular) evolution. A scenario for the transition from earlier self-replicating RNA events to the translation of proteins from mRNAs is illustrated here.



Adaptor RNAs in the illustration will become tRNAs. The novel, relatively unfolded RNA depicts a presumptive mRNA. Thus, even before the entry of DNA into our RNA world, it is possible to imagine the selection of the defining features of the genetic code and mechanism of translation (protein synthesis) that characterizes all life on the planet. Next, we consider "best-speculations" of how RNA-based information storage and catalytic chemistries might have made the evolutionary transition to DNA-based information storage and predominantly protein based enzyme catalysis.

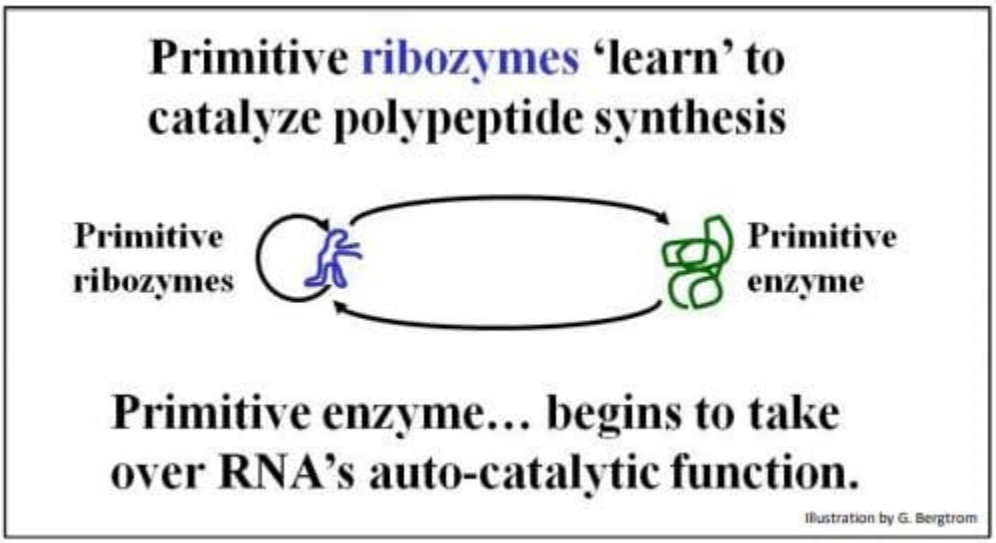
D. From Ribosomes to Enzymes; From RNA to DNA

The term *co-catalysis* could very well describe biochemical reactions in which a catalyst accelerates a chemical reaction whose product feeds back in some way on its own synthesis. We saw this in action when we discussed allosteric enzyme regulation and the control of biochemical pathways. Catalytic feedback loops must have been significant events in the evolution of the *intermolecular communication* and *metabolic coordination* required for life.

Here we will consider some scenarios for the transition from an RNA world to something more recognizable as today's nucleic acid information storage and proteinbased catalytic metabolism.

1. Ribozymes Branch Out: Replication, Transcription, and Translation

If RNAs catalyzed their own replication, it may have resembled the autocatalytic replication of AATE. At the same time, some RNAs may also have attracted amino acids to their surfaces and catalyzed peptide bond formation, as already described. Shapely prebiotic RNAs may therefore have catalyzed synthesized peptides, some of which would eventually take over catalysis of RNA synthesis! The scenario is summarized below.



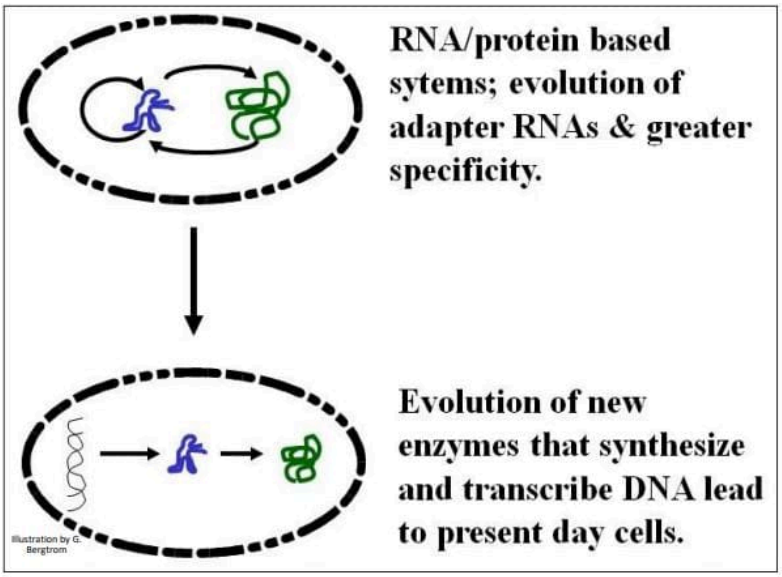
356 Information Storage and Retrieval in an RNA World

