CELLS: MOLECULES AND MECHANISMS



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Cells: Molecules and Mechanisms

E. V. Wong

Axolotl Academica Publishing

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Preface

Yet another cell and molecular biology book? At the very least, you would think that if I was going to write a textbook, I should write one in an area that really needs one instead of a subject that already has multiple excellent and definitive books. So, why write this book, then? First, it's a course that I have enjoyed teaching for many years, so I am very familiar with what a student really needs to take away from this class within the time constraints of a semester. Second, because it is a course that many students take, there is a greater opportunity to make an impact on more students' pocketbooks than if I were to start off writing a book for a highly specialized upper level course. And finally, it was fun to research and write, and can be revised easily for inclusion as part of our next textbook, High School Biology.

As with every textbook, this one owes a huge debt to the many excellent textbooks that came before it. And in fact, because I consider this more of a teaching text than a reference text, if a student is serious about this subject area, and has the funds, I very strongly recommend picking up one of the classic tomes in this field of biology, either *Molecular Biology of the Cell* by Bruce Alberts and colleagues (Garland Science), or *Molecular Cell Biology*, by Harvey Lodish and colleagues (W.H. Freeman & Co.). But to come back to a reason for writing my own text, as wonderful as those two books are, they are actually *over*loaded with information for an average introductory (sophomore-level) course in cell biology.

One of the areas that was carved out of the primary text is the historical perspective and experimental design that led to the discovery of the molecules and mechanisms described. This was a difficult decision because I am a strong believer in understanding the scientific thought process and learning from history. In the end though, I decided to provide that kind of information as ancillary material that can be included by course instructors at their discretion, because long experience and many conversations with students indicate that even if they thought it was going to be "on the test", they tended to gloss over the "names and dates" stuff while studying, and on my end, I would rather use exam "space" to test them on information more relevant to success and understanding of more complex concepts later in the course (or in more advanced coursework).

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Axolotl Academic Publishing Company is devoted to the idea that only a scientifically literate general population can make sound decisions on a host of important governmental and non-governmental matters that involve the many advances in science and technology that have occurred in the past few decades. Paradoxically, the science literacy of the average citizen did not keep pace with development by our leading scientists and engineers, and the widening gap has led to many conflicts that, in our opinion, may not exist if everyone was fully versed in the science. Complex issues such as genetic engineering or stem cell research have been boiled down to erroneously simplified sound bite definitions. Providing free textbooks is just one step of many needed to lower some of the economic barriers to quality science education.

Although I must claim responsibility for much of the book (for better or worse), I can only take credit for a little of the art. My talented illustrator, Patrick J. Burton, did most of the original artwork, and served as a reviewer of the text as well. Sources for all other Figures are acknowledged in the Figure caption.

Finally, this book would not have come about had it not been for the unflagging support from my wife and children, who are ultimately the reason for everything I do.

E.V.Wong

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1: Anatomy of a Cell - A Very Brief Overview

Since this entire course is devoted to understanding the workings of the cell, it is almost superfluous to dedicate a chapter to identifying the parts of the cell and their functions. However, because it is easy to get lost in the intricacies of the molecules and chemical reactions within the cell, consider this chapter more of a framework or map for the course, giving context to the minutiae.

The cell is the smallest unit of life, so all cells, whether they are unicellular organisms or just a tiny part of a multicellular organism, have certain characteristics in common: they must contain genetic information and the mechanisms to regulate and use that information to produce its own parts and to reproduce new cells, they must be able to use energy in chemical reactions and physical actions, they must be able to regulate those activities, and they must respond to stimuli.

Cells use DNA (deoxyribonucleic acid) for their genetic material, and all cells contain the transcriptional and translational enzymes to read it and use the information to construct more cell components. However, simply having genetic material does not define life: viruses have genetic material containing all the information necessary to make a complete virus, but it does not contain the enzymes necessary to do so, nor the ability to obtain the raw molecular material needed to do so. It is absolutely dependent on the machinery inside whatever cell it infects. Therefore, a virus is not a living organism.

The genome is not only an instruction set for making a cell (or an organism, for that matter); it is also replicable itself. Roughly speaking, during part of its life cycle, the cell makes an extra copy of its genome and increases the numbers of all the other “stuff” (proteins, fats, etc.) of which it is made, and then it reproduces by division. The mother cell splits into two daughter cells, each with the same complement of genetic information, and with approximately the same cellular components. Thus we see that while the genome is often considered the blueprint for a cell/organism, in fact cells are not built up from scratch directly from DNA. Every cell comes from another cell. The DNA can then be used to customize that cell for specific purposes as determined by its environment. When a particular component of the cell is needed, the information for making that component is read from the DNA and copied into RNA which is used as a program from which ribosomes can manufacture the proteins needed. A living cell needs all these things: the genetic information, the mechanisms and machinery to use the information to build cell parts, and the ability to harness energy to do so.

As we will see in chapter 3, the physical laws of nature require that everything tends towards its simplest, least organized, state unless there is an input of energy to work against that tendency. Since cells are a highly ordered collection of very complex molecules, they must therefore require energy to remain as cells. Thus, life requires the ability to obtain energy, either from sunlight or food, and the ability to convert that energy into forms that can be readily used by the cell to maintain itself by building or rearranging necessary molecules and macromolecular structures.

How do cells know when to carry out these activities? This leads us to the next characteristic of living cells: the ability to respond to stimuli. In other words, they are self-regulating. If glucose levels run low and the cell needs energy, glucose transport proteins are made, or if the cell needs to move to an area of higher food concentration, the cell cytoskeleton rearranges to move the cell. The cell has the ability to initiate repair processes if it detects lesions in its genome, it can pause the cell cycle to allow such repair processes time, and it can even initiate its own death if repairs are repeatedly unsuccessful.

In addition to responding to internal signals, living cells are also able to respond to external stimuli. Whether it is contact with a neighboring cell, binding a hormone released from a cell far away, or simply interacting with non-cellular environmental objects, a cell is able to respond to such stimuli. Responses may include making new proteins, destruction of existing proteins, moving away from the stimulus, moving towards the stimulus, initiation of reproduction, and many other possibilities.

There are two basic types of cells: prokaryotes and eukaryotes. The difference is simple and readily recognizable under light microscopy. Eukaryotic cells contain intracellular membrane-bound compartments (called organelles). Prokaryotic cells do not contain any such compartments (Figure 1.1).

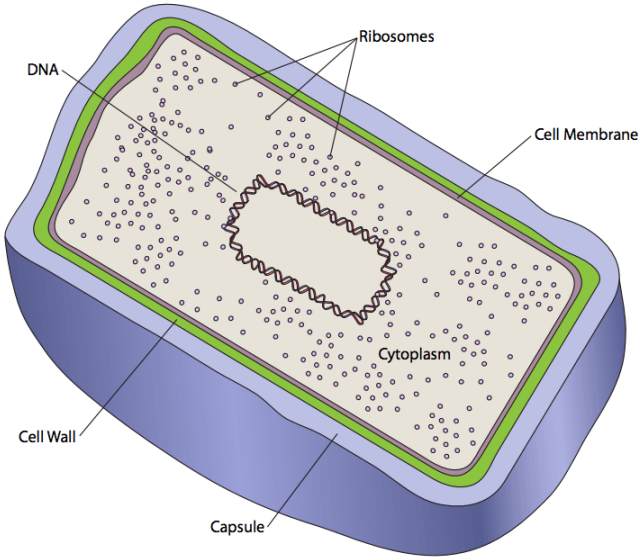


Figure 1.1. A prokaryotic cell

There is only one membrane in prokaryotes, the cell membrane, and only one compartment in prokaryotic cells, the cytoplasm. That does not preclude a certain level of organization in prokaryotes, but it is not as complex as eukaryotes. The genomic DNA is usually organized in a central nucleoid. There are not intracellular membranous organelles, but the cell is defined by a cell membrane. Outside of the cell membrane, prokaryotes have a cell wall. This wall is relatively rigid and confers shape to the cell. Depending on the type of bacteria, the thickness of the wall varies (thick = “gram positive”, thin = “gram negative”). Some, but not all bacteria also secrete another layer outside of the cell wall. This is a relatively tight matrix called a capsule that helps protect the cell from dessication in dry environments. A comparatively loose matrix of the same types of molecules may be secreted, and instead of the capsule, the result is called a slime layer. The slime layer is important in bacterial attachment and formation of biofilms (see chapter 13, Extracellular Matrix).

Eukaryotic cells are considerably more complex. Eukaryotic organisms are currently classified into four kingdoms: animal, plant, fungus, and protists. The animal cell in Figure 1.2 depicts has many features in common with cells of the other three kingdoms.

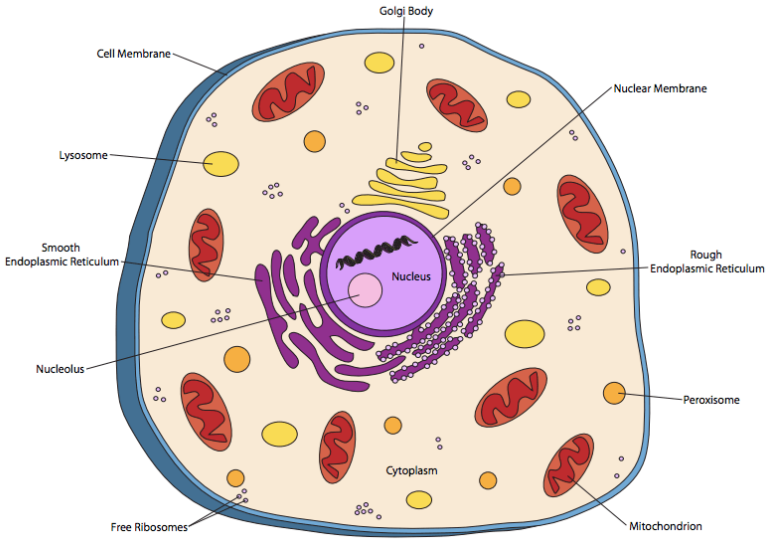


Figure 1.2. A prototypical animal cell

Obviously, the biggest difference between the animal cell (or any eukaryotic cell) and prokaryotic cells is the presence of internal membrane-bound compartments, or organelles. The most prominent of these is the nucleus, which houses the DNA. Traditionally, it has been assumed that most eukaryotic genomes can range from 10 to 100 x 10^6 nucleotides (10-100 Mb) in total length,

over two or more chromosomes (DNA molecules) of roughly similar size. In contrast, prokaryotic genomes have traditionally been viewed as a single circular chromosome, and mostly under a megabase (10⁶ nucleotides) in length.

> Recent and better methods for genome mapping and sequencing, and a broadening of the sample organisms has shown those numbers to be inaccurate. In fact, eukaryotic genomes range from ~3 Mb to over 40000 Mb. Prokaryotic genomes vary from 0.5 Mb to a little over 10 Mb (0.5 to 6 Mb for Archaea, 0.6 to 10 Mb for Bacteria) and may be spread over multiple DNA molecules that may be either linear or circular.

The nucleus is bounded by a double-layered membrane (most other organelles are bounded by a single membrane) that is continuous with the the endoplasmic reticulum (ER). The endoplasmic reticulum is subdivided into the rough ER (RER) and the smooth ER (SER) based on appearance in electron micrographs. The “studs” on the RER are ribosomes, which are the molecular machinery for making proteins in the cell. There are also free-floating ribosomes - the difference is that the free ribosomes make proteins that stay in the cytoplasm, while ribosomes attached to the RER are synthesizing proteins that are destined to insert into a membrane, localize inside an organelle, or be secreted out of the cell entirely. The RER makes modifications to the proteins as well as compartmentalizing them. The SER counts lipid synthesis (e.g. to make membranes) and detoxification reactions among its duties. It should be noted that ribosomes on the RER are not permanently attached, and after they have produced a protein, they dissociate from the RER and rejoin the general pool of free ribosomes in the cytoplasm.

The Golgi complex, or Golgi bodies, while physically independent, are a functional extension to the protein processing and sorting that occurs in the ER. Proteins leave the Golgi in vesicles bound for the cell membrane or other organelles. Vesicles, while membrane-bound, are not generally counted as organelles: they are simply small transport packages.

Mitochondria are complex organelles that are not only bounded by a membrane, but also contain a second membrane that is highly crenulated. Mitochondria make aerobic respiration possible, using oxygen as an oxidizer to produce chemical energy (i.e. ATP) far more efficiently than the anaerobic processes used by most prokaryotes. This ability to produce more energy from the same amount of food allows eukaryotic cells to grow larger than prokaryotes.

Lysosomes are acidic and contain digestive enzymes that break down large food molecules — particularly proteins and fats — to make them usable by the rest of the cell. These enzymes work optimally in acidic conditions, which acts as a sort of safety mechanism: if a lysosome breaks and releases its enzymes into the cytoplasm, they will not break down cellular components willy-nilly because the cytoplasmic pH is close to neutral and the enzymes do not work well. Once thought to be exclusive to animal cells, lysosomes have now been described in all cells from all eukaryotic kingdoms.

Peroxisomes also break down or convert molecules, but they generally act on smaller molecules by oxidation. For example, some peroxisomes in human liver cells are used to break down alcohol (ethanol). Processes like this often produce H_2O_2, hydrogen peroxide, as a byproduct. Since H_2O_2 in high concentrations is harmful, peroxisomes often contain an enzyme, catalase, that converts it into water and molecular oxygen.

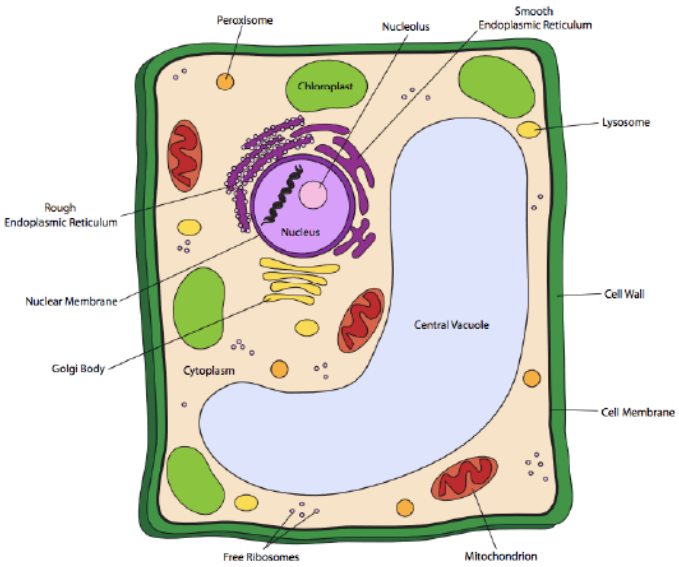


Figure 1.3. A typical plant cell

Plant cells have all of the above named organelles, but additionally may also bear two other types of organelles: chloroplasts and vacuoles. In addition to this, plant cells also have a rigid cell wall external to the cell membrane. Chloroplasts are similar to mitochondria in shape and structure (membranes within the outer membrane). However, its function is very different: chloroplasts absorb light energy from the sun (or other light sources) and convert them into chemical energy in the form of simple sugars for the cell to store and use later, in essence turning the cell into a solar energy warehouse and distribution center. Since plants and some photosynthetic bacteria are the only organisms capable of converting solar energy into a form useful to living cells, they are crucial to the survival of all other life.

Vacuoles are essentially storage units. They may store starches for use as energy sources when sunlight is unavailable or when immediate photosynthesis alone is not sufficient to provide for the energy needs of the cell. Other vacuoles, such as the one depicted above in Figure 1.3, store water, which helps the cell to maintain rigidity in combination with the cell wall.

Plant cell walls are composed of very different materials than the previously mentioned bacterial cell walls. Plant cell walls are primarily composed of the glucose polymer, cellulose, but contain other polysaccharides as well. Depending on the type of plant cell, there may be multiple layers of cellulose composing the cell wall. The wood and bark of trees, for example have both a primary (thin) cell wall and a secondary (thick) wall, while the leaves would have only a primary wall. Fungi also have cell walls, and they too are different from bacterial cell walls. True fungi have cell walls that are composed primarily of the polysaccharide chitin, and no cellulose.

> Interestingly, two groups once classified as fungi: oomycetes and dictyostelids, have cell walls composed of cellulose (and some have both). These organisms have been reclassified in Protista.

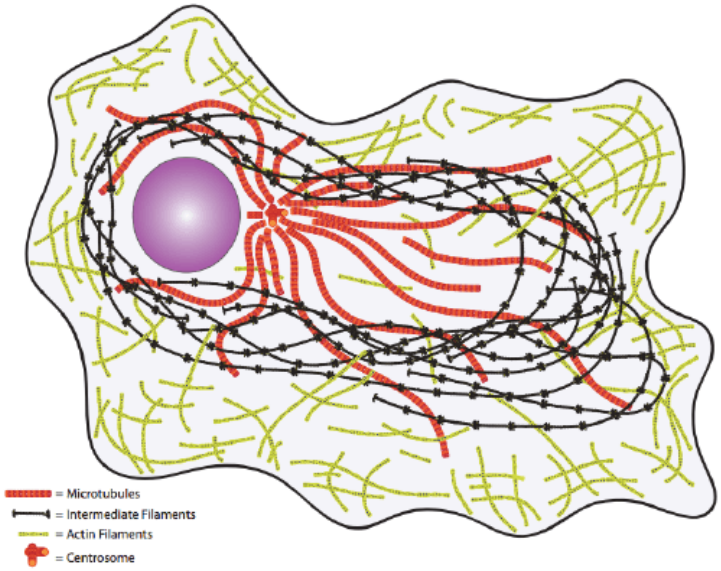


Figure 1.4. Animal cell cytoskeleton

Finally, consider the cytoplasm. Once considered merely the aqueous environment in which the "important" molecules or organelles floated, it is now better understood to be filled with important structural and transport elements (Figure 1.4). The cytoskeleton provides not only an internal physical structure but also a transport system to move molecules, vesicles, and even organelles to where they are needed.

All of the cell parts introduced in this chapter will be explained in much greater detail in subsequent chapters. More importantly, the intertwined relationships between many of the molecules and organelles will be discussed and elucidated. As you go through this course, you will notice that the same species come up over and over as examples. These are the model organisms upon which the great majority of molecular cell biology research is based. Most prokaryotic research has been based on *Escherichia coli* (*E. coli*), which is a Gram-negative rod-shaped bacterium commonly found in the gut of many higher animals. The Gram-negative soil bacteria, *Bacillus subtilis*, is a sporeforming organism that has also been used in research because, like *E. coli*, its genome is easily manipulated for experimentation, and is also relatively easy to grow in the lab. On the eukaryotic side, yeast (*Saccharomyces cerevisiae*, or *Schizosaccharomyces pombe*) are very commonly used for simpler intracellular processes due to simple genetics and very fast generation times. *Caenorhabditis elegans* (a nematode) and *Drosophila melanogaster* (fruit fly) are popular invertebrate model organisms, especially for developmental and genetic studies due to the small number of cells, mostly with traceable lineage, and fast generation time (for metazoans). Frogs, particularly the South African clawed frog, *Xenopus laevis*, and the Northern Leopard frog, *Rana pipiens*, are popular for certain types of developmental and cell cycle studies because they have huge oocytes that are amenable to many kinds of genetic and physiological manipulation not possible in other cells. *Arabidopsis thaliana* is the most commonly used model organism for the study of plant genetics. Finally, because they are mammals like us (humans), but breed quickly and can be genetically manipulated with relative ease, mice (*Mus musculus*) are very commonly used in the study of more complex intra- or inter-cellular mechanisms. More recently, the near complete sequencing of the genome and development of techniques to manipulate it, have made the rat (*Rattus norvegicus*) another viable research organism for the study of mammalian genes.

> Further information on model organisms can be found at the United States' National Institutes of Health web site: www.nih.gov/science/models/

The commonalities that make all of these organisms excellent models for the study of the molecules of the cell and the interactions between them that constitute life, are a relatively short generation time, well-described (and in most cases fully sequenced) genome, and ease of experimental manipulation. Most of the molecules and mechanisms you will learn in the course were discovered in the simpler model mechanisms, and then found again, often with elaboration in the more complex ones.



Figure 1.5. Model Organisms. The Northern Leopard frog, Rana pipiens (left), the mustard-family plant, Arabidopsis thaliana (center), and the common rat, Rattus norvegicus (right). All images released to the public domain by the United States Goverment (USGS, NPS, NIH).

Thumbnail: A diagram of a typical prokaryotic cell. (Public Domain; LadyofHats).

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2: BASIC CELL CHEMISTRY - CHEMICAL COMPOUNDS AND THEIR INTERACTIONS

2.1: Water

2.2: Acids and Bases

2.3: Carbon

2.4: Sugars

2.5: Nucleotides

2.6: Amino Acids

2.7: Fatty Acids

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2.1: Water

There is no life without water. In this chapter, water will be used to review some very basic ideas in chemistry, particularly as applies to cell and molecular biology. What is water? H_2O. Two hydrogen atoms and one oxygen atom (Figure 2.1.1). Together they form a molecule of water. They are defined as a molecule by the presence of strong chemical bonds connecting each atom.

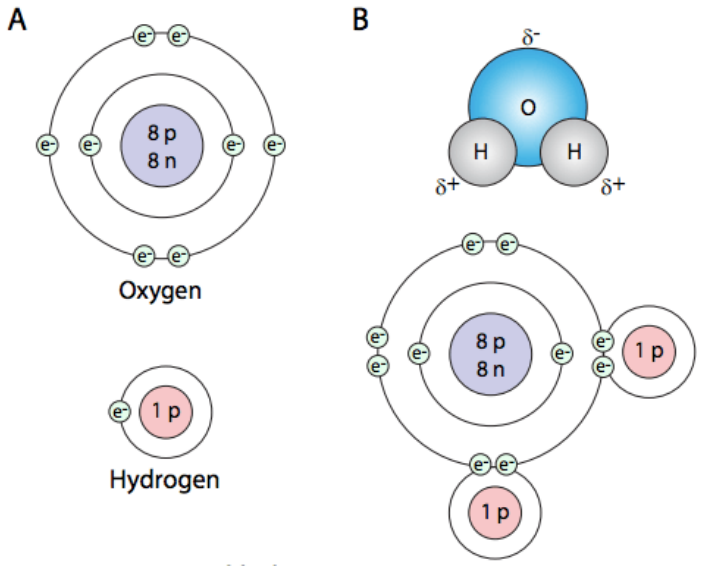


Figure 2.1.1. (A) Oxygen and hydrogen (B) water

In this case, each atom is connected to another by a *covalent* bond. These are the strongest type of chemical bonds, and form when two atoms are *sharing electrons* in order to fill their outermost (valence) electron shell and increase stability.

> The volume of an atom is defined by electrons in a very fast and energetic obit around a nucleus. The electrons are very small negatively charged particles, and the nucleus is composed of neutrons (electrically neutral) and protons (positively charged), both relatively massive in comparison to electrons. The electrons' orbits around the nucleus can be approximated by "shells" or levels. These shells characteristically have limitations on the number of electrons that can fit within them: the first shell (closest to nucleus) holds only 2 electrons, while the second shell holds 8, and the third shell holds 18. The atom is most stable when its outer shell (and by extension, all inner ones also) is filled. The energy of the electrons also varies by level - innermost electrons have the least energy while the outermost electrons have the most.

In the case shown here, hydrogen (H) has only one electron, and for maximal stability of that electron shell, it should have two. Oxygen, on the other hand, has six electrons in its outer shell, and a filled shell would have eight. Thus, it would "like" to pull in two more electrons for maximal stability. As shown in Figure 2.1.1B, both of those requirements are fulfilled when each of the hydrogen atoms shares an electron with the oxygen, which also shares an electron each with the hydrogen. The water molecule can also be written as H—O—H, in which the single solid line indicates a pair of shared electrons, i.e. a single covalent bond. The energy of an average single covalent bond is about 80 kcal/mol. However, as shown below, double and even triple covalent bonds are possible. The strength of those types of bonds is slightly less than double (~150 kcal/mol) or triple (~200 kcal/mol) the energy of the single bonds.

> Bond energy is a measure of the strength of the bond between two covalently joined atoms, and is proportional to the bond distance, which is determined by the atomic radii. It is not the same thing as bond dissociation energy, which is the energy released in a homolytic reaction (bond is split with electrons equally distributed) taking place at absolute zero, but they are similar in being measures of bond strength.

Sharing electrons is not the only way to create bonds between atoms. *Ionic* bonds are created when an atom donates or receives an electron, rather than sharing one. When an atom gives up an electron, the electrical balance between the numbers of positively charged protons in its nucleus and negatively charged electrons is upset, and the overall atom now has a positive electrical charge. Similarly when an atom receives an extra electron, the balance in a neutral atom is upset, and the atom becomes negatively charged. An ionic bond is formed when one atom donates an electron to an adjacent atom, creating an ionic pair, one positively and one negatively charged. The electrical attraction between the oppositely charged atoms holds them together.

> Although salts (such as NaCl) are ionic compounds, not all ionic compounds are salts. The chemical definition of a salt requires that the compound be formed by the substitution of a hydrogen ion (H+) in the original compound. This usually occurs in neutralization reactions, such as the neutralization of hydrochloric acid, HCl (or H^+ Cl^-) with sodium hydroxide (Na^+ $(OH)^-$), which yields the salt NaCl, and water (HOH = H_2O).

Ionic bonds are weaker than covalent bonds, with an average bond energy of ~5.5 kcal/mol. Both covalent and ionic bonds are thermodynamically stable in dry, room temperature conditions (25°C, 298 K, 77°F). The average energy imparted when molecules collide at this temperature is only ~0.6 kcal/mol, far less than the energy needed to break a covalent or ionic bond.

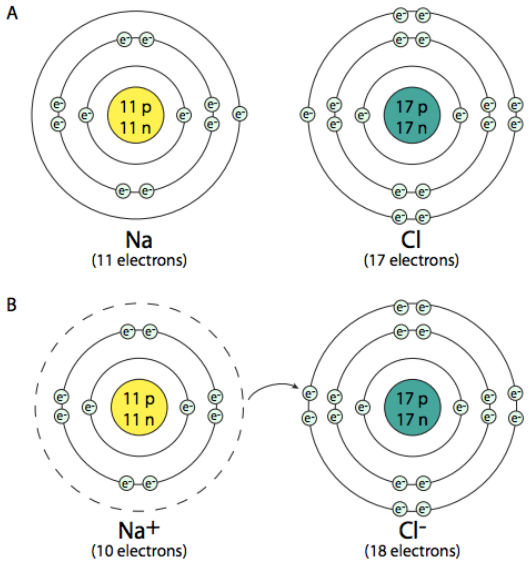


Figure 2.1.2. (A) Individually, the Na atom and the Cl atom are electrically neutral. However, they are both very reactive chemically because both need only get rid of (Na) or take in (Cl) one electron to have a full outer shell. (B) Because an electron is completely transferred, the Na becomes Na+ and Cl becomes Cl- , reflecting the new charge imbalance. Although electrically no longer neutral, the thermodynamic enhancement from filling the outer shells makes both of these ions very stable.

Covalent and ionic bonds between atoms are the only way to make molecules, which are stable collections of chemically bonded atoms. However, other attractive interactions between atoms and molecules exist, but they are significantly weaker, and can be disrupted with relatively small changes in temperature or environmental conditions. These are van der Waal's forces. They are very short-range interactions, requiring close apposition of the two atoms. As mentioned, an individual hydrogen bond (a specific type of van der Waal's force described below) or other van der Waal's interaction can be easily disrupted, but these types of interactions generally occur en masse. In a sense, they are like molecular Velcro® - each individual little plastic hook and individual loop of nylon could barely hold two hairs together, but a suit of velcro can hold a person on a vertical wall (a la Late Night with David Letterman, 1984).