Selection favoring the synthesis of short oligopeptides and polypeptides is consistent with a catalytic diversification that led to the dominance of protein catalysts, i.e., enzymes. The primitive enzyme shown here must have been selected because at first, it assisted the autocatalytic replication of the RNA itself! Over time, the enzyme would evolve along with the RNA. This co-evolution then eventually replaced autocatalytic RNA replication with the enzyme-catalyzed RNA synthesis we recognize as transcription today. In this scenario, self-splicing premRNAs and ribozymes are surviving remnants of an RNA world!

Let's turn now to some ideas about how an RNA world could make the transition to the DNA-RNA-protein world we have today

2. Transfer of Information Storage from RNA to DNA

The transfer of function from RNA to DNA is by no means a settled issue among students of life origins and early evolution. A best guess is that the elaboration of protein enzymes begun in the RNA world would lead to reverse transcriptase-like enzymes that copied RNA information into DNA molecules. DNA information may have been selected because DNA is chemically more stable than RNA. The basic transfer scenario is illustrated below.



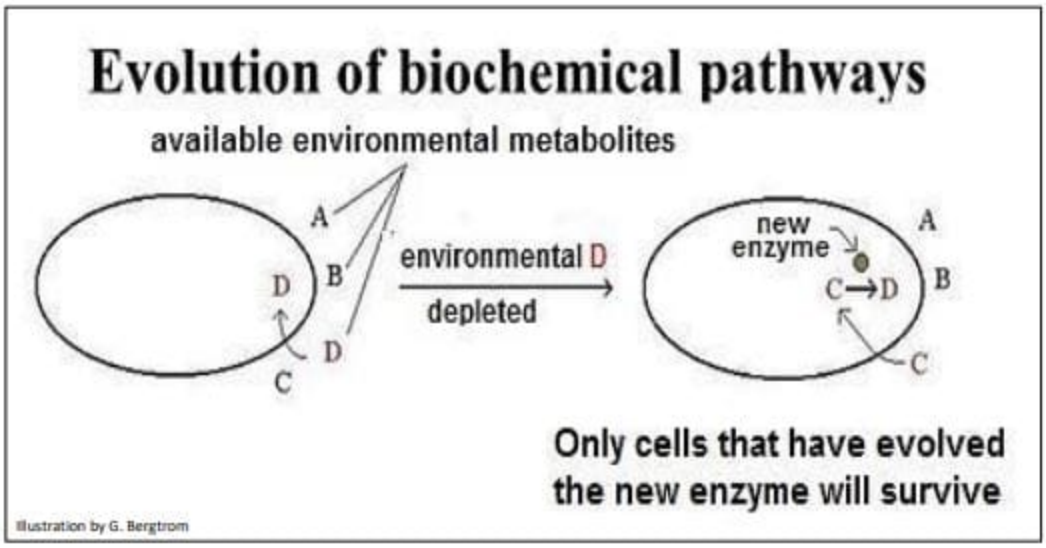
All cells alive today store information in DNA (only some viruses have an RNA genome). Therefore, transition to the use of DNA as an information molecule must have preceded the origin of life. At least, it must have occurred in the cells from which the LUCA arose. Details of this key change involve evolutionary steps yet to be worked out to everyone's satisfaction!

357 The Transition from an RNA World to a DNA World

E. The Evolution of Biochemical Pathways

The tale of the evolution of enzymes from ribozymes and of informational DNA from RNA, and the other metabolic chemistries behind prebiotic semipermeable boundaries is ongoing in cells today. Undoubtedly, early cellular metabolism involved only reactions crucial to life, catalyzed by a limited number of enzymes. But, if evolution inexorably trends towards greater complexity of molecular communication and coordination, in other words, towards increasingly refined regulation of metabolism, how did the repertoire of enzymes get larger, and how did biochemical pathways become more elaborate? We answered the first question elsewhere, when we discussed gene duplication (e.g., by unequal crossing over). The duplicate genes encoding the same enzyme provided the raw material for new enzymes and new enzymatic functions.

Whether in cells or in prebiotic structures, we can hypothesize how a new chemical reaction could evolve. For example, assume that a cell acquires molecule **D** required for an essential function, from an external, environmental source. What happens if levels of **D** in the environment become *limiting*? Clearly, cells would die without enough **D**. That is, unless cells that already have a duplicated, redundant gene that has mutated and now encodes an enzyme with the ability to make **D** in the cell. Such a cell might have existed with other cells without the mutation, but a D-limited environment would select the mutant cell for survival and reproduction. Imagine the scenario illustrated below.



358 Origins and Evolution of Biochemical Pathways

In a similar scenario, a mutation in a duplicated gene could result in a novel enzyme activity that can convert some molecule (e.g., C or D) in the cell into a new molecular product. If the new enzyme and molecular product do not kill or debilitate the cell, the cell might survive to be selected by some future exigency.

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20.7: A Summary and Some Conclusions

Our consideration of how life began on earth was intentionally placed at the end of this textbook, after we tried to get a handle on how cells work. Clearly any understanding of life origins scenarios is very much a matter of informed, if divergent speculations. Alternative notions for the origins of life entertained here all address events that presaged life under 'best-guess' hypothetical conditions. After trying to get a grip on prebiotic events, we asked how we got from what could have happened under a given set of prebiotic conditions to the cellular life we recognize today. All proposals recognize that the first cells had all of the properties of life (including evolution itself). Starting with that common understanding, all arguable scenarios try to navigate pathways from primitive, less controlled chemistries to more regulated and coordinated metabolisms, in other words from chemical simplicity to biochemical complexity. The chemical and metabolic evolution that began before life may have overlapped in time with cellular evolution, at least until the LUCA. While chemical evolution was mainly a series of selections by the physicality of a prebiotic world, the evolution of life contends with both that physical world, and with life itself. LUCA, the universal common ancestor, had already escaped the RNA world, replicating DNA, transcribing RNA and translating mRNAs into polypeptides, all behind a semipermeable phospholipid bilayer. Whether a heterotroph or (increasingly more likely) an autotroph, LUCA used the energy of ATP to power all of its cellular work, as do its descendants. Thus, cellular evolution, in fact all life after the LUCA, is focused on continued selection of the complexities of metabolism that enables the spread and diversification of life from wherever it started.

The selection of chemistries and traits encoded by already existing, accumulated random, neutral genetic changes, continue to this day, increasing the diversity of species and their spread to virtually every conceivable ecological niche on the planet. The overall takehome message of this chapter should be an understanding of how the molecular basis of evolution can help us understand how life may have begun on earth (or anywhere for that matter!). In turn, speculation about life's origins informs us about how the properties of life were selected under a set of prebiotic physical and chemical conditions.

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20.8: Key Words and Terms

AATE	deep sea hydrothermal vent	progenote
abiogenesis	Hadean eon	proteinoid microsphere
adapter RNA	heat of baking	protocell
alkaline hydrothermal vent	heterotrophs-first	reducing atmosphere
aminoadenosine triacid ester	ionizing radiation	retroviruses
Archean eon	last universal common ancestor	ribonucleoproteins
autocatalysis	liposome	ribozymes
autotrophs-first	LUCA	RNA world
biofilm	metabolic environment	Serpentinite
biogenesis	molecular communication	serpentinization
black smoker	non-reducing atmosphere	spontaneous generation
chemoautotrophs	ozone layer	tidal pool scenario
coacervate	Panspermia	white smoker
co-catalysis	photoautotrophs	zircon
	primordial soup	

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INDEX

D

Dark Proteome
15.7: Ome-Sweet Ome

DNA microarray
15.6: Genomic Approaches- The DNA Microarray

E

Eulogy for Junk DNA.
14.7: Coping with the Dangers of Rampant Transposition

G

glycolipids
16.7: Glycolipids

H

Human Transcriptome Study
15.7: Ome-Sweet Ome

L

Last Universal Common Ancestor (LUCA)
20.1: Introduction

M

Mapping Protein Networks
15.7: Ome-Sweet Ome

N

Nature vs. Nurture: Influences on Epigenome and Methylome
15.7: Ome-Sweet Ome

Not junk after all?
14.7: Coping with the Dangers of Rampant Transposition

P

polymerase chain reaction (PCR)
15.5: The Polymerase Chain Reaction (PCR)