In the case of hydrogen bonds, these occur when there is permanent asymmetric electron sharing within a covalently bonded molecule so that the shared electrons spend more time around one nucleus (thus imparting a negative character), than the other (which is therefore somewhat positive in character) to create a permanent electrical dipole. These dipole moments can interact with oppositely charged moments on other molecules or the same molecule. Van der Waals forces also include induced (nonpermanent) dipole-dipole interactions in which a temporary shift in electron density as they orbit the nucleus forms a minute charge differential, that can induce an opposite and attractive charge differential in a very close neighboring atom. In fact, some texts define van der Waals forces exclusively as such, leaving hydrogen bonds as a separate category altogether. One of the

arguments for that idea is that the bond length of the average H-bond is smaller than the sum of the van der Waal's radii of the two atoms.

As noted above, hydrogen bonds result from severely uneven sharing of electrons that generate permanent dipoles. In biological systems, this generally means that a hydrogen is covalently bound to either an oxygen or a nitrogen atom, which are both highly electronegative atoms, strongly attracting the shared electrons away from the hydrogen. Common hydrogen-bonding pairs are OH· · ·:O, OH· · ·:N, NH· · ·:N, and NH· · ·:O. Dotted lines are a common method for depicting hydrogen bonds in printed text and diagrams.

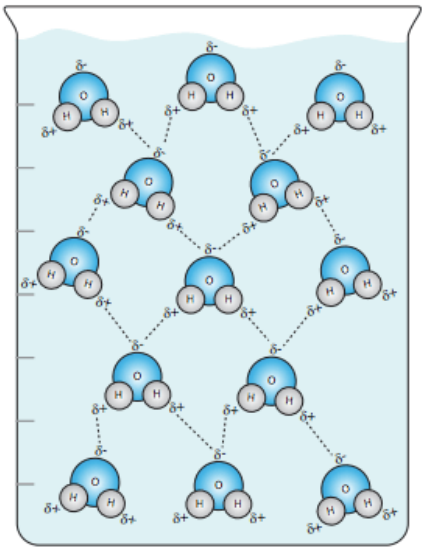


Figure 2.1.3. The hydrogen bonding of water molecules to one another is an important determinant of the physical properties of water.

Water is a molecule that has a permanent dipole (i.e. it is a polar molecule), with the highly electronegative oxygen nucleus taking the lion's share of the shared electrons' time, leaving the hydrogen nuclei stripped bare down to their protons. The geometry of the water molecule (Figure 2.1.1B) makes one side of the molecule somewhat negative with two pairs of free electrons, and the opposite side positive, because the shared electrons are only rarely near the hydrogen nuclei. This gives water the ability to hydrogen bond, and is the basis for several of water's most important qualities. The ability to form many hydrogen bonds leads to a high specific heat of water, and enables it to act as a generous heat buffer. In order to get enough molecules of water moving faster and increase the temperature of the water, the energy put into the water must first be used to break apart the hydrogen bonds without generating heat. This is unlike most other liquids, which do not link internally with H-bonding. So the water is able to absorb more heat (energy) without a phase change than many other liquids.

Another important and unique characteristic of water is that the solid phase (ice) is less dense than the liquid phase. With most other liquids, as the temperature drops, the molecules have less energy, so they move less, and they stay closer together, increasing the density. Only part of that holds true with water. Again, the ability to form hydrogen bonds is directly related to this: as the temperature is lowered, the molecules move around less, affording them more opportunities to form hydrogen bonds. However, even though they are attractive, the H-bonds also act as spacers separating the water molecules more than if they were allowed to tumble about together in a liquid without forming H-bonds.

> This aspect of water chemistry is actually more important to life in a geologic sense than at the cellular level. At the cellular level, the consequence is that freezing cells causes the water in them to expand and burst, killing them at low temperatures unless the cell has chemicals that act as antifreeze and lower the freezing temperature of the cytoplasm. On the other hand, at the geological level, when a pond or lake freezes in winter, the ice is less dense than water, thus staying on top of the pond, insulating deeper layers, and helping them stay liquid and able to support life (many organisms migrate deeper down in the winter). If water became more dense as it froze, as many other molecules, ice would sink, and eventually the entire pond would be completely solid, killing off most life in it once a year!

From a chemical standpoint, the polar nature of water makes it an excellent solvent for ionic and polar molecules. As you can

see in the Figure, the hydrogen side of water interacts with the negatively charged chloride ion, while the oxygen side of water interacts with the positively charged sodium ion, thus easily dissolving the salt. However, the polarity of water also makes it repel nonpolar molecules or by non-polar regions of molecules. This property, known as hydrophobicity, is crucial to life, since it is the basis for the formation of the biological membranes that define a cell. In general terms, the H-bonding between water molecules is very stable. Non-polar molecules cannot participate in H-bonding, and therefore create areas of instability wherever they are touching aqueous (water-based) solutions. The resolution to this problem is for hydrophobic molecules to aggregate, thus lowering the total surface area in contact with water. In living organisms, many protein and lipid molecules are amphipathic, with some portions hydrophobic, while other parts of the molecule are hydrophilic.

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2.2: Acids and Bases

While it is easiest to think of water as H_2O, it is in fact in an equilibrium between the ionized molecules H^+ (which is simply a proton) and OH^- (the hydroxyl ion). The H^+ itself can be subsequently bound to a water molecule to form a hydronium ion, H_3O^+.

> Water can dissociate from H_2O into the ions H^+ and OH^-, in which the departing hydrogen leaves its electron with the oxygen. However, H^+ is extremely reactive and almost immediately attaches to a nearby water molecule, forming the hydronium ion, H_3O^+.

The release of H^+ and OH^- are not limited to water molecules, and many compounds do so in aqueous solutions. These compounds can be classified as acids (raising the free H^+ concentration) or bases (increasing the free hydroxyl concentration]. The extent to which acids and bases donate or remove protons is measured on the pH scale, which is a logarithmic scale of relative H^+ concentration. Thus the Coca-Cola® that I am drinking, and which counts phosphoric, carbonic, and various other acids among its ingredients, has a pH around 3, which means that it liberates 10^4 times more H+ than water, which has a pH of 7. Inside cells, the pH range is tightly restricted to slightly above neutral (neutral = pH 7), although in eukaryotes, various intracellular organelles (e.g. lysosomes) may have significantly different internal acidity/alkalinity. This is important biologically because changes in acidity or alkalinity can alter hydrogen and ionic bonds, thus potentially changing the shape and activity of enzymes and other biomolecules.

Sometimes, this can be used to an organism's advantage. For example, cells lining the stomach of an animal such as yourself secrete the enzyme pepsin into the stomach to help digest proteins. Pepsin has a pH optimum close to pH 2, which is great because stomach pH is also around 2. However, considering that cells themselves contain a lot of proteins, and we don't want pepsin-containing cells to digest themselves away, what is the solution? Because the pH inside the cell is close to 7.2, far above the pH optimum for pepsin, it is inactive inside the cell, and only works after it has been secreted into an acidic environment.

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2.3: Carbon

The major constituent molecules in all living organisms are based on carbon. Carbon has versatility stemming from its four outer shell electrons, allowing the possibility of four covalent bonds with a variety of partners, including very stable carbon-carbon covalent bonds. Because of this, long carbon chains can form the backbone of more complex molecules and makes possible the great diversity of macromolecules found in the cell. The carbon chains themselves are not very reactive, but they often have reactive chemical groups attached to them.

Common groups are the hydroxyl (—OH), carbonyl (—CO), carboxyl (—COOH), and phosphate ($—PO_4$). Carbon chains may even have other carbon chains attached to them. The smaller ones behave and are named as groups also: methyl ($—CH_3$), ethyl, ($—C_2H_5$), propyl ($—C_3H_7$), and so forth. Figure 2.3.4B (below) depicts several functional groups that can be found in the simple molecule acetic acid (very dilute acetic acid is the primary component of vinegar).

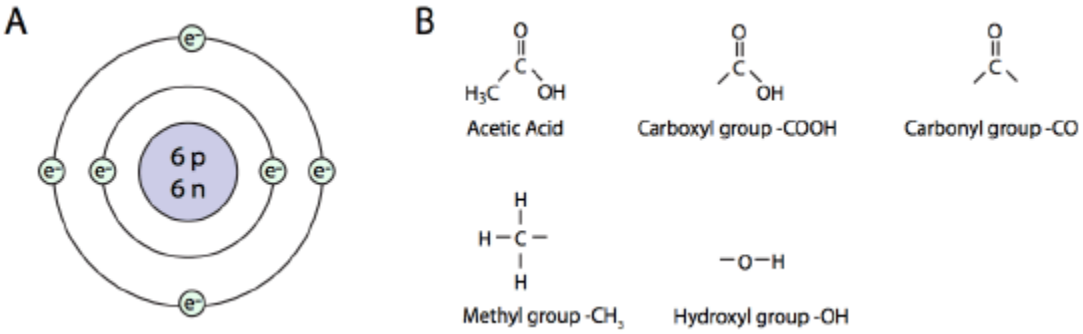


Figure 2.3.4. (A) The carbon atom has four electrons in its outer shell. (B) Functional groups that can be identified from a molecule of acetic acid.

Carbon is also the basis for the four major classes of biological molecules: sugars, nucleotides, amino acids, and fatty acids. The first three are classes of molecules that can be strung together by covalent bonds to make important large biomolecules: simple sugars can form large polysaccharides such as starch, cellulose, or glycogen, nucleotides can form RNA (ribonucleic acids) or DNA (deoxyribonucleic acids), and amino acids can form proteins. Fatty acids, on the other hand, are acid derivatives of long chains of carbons linked to one another, with hydrogens taking up most of the other bonding positions.

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2.4: Sugars

Sugars, and glucose in particular, are important molecules for cells because they are the primary energy source. Sugars have the general chemical formula CH_2O and can be joined together almost infinitely for storage. However, because they are hydrophilic, they allow water molecules to intercalate between them, and cannot pack as efficiently as fats, which are hydrophobic and thus exclude water. On the other hand, the sugars can be mobilized for use more quickly. Therefore, polysaccharides are usually short-term reservoirs of energy for an organism, while fats are used for longer-term storage.

The general chemical formula cannot fully define a particular sugar, because the same set of atoms, e.g. $C_6H_{12}O_6$ can refer to glucose, fructose, mannose, or galactose, and that doesn't even include the stereoisomers. Isomers are rearrangements of the same atoms, such as with glucose and fructose (Figure 2.4.5), while stereoisomers are much more similar: they are mirror-images of one another. Thus glucose can exist as L-glucose or D-glucose, depending on whether it is a "left-handed" or "right-handed isomer. This may seem like an esoteric distinction, but it becomes important in intermolecular interactions, because many are based on recognition of specific shapes, so an l-conformation molecule may not be recognized by an enzyme that recognizes its d-isomer.

Another important aspect of sugar chemistry is whether it is an aldose or a ketose, based on the type of carbonyl group it carries. This is easiest to understand looking at the position of the carbonyl group in the linear structure: put simply, an aldehyde is a terminal carbonyl group, while a ketone is an internal carbonyl group. Sugars in aqueous solution exist in an equilibrium between the linear form and the ring form, which is formed by intramolecular attack by a hydroxyl group on the carbonyl. Technically, the cyclic sugar is a pyranose (6-membered ring) or a furanose (5-membered ring), so that D-glucose cyclizes into D-glucopyranose. However, in most cell biology courses, the cyclic sugar will still be referred to as its non-cyclic alter ego. Note that due to the difference between the $C_6H_{12}O_6$ aldose glucose, and the $C_6H_{12}O_6$ ketose fructose, cyclization generates a pyranose in one case, and a furanose in the latter (Figure 2.4.5), although the number of carbons (and other atoms) are the same. These two molecules are therefore recognized differently by the enzymes of the cell, leading to different metabolic pathways.

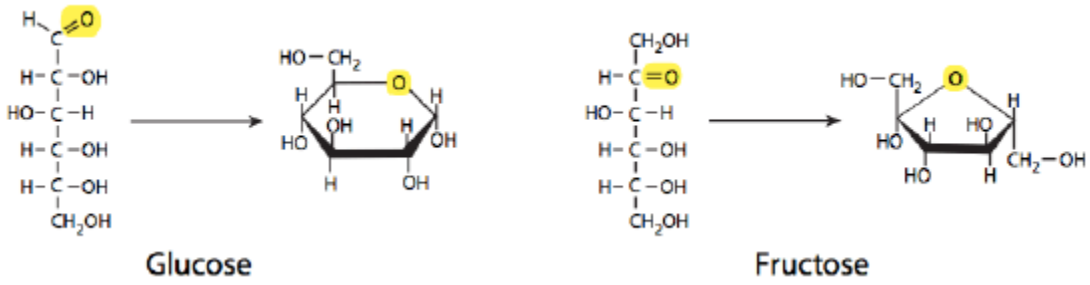


Figure 2.4.5. Glucose is an aldose (terminal carbonyl) that cyclizes into a pyranose, fructose is a ketose (internal carbonyl) that cyclizes into a furanose.

Simple sugars can be joined together by condensation reactions to form glycosidic bonds. These reactions are called condensation reactions because they form water as a byproduct. The glycosidic bond is an —O— linkage between carbons of two sugars. The bond is usually named with the specific linkages: for example in cellulose, glucoses are linked by β(1,4) linkages, which means in a standard ring diagram, the upward-facing β-hydroxyl on the 1-carbon interacts with the —OH on the 4-carbon of a neighboring glucose (Figure 2.4.6B). [Technically, since only two glucoses are shown here, this is a molecule of cellobiose, not cellulose.] In contrast, the maltose shown in the same Figure (Figure 2.4.6A), while also showing two glucoses linked together, is an α(1,4) linkage, with a downward-facing a-hydroxyl on the 1-carbon.

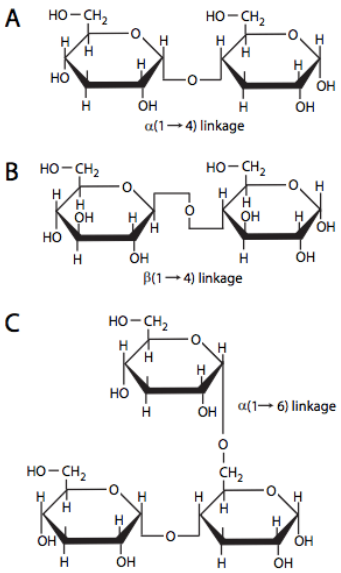


Figure 2.4.6. (A) the α*(1,4) glycosidic bond of maltose, (B) the* β*(1,4) bond of cellobiose, and (C) the* α*(1,6) bond in branching glycogen.*

Large polysaccharides generally have one of two functions: as a very strong structural component of a cell, and as a storage molecule for readily accessible energy. The two major structural polysaccharides made by cells are cellulose and chitin. Cellulose is primarily synthesized by plants, while chitin is mostly synthesized by invertebrates (think crab shells), though it is also made by many fungi and algae. As we just saw, cellulose is an array of parallel lengths of glucose monomers joined together by β(1,4) glycosidic bonds (Figure 2.4.7). These long glucans are stacked closely on one another so that many H-bonds can form along their lengths, which are virtually limitless, determined by the needs of the organism. Interestingly, chitin is also a homopolymer linked by β(1,4) glycosidic bonds, but instead of glucose, the monosaccharide used is N-acetylglucosamine (often abbreviated GlcNAc, see chapter 11). However, the macromolecular structure is very similar to cellulose, and like cellulose, it is very strong.

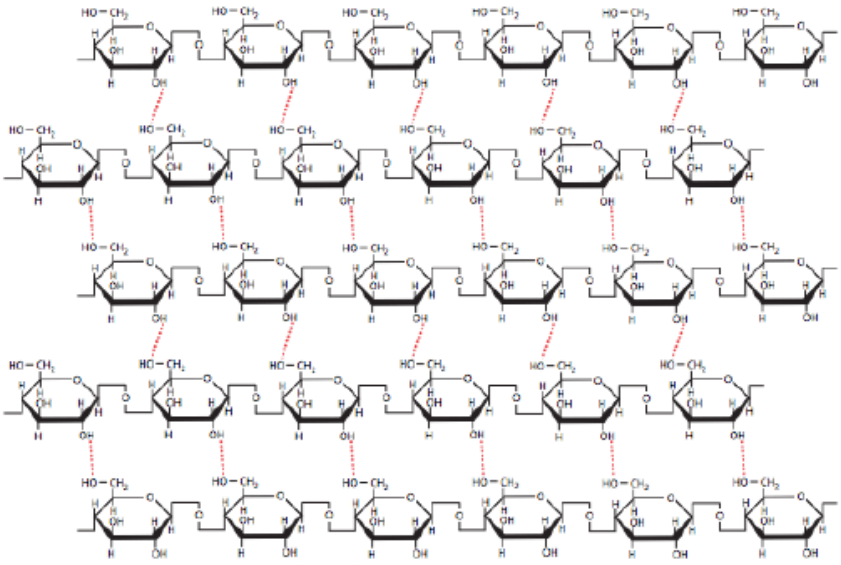


Figure 2.4.7. Cellulose is a very strong material due to the many hydrogen bonds (in red) possible when strands of β(1,4)-linked glucoses are aligned.

As with structural polysaccharides, there are also two primary energy-storage polysaccharides: starch, which is synthesized by plants, and glycogen, which is synthesized by animals. Starch is actually a mixture of two slightly different polysaccharides. One is a-amylose, which is a glucose homopolymer like cellulose, but connected by α(1,4) glycosidic linkages, which makes it completely different structurally. Unlike the linear and highly stackable cellulose polysaccharides, a-amylose takes on a twisting a-helical shape. The other starch polysaccharide is amylopectin, which is like a-amylose with the addition of branches formed

from α(1,6) glycosidic bonds every 24-30 residues (Figure 2.4.6C). The storage polysaccharide for animals, glycogen, is essentially amylopectin with a higher frequency of branching, approximately every 8-14 residues. Whereas the tight packing of the structural polysaccharides renders them waterproof, this is certainly not the case for starch or glycogen, both of which can interact with many water molecules simultaneously, and swell up with the hydration, as any cook who has ever made a pudding (the thickening ingredient is starch from corn) can attest.

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2.5: Nucleotides

Nucleotides, the building blocks of RNA and DNA, are themselves composed of a pentose sugar attached to a nitrogenous base on one side and a phosphate group on another. The sugar is either the 5-carbon sugar ribose or its close cousin, deoxyribose (the "deoxy" refers to a "missing" hydroxyl group on the 2-carbon, which has an H instead). The attached nitrogenous base can be a purine, which is a 6-member ring fused to a 5-member ring, or a pyrimidine, which is a single 6-membered ring. These bases are usually adenine (purine), guanine (purine), thymine (pyrimidine), and cytosine (pyrimidine) for DNA, with a substitution of uracil for thymine in RNA bases. However, there are also some unconventional and modified bases that show up in special situations, such as in tRNAs. In addition to being the monomer components of DNA and RNA, nucleotides have other important functions as well. The best known, adenosine triphosphate, or ATP, is the primary "instant" energy source for the cell by the energy released through hydrolysis of its terminal phosphate group.

DNA or RNA are built from nucleotides through linkages of the sugars, and the polymerization occurs by condensation reactions, but these bonds are not glycosidic bonds like with polysaccharides. Instead, bonds form between the 5' phosphate group of one nucleotide and the 3' hydroxyl group of another. These are phosphodiester bonds, and a quick glance at the structure (Figure 2.5.8) explains the naming: an ester bond is a carbonoxygen linkage, and the phosphodiester bond is a C-O-P-O-C, so there are two esters with a phosphorus linking them. With the purine or pyrimidine base on the 1-carbon, this arrangement places the bases on the opposite side of the sugar from the polymerizing phosphodiester bonds. This forms a sugar-phosphate backbone to the DNA/RNA, which then has the bases projecting out from it.

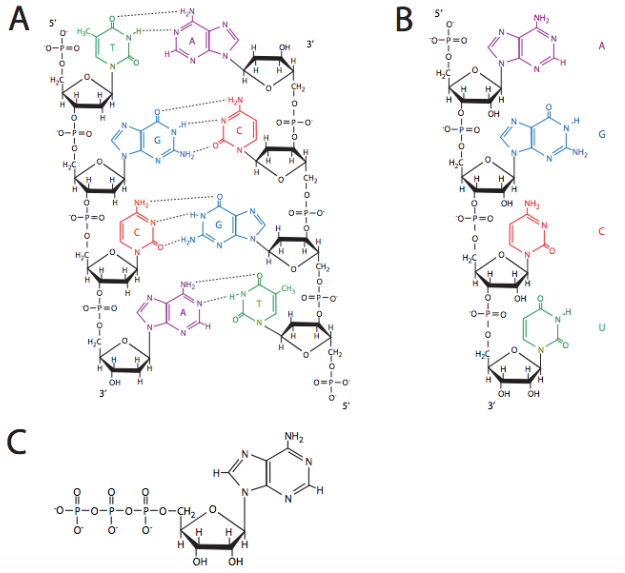


Figure 2.5.8. (A) DNA and (B) RNA differ by the presence of —OH on the 2-carbon of ribose but not deoxyribose and the use of uracil in RNA instead of thymine. Both are constructed from nucleotides like adenosine triphosphate (C).

The bases will then likely interact with the bases of other nucleotides, whether part of another nucleic acid strand or free-floating. Not only do they interact, but they interact with great specificity and consistency: adenines base-pair with thymines (or uracils) through two hydrogen bonds, while guanines interact with cytosine through three H-bonds. Note that while one extra hydrogen bond does not appear to be particularly significant, the attraction between G-C is 50% stronger than between A-T, and over long stretches of DNA, areas high in G-C content are significantly more difficult to unzip (separate strands) than areas high in A-T pairs. This specific base-pairing, known as *Chargaff's rules*, is the basis for life: base-pairing is needed to make DNA double stranded, which gives an organism a built-in backup of genetic information and it is also the basis for transforming that information into proteins that form the bulk of a cell.

Nucleic acids, the long polymers of nucleotides, exist in either single or double stranded forms in vitro. However, in the cell, most RNA is single-stranded, and most DNA is double-stranded. This difference is important to their function: RNA is a temporary information transfer molecule for a particular gene, DNA is the permanent repository of all genetic information needed to make an organism. Therefore, RNA needs to be easily read, meaning that the bases need to be accessible, and not locked to a complementary strand. Its long-term stability is not particularly important because when it is made, usually many copies are made

at the time, and it is only needed while the cell needs to make the protein it encodes. Conversely, the same strand of DNA is read over and over to make the RNA, and since there are only two copies of each chromosome (a chromosome is a single double-stranded DNA molecule) in a cell, the ability to maintain the integrity of the DNA is crucial. Because of base pairing, each strand of DNA contains all the information necessary to make a complete exact copy of its complementary strand.

Of course, the point of the genetic information in DNA is to encode the production of proteins that can then carry out the functions that define cellular life. Some of those functions, such as DNA replication, gene regulation, transcription, and translation, require the proteins to interact with a nucleic acid. Usually, part of the recognition process involves apposition of a positively charged region of the protein to the DNA (or RNA), which is a very negatively charged molecule, as expected from all the phosphates in the sugar-phosphate backbone. RNA, but not DNA (with some exceptions), can also interact with itself by complementary base-pairing. If a stretch of RNA sequence comes into contact with a stretch of RNA with a complementary sequence on the same molecule, then base-pairing can occur. Depending on the number of nucleotides between the complementary areas, secondary structures such as stem-and-loops and hairpins can form.

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2.6: Amino Acids

Most of the major molecules of the cell - whether structural, like cellular equivalents of a building's girders and beams, or mechanical, like enzymes that take apart or put together other molecules, are proteins. Proteins interact with a wide variety of other molecules, though any given interaction is usually quite specific. The specificity is determined in part by electrical attraction between the molecules. So, what determines the charge of different regions of a protein?

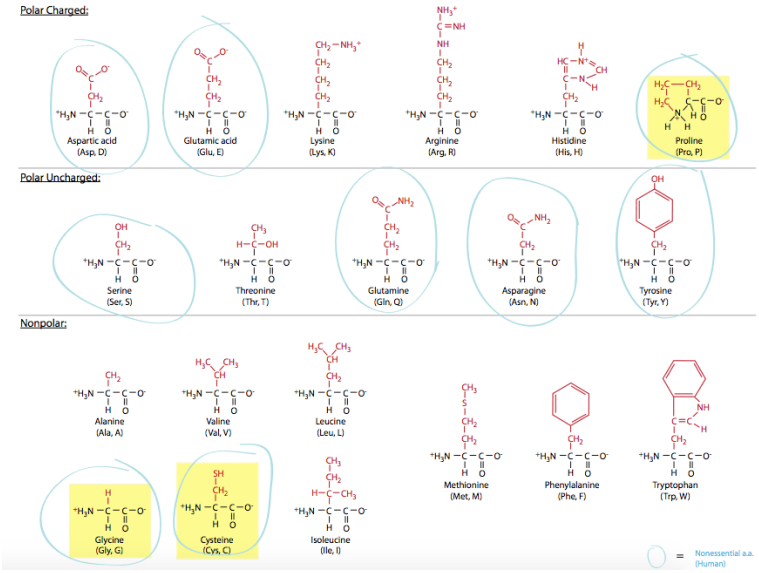


Figure 2.6.9. The Amino Acids. The backbone is shown in black, while the side chains are colored red. Amino acids circled in blue can be synthesized by humans, while the uncircled amino acids must be ingested. Amino acids with a yellow background have unique structural considerations: the extremely small side chain of glycine allows it to fit into tight spaces, the sulfhydryl group of cystein allows the formation of disulfide bonds, and the cyclic structure of proline introduces a forced bend in the polypeptide chain.

Amino acids (Figure 2.6.9), which are joined together to make proteins, may be positively charged (basic), negatively charged (acidic), polar, or nonpolar, based on the characteristics of their side chains. The charge on the amino or carboxyl end of each amino acid does not play a role in the overall character of any particular region of the protein, because they are effectively neutral, having been linked, the amino group of one amino acid to the carboxyl group of another, by a peptide bond. Note the Figure of the amino acid: it is one carbon, called the a carbon, linked to amino and carboxyl groups on opposite sides, and to hydrogen, and a side chain, denoted by R. These side chains, of which there are twenty common ones, can be as simple as a hydrogen atom (glycine), or could be quite complex, involving extended ring structures (histidine, phenylalanine). The variety in their size, shape, and charge all add up to an extremely versatile set of building blocks for some of the most important working molecules of the cell.

chirality

Almost all amino acids (glycine is the exception) are optically active, which means that they are asymmetric in such a way that it is impossible to superimpose the original molecule upon its mirror image. There is a "handed-ness" about them, much as your right hand cannot be superimposed on your left hand if both palms must face the same direction. In fact, in the Figure here, you can also understand why glycine is an exception, since its R-group is a simple hydrogen atom.

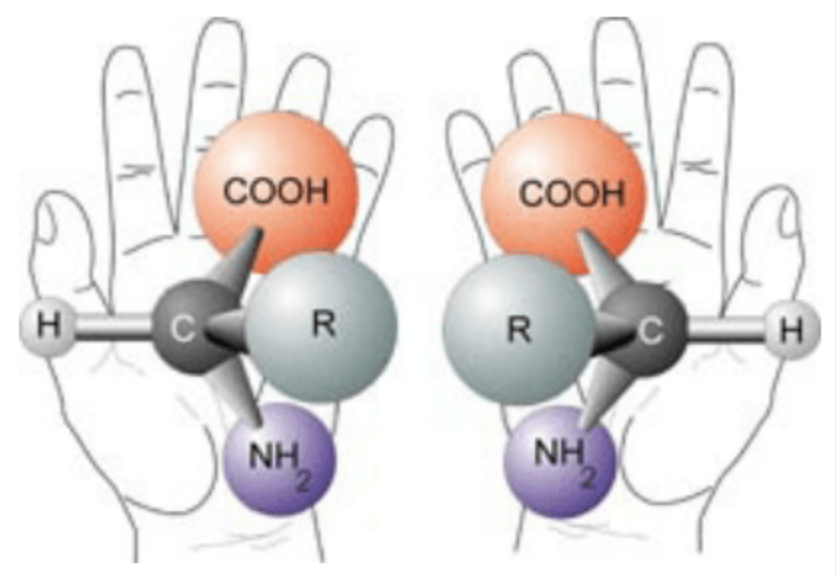


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Chiral pairs, or enantiomers, not only have the same atomic components like all isomers, they also have the same bonds and bond order. The term “optically active” comes from the discovery that polarize light is rotated in different directions by enantiomers. Amino acids are often labeled as either d- (dextrorotatory) or l- (levorotatory) depending on their atomic configuration in relation to the enantiomers of glyceraldehyde. This is a common naming system, but not always logical, in that almost half of the l-amino acids are in fact dextrorotatory (clockwise rotation of light), but their molecular configurations resemble the levorotatory isomer of glyceraldehyde.