S

see Mining Mitochondrial Genes
15.7: Ome-Sweet Ome

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    - 1.3: Domains of Life - *CC BY 4.0*
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    - 1.6: The Origins, Evolution, Speciation, Diversity and Unity of Life - *CC BY 4.0*
    - 1.7: Microscopy Reveals Life's Diversity of Structure and Form - *CC BY 4.0*
    - 1.8: Key Words and Terms - *CC BY 4.0*
  - 2: Basic Chemistry, Organic Chemistry and Biochemistry - *CC BY 4.0*
    - 2.1: Introduction - *CC BY 4.0*
    - 2.2: Atoms and Basic Chemistry - *CC BY 4.0*
    - 2.3: Chemical bonds - *CC BY 4.0*
    - 2.4: A Close Look at Water Chemistry - *CC BY 4.0*
    - 2.5: Some Basic Biochemistry- Carbon, Monomers, Polymers and the Synthesis and Degradation of Macromolecules - *CC BY 4.0*
    - 2.6: Key Words and Terms - *CC BY 4.0*
  - 3: Details of Protein Structure - *CC BY 4.0*
    - 3.1: Introduction - *CC BY 4.0*
    - 3.2: Levels (Orders) of Protein Structure - *CC BY 4.0*
    - 3.3: Changes in Protein Shape Can Cause Disease - *CC BY 4.0*
    - 3.4: Quaternary Structure - *CC BY 4.0*
    - 3.5: Some Proteins are Chemically Modified and Others Require Prosthetic Groups to be Biologically Active - *CC BY 4.0*
    - 3.6: Protein Domains, Motifs, and Folds in Protein Structure - *CC BY 4.0*
    - 3.7: Proteins, Genes and Evolution- How Many Proteins are We? - *CC BY 4.0*
    - 3.8: View 3D Animated Images of Proteins in the NCBI Database - *CC BY 4.0*
    - 3.9: Key Words and Terms - *CC BY 4.0*
  - 4: Bioenergetics - *CC BY 4.0*
    - 4.1: Introduction - *CC BY 4.0*
    - 4.2: Kinds of Energy - *CC BY 4.0*
    - 4.3: Deriving Simple Energy Relationships - *CC BY 4.0*
    - 4.4: Key Words and Terms - *CC BY 4.0*
  - 5: Enzyme Catalysis and Kinetics - *CC BY 4.0*
    - 5.1: Introduction - *CC BY 4.0*
    - 5.2: Enzymes - *CC BY 4.0*
    - 5.3: Key Words and Terms - *CC BY 4.0*
  - 6: Glycolysis, the Krebs Cycle and the Atkins Diet - *CC BY 4.0*
    - 6.1: Introduction - *CC BY 4.0*
    - 6.2: Glycolysis (from the Greek glyco (sugar) lysis (Separation), or Sugar Breakdown - *CC BY 4.0*
    - 6.3: Some Details of Glycolysis - *CC BY 4.0*
    - 6.4: Gluconeogenesis - *CC BY 4.0*
    - 6.5: The Atkins Diet and Gluconeogenesis - *CC BY 4.0*
    - 6.6: The Krebs/TCA/Citric acid cycle - *CC BY 4.0*
    - 6.7: Key Words and Terms - *CC BY 4.0*
  - 7: Electron Transport, Oxidative Phosphorylation, and Photosynthesis - *CC BY 4.0*
    - 7.1: Introduction - *CC BY 4.0*
    - 7.2: The Electron Transport Chain (ETC) - *CC BY 4.0*
    - 7.3: Oxidative Phosphorylation - *CC BY 4.0*
    - 7.4: Photosynthesis - *CC BY 4.0*
    - 7.5: More Thoughts on the Mechanisms and

Evolution of Respiration and Photosynthesis - *CC BY 4.0*
- 7.6: Key Words and Terms - *CC BY 4.0*