Ribosome-created proteins and peptides are all constructed with l-amino acids. However, d-amino acids do exist in nature, and can be incorporated into peptides through non-ribosomal means. An excellent example is found in the cell walls of some bacteria. Because most proteolytic enzymes only act on proteins with lamino acids, the incorporation of d-amino acids into the cell wall can protect the bacteria from harm. These D-amino acids are incorporated by transpeptidase. Transpeptidase is also the target of the antibiotic, penicillin, which is an irreversible inhibitor of that enzyme.

In the cell, a peptide bond is formed between two amino acids with enzymatic help from the ribosome. Like the previous two polymerizing reactions, formation of peptide bonds is a condensation reaction in which the carbon of the carboxyl group and the nitrogen from the amino group of their respective amino acids are bonded together (Figure $2.6.10$). This is a very stable bond due to resonance of the amide group. In the cell, peptide bonds are mostly nonreactive, except when attacked by proteolytic enzymes.

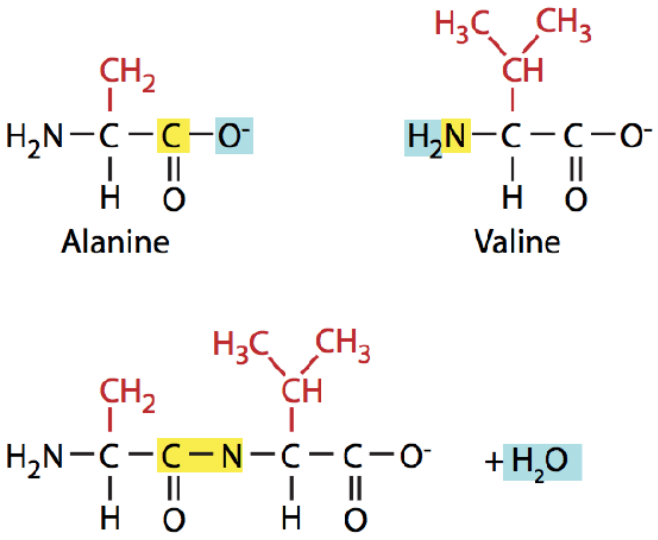


Figure 2.6.10. Peptide Bond Formation. A condensation reaction between the carboxyl group of alanine and and the amino group of valine generates a peptide bond linking the two amino acids, with a molecule of water as a byproduct.

A peptide is an inexact term used for relatively few (usually <30) amino acids joined together. Each amino acid in a polypeptide or protein may also be referred to as a "residue" which can sometimes be confusing because the same term is also applied to monomers of nucleic acids and of polysaccharides. Larger polymers are known as polypeptides or as proteins, although polypeptide has more of a structural connotation and may be used to indicate an unfinished or not-yet-functional state, whereas protein generally implies some physiological function. On of the key characteristics of proteins is the ability to form secondary, tertiary, and for proteins, quaternary structure by means of specific folding patterns. If you think of a long piece of thread, yarn, or rope, you can probably imagine an infinite number of different ways to arrange it, from spirals to loops to random tangles. This is essentially what can happen with a protein with the constraints put upon it by the size and charge of the amino acids that compose it.

The *primary structure* of a protein is simply the sequence of amino acids that compose the protein. These amino acids are joined by peptide bonds from the carboxyl terminal of one amino acid to the amino terminal of the next. Secondary structure refers to the localized, simple, shapes that can be formed, such as alpha-helices, or beta-sheets. These come about primarily through hydrogen bonding to nearby (relative to the primary structure) residues.

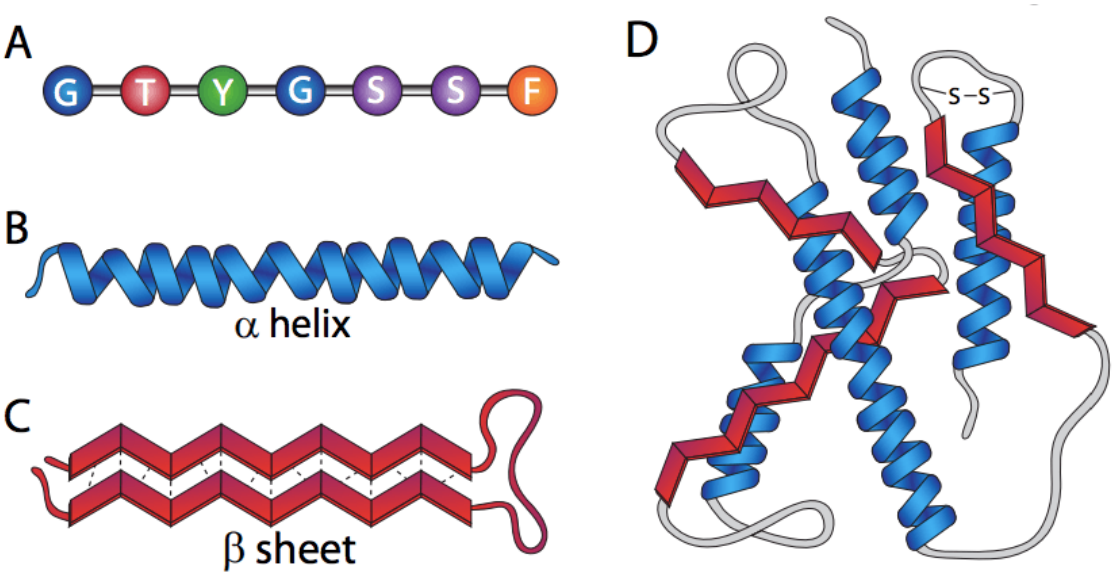


Figure 2.6.11. (A) Primary structure, (B) Secondary structure: an a-helical region, (C) Secondary structure: a b-pleated sheet region, (D) Tertiary structure.

Tertiary structure is 3-dimensional structure that is built upon arrangements of second ary structures, often through disulfide bonds and hydrophobic interactions in addition to hydrogen bonding. In the context of structural stability, cysteine plays a special role. Beyond the primary structure, most protein folding is held in place by hydrogen bonds. Although strong enough in most situations, they can be disrupted without extraordinary energy. Disulfide bonds (—S—S—) are covalent bonds that form between the sulfhydryl groups of two cysteines that effectively locks the local protein structure in place, making the protein extremely stable.

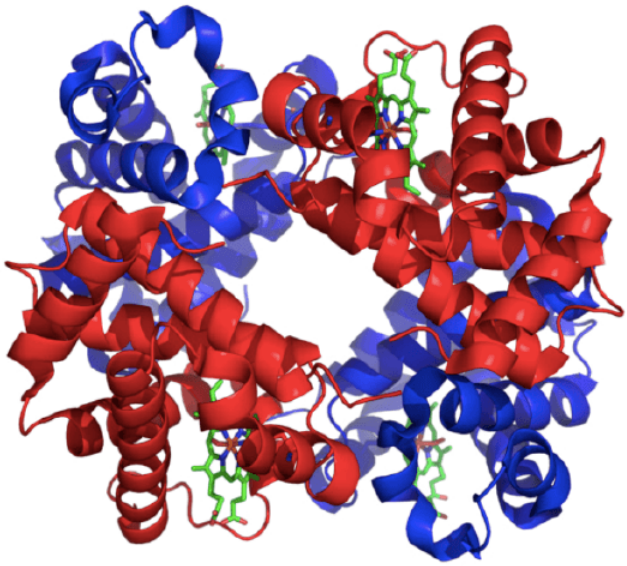


Figure 2.6.12. Quaternary structure is illustrated here by hemoglobin, which is composed of four independent polypeptide subunits that come together in a specific conformation to make a functional hemoglobin protein.

Finally, quaternary structure is the arrangement of different individual polypeptides (subunits) into a functional protein. Obviously, only multi-subunit proteins have a quaternary structure.

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2.7: Fatty Acids

Unlike monosaccharides, nucleotides, and amino acids, fatty acids are not monomers that are linked together to form much larger molecules. Although fatty acids can be linked together, for example, into triacylglycerols or phospholipids, they are not linked directly to one another, and generally no more than three in a given molecule. The fatty acids themselves are long chains of carbon atoms topped off with a carboxyl group. The length of the chain can vary, although most are between 14 and 20 carbons, and in higher order plants and animals, fatty acids with 16 and 18 carbons are the major species.

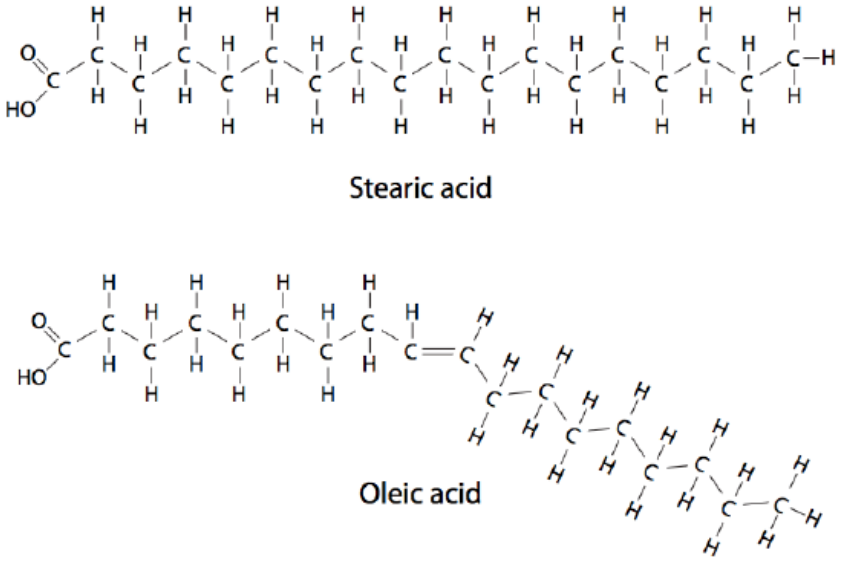


Figure 2.7.13. Fatty acids. (Top) Stearic acid is a fully saturated fatty acid with no carbon-carbon double bonds. (Bottom) Oleic acid is an unsaturated fatty acid.

Due to the mechanism of synthesis, most fatty acids have an even number of carbons, although odd-numbered carbon chains can also be generated. More variety can be generated by double-bonds between the carbons. Fatty acid chains with no double bonds are saturated, because each carbon is saturated with as many bonded hydrogen atoms as possible. Fatty acid chains with double bonds are unsaturated (Figure 2.7.13). Those with more than one double bond are called polyunsaturated. The fatty acids in eukaryotic cells are nearly evenly divided between saturated and unsaturated types, and many of the latter may be polyunsaturated. In prokaryotes, polyunsaturation is rare, but other modifications such as branching and cyclization are more common than in eukaryotes. A table of common fatty acids is shown below.

Myristic Acid	14:0 (14 carbons, no double bonds
Palmitic Acid	16:0
Stearic Acid	18:0
Arachidic Acid	20:0
Palmitoleic Acid	16:1
Oleic Acid	18:1
Linoleic Acid	18:2
Arachidonic Acid	2:4

There are significant physical differences between the saturated and unsaturated fatty acids due simply to the geometry of the double-bonded carbons. A saturated fatty acid is very flexible with free rotation around all of its C-C bonds. The usual linear diagrams and formulas depicting saturated fatty acids also serve to explain the ability of saturated fatty acids to pack tightly together, with very little intervening space. Unsaturated fatty acids, on the other hand are unable to pack as tightly because of the rotational constraint impoarted by the double bond. The carbons cannot rotate around the double bond, so there is now a “kink”

in the chain. Generally, double-bonded carbons in fatty acids are in the cis- configuration, introducing a 30-degree bend in the structure.

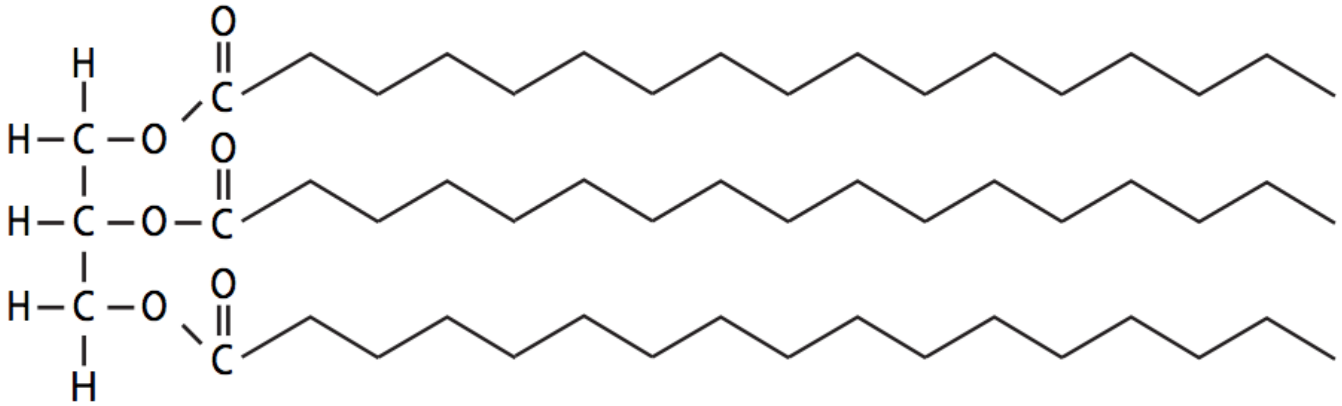


Figure 2.7.14. Triglycerides. These lipids are formed by conjugation of a glycerol to three fatty acyl chains through ester bonds from each glycerol oxygen.

Fatty acids inside cells are usually parts of larger molecules, rather than free acids. Some of the most common lipids derived from fatty acids are triacylglycerols, phosphoglycerides, and sphingolipids. Triacylglycerols, as the name implies, is three fatty acid (acyl) chains connected to a glycerol molecule by ester bonds (Figure 2.7.14). Triacylglycerols, also known as triglycerides, may have fatty acids of the same (simple triacylglycerols) or varying types (mixed triacylglycerols). Mixtures of these are the primary long-term energy storage molecules for most organisms. Although they may be referred to colloquially as fats or oils, the only real difference is the degree of saturation of their constituent fatty acids. Mixtures with higher percentages of saturated fatty acids have a higher melting point and if they are solid at room temperature, they are referred to as fats. Triacylglycerol mixtures remaining liquid at room temperature are oils.

In human medicine, a common test for heart disease risk factors is measurement of triglyceride levels in the blood. Although various cell types can make and use triglycerides, most of the triglycerides in people are concentrated in the adipose tissue, which is made up of adipocytes, or fat cells, though liver is also a significant fat store. These cells have specialized to carry fat globules that take up most of the volume of the cell. When triglyceride levels in the blood are high, it means that fat is being produced or ingested faster than it can be taken up by the adipocytes.

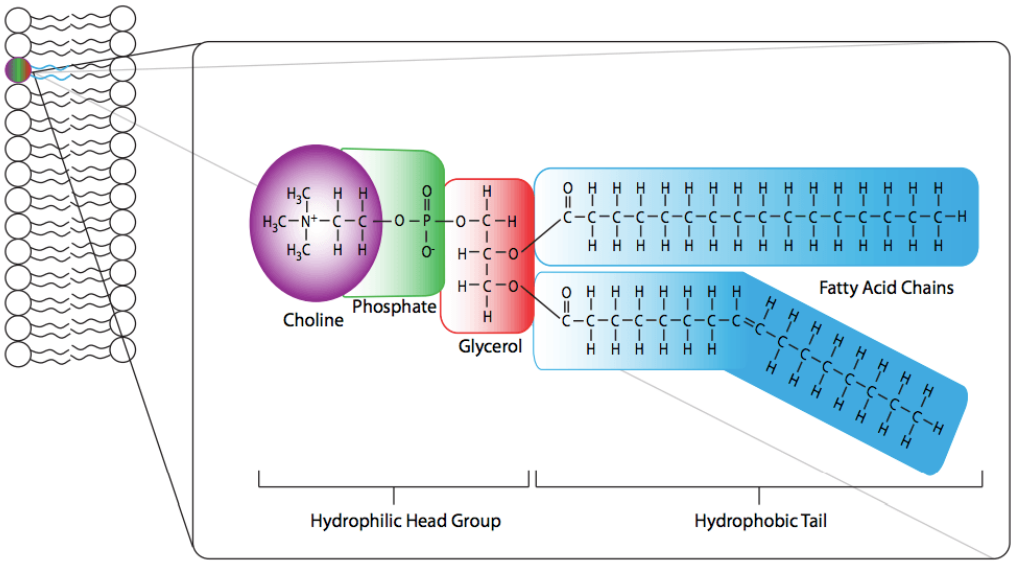


Figure 2.7.15. A phospholipid: the glycerol backbone (red) connects to two fatty acids and to a phosphate and polar head group.

Phospholipids (also called phosphoglycerides or glycerophospholipids), are also based on attachment of fatty acids to glycerol. However, instead of three fatty acyl tails, there are only two, and in the third position is a phosphate group (Figure 2.7.15). The

phosphate group also attaches to a “head group” . The identity of the head group names the molecule, along with the fatty acyl tails. In the example Figure, 1-stearoyl refers to the stearic acid on the 1-carbon of the glycerol backbone; 2-palmitoyl refers to the palmitic acid on the 2-carbon of the glycerol, and phosphatidylethanolamine refers to the phosphate group and its attached ethanolamine, that are linked to the glycerol 3-carbon. Because of the negatively-charge phosphate group, and a head group that is often polar or charged, phospholipids are amphipathic - carrying a strong hydrophobic character in the two fatty acyl tails, and a strong hydrophilic character in the head group. This amphipathicity is crucial in the role of phospholipids as the primary component of cellular membranes.

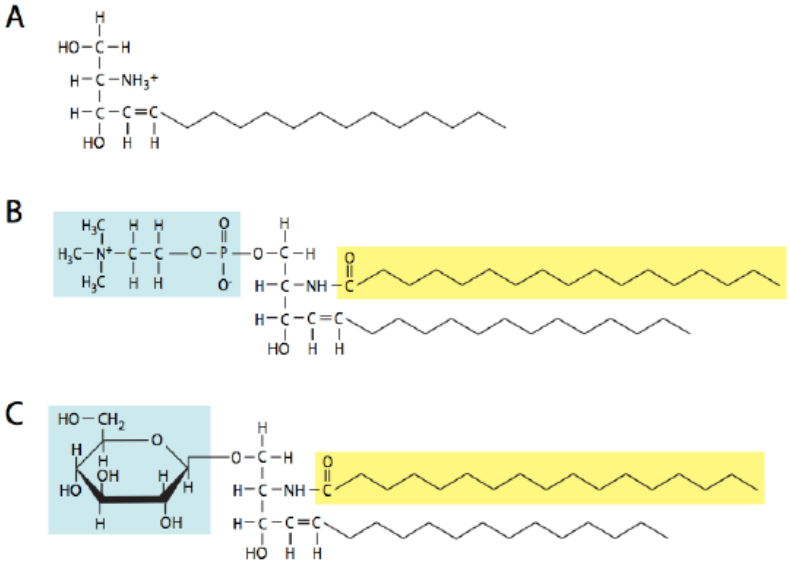


Figure 2.7.16. Sphingolipids are based on the amino alcohol, sphingosine (A). Ceramides have a fatty acid tail attached, and a ceramide with a phosphocholine head group is a sphingomyelin (B). If the head group is a sugar, then the molecule is a cerebroside. (C)

Sphingolipids (Figure 2.7.16) are also important constituents of membranes, and are based not upon a glycerol backbone, but on the amino alcohol, sphingosine (or dihydrosphingosine). There are four major types of sphingolipids: ceramides, sphingomyelins, cerebrosides, and gangliosides. Ceramides are sphingosine molecules with a fatty acid tail attached to the amino group. Sphingomyelins are ceramides in which a phosphocholine or phosphoethanolamine are attached to the 1-carbon. Cerebrosides and gangliosides are glycolipids - they have a sugar or sugars, respectively, attached to the 1-carbon of a ceramide. The oligosaccharides attached to gangliosides all contain at least one sialic acid residue. In additional to being a structural component of the cell membrane, gangliosides are particular important in cell to cell recognition.

Lipids are vaguely defined as biological compounds that are insoluble in water but are soluble in organic solvents such as methanol or chloroform. This includes the fatty acid derivatives listed above, and it includes the final topic for this chapter, cholesterol. Cholesterol (Figure 2.7.17) is the major biological derivative of cyclopentanoperhydrophenanthrene, a saturated hydrocarbon consisting of four fused ring formations. It is an important component of plasma membranes in animal cells, and is also the metabolic precursor to steroid hormones, such as cortisol or b-estradiol. Plant cells have little if any cholesterol, but other sterols like stigmasterol are present. Similarly, fungi have their particular sterols. However, prokaryotes do not, for the most part, contain any sterol molecules.

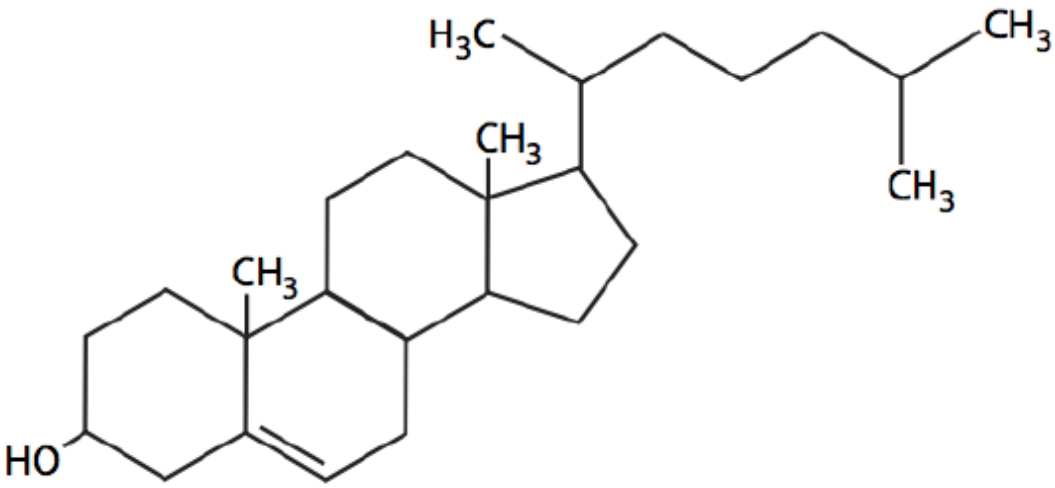


Figure 2.7.17. Cholesterol is an important lipid both as a membrane component and as a steroid precursor.

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3: BIOENERGETICS - THERMODYNAMICS AND ENZYMES

3.1: The Laws of Thermodynamics
3.2: Enzymes
3.3: Enzyme Kinetics
3.4: Regulation of Enzyme Activity

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3.1: The Laws of Thermodynamics

Two fundamental concepts govern energy as it relates to living organisms: the First Law of Thermodynamics states that total energy in a closed system is neither lost nor gained — it is only transformed. The Second Law of Thermodynamics states that entropy constantly increases in a closed system.

More specifically, the First Law states that energy can neither be created nor destroyed: it can only change form. Therefore, through any and all processes, the total energy of the universe or any other closed system is constant. In a simple thermodynamic system, this means that the energy is transformed either by the transfer of heat energy (i.e. heating and cooling of a substance) or by the production of mechanical work (i.e. movement). In biological and chemical terms, this idea can be extended to other forms of energy such as the chemical energy stored in the bonds between atoms of a molecule, or the light energy that can be absorbed by plant leaves.

> Work, in this case, need not imply a complicated mechanism. In fact, there is work accomplished by each molecule in the simple expansion of a heated mass of gaseous molecules (as visualized by expansion of a heated balloon, for example). This is expressed mathematically as the Fundamental Thermodynamic Relation:
>
> in which E is internal energy of the system, T is temperature, S is entropy, p is pressure, and V is volume.
>
> Unlike the First Law which applies even to particles within a system, the Second Law is a statistical law — it applies generally to macroscopic systems. However, it does not preclude smallscale variations in the direction of entropy over time. In fact, the Fluctuation theorem (proposed in 1993 by Evans et al, and demonstrated by Wang et al in 2002) states that as the length of time or the system size increases, the probability of a negative change in entropy (i.e. going against the Second Law) decreases exponentially. So on very small time scales, there is a real probability that fluctuations of entropy against the Second Law can exist.

The Second Law dictates that entropy always seeks to increase over time. Entropy is simply a fancy word for chaos or disorder. The theoretical final or equilibrium state is one in which entropy is maximized, and there is no order to anything in the universe or closed system. Spontaneous processes, those that occur without external influence, are always processes that convert order to disorder. However, this does not preclude the imposition of order upon a system. Examining the standard mathematical form of the Second Law:

$\Delta S_{system} + \Delta S_{surroundings} = \Delta S_{universe}$,

where $\Delta S_{universe} > 0$

shows that entropy can decrease within a system as long as there is an increase of equal or greater magnitude in the entropy of the surroundings of the system.

The phrase “in a closed system” is a key component of these laws, and it is with the idea encapsulated in that phrase that life can be possible. Let’s think about a typical cell: in its lifetime, it builds countless complex molecules - huge proteins and nucleic acids formed from a mixture of small amino acids or nucleotides, respectively. On its surface, this example might seem to be a counterexample to the second law - clearly going from a mixture of various small molecules to a larger molecule with bonded and ordered components would seem to be a decrease in entropy (or an increase in order). How is this possible with respect to the second law? It is, because the second law applies only to closed systems. That is, a system that neither gains nor loses matter or energy.

> The “universe” is a closed system by definition because there is nothing outside of it.

A living cell is not a closed system: it has inputs and outputs. However, the second law is still useful if we recognize that the only way that it can be bypassed is through the input of energy. If a cell cannot take in food (input of matter and energy into the

system) it dies, because the second law requires that everything eventually breaks down into more random/chaotic collections of smaller components. The order required to sustain life (think about all the different complex molecules that were mentioned in the previous chapter) is phenomenal. The same thing applies on the organismal level (Figure 3.1.1) - without an input of energy (in the form of food molecules for animals or in the form of light for plants), the organism will die and subsequently decompose.

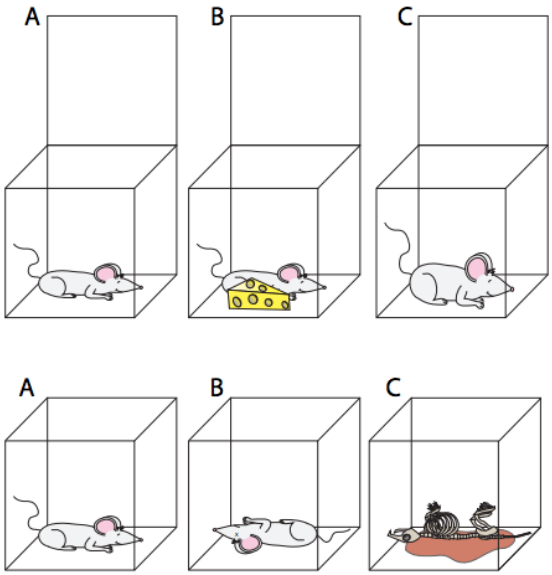


Figure 3.1.1. In the top panel, depicting an open system in which there are inputs for matter and energy (in the form of food), the box is open and air can be exchanged, food dropped in, etc, thus allowing the mouse to grow. However, in the bottom panel, depicting a closed system, the mouse does not have ready access any more oxygen than is in the box, nor does it have access to food. Without these inputs, the second law takes effect, and the mouse dies and decomposes into many smaller molecules.