- 8: DNA, Chromosomes and Chromatin - *CC BY 4.0*
  - 8.1: Introduction - *CC BY 4.0*
  - 8.2: The Stuff of Genes - *CC BY 4.0*
  - 8.3: DNA Structure - *CC BY 4.0*
  - 8.4: Genes and Chromatin in Eukaryotes - *CC BY 4.0*
  - 8.5: Structure and Organization of DNA in Bacteria - *CC BY 4.0*
  - 8.6: Key Words and Terms - *CC BY 4.0*
- 9: Details of DNA Replication and Repair - *CC BY 4.0*
  - 9.1: Introduction - *CC BY 4.0*
  - 9.2: DNA Replication - *CC BY 4.0*
  - 9.3: DNA Repair - *CC BY 4.0*
  - 9.4: Key Words and Terms - *CC BY 4.0*
- 10: Transcription and RNA Processing - *CC BY 4.0*
  - 10.1: Introduction - *CC BY 4.0*
  - 10.2: Overview of Transcription - *CC BY 4.0*
  - 10.3: Details of Transcription - *CC BY 4.0*
  - 10.4: Details of Eukaryotic mRNA Processing - *CC BY 4.0*
  - 10.5: Ribosomal RNA Processing in Eukaryotic Nuclei - *CC BY 4.0*
  - 10.6: tRNA Processing in Eukaryotic Nuclei - *CC BY 4.0*
  - 10.7: RNA and Ribosome Export from the Nucleus - *CC BY 4.0*
  - 10.8: Key Words and Terms - *CC BY 4.0*
- 11: The Genetic Code and Translation - *CC BY 4.0*
  - 11.1: Introduction - *CC BY 4.0*
  - 11.2: An Overview of the Genetic Code - *CC BY 4.0*
  - 11.3: Gene and Protein Colinearity and Triplet Codons - *CC BY 4.0*
  - 11.4: Translation - *CC BY 4.0*
  - 11.5: Key Words and Terms - *CC BY 4.0*
- 12: Regulation of Transcription and Epigenetic Inheritance - *CC BY 4.0*
  - 12.1: Introduction - *CC BY 4.0*
  - 12.2: Gene Regulation in Prokaryotes - *CC BY 4.0*
  - 12.3: The Problem with Unregulated (Housekeeping) Genes in All Cells - *CC BY 4.0*
  - 12.4: Gene Regulation in Eukaryotes - *CC BY 4.0*
  - 12.5: Epigenetics - *CC BY 4.0*
  - 12.6: Key Words and Terms - *CC BY 4.0*
- 13: Post Transcriptional Regulation of Gene Expression - *CC BY 4.0*
  - 13.1: Introduction - *CC BY 4.0*
  - 13.2: Post-transcriptional Control of Gene Expression - *CC BY 4.0*
  - 13.3: Eukaryotic Regulation of Translation - *CC BY 4.0*
  - 13.4: Key Words and Terms - *CC BY 4.0*
- 14: Repetitive DNA - A Eukaryotic Genomic Phenomenon - *CC BY 4.0*
  - 14.1: Introduction - *CC BY 4.0*
  - 14.2: The Complexity of Genomic DNA - *CC BY 4.0*
  - 14.3: The 'Jumping Genes' of Maize - *CC BY 4.0*
  - 14.4: Transposons Since McClintock - *CC BY 4.0*
  - 14.5: On the Evolution of Transposons, Genes, and Genomes - *CC BY 4.0*
  - 14.6: Roles of Transposition in Evolution and Diversity - *CC BY 4.0*
  - 14.7: Coping with the Dangers of Rampant Transposition - *CC BY 4.0*
  - 14.8: Key Words and Terms - *CC BY 4.0*
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  - 15.1: Overview - *CC BY 4.0*
  - 15.2: Make and Screen a cDNA Library - *CC BY 4.0*
  - 15.3: DNA Sequencing - *CC BY 4.0*
  - 15.4: Genomic Libraries - *CC BY 4.0*
  - 15.5: The Polymerase Chain Reaction (PCR) - *CC BY 4.0*
  - 15.6: Genomic Approaches- The DNA Microarray - *CC BY 4.0*
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  - 15.8: From Genetic Engineering and Genetic Modification - *CC BY 4.0*
  - 15.9: Key Words and Terms - *CC BY 4.0*
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  - 16.1: Overview - *CC BY 4.0*
  - 16.2: Plasma Membrane Structure - *CC BY 4.0*
  - 16.3: Membrane Proteins - *CC BY 4.0*
  - 16.4: How Membrane Proteins are Held in Membranes - *CC BY 4.0*
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  - 16.8: Glycoproteins and Human Health - *CC BY 4.0*
  - 16.9: Key Words and Terms - *CC BY 4.0*
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  - 17.1: Introduction - *CC BY 4.0*
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  - 19.1: Introduction - *CC BY 4.0*
  - 19.2: Bacterial Cell Division and the Eukaryotic Cell Cycle - *CC BY 4.0*
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  - 20.2: Thinking about Life's Origins- A Short Summary of a Long History - *CC BY 4.0*
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  - 20.4: Origins of Organic Molecules in a Non-Reducing Atmosphere - *CC BY 4.0*
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