Creating molecules from atoms costs energy because it takes a disordered collection of atoms and forces them, through chemical bonds, into ordered, non-random positions. There is likewise an energy cost to formation of macromolecules from smaller molecules. By imposing order in the system, there must be an associated input of energy. This happens at every level of the system: atoms to molecules, small molecules to macromolecules, groups of molecules to organelles, etc.

> As the polymerizing reaction reduces entropy, it requires energy generated (usually) by the breakdown of ATP into AMP and PPi, which is a reaction that increases entropy.

Where does that energy go? It ends up in the bonds that are holding the molecules or macromolecules in their ordered state. When such a bond is broken, and a molecule is turned back into a collection of atoms, energy is released. The energy in a chemical bond is thus potential energy - it is stored energy that, when released, has the ability to do work. This term, if you recall your high school physics, is usually learned along with kinetic energy, which is energy that is being used in the process of actually doing work (i.e. moving an object from one place to another). The classic example is the rock on the top of a hill: it has potential energy because it is elevated and could potentially come down. As it tumbles down, it has kinetic energy as it moves. Similarly in a cell, the potential energy in a chemical bond can be released and then used for processes such as putting smaller molecules together into larger molecules, or causing a molecular motor to spin or bend - actions that could lead to pumping of protons or the contraction of muscle cells, respectively.

Coming back to the second law, it essentially mandates that breaking down molecules releases energy and that making new molecules (going against the natural tendency towards disorder) requires energy. Every molecule has an intrinsic energy, and therefore whenever a molecule is involved in a chemical reaction, there will be a change in the energy of the resulting molecule(s). Some of this change in the energy of the system will be usable to do work, and that energy is referred to as the free energy of the reaction. The remainder is given off as heat.

The Gibbs equation describes this relationship as

$$\Delta G = \Delta H - T\Delta S \tag{3.1.1}$$

where ΔG is the change in free energy, ΔH is the change in enthalpy (roughly equivalent to heat), T is the temperature at which the reaction takes place, and ΔS is the change in entropy. As a matter of convention, release of free energy is a negative number, while a requirement for input of energy is denoted with a positive number. Generally, a chemical reaction in which ΔG < 0 is a spontaneous reaction (also called an exergonic reaction), while a chemical reaction in which ΔG > 0 is not spontaneous (or endergonic). When ΔG = 0, the system is in equilibrium. ΔG can also be expressed with respect to the concentration of products and reactants:

$$\Delta G = \Delta G^o + RT \ln \left(\frac{[P_1][P_2][P_3]...}{[R_1][R_2][R_3]...} \right) \tag{3.1.2}$$

Terms in square brackets denote concentrations, ΔG° is the standard free energy for the reaction (as carried out with 1M concentration of each reactant, at 298K and at 1 atm pressure), R is the gas constant (1.985 cal $K^{-1}mol^{-1}$), and T is the temperature in Kelvin. In a simpler system in which there are just two reactants and two products:

$$a\,\mathrm{A} + b\,\mathrm{B} \rightleftharpoons c\,\mathrm{C} + d\,\mathrm{D} \tag{3.1.3}$$

the equation for free energy change becomes

$$\Delta G = \Delta G° + RT\ln([C]^c[D]^d/[A]^a[B]^b)$$

This is important to us as cell biologists because although cells are not very well suited to regulating chemical reactions by varying the temperature or the pressure of the reaction conditions, they can relatively easily alter the concentrations of substrates and products. In fact, by doing so, it is even possible to drive a non-spontaneous reaction (ΔG > 0) forward spontaneously (ΔG < 0) either by increasing substrate concentration (possibly by transporting them into the cell) or by decreasing product concentration (either secreting them from the cell or by using them up as substrates for a different chemical reaction).

Changes in substrate or product concentration to drive a non-spontaneous reaction are an example of the more general idea of coupling reactions to drive an energetically unfavorable reactions forward. Endergonic reactions can be coupled to exergonic reactions as a series of reactions that ultimately is able to proceed forward. The only requirement is that the overall free energy change must be negative (ΔG < 0). So, assuming standard conditions (ΔG = ΔG°'), if we have a reaction with a free energy change of +5 kcal/mol, it is non-spontaneous. However, if we couple this reaction, to ATP hydrolysis for example, then both reactions will proceed because the standard free energy change of ATP hydrolysis to ADP and phosphate is an exergonic -7.3 kcal/mol. The sum of the two ΔG values is -2.3 kcal/mol, which means the coupled series of reactions is spontaneous.

In fact, ATP is the most common energy "currency" in cells precisely because the -7.3 kcal/mol free energy change from its hydrolysis is enough to be useful to drive many otherwise endergonic reactions by coupling, but it is less costly (energetically) to make than other compounds that could potentially release even more energy (e.g. phosphoenolpyruvate, PEP). Also, much of the -14.8 kcal/mol (ΔG°') from PEP hydrolysis would be wasted because relatively few endergonic reactions are so unfavorable as to need that much free energy.

> Why is ATP different from other small phosphorylated compounds? How is it that the γ-phosphoanhydride bond (the most distal) of ATP can yield so much energy when hydrolysis of glycerol-3-phosphate produces under a third of the free energy? The most obvious is electrostatic repulsion. Though they are held together by the covalent bonds, there are many negative charges in a small space (each phosphate carries approximately 4 negative charges). Removing one of the phosphates significantly reduces the electrostatic repulsion. Keeping in mind that DG is calculated from the equilibrium of both reactants and products, we also see that the products of ATP hydrolysis, ATP and phosphate, are very stable due to resonance (both ADP and Pi have greater resonance stabilization) and stabilization by hydration. The greater stability of the products means a greater free energy change.

Even when a reaction is energetically favorable (ΔG < 0), it may not occur without a little "push", chemically speaking. The "push" is something called *activation energy*, and it overcomes thermodynamic stability. Consider glucose, for instance. This simple sugar is the primary source of energy for all cells and the energy inherent within its bonds is released as it breaks down into carbon dioxide and water. Since this is large molecule being broken down into smaller ones, entropy is increased, thus energy is released from reaction, and it is technically a spontaneous reaction. However, if we consider a some glucose in a dish on the lab bench, it clearly is not going to spontaneously break down unless we add heat. Once we add sufficient heat energy, we can remove the energy source, but the sugar will continue to break down by oxidation (burn) to CO_2 and H_2O.

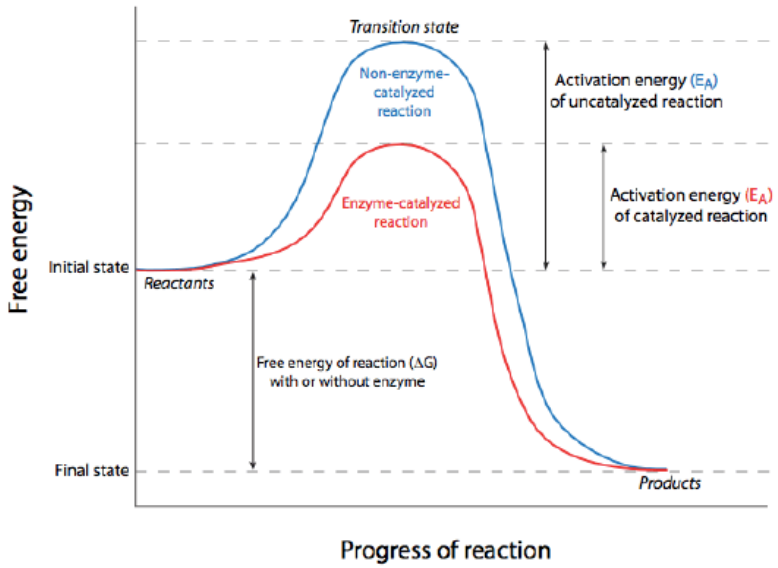


Figure 3.1.2. Catalysts lower the activation energy barrier to chemical reactions without altering the free energy change for that reaction.

Put another way, the reactant(s) must be brought to an unstable energy state, known as the transition state (as shown at the peak of the graphs in Figure 3.1.2). This energy requirement barrier to the occurrence of a spontaneous thermodynamically favored reaction is called the activation energy. In cells, the activation energy requirement means that most chemical reactions would occur too slowly/infrequently to allow for all the processes that keep cells alive because the required energy would probably come from the chance that two reactants slam into one another with sufficient energy, usually meaning they must be heated up. Again, cells are not generally able to turn on some microscopic Bunsen burner to generate the activation energy needed, there must be another way. In fact, cells overcome the activation energy problem by using catalysts for their chemical reactions. Broadly defined, a catalyst is a chemical substance that increases the rate of a reaction, may transiently interact with the reactants, but is not permanently altered by them. The catalyst can be re-used because it is the same before the reaction starts, and after the reaction completes. From a thermodynamic standpoint, it lowers the activation energy of the reaction, but it does not change the ΔG. Thus it cannot make a non-spontaneous reaction proceed; it can only make an already spontaneous reaction occur more quickly or more often.

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3.2: Enzymes

Biological catalysts are called enzymes, and the overwhelming majority of enzymes are proteins. The exceptions are a class of RNA molecules known as ribozymes, of which most act upon themselves (i.e. part of the RNA strand is a substrate for the ribozyme part of the strand). In this book (and most textbooks in this field), unless otherwise specified, the term enzyme refers to one made of protein. Enzymes confer extraordinary specificity to a chemical reaction: a reaction that might occur between a variety of potential substrates in an uncatalyzed situation may only be allowed between two specific substrates when catalyzed by an enzyme. Enzymes allow cells to run chemical reactions at rates from a million to even a trillion times faster than the same reactions would run under similar conditions without enzymes. In some cases, the enzymes al - low reactions to proceed that would normally (i.e. sans enzyme) require more extreme temperature, pressure, or acidity/alkalinity. Finally, and perhaps most importantly for life, enzymes can be regulated. This is crucial for the cell, since it must be able to react to different situations, such as availability of energy, accumulation of toxic byproducts, the need to reproduce, etc. Not only can enzymes be modified either covalently or noncovalently to increase or decrease their activity, the cell can also regulate production of the enzymes, providing another level of control over particular cellular biochemical reactions.

enzyme classification

Enzymes have been cataloged and classified since the 1950's, during which time there was an explosion of enzyme discoveries and a need for a unified nomenclature and catalog. An International Commission on Enzymes was established and thus started the Enzyme List. All enzymes now have both recommended names for common usage, often reflecting historical naming, and a systematic name, which is highly specific. They also have a classification number based on their activity. The major classes of enzymes are

1. *Oxidoreductases* that carry out oxidation-reduction reactions,
2. *Transferases* that transfer functional groups,
3. *Hydrolases* that carry out hydrolysis reactions,
4. *Lyases* that eliminate groups to form double bonds,
5. *Isomerases* that rearrange the bonds in a molecule but do not add or remove atoms, and
6. *Ligases* that form bonds in reactions coupled to ATP hydrolysis.

As an example, DNA ligase (recommended name) catalyzes the formation of a phosphodiester bond between the 3' end of one DNA fragment and the 5' end of another. Its rather long and tedious systematic name is "poly(deoxyribonucleotide):poly(deoxyr ibonucleotide) ligase (AMP-forming)" and its classification number is 6.5.1.1. As a ligase, it is class 6; because it forms phosphoric ester bonds, it is subclass 5; the sub-subclass of 1 in this case is meaningless because it is the only sub-subclass of phosphoricester bond-forming ligases, but the final number designates the DNA ligase separately from other 6.5.1 enzymes such as RNA ligase, which is 6.5.1.3.

Enzymes are the most diverse type of protein in a cell. They vary not only in size, but also in the number of independently manufactured subunits that must come together to form an active enzyme, or holoenzyme. Part of the reason for requiring so many different enzymes is that they are usually very specific for their substrate molecules, and that specificity is based upon a combination of shape and charge. The interactions between substrate and enzyme are often likened to a lock and key or pieces of a jigsaw puzzle. If the substrate fits the shape of the enzyme's active site (the part of the enzyme that carries out the actual catalytic reaction), and the charges interact (e.g. positively charged amino acids on the enzyme lining up with negative charges on the substrate), then there may be further stabilization of the interaction by Van der Waals and hydrogen bond interactions. In fact, formation of a stable Enzyme-Substrate (ES) intermediate is energetically analogous to the transition state (Figure 3.2.2) of reactions.

The specificity of enzymes is such that stereoisomers may not be recognized by some enzymes: for example, a protease (enzymes that chop up proteins into smaller pieces by hydrolyzing the peptide bonds between specific amino acids) such as trypsin can be stymied by the presence of a D-amino acid in place of the usual L-amino acid in a protein, even though it is a mirror image of the very same amino acid. This specificity means that enzymes are highly selective with respect to the reactions they catalyze, which means that specific reactions can be greatly enhanced without causing a general increase in many related chemical reactions. Another implication of the high specificity is that enzymes can (and often do) have high affinity for their substrates without the problem of binding non-substrate molecules (other than specific inhibitors - see below).

If most biochemical reactions would proceed extremely slowly, if at all, without catalysis, enzymes are needed to lower the activation energy needed for chemical reactions to support life. Exactly how does an enzyme lower the activation energy of a reaction? What exactly does “activation energy” mean in the context of a cell? To understand this, there are two principles to keep in mind: first, when we talk about chemical reactions, generally, we are concerned with populations of substrate, product, and enzyme molecules, not individuals; and second, the reactions are generally taking place between molecules dissolved in the aqueous cytoplasm of the cell.

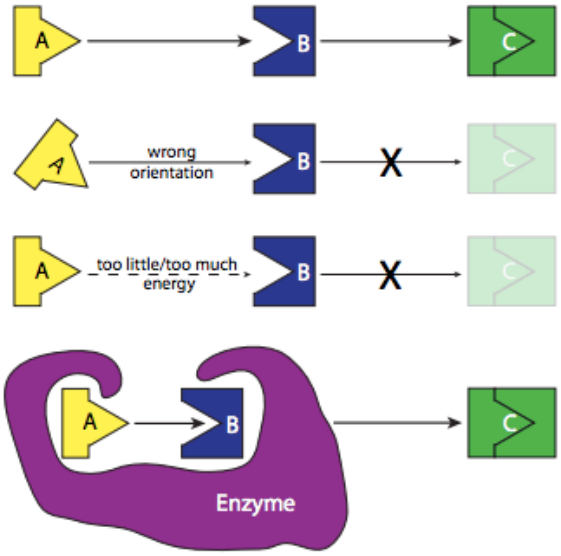


Figure 3.2.3. Enzymes can lower activation energy by binding substrates individually and bringing them together under optimal conditions to react.

Consider a reaction in which substrates A and B interact to form product C (Figure 3.2.3). If this reaction is not catalyzed, it depends on the happenstance that a molecule of A runs into a molecule of B in just the right orientation, and with the right amount of energy, to react and form the new molecule. We can conceptualize “activation energy” as the difficulty in getting A and B together perfectly so the reaction can proceed. How might an enzyme lower this activation energy? By making it easier for A and B to find each other with the right orientation and energy. So it could have binding sites for molecule A and molecule B, and once it has bound these two molecules, it changes its conformation, bringing A and B together under exactly the right conditions to react and form C. Once the reaction is complete, the product floats off because the enzyme has no affinity for it, and the enzyme returns to its initial shape, ready to bind more substrates.

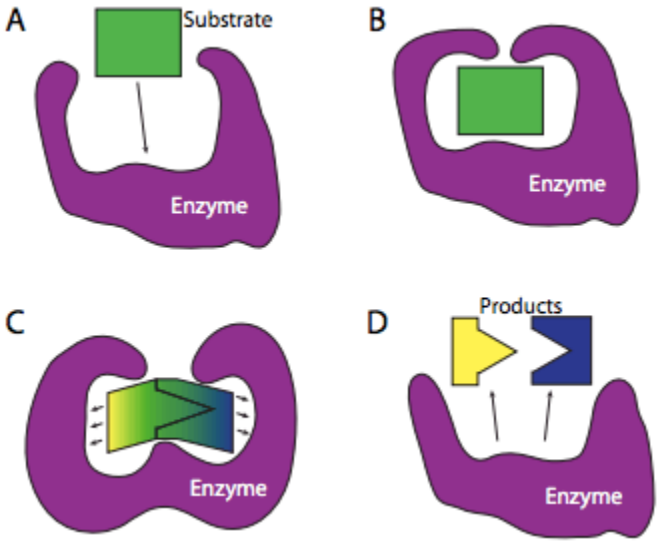


Figure 3.2.4. The enzyme (A) binds a specific substrate, leading to a conformational change (B) that stresses the molecule (C) until it breaks down into two smaller component molecules (D), which are released.

Enzymes may also facilitate a chemical reaction by acting as a temporary holding site for an active group being transferred from one substrate to another. Alternatively, temporary formation of hydrogen bonds or even covalent bonds between the enzyme and substrate can alter chemical characteristics of the substrate to make it react more easily. An example of enzyme mechanisms on the molecular level is shown in Chapter 5: Figure 3.2.1.

Another example may be found with enzymes that break apart a molecule (Figure 3.2.4). In order for a molecule to break apart, it may need to collide with another molecule with sufficient energy to break one or more of its covalent bonds. An enzyme that catalyzes the breakdown reaction might bind to the molecule, and in binding it, undergoes a conformational shift that bends or twists the molecule in such a way that the bonds in the substrate molecule are weakened or broken. These two examples oversimplify the chemistry of enzyme activity into a mechanical idea, but the general relationship in how an enzyme lowers activation energy for a reaction is accurate.

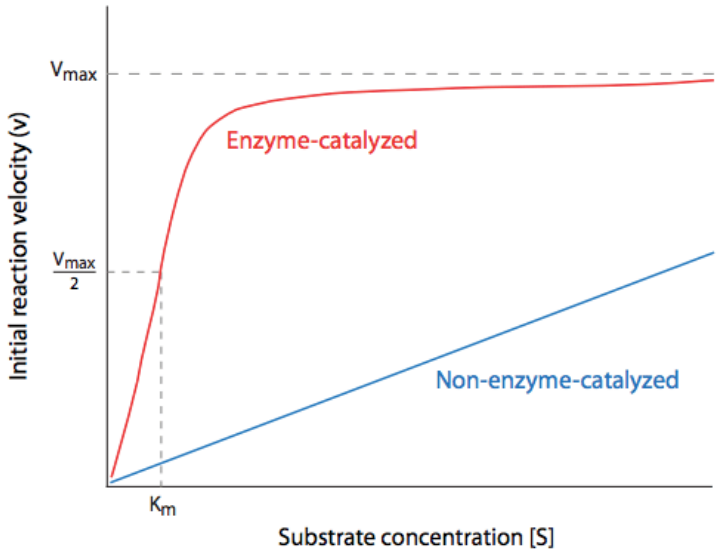


Figure 3.2.5. Saturation curve for a single-substrate enzyme-catalyzed reaction.

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3.3: Enzyme Kinetics

Unlike uncatalyzed (but readily occurring) reactions, in which the rate of the reaction is dependent only on the concentration of the reactants, the rate of enzyme-catalyzed reactions is limited by the number of enzyme molecules available. This maximal rate of turnover from substrate to product is a function of the speed of the enzyme and the number of enzyme molecules. V_{max}, this theoretical maximal rate or reaction, is approached when there is such a high concentration of substrate molecules that not only is every available enzyme at a given time occupied, but as soon as an enzyme finishes converting substrate to product, it immediately binds a new substrate. Another term, Km, is related to V_{max} in that K_m (the Michaelis constant) is the concentration of substrate at which half-maximal reaction rate $V_{max}/2$ occurs. These two terms are related in the Michaelis-Menten equation, which describes the reaction rate v with respect to the substrate concentration [S].

$$v = \frac{V_{\max}[\mathrm{S}]}{\mathrm{K_M} + [\mathrm{S}]} \tag{3.3.1}$$

The Michaelis-Menten equation was derived by Leonor Michaelis and his graduate student Maud Menten in 1913, based on work by Victor Henri, and is applicable only to simple enzyme kinetics in which there is only one substrate that is changed immediately to a product during the reaction without forming any intermediate compound, the enzyme in question shows no allostericity, and the reaction is unidirectional.

It should be noted that the reaction rate *v* is actually the initial reaction rate at a particular substrate concentration, and is sometimes denoted *vo*. Naturally, as the reaction continues, the substrate concentration decreases, along with the reaction rate.

The Michaelis-Menten equation assumes a simple reaction of the form:

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \longrightarrow \mathrm{E} + \mathrm{P} \tag{3.3.2}$$

where E is an enzyme, S is the substrate, and P is the product. Note the formation of the intermediate enzyme-substrate complex, ES, which is a transition state (recall Figure 3.3.2) in which the substrate is unstable and associated with the enzyme. In fact, ES could as easily be considered EP, since this state is essentially the tipping point between the conversion from substrate to product. In this construction, the **Michaelis constant**, K_M, of an enzyme-catalyzed reaction is (k_2 + k_3)/k_1. That is the rate of ES dissociation over the rate of ES association. K_M, of course, varies not only depending on the enzyme, but also with respect to the identity of the substrate. Some enzymes can work with multiple substrates, and the K_M of that enzyme for the different substrates is usually different. Because the saturation curve in Figure 3.3.5 can be difficult to work with, linearizations of the Michaelis-Menten equation (Equation 3.3.1) were developed. The most common is the **double reciprocal plot**, better known as the Lineweaver-Burk plot. On this type of graphical representation of enzyme kinetics, the reciprocal of the substrate concentration is plotted against the reciprocal of the reaction velocity. This generates a line in which the x-intercept is then $-1/K_m$, the y-intercept is $1/V_{max}$, and the slope of the line is K_m/V_{max}.

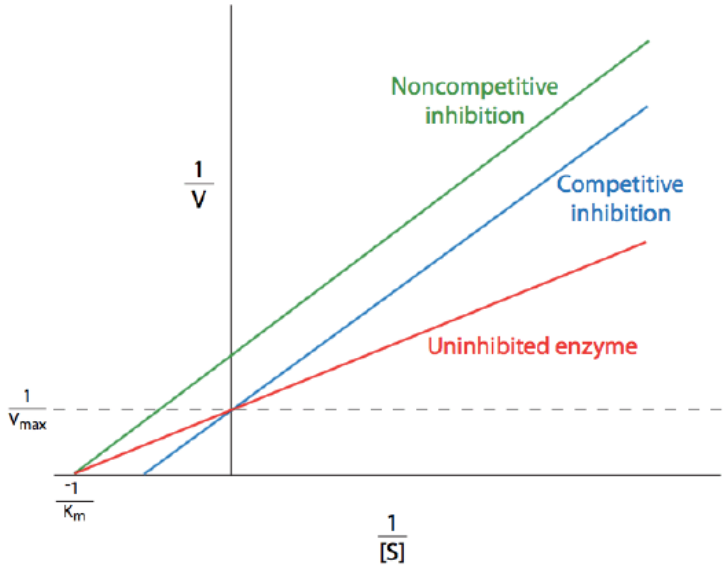


Figure 3.3.7. Lineweaver-Burk plot of enzyme kinetics and the effect of competitive and non-competitive inhibitors at constant concentrations.

The Lineweaver-Burk plot

Obtaining V_{max} and K_m from a direct plot of v against [S] can be difficult because even at very high substrate concentrations, experimental data may still be significantly under the V_{max}. This leads to underestimation of the V_{max}.

The Lineweaver-Burk plot addresses this concern, but has some shortcomings of its own. Because it is easier to obtain data at high concentrations, most of the data points are near 0, and fewer data points are available further out (to the right of the graph). Because these are reciprocals, under these low [S] conditions, small errors in measured values of \(*v*\) turn into large errors in 1/*v*, and therefore large errors in K_M and V_{max}. This is evident on examination of the Lineweaver-Burk equation:

$$\frac{1}{v} = \left(\frac{K_M}{V_{\max}}\right)\frac{1}{[S]} + \frac{1}{V_{\max}} \tag{3.3.3}$$

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3.4: Regulation of Enzyme Activity

Figure 3.4.7 (and 9) also illustrates the effects of two different types of inhibition on the different components of enzyme kinetics. Enzymes can be slowed down or even prevented from catalyzing reactions in many ways including preventing the substrate from entering the active site or preventing the enzyme from altering conformation to catalyze the reaction. The inhibitors that do this can do so either reversibly or irreversibly. The irreversible inhibitors are also called **inactivators**, and either bind to the enzyme with such high afinity as to be virtually irreversible, or they actually form covalent bonds with the enzyme. Reversible inhibitors are generally grouped into two basic types: competitive and non-competitive.

Finasteride (trade names include Propecia and Proscar) is an irreversible inhibitor that binds very tightly to the enzyme 5-a-re- ductase, used in converting testosterone to dihydrotestosterone. It is used in the treatment of male pattern baldness, benign pro- static hyperplasia, and prostate cancer.

Aspirin is an example of an irreversible inhibitor that actually forms a covalent bond with the enzyme. The aspirin (acetylsalicylic acid) transfers its acetyl group onto a serine residue on cyclooxygenase-2 (COX-2). This stops the production of in ammation-producing prostaglandins and thromboxanes by COX-2.

Methotrexate is a competitive inhibitor of dihydrofolate reductase (DHFR), an enzyme that synthesizes tetrahydrofolate, which is a precursor for purine synthesis, and therefore for DNA and RNA. It has a very similar molecular structure for folic acid, the natural substrate of DHFR. Methotrexate is used as an anti-cancer drug because it affects rapidly reproducing cells (which need to make DNA sooner than other cells) more than non-cancerous cells.

Competitive inhibition is perhaps the simplest to understand. The inhibitor molecule competes directly with the substrate for the active site of an unbound enzyme. If an inhibitor binds to the active site, the substrate is unable to do so until the inhibitor has vacated the site. Thus, one could potentially overwhelm competitive inhibition with sufficiently larger concentrations of substrate so that the probability that the enzyme bumps into a substrate to bind becomes exceeding large compared to the probability of bumping into an inhibitor. Normal, uninhibited V_{max} is then achieved despite the presence of the competitive inhibitor, which has only affected the K_m, that is, the concentration of substrate needed to reach $V_{max}/2$. This is the kinetic signature of competitive inhibitors: with increasing inhibitor concentrations, K_M is increased but V_{max} is unaffected.

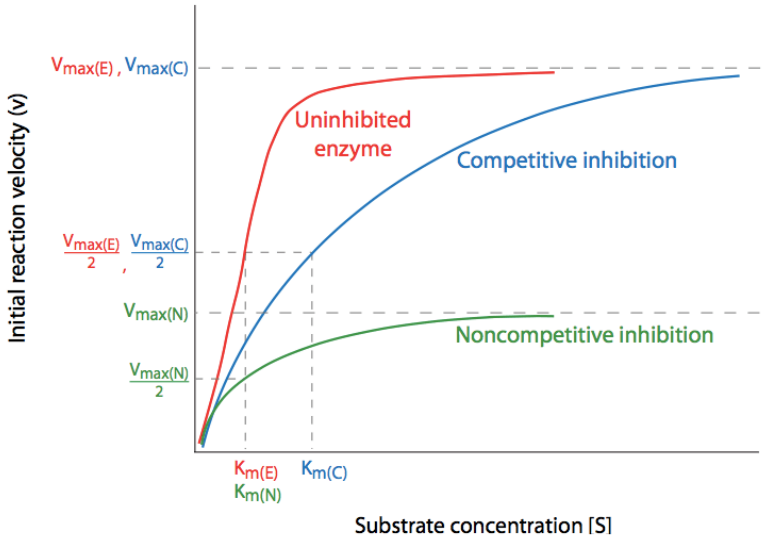


Figure 3.4.9. Saturation curves for enzyme-catalyzed reactions without inhibitor (red) with competitive inhibitor (blue) at a constant concentration, and with noncompetitive inhibi- tor (green) at a constant concentration.

Non-competitive inhibition involves inhibiting the enzyme by altering its ability to complete the catalyzed reaction through binding of the enzyme at a position that is not the active site. When the inhibitor binds to the enzyme, it causes a change, usually

con- formational, that may either prevent the enzyme from binding the substrate, or prevent the enzyme from acting upon a bound substrate. In either case, increasing the availability of substrate will not ultimately overcome the effect of the inhibitor. Thus, V_{max} is reduced not because some proportion of the enzymes are no longer usable, but because the enzymes that are available have the same access to substrate as it would without inhibitor (that is, it is not in competition with an inhibitor), the K_m is not affected.

Allostery

Non-competitive regulation is one example of *allosteric regulation* of enzymes. Allosteric interactions occur when the binding of a ligand (not necessarily a substrate) to a protein influences the binding of another ligand to the protein at a separate binding site. These kinds of interactions can be either positive (activating) or negative (inhibitory), and either homotropic (both ligands are identical) or heterotropic (ligands are different). Interestingly, sometimes the regulator ligand may actually be a product of the catalyzed reaction. In this kind of feedback mechanism, the progress of a reaction is self-regulating.

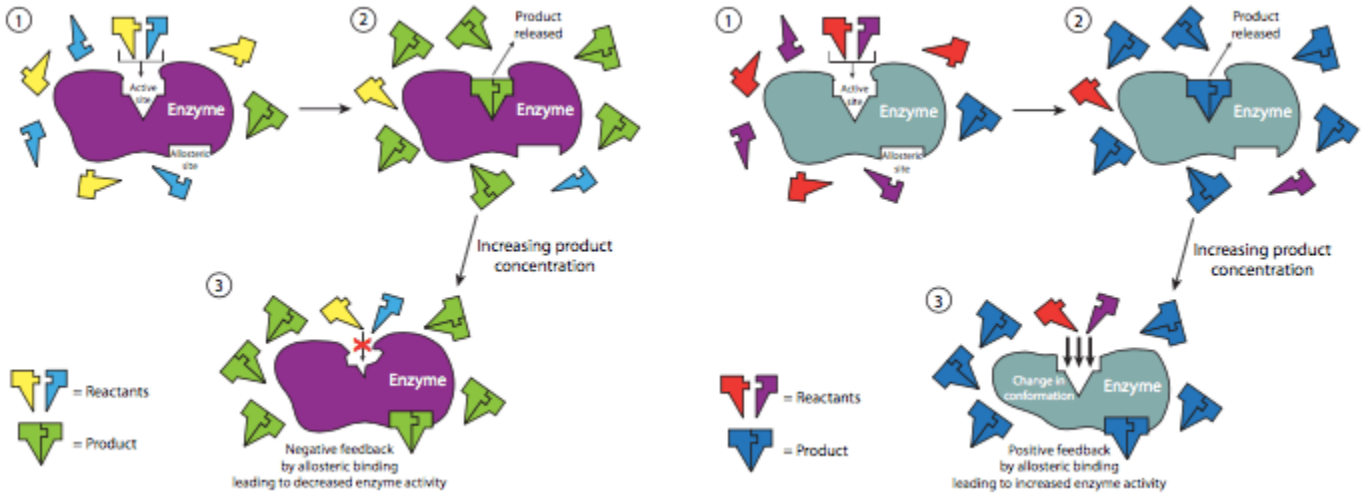


Figure 3.4.10. Feedback control loops. (Left) Negative feedback is a common biological mechanism for self-regulating processes. The product of the reaction, either by itself or inconjunction with other molecules, acts as an allosteric inhibitor of the enzyme. (Right) Positive feedback is less common because it can lead to rapid expansion of the scope of a reaction, and requires an external (relative to the enzymatic reaction) mechanism for slowing or stopping the reaction.

Many of the enzymes in metabolic pathways (chapters 5 and 6) are regulated by a naturally occurring non-competitive inhibitor. One example is phosphofructokinase (PFK), which is involved in glycolysis, which produces ATP for the cell. However, if there are high enough levels of ATP in the cell that other cellular processes aren't using, then it can bind to phosphofructokinase outside of its active site (it binds fructose-6-phosphate) and turn it off. This blocks glycolysis and production of excess ATP when the cell does not need it. As ATP is used up, there is less available to inhibit PFK and glycolysis starts back up.

Modeling Allosteric Behavior

There are two models for such allosteric interactions.

- The **symmetry model**, also known as the concerted model, or MWC model (Monod, Wyman, and Changeux, 1965), proposes that the allosteric enzyme is an oligomer of several subunits, each of which are symmetrically related, and can be in either a "tensed" or "relaxed" state, but all of the subunits are in the same state and at equilibrium. When a ligand binds, it changes the state of the subunit(s) to which it binds, and to maintain equilibrium, that in turn causes the state of the other subunits to match, thus altering binding properties for a subsequent ligand.
- The **sequential model**, or the KNF model (Koshland, Nemethy, and Filmer, 1966), proposes something quite different: although it also supposes subunits in either tensed or relaxed states, it does not require all subunits to be connected in such a way as to mandate that all subunits be in the same state, and therefore conformational changes in one subunit need not be propagated to all. Instead, using an induced-fit model of ligand binding rather than the more rigid basic lock and key mechanism, it suggests that when a ligand binds to the enzyme, it induces a slight conformational change

in the active site that increases its affinity for the ligand. The conformational change may slightly alter the conformation of other subunits of the enzyme, but may not constitute a state change between relaxed and tensed depending on how tightly the subunits are interacting. However, the change is enough to increase substrate affinity in adjacent subunits.

Advanced: Uncompetitive and Mixed inhibition

Most cell/molecular courses stop the discussion of enzyme inhibitors at competitive vs non-competitive based on their kinetic profiles. However, it should be noted that if you take a biochemistry course, you may encounter the terms *uncompetitive* inhibitor and *mixed* inhibitor. These terms are defined not just by the enzyme kinetics, but the mechanism of interaction:

- *Uncompetitive inhibitors* bind only to the enzyme-substrate (ES) complex, and not to the enzyme before it has encountered substrate. This leads to decreased V_{max} and decreased K_m.
- *Mixed inhibition* means that the inhibitor can bind to either enzyme alone or the enzyme-substrate complex. Because the affinities of the inhibitor for the two forms of the enzyme are different, and because part of it depends on substrate concentration while the other kind of binding does not, generally, V_{max} decreases and K_m increases. Non-competitive inhibition is a special case of mixed inhibition in which the catalytic activity of the enzyme is diminished or abolished, but the ability to bind substrate is unaltered.

Other Mechanisms of Inhibition

While important, especially pharmaceutically, the use of enzyme inhibitors is not the only way to regulate enzymes. There are numerous examples of one type of enzyme activating or inhibiting another. The most common general example are the protein kinases. These enzymes phosphorylate (transfer phosphate group to) other enzymes and thereby activate them. Kinases are generally fast and very specific, and this is an efficient method for activating large numbers of particular enzymes quickly. Conversely, protein phosphatases are enzymes (also quite fast, but much less specific than kinases) that remove the phosphate groups from phosphorylated proteins, thereby turning off those enzymes. Keep in mind that this is a generalization, and that not all phosphorylations are activating. In addition to enzymatic inhibition of enzymes, there is also inhibition by binding and sequestration of the substrates. In fact, the antibiotic vancomycin works just this way, binding to the substrate peptide for transpeptidase and preventing the enzyme from recognizing it. Transpeptidase normally helps stabilize the cell wall of certain bacteria by altering some of the proteins, and without its activity, the protection of the cell wall is compromised and the bacteria may be more easily killed.

Well over half of the enzymes discovered so far do not act in the simplistic Michaelis-Menten one-substrate-one-product mechanism, but rather operate with two substrates and two products, usually with the transfer of an active group. These types of reactions are sometimes known as Bi Bi reactions. There are two major classes of these reactions: the *sequential reactions*, in which all substrates bind with the enzyme before the reaction proceeds, and the *ping pong reactions*, in which one or more products are created and released before all of the substrates have been bound. In fact, unlike sequential reactions, the two substrates do not interact with one another while bound to the enzyme.

Optimal Conditions

The activity of enzymes is greatly influenced by both pH and temperature, as expected from the discussion of protein structure in the previous chapter. Activity profiles of most enzymes show a peak of activity that tails off on either side, whether it is pH or temperature. This is an innate characteristic of the enzyme. For example, pepsin, a digestive enzyme secreted into the stomach (pH 2) does not function when the pH > 5. On the other hand, another digestive enzyme, trypsin, which is secreted into the duodenum (proximal small intestine) where the pH is ~8, does not work in acidic environments. Changes in pH can change ionization of amino acid side chains that can thereby alter interaction with the substrate, or lead to changes in tertiary structure.

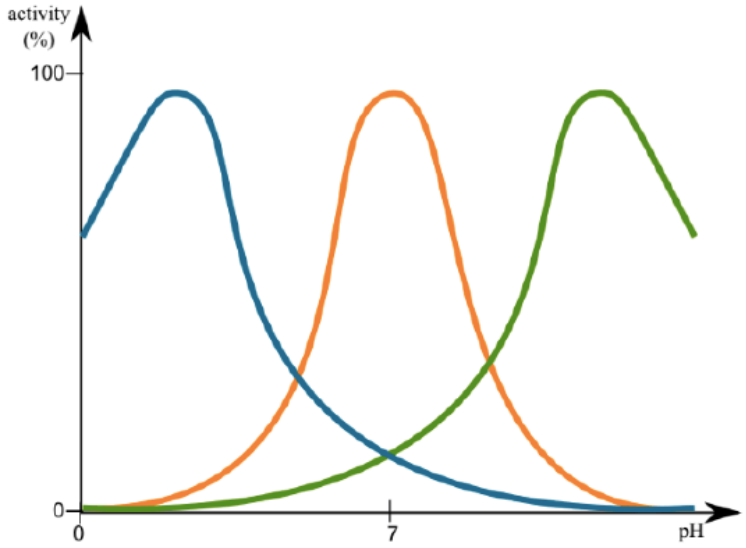


Figure 3.4.11. pH dependence of enzymatic activity. This graph depicts three hypothetical enzymes with acidic, neutral, and basic pH optima.

Similarly, at suboptimal temperatures, the likelihood of protein-substrate interaction is low but above the optimal temperature, the increased energy can lead to breaking of hydrogen bonds within the structure of the enzyme, resulting in changes that inactivate the catalytic ability of enzyme or prevent it from binding substrate with sufficient affinity. The temperature optimum of most enzymes is very close to its typical environment. Thus, a human enzyme would operate optimally around 37°C, while an enzyme from bacteria that live in deep-sea volcanic vents (e.g. *Thermophilus aquaticus*) might have temperature optima over 90°C. This is one of the reasons that refrigeration can slow down growth of microorganisms (which obviously have no ability to regulate their temperature), and why most microorganisms are killed (enzymes permanently denatured) when put into sustained high temperature environments. Interestingly, the DNA polymerase from the *T. aquaticus* bacteria, also commonly called Taq polymerase, is used in a rapid DNA-amplifying lab technique known as PCR (polymerase chain reaction, see Methods chapter) in which samples are repeatedly heated to high temperatures to separate DNA strands in preparation for making copies of them. DNA polymerases from most prokaryotic or eukaryotic species would be denatured and in- activated by the high heat, but Taq has evolved (with respect to its tertiary structure) for extraordinary structural stability even in heat extremes.

Finally, many enzymes require a molecular partner that has no catalytic activity of its own, but like a catalyst, is not permanently altered by the chemical reaction. These molecules are *cofactors*. Some are simple: elemental, in fact, including metal ions such as Zn^{2+} or Ca^{2+}. Others are slightly more complex: small organic cofactors are called *coenzymes*, and accomplish the same thing, acting as a required partner to the enzyme in catalyzing a reaction. The interaction with the enzyme itself varies and may be only transient, as in NAD^+/NADH which are coenzymes used in redox reactions, or permanently bound to the enzyme by covalent bond like the heme group of hemoglobin. Often the function of the coenzyme is to provide an active group to facilitate the catalyzed reaction. Coenzyme A, in various metabolic pathways such as glycolysis or the tricarboxylic acid cycle, can be bound to a substrate to form a stable product that then acts as an intermediate. The Co-A is released from the molecule as it undergoes the next step in a series of reactions in the metabolic pathway (see Chapter 5).

From a human health standpoint, it is interesting to note that many coenzymes are vitamins, or derived from vitamins. These are the B vitamins biotin (B_7), cobalamin (B_{12}), folic acid (B_3), niacin/nicotinamide (B_9), pantothenic acid (B_5), pyridoxine (B_6), riboflavin (B_2), and thiamine (B_1). Vitamins are small organic compounds that are not synthesized by an organism and must therefore be ingested. They are generally needed only in small quantities, but necessary nonetheless. Naturally, the vitamins we are familiar with are those required by humans. The specific roles of these vitamins and the con- sequences of not having enough of them are discussed later in this textbook, as the enzymes that they work with are introduced in detail.

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4: MEMBRANES - STRUCTURE, PROPERTIES AND FUNCTION

Biological membranes are the basis for many important properties of the cell, not the least of which is to physically define the cell boundary, and in eukaryotes, the boundaries of each intracellular organelle. However, they are not completely impermeable boundaries, and through embedded proteins, the membrane serves as the gatekeeper for the passage of specific molecules into (e.g. nutrients) and out of (e.g. waste) the cell. Other embedded proteins can identify the cell to other cells, and participate in numerous interactions with the environment or other cells. Finally, the membrane, or more precisely, the chemical gradients across the membrane, is an important energy source for the cell.

4.1: Membrane Structure and Composition

4.2: Membrane Permeability

4.3: Membrane Transport Proteins

4.4: The Action Potential in Neurons

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4.1: Membrane Structure and Composition

Since most cells live in an aqueous environment and the contents of the cell are also mostly aqueous, it stands to reason that a membrane that separates one side from the other must be *hydrophobic* to form an effective barrier against accidental leakage of materials or water. In the earlier chapter on the basic biomolecules, cellular membranes were partially defined as being composed primarily of phospholipids: molecules consisting of a phosphorylated polar head group attached to a glycerol backbone that has two long hydrocarbon tails. The composition of the hydrocarbons can vary in length and degree of saturation, and there is also variation in the head groups. It is also important to remember that although we concentrate on the phospholipids as the primary components of the membrane, there are other significant parts: other lipids, including cholesterol, integral and peripheral membrane proteins, and glycosylated lipids and proteins.

Because the phospholipids are amphipathic, meaning they have a hydrophilic head and hydrophobic tail, the simplest conformation for a small group of phospholipids in aqueous solution might be expected to be a micelle (Figure 4.1.1A), but is this actually the case? Mixtures of hydrophobic molecules and water are thermodynamically unstable, so this structure would protect the hydrophobic fatty acyl tails from the aqueous environment with which the head groups interact. Micelles can form with other amphipathic lipids, the most recognizable being detergents such as SDS (sodium dodecyl sulfate, also called sodium lauryl sulfate), used in common household products such as shampoos. The detergents act by surrounding hydrophobic dirt (1B) and holding it in solution within the micelle to be rinsed away with the water. At smaller sizes, the micelle is fairly stable; however, when there are a large number of phospholipids, the space inside the micelle becomes larger and can trap water in direct contact with the hydrophobic tails (1C). This renders the micelle unstable, so a large single layer of phospholipid is unlikely to serve stably as a biological membrane. Micelles form easily with SDS and other single- tailed lipids because their overall shape (van der Waals envelope) is conical (1D), which lends itself to fitting tight curvatures. However, phospholipids are more cylindrical, and it is harder to t them into a tight spherical micelle. If they do form micelles, they tend to be larger, and likely to collapse.

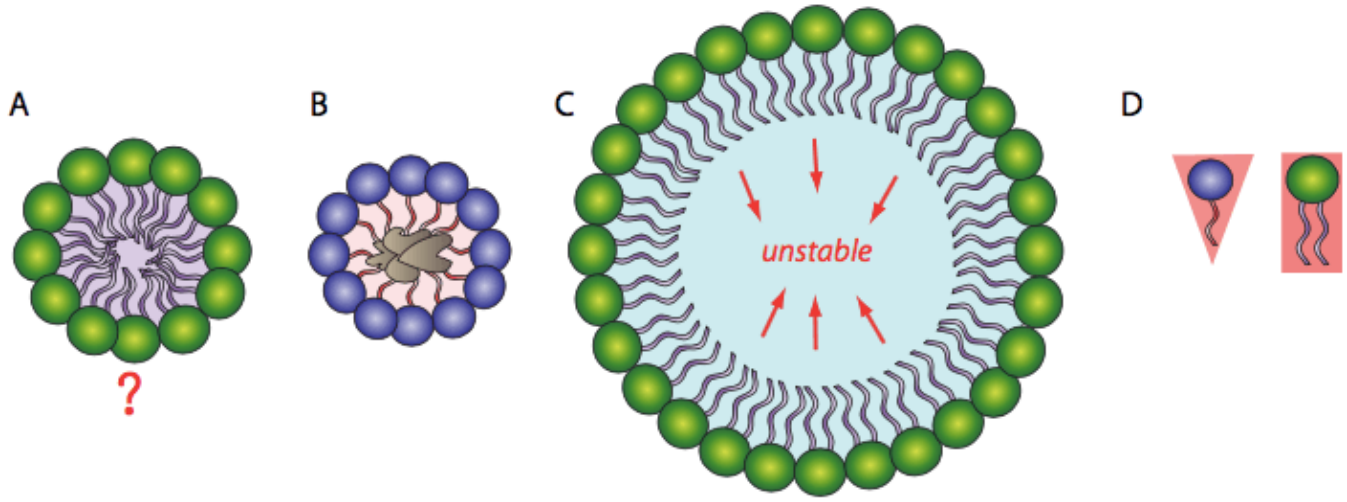


Figure 4.1.1.

On the other hand, a phospholipid bilayer (Figure 4.1.2A) could form a fatty acyl sandwich in which the polar head groups face outward to interact with an aqueous environment, and the fatty acids are sequestered in between. However, this does not resolve the problem on the edges of the sandwich. Sometimes, a collapsed micelle can form a closed bilayer in which the edges appear to be sealed, but due to the shape of the phospholipids, there is poor contact between acyl chains. Such a tight bend is unstable and the edge phospholipids are likely to break apart from one another. So, the solution to the ideal phospholipid structure in an aqueous environment is a spherical phospholipid bilayer (2B and cutaway in 2C): no edges mean no exposed hydrophobicity.

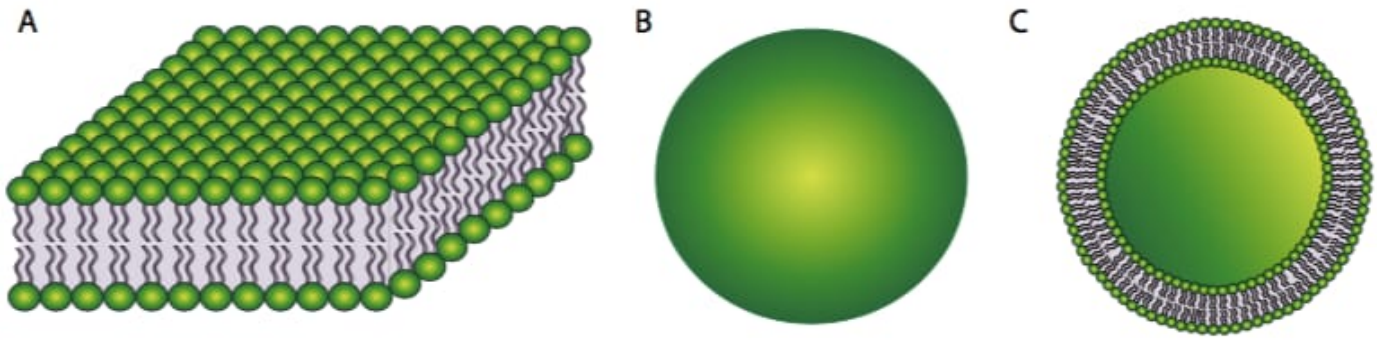


Figure 4.1.2.

The stability of the spherical phospholipid bilayer does not imply that it is static in its physical properties. In most physiologically relevant conditions, the membrane is cohesive, but fluid. It can mold its surface to the contours of whatever it is resting on, and the same thermodynamic and hydrophobic properties that make it so stable also allow it to seal up minor tears spontaneously. Maintaining a working range of fluidity is important to the cell: if the membrane is too rigid, then it may be unable to move or undergo necessary processes such as endocytosis, in which a cell takes up large extra-cellular molecules by enveloping them with the cell membrane and pinching it off in a vesicle; while if it becomes too fluid, it may lose integrity and fall apart.

There are three major factors that govern the fluidity of the membrane:

1. degree of saturation of the fatty acyl chains,
2. the temperature, and
3. the concentration of cholesterol.

Just as overly large micelles will be unstable, there is also a minimal concentration of lipid needed for a micelle to form. This is known as the critical micelle concentration (cmc) and is a property of each particular lipid. Below the cmc, there are not enough amphipathic lipids to mutually shield their hydrophobic tails from the water, and the more likely position of the lipids is on the surface of the aqueous solution, hydrophilic heads in contact, with the hydrophobic tails in the air.

Liposomes are artificial spherical bilayers (Figure 4.1.2) that are used both in research for study of both membrane lipids and integral membrane proteins. They are also used to deliver drugs and other macromolecules into cells for either research of therapeutic purposes. A common method for getting DNA, a large molecule that is not normally transported into cells, into a cell is called lipofection. This technique involves creating liposomes within a solution containing the DNA of interest. This traps the DNA into the liposome, which can then be applied to cells. The liposomes then fuse with the plasma membrane and deliver the DNA into the cell.

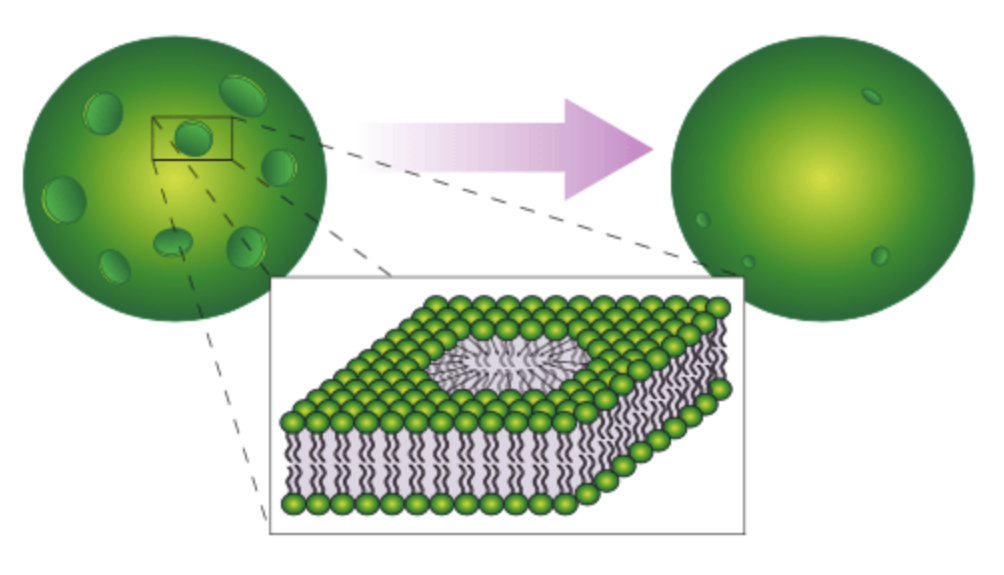


Figure 4.1.3.

Another technique for introducing foreign DNA into a cell is **electroporation**, which illustrates the self-sealing property of the phospholipid bilayer (Figure 4.1.3). The cell (usually thousands of cells, actually) is placed in a DNA solution and subjected to an electric current. This transiently pulls on the cell membrane in all directions, causing many small holes to open up. DNA then moves into the cell, and the holes spontaneously heal. Of course, the process is not perfect, and due to variations in the cells and in the electric field, some cells will be unable to reseal the holes, or for other reasons may not survive the process.

Fully saturated fatty acyl chains can rotate freely around any bond and therefore can pack together very tightly, thus decreasing membrane uidity. As more unsaturated fatty acyl chains are introduced into the membrane, the more space there is between some of the fatty acyl tails, and there is an increase in fluidity. Similarly at higher temperatures, even saturated fatty acyl chains, with their increased energy, move more and create more space between the chains, also increasing fluidity. Finally, cholesterol, as a small planar lipid molecule, can intercalate between the fatty acyl tails. Interestingly, in normal physiological temperatures, the effect of cholesterol is dependent on its concentration. At normal concentrations, the cholesterol restricts acyl tail movement and decreases fluidity. However at very low concentrations, cholesterol has the opposite effect, separating the hydrophobic tails and slightly increasing fluidity, especially in the innermost regions of the membrane. [In case you were wondering, human medical problems with cholesterol are unrelated to the cholesterol in the cell membranes, and refers to cholesterol (bound to lipoprotein carriers) in the bloodstream. That cholesterol can build up in the blood vessels, decreasing the internal diameter and along with it, blood flow. When this happens to the heart, which uses a lot of oxygen, the oxygen deprivation can cause necrosis and a heart attack.]

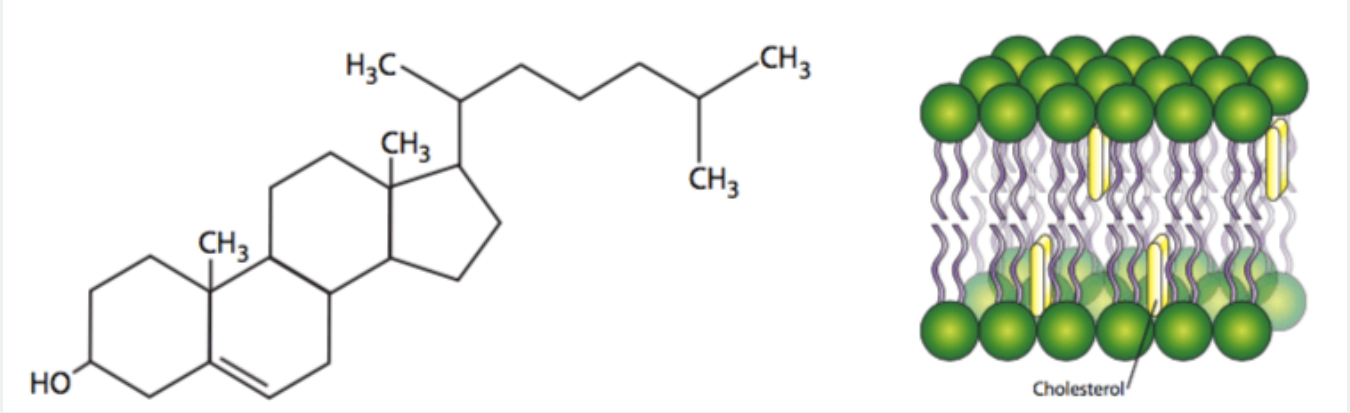


Figure 4.1.4. Cholesterol is a small planar molecule that can fit in between the fatty acyl tails of phospholipids in the membrane.

The effect of cholesterol is actually a little more complicated than explained above. Cholesterol is not only planar but rigid, and it intercalates close between acyl tails near the head region. Be- cause cholesterol is somewhat shorter than many acyl tails, while the parts of the hydrocarbon chains nearer the head groups are stabilized and restricted in movement, the cholesterol actually acts as a spacer for the other (methyl) end of each chain, so in the center domain of the hydrophobic core of the bilayer, there is actually increased fluidity around cholesterols. Cholesterol concentration varies greatly among organelles of the same cell, and can even be dynamically regulated in response to temperature changes. Membrane samples taken from fish living in warmer temperatures contain more cholesterol than samples from the same species acclimatized to a lower temperature.

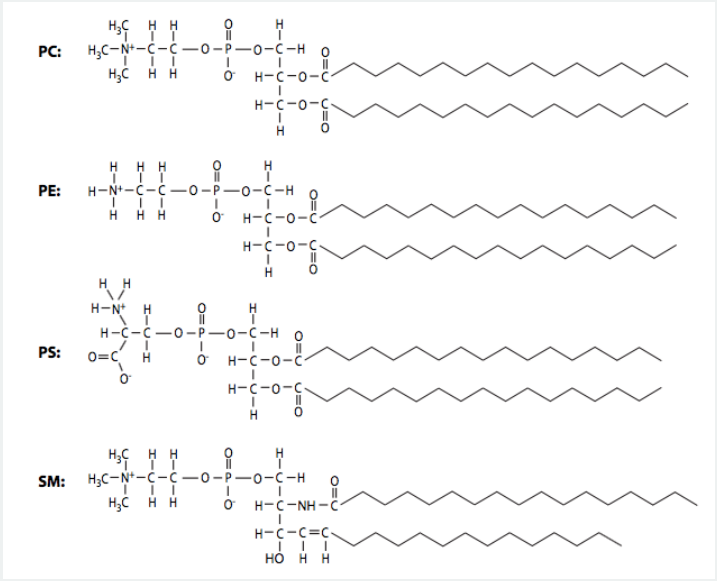


Figure 4.1.5. The molecular structures of phosphatidylcholine (PC), phosphatidyletha- nolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM). The acyl chains of all four molecules are of variable length except the 13-C chain of SM.

In addition to the three factors noted above, phospholipid composition can also alter membrane fluidity: shorter acyl chains lead to greater fluidity, while longer chains, with more surface area for interaction, generate membranes with higher viscosity. The phospholipid composition of biological membranes is dynamic and can vary widely. The table below shows the differences in the ratios of major phospholipid species phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) in plasma membranes from two different cell types. As you might expect based on their differing functions, the ratios of plasma membrane lipids of a myelinating Schwann cell are very different from the lipids in the plasma membrane of a red blood cell.

Lipid	*Schwann Cell PM*	*Erythrocyte PM*
Phosphatidylcholine	44%	19%

Lipid	Schwann Cell PM	Erythrocyte PM
Phosphatidylethanolamine	14%	18%
Phosphatidylserine	3%	8%
Sphingomyelin	29%	17%

Even within a single cell, the composition of the plasma membrane differs from that of intracellular organelles. There is even heterogeneity within a membrane itself - the lipids are not simply distributed randomly in the membrane. Research over the last two decades have identified lipid “rafts” that appear to be speci c for embedding particular proteins. Since they are unanchored lipids, the rafts can move laterally within the membrane just like most individual lipid molecules. Finally, there are different ratios of the lipids between the two layers of the bilayer. The cytoplasmic face of every membrane will have different associations and functions than the non- cytoplasmic face, so why should we expect the lipid composition to be the same?

> Though lipid rafts were considered a possibility in the fluid-mosaic membrane model proposed by Singer and Nicholson in 1972, only in the last two decades has the idea been researched seriously, but there have been technical difficulties with visualizing a small domain of lipids within a virtual ocean of lipids. In broad terms, the rafts are considered small areas of ordered lipids within a larger undirected membrane. Lipid rafts most often form in association with specific membrane proteins while excluding others. Some of the proteins are peripheral membrane proteins such as src or the GPI-linked adhesion molecule Thy-1, while others are transmembrane proteins such as the T-cell receptor. Usually the included proteins have signaling-related functions, and one model proposes that these proteins may direct the organization of selected lipids around them, rather than the other way around.

Although some phospholipids are directly linked to proteins and the cytoskeleton, most are not, and are therefore free to move within the plane of its layer of the bilayer. In the experiment shown in Figure 4.1.6 below, the cell surface has been labeled with a covalently bound dye that fluoresces red when excited by green light. When the dye molecules are exposed to high intensity light for an extended period, they no longer fluoresce, a phenomenon known as **photobleaching**. Photobleaching is a permanent effect, and therefore it can be inferred that if a photobleached spot fluoresces again, then other, non-bleached, phospholipids must have moved into the spot. This experiment clearly proves the lateral mobility of phospholipids in a membrane.

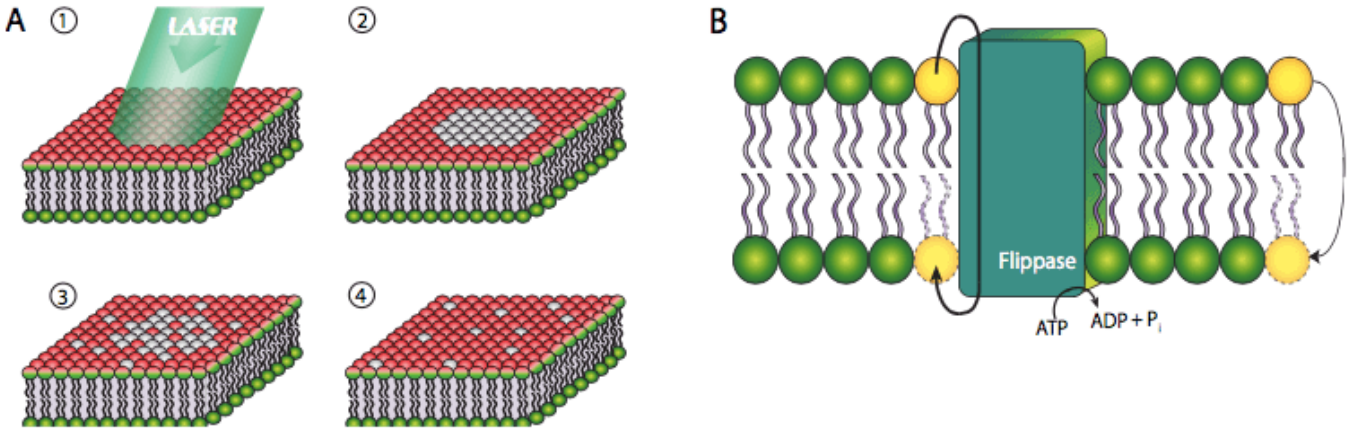


Figure 4.1.6. (A) photobleaching experiments can demonstrate lateral mobility of lipids in a bilayer. But, transverse mobility is severely limited and is usually facilitated by a flippase enzyme (B).

However, this is not the case with transverse mobility from one face of the membrane to the other. Due to the highly unfavorable energetics of pushing a polar head group through the lipid tail layers, phospholipids very rarely move from one layer to the other within a membrane (Figure 4.1.6B, far right yellow phospholipid). Such movement is greatly facilitated by a ippase enzyme, which hydrolyzes a molecule of ATP for the energy to push a phospholipid from one face of the bilayer to the other.

In a pure phospholipid bilayer, membrane proteins as well as lipids have lateral mobility, but in living cells, the proteins are generally constrained either by preferential association with certain kinds of lipids, by direct attachment to cytoskeletal elements, by in- direct attachment to the cytoskeleton, or by being “fenced in” by cytoskeletal gridwork directly underlying the

cell membrane. Since 1972, the Singer-Nicholson “fluid mosaic” model of membrane structure has been accepted as a general model for biological membranes. It proposes that integral membrane proteins as well as membrane lipids have lateral freedom of movement. This has since been re ned with the recognition of lipid rafts and clustered membrane protein patches, but is still viable as a basic model.

> For over a century since Meyer (1899) and Overton (1901) first suggested it, the prevailing theory for the mechanism of gaseous general anesthesia (e.g. ethyl ether, halothane, nitrous oxide, cyclopropane) has been that they partition into and interact with the lipids of the plasma membranes of neurons and by altering the physical membrane properties accomplish the anesthesia. However, in 1985, Franks and Lieb published a report in Nature that, for the first time, showed that an enzyme could be directly affected by a gaseous anesthetic. Since that report, the evidence has been building against the old model of altering membrane lipid properties, and now the current model of direct gas-protein interaction is assumed.

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4.2: Membrane Permeability

A pure phospholipid bilayer, whatever the lipid composition, is a semi-permeable membrane that is generally repellent to large molecules and to ions. Small polar molecules can sometimes pass easily (e.g. ethanol), but more often pass at low rates if at all (e.g. water). However, small nonpolar molecules are able to pass through the membrane with relative ease. The reasons should be self-evident: larger molecules simply cannot fit between the lipid molecules to make their way through. Small molecules that can fit must be hydrophobic, otherwise the fatty acyl core of the membrane will repel them and block them from proceeding. Higher concentrations of cholesterol, by filling in gaps between phospholipid tails, decreases permeability even for small molecules that can normally pass through the membrane easily. Cells need far more than small nonpolar molecules for their material and energy requirements. Fortunately for life on Earth, the membranes of living cells are not purely phospholipids, and as we will see, proteins embedded in the phospholipid bilayer can form conveyances for the transport of many different molecules in and out of the membrane.

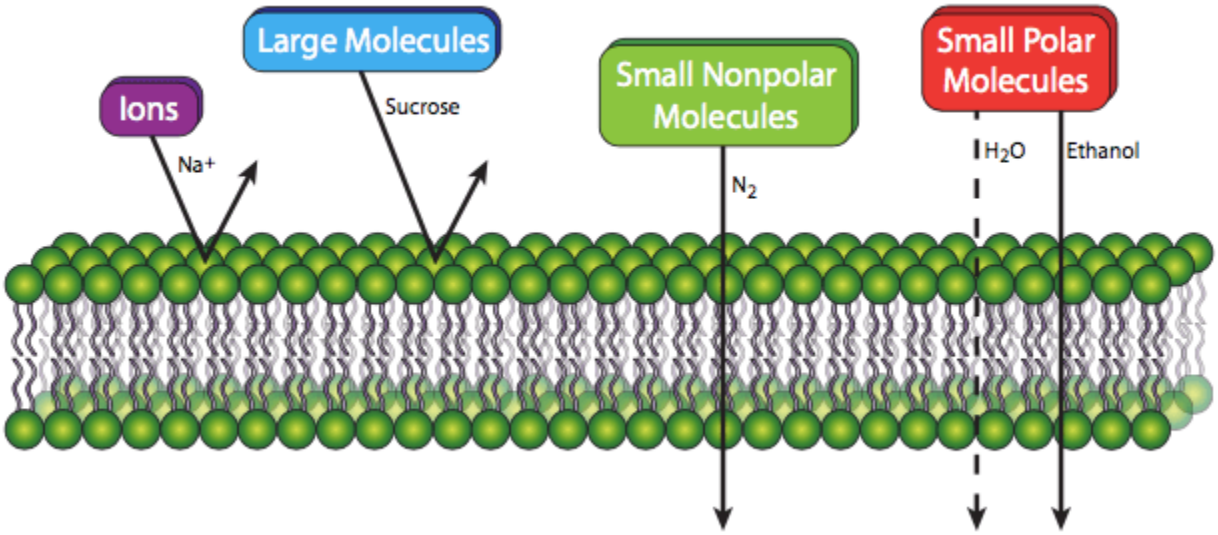


Figure 4.2.7. A pure phospholipid bilayer is inherently semi-permeable.

> In fact, the observation of saturation kinetics in glucose transport in erythrocyte membranes was the first indication of protein-mediated transport (the $GLUT_1$ glucose transporter). Another telling observation was the finding that glucose permeability through erythrocyte membranes is a million times greater than that through an artificial lipid bilayer. The concentration of glucose in the blood is relatively high compared to that inside of most cells, so this is mediated transport, but passive transport since it is going down the concentration gradient. To facilitate the process by preventing a buildup of glucose concentration in the cell, the first step of glucose metabolism is phosphorylation to convert it into a different molecule, glucose-6-phosphate. Thus the concentration of glucose stays very low, and it flows readily from the bloodstream into the cell.

There are some obvious differences between transport of molecules directly through the lipid bilayer (nonmediated transport) and transport using a protein facilitator embedded in the membrane (mediated transport). *Nonmediated transport* is governed by diffusion: the solute moves from areas of high concentration to areas of low concentration, thereby eliminating the gradient. As long as a solute (A) can get through the membrane, its flux (J) is determined solely by concentration difference and the permeability (P) of the membrane: $J_A = P_A ([A]_{out} - [A]_{in})$ and the relationship between the ux across the membrane and the concentration differential is linear.

This is not the case in *mediated transport*. As the name implies, a protein intermediary is required, and alarm bells should be going off in your head saying, “there’s a limit,” to the number of available transport proteins at any given time. Therefore, just as we saw with enzyme kinetics in chapter 3, the ux of solutes going through a transporter is not linearly related to the concentration differential across the membrane, though there is still a concentration effect. Instead, the relationship is logarithmic, reaching a

saturation plateau once all the available transport proteins are in use. At that point, increasing the concentration of the solute will not increase its flux across the membrane. Thus for simple unidirectional mediated transport of a solute (B), the flux (J) can be expressed as a value of the affinity of the transporter for the solute (K_M) and the concentration of the solute:

$$J_B = \frac{J_{max}[B]}{K_M + [B]} \tag{4.2.1}$$

Membrane permeability allows for the possibility of concentration gradients across membranes, which in turn have potential energy associated with the concentration dif- ferential across the membrane. This turns out to be a phenomenally important source of cellular energy, and is the basis for aerobic synthesis of ATP by oxidative phosphorylation (chapter 5). However, to have a meaningful discussion of how concentration differences across semipermeable membranes store energy, we should review some basic concepts rst.

If a point source (e.g. a “glob”) of a solute (e.g. honey) is placed into a solvent (e.g. tea), it starts to dissolve, and as it does so, the concentration of solute near the point source will start out much higher than the concentration towards the periphery of the container (e.g. teacup). Over time, the solute then diffuses from the point source outward in all avail- able directions, and eventually the concentration of solute is equal at any point in tea- cup-space. This behavior is governed by the Second Law of Thermodynamics. The solute is initially concentrated, which means that its constituent molecules are relatively orga- nized. By the second law, these molecules will tend toward chaos, moving away from the constraints of the initial point toward an area with lower concentrations of the solute.

Now, imagine a temporary wall around the point source. The natural tendency is for the solutes to spread out, so by preventing that movement, you have bottled up some potential energy. Of course, this is only potential energy if there is some chance that the solutes can eventually go through the barrier (e.g. the wall has windows that can be opened). If the solutes have absolutely zero chance of passing through, then there is no potential energy because there is no potential to get out and about. Recalling the Energy chapter in which the second law was introduced, the chemical potential energy of a solute is

$$G = RT\ln[A] + G' \tag{4.2.2}$$

so the *chemical potential* difference across a membrane is then

$$\Delta G = RT\ln\left(\frac{[A_i]}{[A_o]}\right). \tag{4.2.3}$$

Now imagine this as something like a hydroelectric dam, where there is a great deal of pressure building up behind the dam, which can be utilized when some of the water is allowed through, powering turbines that generate electricity. In the biological case, there is concentration pressure building up both inside and outside the cell because the natural thermodynamic tendency is to bring the inside and outside concentrations of each solute to equilibrium. When this pressure is released by allowing the ions or other molecules to ow across the membrane, energy is released, and may be captured and used. The most direct example of this the proton-gradient-driven ATP synthase in the inner mitochondrial membrane (Chapter 5), which contains a direct molecular equivalent to the turning of a water wheel with the ow of water. For another example, if we look at [Na^+] in an animal cell, the extracellular concentration is much higher than that intracellularly. When a Na^+ channel is opened, the Na^+ ions rush inward to try to equalize the concentration of Na^+ inside and outside of the cell. Equilibrium is not actually reached in a living cell because Na^+ channels are tightly regulated and only open for short periods of time.

In cells, concentration gradients of ions are great energy sources because the lipid part of the membrane is strongly repellent to ions, preventing them from passing through, but the membrane is embedded with channels and transporters that can allow the ions through if and when they are open. Because ions have both concentration differentials and charge differentials across the membrane, the *electrochemical potential* difference across the membrane is represented by a modification of the chemical potential difference equation with a term that takes that electrical charge into account:

$$\Delta G = RT\ln\left(\frac{[A_i]}{[A_o]}\right) + ZF\Delta\Psi \tag{4.2.4}$$

Z is the charge of the ion (e.g. +1 for Na^+, -1 for Cl^-, +2 for Ca^{2+}), F is the Faraday constant (9.6485 x 10^5 C/mol), and $\Delta\Psi$ is the membrane potential. In an average animal cell, the membrane potential is approximately -70mV. The number is negative to show that the inside of the cell is negative with respect to the outside. Thus, again considering Na^+, not only is there a chemical gradient of more Na^+ ions outside the cell than inside, there is also a charge gradient of more positive charges outside the cell

to inside, so both forces contribute to the energy of Na^+ flow into the cell. The equilibrium potential of one ion (e.g. Na^+) across a membrane is determined by the Nernst equation:

$$E_m = \left(\frac{RT}{zF}\right) \ln \left(\frac{[Na^+]_{out}}{[Na^+]_{in}}\right) \tag{4.2.5}$$

which is extended in the Goldman equation (also Goldman-Hodgkin-Katz equation) that calculates membrane potential based on multiple ion gradients. For most ani- mal cells, a good approximation of the overall membrane potential can be calculated using the three major gradients: Na+, K+, and Cl-. There are, of course, other ion gradients, but their contributions are normally much smaller than these three.

The membrane potential is relatively stable in non-excitable cells, but in neurons and muscle cells, the membrane potential is quite dynamic, so the membrane potential in a non-excited state is referred to in these cells as the resting potential. The membrane (resting) potential in most animal cells is around -70mV. This is due in large part to the presence of K^+ leak channels. These channels leak K^+ from the cell down the concentration gradient until the chemical potential difference of K+ is at equilibrium with the membrane potential. In other words, the gradient pushing K^+ out will eventually be stopped by an equal force from the gradient pushing positive ions (including K^+) back in. There are also Na^+ and Cl^- leak channels, but there are far fewer and they contribute far less to the resting potential than K^+.

> Potassium leak channels are structurally as well as functionally different from other potassium channels. Where most K^+ channels have one pore domain, the leak channels have two. While leak channels, by definition are not voltage gated, nor appreciably activated or inactivated, this is not true of all members of the tandem pore domain family of potassium channels. Interestingly, some (e.g. TASK-1) are mechanoreceptors, opening in response to membrane stretch, and others act as thermoreceoptors, with heat-sensitive activation (e.g. TREK-1).

Although we most commonly think of water as the solvent in which “interesting” molecules (e.g. ions) diffuse, its concentration and movement across membranes has important biological consequences. *Osmosis* is a term that specifically refers to the diffusion of water across a membrane. In this case, water is considered a solute rather than a solvent, so that if a water-permeant liposome, embedded with aquaporin channels to allow the passage of water, is placed in a very salty saline solution, the cell will shrink because there is a lower ratio of water to dissolved salts outside of the cell than inside. This is a *hypertonic* solution relative to the cell. Therefore the water flows from the cell (higher water concentration) out into the saline (with lower water concentration). Conversely, a cell placed in distilled and deionized water will swell and potentially burst because the water rushes from the highest possible concentration (pure water) to a cytoplasm with lower water concentration (because dissolved in it are various ions and other molecules). This is an example of a *hypotonic* solution. An *isotonic* solution will have the same concentration of water inside and outside of the cell.

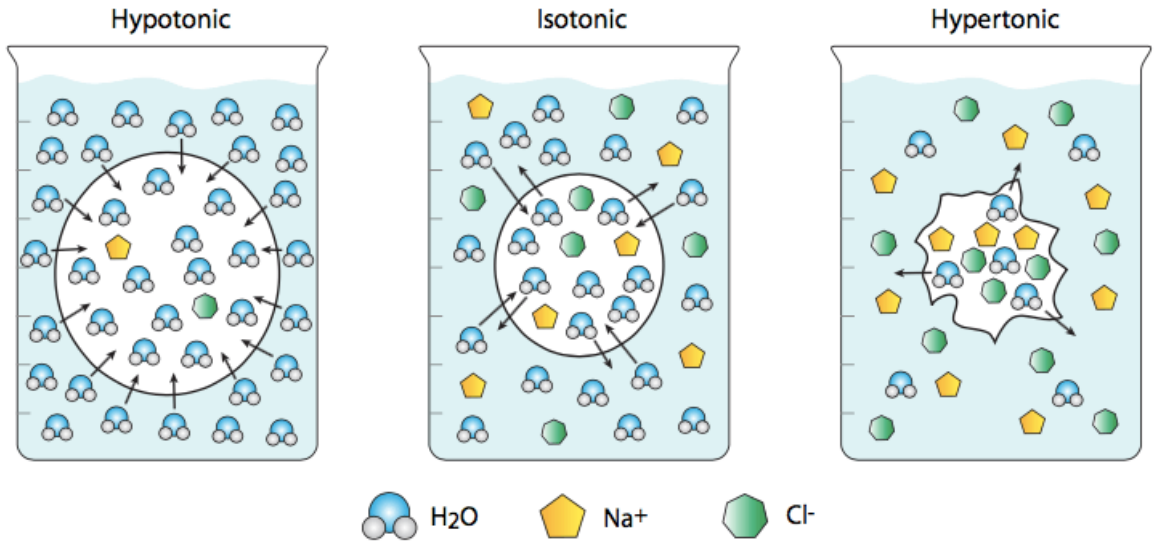


Figure 4.2.8. Osmosis. An artificial "cell" that is permeable to water but not to Na^+, K^+, or Cl^- is placed in a aqueous solutions of varying salinity.

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4.3: Membrane Transport Proteins

Membrane proteins come in two basic types: integral membrane proteins (sometimes called intrinsic), which are directly inserted within the phospholipid bilayer, and peripheral membrane proteins (sometimes called extrinsic), which are located very close or even in contact with one face of the membrane, but do not extend into the hydrophobic core of the bilayer. Integral membrane proteins may extend completely through the membrane contacting both the extracellular environment and the cytoplasm, or they may only insert partially into the membrane (on either side) and contact only the cytoplasm or extracellular environment. There are no known proteins that are completely buried within the membrane core.

Integral membrane proteins (Figure 4.3.9) are held tightly in place by hydrophobic forces, and purification of them from the lipids requires membrane-disrupting agents such as organic solvents (e.g. methanol) or detergents (e.g. SDS, Triton X-100). Due to the nature of the bilayer, the portion of integral membrane proteins that lie within the hydrophobic core of the membrane are usually very hydrophobic in character, or have outward-facing hydrophobic residues to interact with the membrane core. These transmembrane domains usually take one of the two forms depicted in Figures 8 and 14: alpha helices - either individually or in a set with other alpha helices, or barrel-shaped insertions in which the barrel walls are constructed of beta-pleated sheets. The hydrophobic insertions are bounded by a short series of polar or charged residues that interact with the aqueous environment and polar head groups to prevent the hydrophobic portion of the protein from sliding out of place. Furthermore, proteins can have multiple membrane- spanning domains.

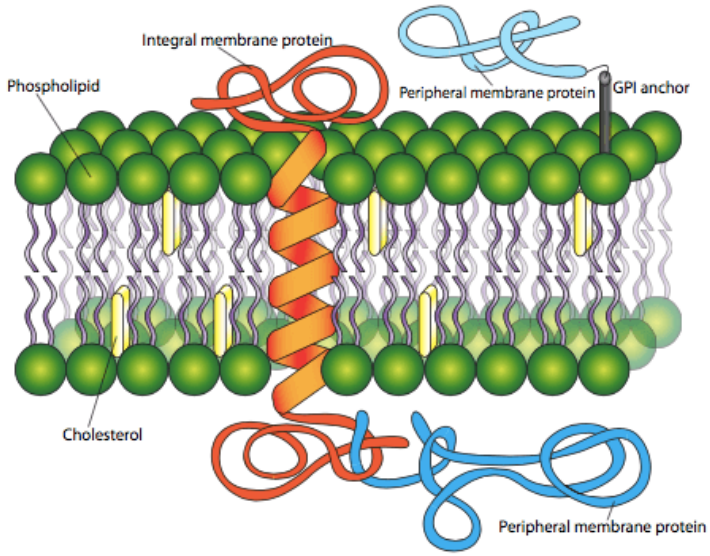


Figure 4.3.9. Integral (orange) and peripheral (blue) membrane proteins embedded in a phospholipid bilayer.

Peripheral membrane proteins (also shown in Figure 4.3.9) are less predictable in their structure, but may be attached to the membrane either by interaction with integral membrane proteins or by covalently attached lipids. The most common such modifications to peripheral membrane proteins are fatty acylation, prenylation, and linkage to glycosylphosphatidylinositol (GPI) anchors. Fatty acylation is most often a myristoylation (a 14:0 acyl chain) and palmitoylation (a 16:0 chain) of the protein. A protein may be acylated with more than one chain, although one or two acyl groups is most common. These fatty acyl chains stably insert into the core of the phospholipid bilayer. While myristoylated proteins are found in a variety of compartments, almost all palmitoylated proteins are located on the cytoplasmic face of the plasma membrane. Prenylated proteins, on the other hand, are primarily found attached to intracellular membranes. Prenylation is the covalent attachment of isoprenoids to the protein - most commonly isoprene (a C5 hydrocarbon), farnesyl (C15), or geranylgeranyl (C20) groups (Figure 4.3.10). GPI anchors (Figure 4.3.11) are found exclusively on proteins on the outer surface of the cell, but there does not appear to be any other commonality in their structures or functions.

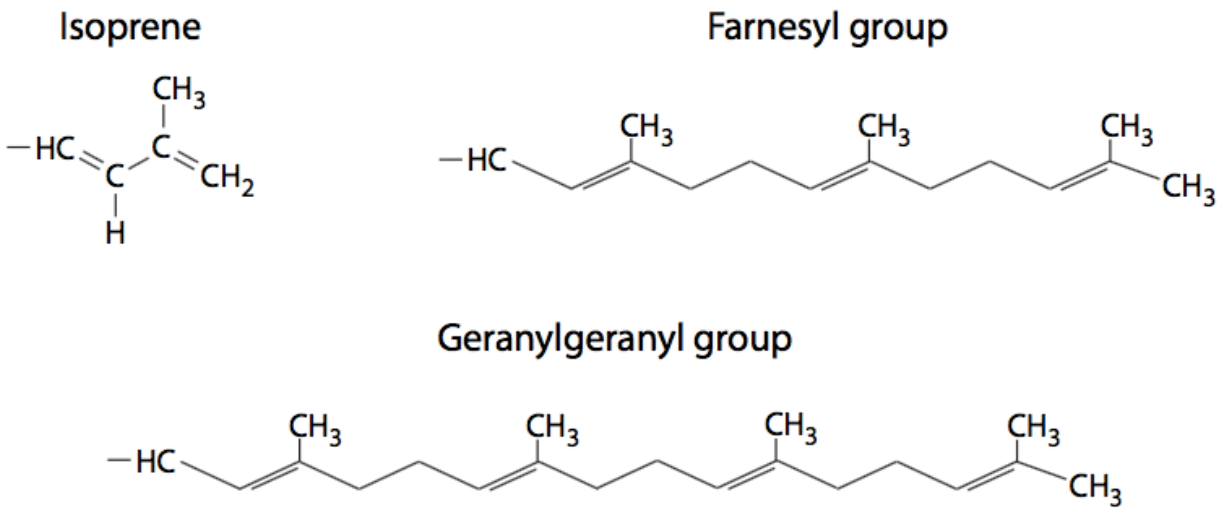


Figure 4.3.10. Prenylation

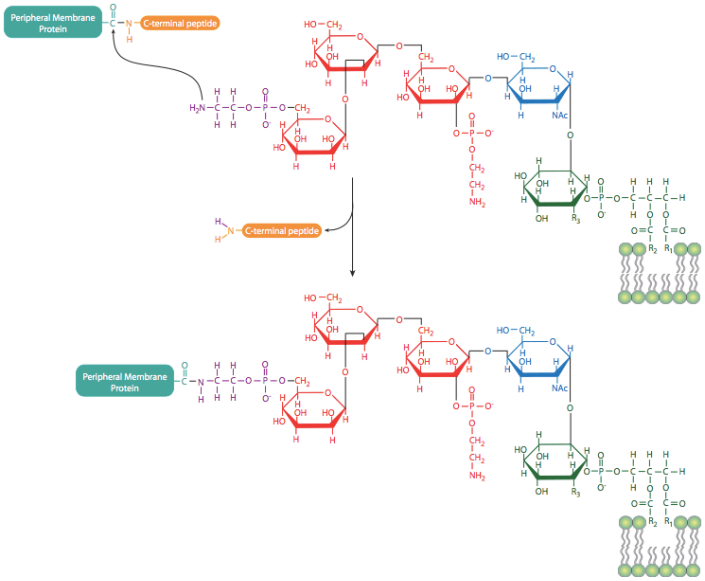


Figure 4.3.11. GPI-linked proteins are connected by the C-terminal carboxyl group to phosphoethanolamine, which is linked to a core tetrasaccharide of three mannose residues and one N-acetylglucoasmine, the latter of which is bound by glycosidic linkage to a phosphatidylinositol.

Of course, not all membrane proteins, or even all transmembrane proteins, are transporters, and the many other functions of membrane proteins - as receptors, adhesion molecules, signaling molecules, and structural molecules - will be discussed in subsequent chapers. The focus here is on the role of membrane proteins in facilitating transport of molecules across the cell membrane.

Transport across the membrane may be either passive, requiring no external source of energy as solute travels from high to low concentration, or active, requiring energy expenditure as solute travels from low to high concentration (Figure 4.3.12).

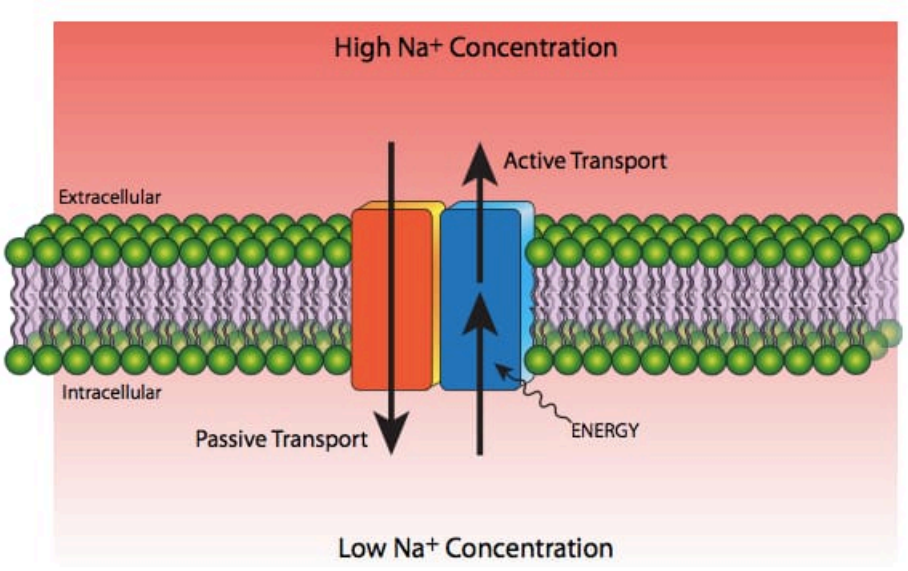


Figure 4.3.12. For Na ions and animal cells, passive transport is inward, sending Na+ from the high concentration outside the cell to the low concentration inside. Active transport requires energy such as ATP hydrolysis to push a Na^+ ion from the low concentration inside the cell to the higher concentration outside.

Passive transport can also be divided into nonmediated transport, in which the movement of solutes is determined solely by diffusion, and the solute does not require a transport protein, and mediated passive transport (aka facilitated diffusion) in which a transport protein is required to help a solute go from high to low concentration. Even though this may sometimes involve a change in conformation, no external energy is required for this process. Nonmediated passive transport applies only to membrane-soluble small nonpolar molecules, and the kinetics of the movement is ruled by diffusion, thickness of the membrane, and the electro-chemical membrane potential. Active transport is always a mediated transport process.

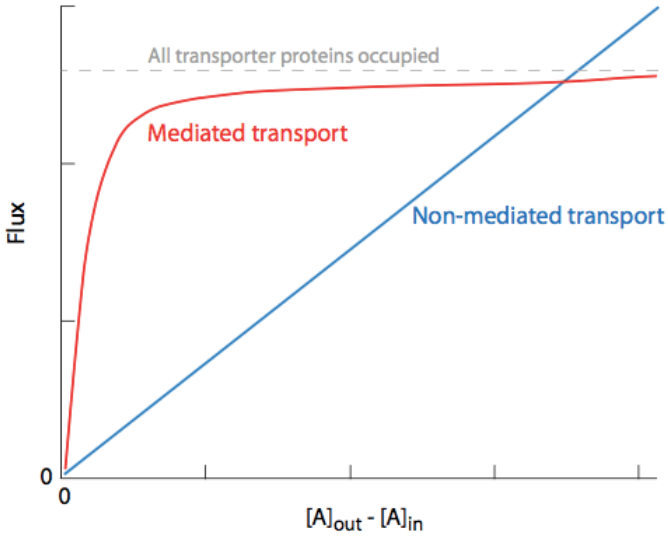


Figure 4.3.13. Non-mediated and Mediated transport: flux vs concentration.

Comparing the solute flux vs initial concentration in Figure 4.3.13, we see that there is a linear relationship for nonmediated transport, while mediated passive transport (and for that matter, active transport) shows a saturation effect due to the limiting factor of the number of available proteins to allow the solute through. Once there is enough solute to constantly occupy all transporters or channels, maximal ux will be reached, and increases in concentration cannot overcome this limit. This holds true regardless of the type of transporter protein involved, even though some are more intimately involved in the transport than others.

In addition to protein transporters, there are other ways to facilitate the movement of ions through membranes. Ionophores are small organic molecules, often (but not exclusively) made by bacteria, that help ions move through membranes. Many ionophores are antibiotics that act by causing the membranes to become leaky to particular ions, altering the electrochemical potential of the membrane and the chemical composition inside the cell. Ionophores are exclusively passive-transport mechanism, and fall into two types.

The first type of ionophore is a small mostly-hydrophobic carrier almost completely embedded in the membrane, that binds to and envelopes a speci c ion, shielding it from the lipid, and then moves it through the cell membrane. The most studied carrier-type ionophore is valinomycin, which binds to K+. Valinomycin is a 12-residue cyclic depsipeptide (contains amide and ester bonds) with alternating d- and l- amino acids. The carbonyl groups all face inward to interact with the ion, while the hydrophobic side chains face outward to the lipid of the membrane. Carrier ionophores are not necessarily peptides: the industrial chemical 2,4-dinitrophenol is an H^+ carrier and important environmental waste concern, and nystatin, an antifungal used to treat *Candida albicans* infections in humans, is a K^+ carrier.

The second type of carrier forms channels in the target membrane, but again, is not a protein. Gramicidin is a prototypical example, an anti-gram-positive antibacterial (except for the source of gramicidins, the gram-positive *Bacillus brevis*) and ionophore channel for monovalent cations such as Na^+, K^+, and H^+. It is im- permeable to anions, and can be blocked by the divalent cation Ca^{2+}. Like valinomycin, gramicidin A is also a made of alternating d- and l- amino acids, all of which are hydrophobic (l-Val/ Ile-Gly-l-Ala-d-Leu-l-Ala-d-Val-l-Val-d-Val-l-Trp-d-Leu-l-Trp-d-Leu- l-Trp-d-Leu-l-Trp). Gramicidin A dimerizes in the membrane to form a compressed b-sheet structure known as a b-helix. The dimerization forms N-terminal to N-terminal, placing the Trp res- idues towards the outer edges of the membrane, with the polar NH groups towards the extracellular and cytoplasmic surfaces, anchoring the pore in place.

Channels are essentially hands-off transport systems that, as the name implies, provides a passage from one side of the cell to another. Though channels may be gated - able to open and close in response to changes in membrane potential or ligand binding, for example - they allow solutes through at a high rate without tightly binding them and without changes in conformation. The solute can only move through channels from high to low concentration. The potassium channel depicted below (Figure 4.3.14A) is an example: there is a selectivity lter (14B) of aligned carbonyl oxygens that transiently positions the K+ ions for rapid passage through the channel, but it does not bind the K^+ for any significant period, nor does the channel undergo any conformational changes as a result of the interaction. Smaller Na^+ ions could (and on rare occasion do) make it through the K+ channel, but because they are too small to be properly positioned by the K^+ filter, they usually pop back out. It should be noted that this channel is a tetramer (14C) and the cutaway diagram in (14A) only shows half of the channel for clarity.

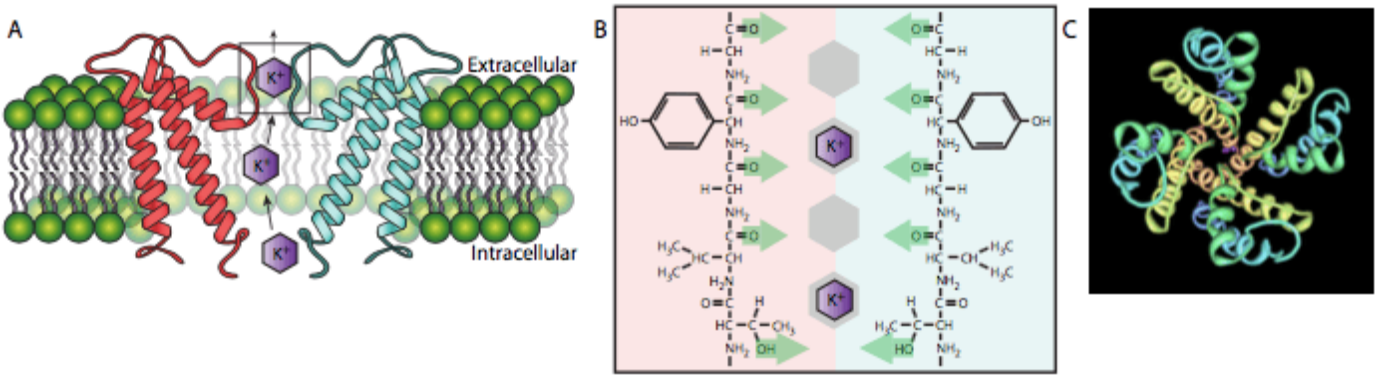


Figure 4.3.14. (A) Half of the tetrameric K^+ channel showing two subunits. (B) Detail of the selectivity lter boxed in A. (C) Top-down image generated from data from the RCSB Protein Data Bank.

While most proteins called “channels” are formed by multiple alpha-helices, the porins are formed by a cylindrical beta sheet. In both cases, solutes can only move down the concentration gradient from high to low, and in both cases, the solutes do not make signi cant contact with the pore or channel. The interior of the pore is usually hydrophilic due to alternating hydrophilic/ hydrophobic residues along the beta ribbon, which places the hydrophobic side chains on the outside, interacting with the membrane core.

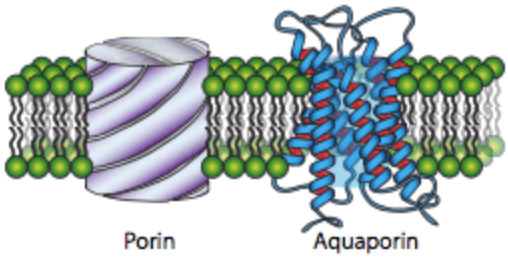


Figure 4.3.15.

Porins are primarily found in gram-negative bacteria, some gram-positive bacteria, and in the mitochondria and chloroplasts of eukaryotes. They are not generally found in the plasma membrane of eukaryotes. Also, despite the similarity in name, they are structurally unrelated to aquaporins, which are channels that facilitate the diffusion of water in and out of cells.

Transport proteins work very differently from channels or pores. Instead of allowing a relatively fast ow of solutes through the membrane, transport proteins move solutes across the membrane in discrete quanta by binding to the solute on one side of the membrane, changing conformation so as to bring the solute to the other side of the membrane, and then releasing the solute. These transport proteins may work with individual solute molecules like the glucose transporters, or they may move multiple solutes. The glucose transporters are *passive transport* proteins, so they only move glucose from higher to lower concentrations, and do not require an external energy source. The four isoforms are very similar structurally but differ in their tissue distribution within the animal: for example, GLUT2 is found primarily in pancreatic b cells, while GLUT4 is found mostly in muscle and fat cells.

On the other hand, the classic example of an *active transport* protein, the Na^+/K^+ ATPase, also known as the Na^+/K^+ antiport, utilizes the energy from ATP hydrolysis to power the conformational changes needed to move both Na^+ and K^+ ions against the gradient. Referring to the Figure 4.3.16, in its resting state, the Na^+/K^+ ATPase is open to the cytoplasm and can bind three Na^+ ions (1). Once the three Na^+ have bound, the transporter can catalyze the hydrolysis of an ATP molecule, removing a phosphate group and transferring it onto the ATPase itself (2). This triggers a conformational change that opens the protein to the extracellular space and also changes the ion binding site so that Na^+ no longer binds with high affinity and drops off (3). However, the ion binding site specificity is also altered in this conformational change, and these new sites have a high affinity for K^+ ions (4). Once the two K^+ bind, the attached phosphate group is released (5) and another conformational shift puts the transporter protein back into its original conformation, altering the K^+ binding sites to allow release of the K^+ into the cytoplasm (6), and revealing Na^+ affinity once again.

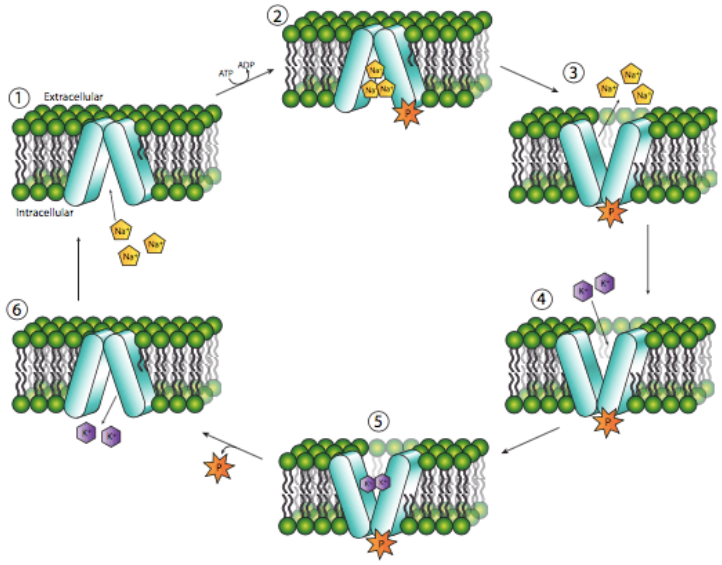


Figure 4.3.16. Active Transport by Na^+/K^+ ATPase. This enzyme pushes three Na^+ ions out of the cell and two K^+ ions into the cell, going against the gradient in both directions and using energy from ATP hydrolysis. [Note: some texts diagram this enzyme activity with separate binding sites for Na^+ and K^+, but recent crystallographic evidence shows that there is only one ion binding site that changes conformation and specificity.]

The Na^+/K^+ ATPase is a member of the P-type family of ATPases. They are named because of the autophosphorylation that occurs when ATP is hydrolyzed to drive the transport. Other prominent members of this family of ATPases are the Ca^{2+}-ATPase that pumps Ca^{2+} out of the cytoplasm into organelles or out of the cell, and the H^+/K^+ ATPase, though there are also P-type H^+ pumps in fungal and plant plasma membranes, and in bacteria.

> Cardiac glycosides (also cardiac steroids) inhibit the Na^+/K^+ ATPase by binding to the extracellular side of the enzyme. These drugs, including digitalis (extracted from the purple foxglove plant) and ouabain (extracted from ouabio tree) are commonly prescribed cardiac medications that increase the intensity of heart contractions. The inhibition of Na^+/K^+ ATPase causes a rise in $[Na^+]_{in}$ which then activates cardiac Na^+/Ca^{2+} antiports, pumping excess sodium out and Ca^{2+} in. The increased $[Ca^{2+}]_{cytoplasm}$ is taken up by the sarcoplasmic reticulum, leading to extra Ca^{2+} when it is released to trigger muscle contraction, causing stronger contractions.

Unlike Na^+ or K^+, the Ca^{2+} gradient is not very important with respect to the electro- chemical membrane potential or the use of its energy. However, tight regulation of Ca^{2+} is important in a different way: it is used as an intracellular signal. To optimize the effectiveness of Ca^{2+} as a signal, its cytoplasmic levels are kept extremely low, with Ca^{2+} pumps pushing the ion into the ER (SR in muscles), Golgi, and out of the cell. These pumps are themselves regulated by Ca^{2+} levels through the protein calmodulin. At low Ca^{2+} levels, the pump is inactive, and an inhibitory domain of the pump itself prevents its activity. However, as Ca^{2+} levels rise, the ions bind to calmodulin, and the Ca^{2+}-calmodulin complex can bind to the inhibitory region of the Ca^{2+} pump, relieving the inhibition and allowing the excess Ca^{2+} to be pumped out of the cytoplasm.

There are three other families of ATPases: the F-type ATPases are proton pumps in bacteria and mitochondria and chloroplasts that can also function to form ATP by running “backwards” with protons owing through them down the concentration gradient. They will be discussed in the next chapter (Metabolism). Also, there are V-type ATPases that regulate pH in acidic vesicles and plant vacuoles, and finally, there are anion-transporting ATPases.

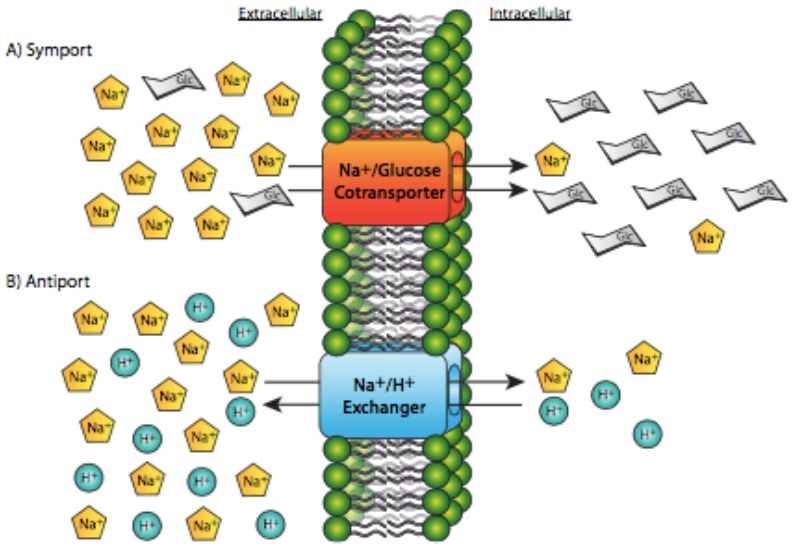


Figure 4.3.17. Symport and Antiport. The terms refer only to direction of solutes in or out of cell, not to energetics. In this symport, the energy release from passive transport of Na^+ into the cell is used to actively transport glucose in also. In the antiport example, Na^+ transport is again used, this time to provide energy for active transport of H^+ out of the cell.

Hydrolysis of ATP, while a common source of energy for many biological processes, is not the only source of energy for transport. The active transport of one solute against its gradient can be coupled with the energy from passive transport of another solute down its gradient. Two examples are shown in Figure 4.3.17: even though one is a *symport* (both solutes crossing the membrane in the same physical direction) and one is an *antiport* (the two solutes cross the membrane in opposite physical directions), they both have one solute traveling down its gradient, and one solute traveling up against its concentration gradient. As it happens, we have used Na^+ movement as the driving force behind both of these examples. In fact, the Na^+ gradient across the membrane is an extremely important source of energy for most animal cells. However this is not universal for all cells, or even all eukaryotic cells. In most plant cells and unicellular organisms, the H^+ (proton) gradient plays the role that Na^+ does in animals.

> Acetylcholine receptors (AchR), which are found in some neurons and on the muscle cells at neuromuscular junctions, are ligand-gated ion channels. When the neurotransmitter (acetylcholine) or an agonist such as nicotine (for nicotinic type receptors) or muscarine (for muscarinic type receptors) binds to the receptor, it opens a channel that allows the ow of small cations, primarily Na^+ and K^+, in opposite directions, of course. The Na^+ rush is much stronger and leads to the initial depolarization of the membrane that either initiates an action potential in a neuron, or in muscle, initiates contraction.

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4.4: The Action Potential in Neurons

The transport of solutes in and out of cells is critical to life. However, in neurons, the movement of ions has another crucial function in metazoan animals: production of action potentials used for neurotransmission. This specialization allows for extremely rapid transmission of information across long distances. An example my mentor would use when teaching basic neuroscience to schoolchildren was a bipolar neuron that extends from the toe to the brain. For information from the toe to be useful, it must reach the brain for processing very quickly. This signal may have to travel several meters in a giraffe, far more in whales. No chemical signal can move that quickly, and despite the popular simplification and dramatic depictions on television, electricity does not flow through neurons as though they were copper wires.

A number of potent toxins have been used in the study of the AchR, including histrionicatoxin, an arrow poison extracted from a South American tree frog, *Dendrobates histrionicus*, curare (d-tubocurarine) isolated from certain plants, and a-bungarotoxin, which is isolated from the Taiwanese snake, the banded krait. All three act by competitive inhibition, blocking the opening of the AchR channel.

Acetylcholine spontaneously dissociates from AchR in about a millisecond, closing the channel. It could then be reactivated by the acetylcholine, but this is tightly regulated by the enzyme acetylcholinesterase (AchE), which is a GPI-anchored protein on the membrane of the target cell, and prevents overstimulation by rapid degradation of the acetylcholine. The military nerve gas sarin, which gained notoriety in a 1995 attack on the Tokyo subway system, works by inactivating AchE, thus causing over- stimulation of AchR.

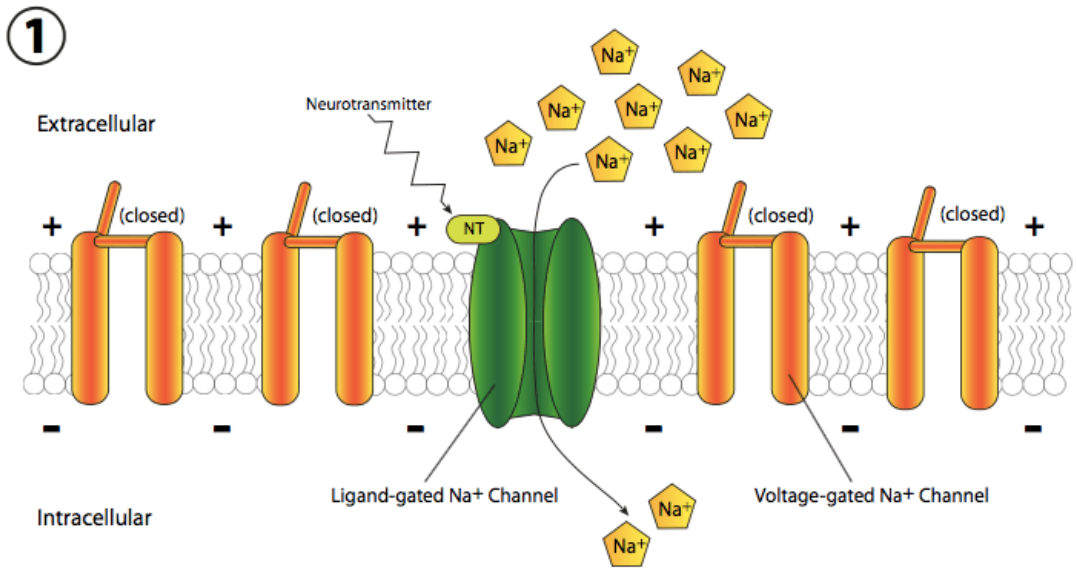


Figure 4.4.18.

The reality does involve rapid changes to membrane potential. Since that is measured in volts and is a difference in electrical charge across the membrane, it is sometimes thought of colloquially as electrical transmission, but the mechanism is completely different from electrons flowing through a wire. Instead, there is a moving change in membrane potential due to sequential opening of ion channels along the length of the axon (the long thin part of the nerve cell). Generally, the signal starts with an excitatory chemical signal, or neurotransmitter (Figure 4.4.18), that binds to a receptor on the neuron. This receptor may be linked to an ion channel or it may itself be a ligand-gated ion channel. In either case, the channel opens and Na^+ comes into the cell.

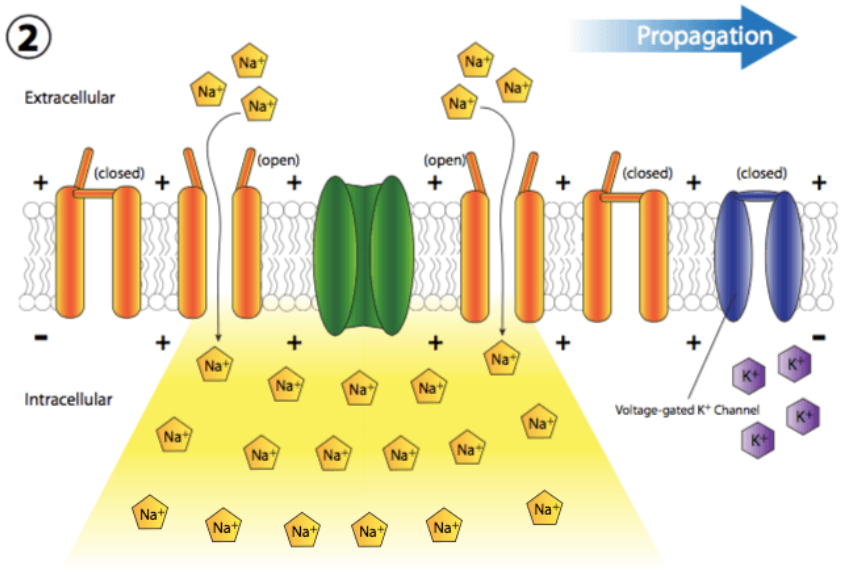


Figure 4.4.19.

The sudden influx of positive charges transiently and locally depolarizes the membrane (more + charges along the inside of the membrane than outside). The depolarization of the membrane near the channel causes all nearby voltage-gated Na^+ channels to transiently open (Figure 4.4.19), thus allowing a new rush of Na^+ ions into the cell that can then depolarize another small section of membrane. Although technically, channels open up on either side of the receptor, normally, the receptor is located on the main cell body, or soma, while most of the ion channels are lined up along the axon. Therefore, there is a de facto directionality to the propagation of the depolarization.

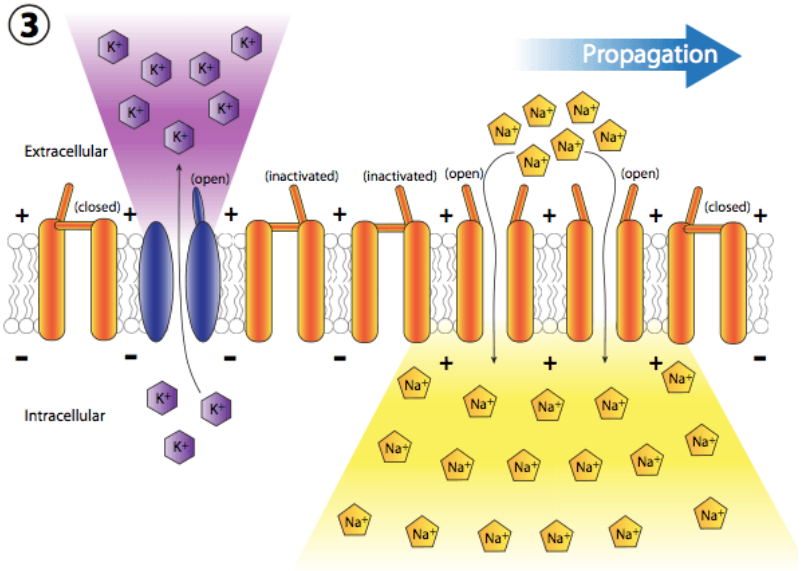


Figure 4.4.20.

More voltage gated Na^+ channels are opened up quickly after their neighboring channels have opened and depolarized their section of the membrane, leading to a long chain reaction of voltage-gated depolarizations all the way down the axon (Figure 4.4.20). Once the signal is propagating down the axon, there are two questions that should come into your head. First, neurons are not disposable cells, so how are all these opened channels reset and the membrane repolarized for the next action potential? And second, why wouldn't the depolarization wave become bidirectional since each little depolarization area spreads out in both directions from the opened Na^+ gate?

In fact, the answers to both questions are closely related, and has to do with the gating mechanism of voltage-gated ion channels (Figure 4.4.21). Voltage-gated ion channels have two gates: one that opens in response to an increase in membrane potential, and

one that closes the channel after a short period of time. This means that there are three potential states for most voltage-gated ion channels: closed, open, and inactivated.

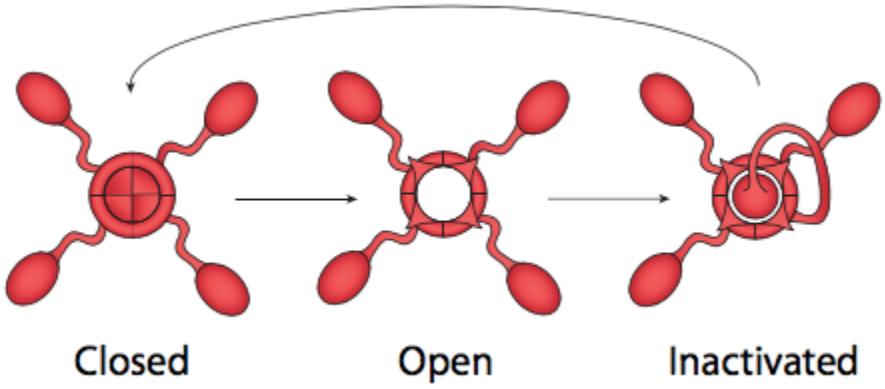


Figure 4.4.21. Three states of a voltage-gated K^+ channel.

The inactivated state occurs when the second gate (more like a plug, really) closes, because this is voltage-insensitive. The plug eventually comes out of the pore and the voltage-sensitive gate is set back in place to the closed state. Voltage-sensitive K^+ channels are tetramers, as shown here, and each subunit carries a potential inactivating domain. The choice of which domain appears to be random. Voltage-sensitive Na^+ channels, on the other hand, also have four transmembrane regions, but they are all part of a single protein, and the channel only has one inactivation gate/domain. However, the three-state gating mechanism is still the same.

Back to the action potential: each new depolarization opens the next adjacent set of voltage gated Na^+ channels, and so on. In a neuronal axon, which is where action potentials occur, the movement of the depolarizations happens very quickly and unidirectionally. It happens quickly because the axon is a very long thin projection of the cell, so the volume is small and therefore the influx of Na^+ can quickly depolarize a long section of membrane. It happens unidirectionally because the previously opened voltage-sensitive Na^+ channels go into a refractory inactivated state that cannot be reopened immediately.

Thus when the adjacent wave of depolarization hits, the previously opened channels do not open again, and the opening of more voltage-gated channels continues unidirectionally away from the start.

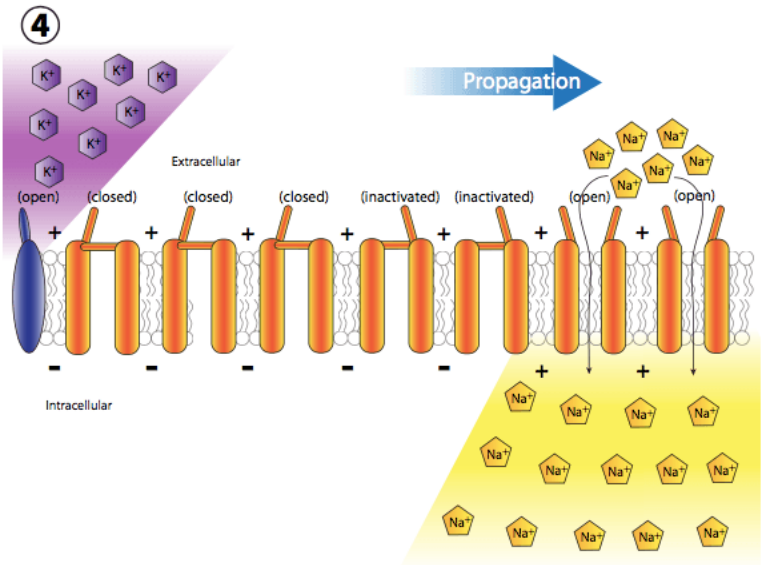


Figure 4.4.22.

But wait, there's more! Just because the Na^+ channels are locked closed doesn't mean that the membrane goes back to normal. What repolarizes the membrane behind the action potential? The voltage-gated potassium channels, which are interspersed

among the Na^+ channels (note purple ions and channels in figs. 20, 22). They open more slowly than the sodium channels (Figure 4.4.23) and thus reach peak flux shortly after the peak flux of Na^+. Thus, the recovery phase, or repolarization back towards the resting potential, is helped by both cessation of the inward Na^+ movement and continued outward K^+ movement.

Since all action potentials behave the same way from the standpoint of changes in membrane potential, the difference between stronger and weaker nerve signals is in the frequency/rate of action potential firing, not in magnitude of the ion flux.

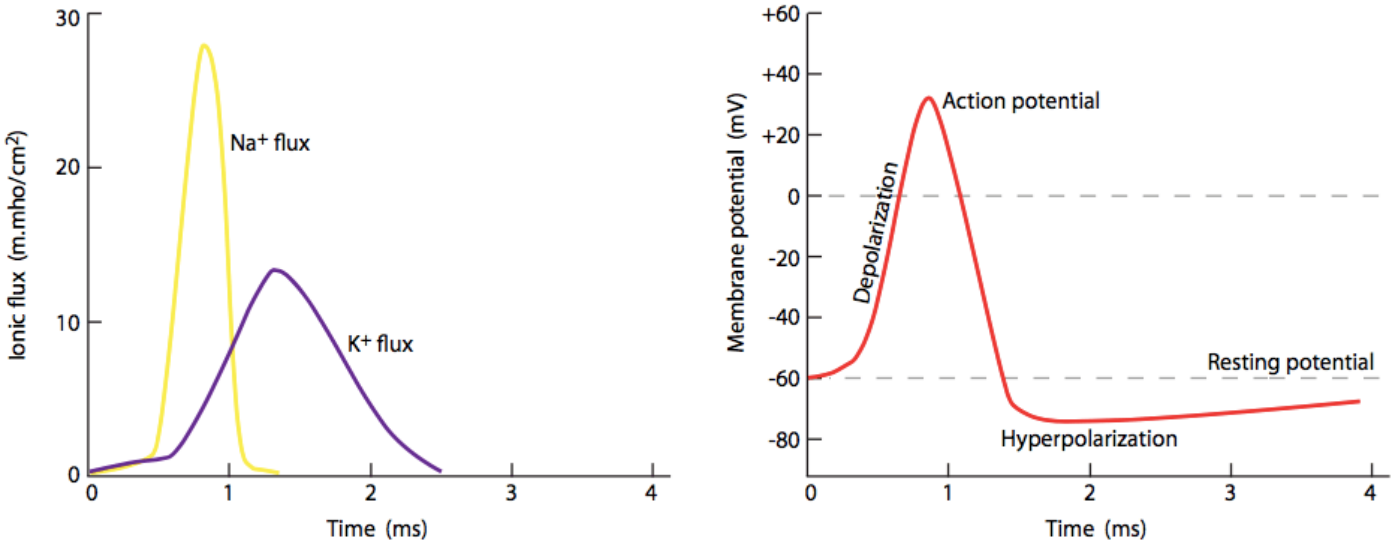


Figure 4.4.23. The contributions of Na^+ flux (depolarizing) and K^+ flux (repolarizing) to the neuronal action potential. Remember that the Na^+ movement is into the cell, while K^+ is moving out.

Since neuronal transmission of information is critical to many systems, it is not surprising that a variety of organisms have evolved toxins that can paralyze or kill an attacker or prey. Interestingly, the primary target of the toxins is the voltage-gated Na^+ channel, and not the K^+ channel.

The toxin most widely used in research is tetrodotoxin (TTX), which is derived from the skin and certain organs of the puffer fish (*Fugu rubripes*). This fish is considered a delicacy (primarily in Japan), and even when prepared by expert and specially-trained chefs, the small amount of residual TTX in the flesh causes a tingling numbing of the tongue as neural excitation is blocked. Interestingly (but unrelated to ion channels) fugu has a highly compressed genome - where most other eukaryotes have large swaths of non-coding regions, fugu has little, and its genes are very close together. In fact, it has almost as many predicted genes as the human genome in only 1/8th the DNA!

Saxitoxin (STX) is another poison that acts on the voltage-gated Na^+ channel. It is produced by the plankton that cause "red tide" (dino agellates of the genus *Alexandrium*, *Gymnodium*, and *Pyrodinium*) and is concentrated by filter-feeders such as mussels, oysters, and other shell fish. Interestingly, STX was once investigated by the US military as a nerve agent for chemical warfare, under the designation TZ. Both STX and TTX bind to the external surface of the closed voltage-gated Na^+ channel near the mouth of the pore, preventing opening, and thereby blocking Na^+ flow.

Batrachotoxin, on the other hand, binds to voltage-gated Na^+ channels when they are open rather than closed, so there is a massive continuous influx of Na^+. A product of another South American tree frog, *Phyllobates aurotaenia*, this toxin is used as a hunting poison by aboriginal tribes in the area, and is the most potent venom currently known. With a lethal dose of less than 2 mg/kg, batrachotoxin is roughly ten times more deadly than TTX. However, to put this in perspective, botulinum toxin, used pharmacologically as Botox® and the cause of botulism, is well over a hundred times more potent (though acting through a completely different mechanism - prevention of neurotransmitter release).

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5: METABOLISM I – CATABOLIC REACTIONS

Life requires energy. As our discussion of biomolecules pointed out, the major functional components of the cell are mostly polymers - long chains of smaller individual molecular units. Each addition of a small link to the chain costs energy. Chemical reactions that build up complex molecules from simple ones are known as *anabolic* reactions. Conversely, heterotrophic organisms such as animals ingest food made up of these large polymers, which, when broken down in the digestive process, release energy for maintaining and building that organism. Such chemical reactions, in which complex molecules are broken down to simpler components, are classified as *catabolic* reactions. Taken as a group of reactions within a cell or even an organism, they can be referred to as the cell's or organism's anabolism or catabolism. The sum total of both types of reactions is the cell's *metabolism*.

5.1: Glycolysis

5.2: Fermentation

5.3: 5.3 The TCA Cycle

5.4: Oxidative Phosphorylation

5.5: Uncoupling Electron Transport from ATP Synthesis

5.6: Structure of Electron Carriers

5.7: 5.7 Starch and Glycogen Depolymerization

5.8: 5.8 Fatty Acid Breakdown

5.9: Amino Acid Degradation

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5.1: Glycolysis

Nearly all metabolic reactions are catalyzed by enzymes in order to keep up with the energy and material demands of the cell. In fact, the discussion of some of the metabolic processes in this chapter will almost seem to be laundry lists of enzymes. We will begin with one such list in describing the catabolism of the simple sugar, glucose, through the process of glycolysis.

Glycolysis

Whether the cell is prokaryotic or eukaryotic, one of its basic methods for generating usable energy is glycolysis. This process uses glucose, which is the most common energy source for most cells. However, glucose cannot be directly broken down to provide energy for the cell: glycolysis is a process that breaks it down in a series of reactions to create adenosine triphosphate (ATP), which is the most common energy "currency" of the cell. That is, ATP *can* release usable energy in a single reaction.

Glucose, being a 6-carbon sugar, has a large amount of potential energy stored in its bonds. However, since it is thermodynamically stable, it would take the investment of a lot of external energy to release the energy of glucose in one step (e.g. lighting it on fire to break it down into CO_2 and H_2O), and not only is it impossible for cells to generate that kind of energy at once, the cell has no mechanism to use all the energy released at one instant in time. Most of it would be wasted as excess heat. Instead, the cell uses enzymes to destabilize and break down the sugar through a series of conversions into intermediate compounds. The basic process and enzymes involved are as follows.

1. Glucose is phosphorylated by hexokinase to make Glucose-6-Phosphate. The enzyme is so named because it is a kinase (puts a phosphate group on) that acts on a hexose (six-carbon sugar). In this case, it places the phosphate on the 6-carbon of glucose. However, hexokinase can also phosphorylate other hexoses such as fructose and mannose (all in the D-conformation). There are two major reasons this is good for the cell. Since glucose concentration is higher inside the cell than outside, there is pressure for it to move back out of the cell. By converting it to G6P, it is no longer part of the glucose concentration gradient, and it has a charged phosphate group, making it nearly impossible to leak out of the membrane. The addition of the phosphate also increases the energy in the molecule, making it less thermodynamically stable, so that it can be broken down. This reaction requires the use of ATP as a phosphate donor and the energy needed to attach it. That is, energy is *used* in this step, not produced. Consider it an investment of energy though, since by the end of glycolysis, more ATP is produced than used.

> Hexokinase requires ATP in the form of a complex (to the 2nd and 3rd phosphate groups) with a divalent cation, typically Mg^{2+} *in vivo*. ATP alone is actually a competitive inhibitor of hexokinase. The product, G6P, also functions as an inhibitor, thus providing some measure of feedback regulation. In fact, muscle cells using glycogen stores convert the glycogen directly to G6P, so hexokinase activity is very low in those cells.

2. Glucose-6-Phosphate is converted to Fructose-6-Phosphate by phosphoglucose isomerase. As the name implies, the isomerase simply rearranges the existing atoms within the G6P to make the F6P without removal or addition of any atoms.

3. Fructose-6-Phosphate is phosphorylated by phosphofructokinase (PFK) to Fructose- 1,6-bisphosphate. There is again an investment of an ATP to provide the phosphate group and the energy to attach it.

> PFK is an important regulator of glycolysis. It is a tetrameric protein, and each subunit has two binding sites for ATP: one is the normal substrate site, the other is an inhibitory site such that binding of ATP lowers the enzyme's affinity for F6P. ATP is not the only regulator of PFK activity: AMP is also a positive regulator of PFK, and can increase it up to 5-fold.

4. The Fructose-1,6-bisphosphate is cut in half by aldolase, yielding a molecule of dihy- droxyacetone phosphate and a molecule of glyceraldehyde-3-phosphate.

There are two classes of aldolases: class I are found in animals and plants, while class II are found in fungi and bacteria. Class I require no cofactors, but class II require a divalent cation (physiologically usually Fe^{2+} or Zn^{2+}).

5. The G3P can participate in the next reaction, but the dihydroxyacetone phosphate, despite its similarity, cannot. So, it needs to be rearranged by triose phosphate isomerase, which converts it to another molecule of glyceraldehyde-3-phosphate.

Triose phosphate isomerase is a "perfect enzyme" that catalyzes the formation of product as fast as the enzyme and substrate can make contact in solution (i.e. rate is purely diffusion-limited).

6. Each of the two molecules of G3P generated from the glucose molecule now undergo oxidation catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of NAD^+ and inorganic phosphate (P_i). Each of these reactions produces 1,3-bisphosphoglycerate, which has a high-energy phosphate group, and NADH. NADH is a high energy electron carrier (electron comes from G3P). In eukaryotes with an
aerobic environment, this NADH will likely be used to help generate ATP through the tricarboxylic acid cycle (aka Krebs cycle or citric acid cycle). In anaerobic situations, the NADH will participate in fermentation for reasons discussed in the next section.

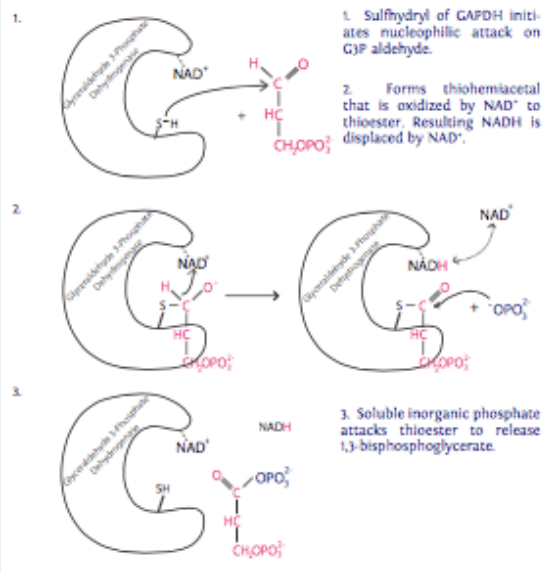


Figure 5.1.1. NAD^+ is reduced by glyceraldehyde-3-phosphate dehtdrogenase to form NADH. The NADH is released by substitution with another NAD^+.

7. The phosphate group on the 1-carbon of 1,3-bisphosphoglycerate is transferred to ADP by phosphoglycerate kinase to make 3-phosphoglycerate and ATP (finally!). From the two molecules of G3P entering step 6, we get two molecules of ATP to provide energy for the cell in this step. Recalling the earlier investment of ATP (in steps 1 and 3), the reaction has only "broken even" at this point. 2 in, 2 out.

The name of the enzyme suggests that a phosphate is added to phosphoglycerate. This is not a mistake: remember that enzymes can catalyze reactions in either direction, depending on reaction conditions. Under conditions of high phosphoglycerate and ATP, phosphorylation of phosphoglycerate would occur. However, the physiological conditions are a relatively high concentration of the 1,3-bisphosphoglycerate in comparison to relatively low levels of phosphoglycerate thus driving the reaction "backwards" with respect to the naming of the enzyme.

8. The 3-phosphoglycerate is then rearranged by phosphoglycerate mutase to make 2-phosphoglycerate. This molecule has a higher free energy of hydrolysis than when the phosphate group is on the 3-carbon.

The action of phosphoglycerate mutase is not just the intramolecular phosphate group transfer that it seems to be at first glance. The enzyme must first be activated by phosphorylation, and it is the enzyme's phosphate that is added to the 2-carbon of 3PG. The doubly-phosphorylated intermediate then transfers its 3-phosphate to the enzyme, and 2PG is released.

9. That energy is used to create ATP, as the 2-phosphoglycerate undergoes dehydration by enolase to make phosphoenolpyruvate (PEP).

PEP is made because hydrolysis of the phosphate from 2PG does not release enough energy to drive phosphorylation of ADP to ATP. PEP hydrolysis, on the other hand, releases significantly more than needed.

10. Pyruvate kinase then transfers a high energy phosphate group from PEP to ADP, producing an ATP for use by the cell, and pyruvate.

Pyruvate kinase requires not only divalent Mg^{2+} as with most other kinases, but also K^{+}. The enzyme works in two steps: the ADP attacks the PEP phosphorus to make ATP and enolpyruvate. Enolpyruvate is then converted to its keto tautomer.

Keeping in mind the doubling of reactions from steps 6-10 (splitting of fructose-1,6- bisphosphate generates two G3P), the total usable energy production from glycolysis of a single molecule of glucose is 4 ATP and 2 NADH. However, the net ATP production is only 2 ATP if we remember the initial investment of two ATP in the early steps. *Not really anything to write home about.* Furthermore, although the NADH and pyruvate can participate in the tricarboxylic acid cycle in aerobic eukaryotic situations to generate a significant amount of ATP, in anaerobic situations, they do not produce usable energy.

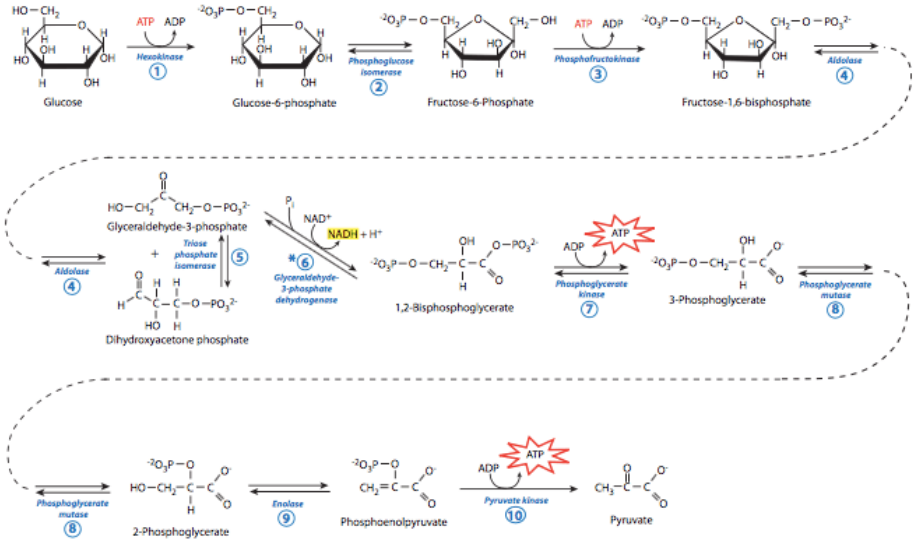


Figure 5.1.2. Overview of Glycolysis.

*Bidirectional arrows indicate enzymes used for both glycolysis and gluconeogenesis. Unidirectional arrows indicate enzymes that only function in glycolysis. *Note that reactions 6-10 are occurring in duplicate (two G3P from one glucose).*

Thus anaerobic ATP production, i.e. glycolysis, is far less efficient at extracting energy from a glucose molecule than aerobic ATP production, which can generate approximately 38 ATP per glucose. On the other hand, when a lot of ATP must be generated quickly, glycolysis is the mechanism of choice, in cells such as the fast-twitch fibers of skeletal muscle. These cells actually have very few mitochondria because glycolysis can produce ATP at a much higher (up to 100 times) rate than oxidative phosphorylation. What happens to the pyruvate and NADH? In aerobically metabolizing cells, they go to the mitochondria for the

TCA cycle and oxidative phosphorylation. In anaerobes, they undergo fermentation.

> Note that the NADH produced by glycolysis in the cytoplasm does not *directly* participate in oxidative phosphorylation in the mitochondria since the inner mitochondrial membrane is impermeable to it, but it sends a "virtual equivalent" into the mitochondria via one of two pathways: the aspartate-malate shuttle combines malate-α-ketoglutarate antiports, aspartate-glutamate antiports, and metabolite interconversion by transaminase with malate dehydrogenase to oxidize NADH cytoplasmically and use the energy generated to reduce NAD^+ in the mitochondrial matrix; the other pathway is a DHAP shuttle system, in which NADH is used to reduce dihydroxyacetone phosphate to glycerol-3-P using a cytoplasmic glycerol-3-phosphate dehydrogenase, and the cycling the DHAP to glycerol-3-P via a flavoprotein dehydrogenase embedded in the inner mitochondrial membrane. This flavoprotein dehydrogenase takes the electrons from glycerol-3-P to make $FADH_2$, which can participate in the electron transport chain.
>
> The DHAP or glycerophosphate shuttle is less efficient than the malate-aspartate shuttle, generating approximately 2 ATP vs 2.7 ATP per NADH. However, it can operate even when the concentration of cytoplasmic NADH is low, as happens in tissues/cells with a very high metabolic rate (including skeletal muscle and brain), while the malate-aspartate shuttle (prevalent in liver and heart) is sensitive to the relative concentration of NADH and NAD^+.

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5.2: Fermentation

When the average person hears the word “fermentation” he probably thinks about alcohol. As you no doubt recall, glycolysis gave us some usable energy in the form of ATP, and then there are the other products, NADH and pyruvate. As we shall see in the next section, if the cell is eukaryotic and oxygen is available, then those molecules can help make more ATP. If no oxygen is available or the cell is just a lowly prokaryote, it undergoes fermentation to produce either lactate or ethyl alcohol. Why does the cell need lactate or ethanol? It doesn’t, although the lactate can contribute to overall metabolism. What the cells do need is NAD^+ so that glycolysis can continue beyond step 6. Without fermentation, continued glycolysis would convert all of the NAD^+ to NADH, and then be stuck, unable to continue. So the primary reason for fermentation, whichever path it takes, is to regenerate NAD^+ from the NADH.

Lactate Fermentation

In **lactate fermentation**, the pyruvate is converted to lactate by lactate dehydrogenase. This reaction requires the oxidation of NADH, which thus provides NAD^+ to the cell for continued glycolysis.

For many cells, the lactate is a waste product and excreted. In fact, this is the case with most muscles: the lactate is carried by the blood from the muscle cells to the liver, where it can be converted to glucose. Thus, although lactate is formed at high rates when muscles are overworked and become fatigued, it is not directly the cause of muscle fatigue. As oxygen availability cannot keep up with aerobic ATP production, and larger and larger proportions of the ATP generated come from glycolysis with fermentation. The current model of muscle fatigue posits that it is due to acidification of the muscle cell as it undergoes rapid glycolysis.

However, in some tissues and cell types, particularly in the heart and brain of higher animals, cell membranes are highly permeable to lactate, the cells can readily convert the lactate to pyruvate, and since these are highly oxygenated tissues, the pyruvate is then used for the TCA cycle and oxidative phosphorylation to generate ATP. In fact, some non-neuronal support cells in the brain (astrocytes) generate and excrete copious lactate which is taken up by the neighboring neurons to fuel ATP production.

Alcohol Fermentation

In **alcohol fermentation**, pyruvate is first acted upon by pyruvate decarboxylase, which liberates a CO_2 molecule and produces acetaldehyde. Acetaldehyde is then acted upon by alcohol dehydrogenase, using NADH, generating NAD^+ and ethanol. Here, like with lactate fermentation, the desired product is the regenerated NAD^+. Ethanol is excreted, and in most animals, is converted to acetaldehyde and then acetic acid, before finally ending up as acetyl-CoA.

As with glycolysis, fermentation can and does take place in cells that are able to make ATP by oxidative phosphorylation. The relative contribution of glycolysis and oxidative phosphorylation to the cellular ATP pool is determined dynamically by physiological conditions.

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5.3: 5.3 The TCA Cycle

So you're a hot young eukaryote sporting all kinds of fancy internal membranous organelles, with a need to prove yourself better than the old guard prokaryotes — what do you do? Well, make scads of ATP, of course! And seemingly effortlessly at that, using only the dregs left over after glycolysis has taken its pass at a glucose molecule: NADH and pyruvate. Glycolysis in eukaryotes, as be ts its prokaryotic origins, happens in the cytoplasm. The TCA cycle (also called the citric acid cycle) happens inside the matrix of the *mitochondria*, a double-membraned organelle.

The pyruvate needs to make its way from the cytoplasm, through both outer and inner mitochondrial membranes, and into the mitochondrial matrix. How does this work? The outer membrane is porous, being riddled with large relatively nonspecific anion channels known as voltage-dependent anion channels (VDACs), and will readily admit pyruvate. In contrast, the inner mitochondrial membrane is highly impermeable, and entry of pyruvate is specifically regulated by a pyruvate transporter protein.

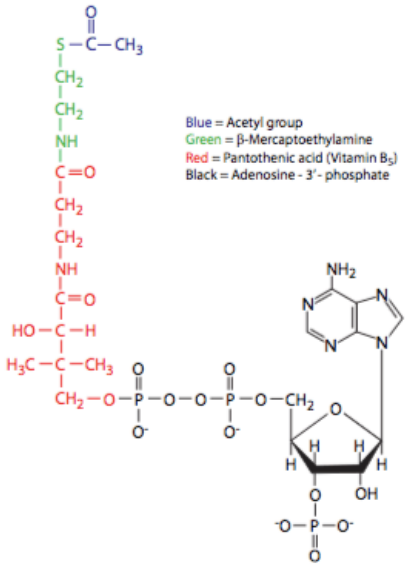


Figure 5.3.3. Acetyl-Coenzyme A is composed of four distinct molecular parts.

1. Once the pyruvate enters the mitochondrial matrix, the pyruvate dehydrogenase complex (consisting of three enzyme subunits E1, E2, and E3) converts it to acetyl-CoA (Figure 5.3.3) for entry into the tricarboxylic acid cycle (TCA). This reaction generates NADH and liberates CO_2.

> Pyruvate dehydrogenase complex (Figure 5.3.4) is actually an amalgamation of three enzymes. That is, there are three subunits to the complex: pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3). These three subunits are associated by noncovalent bonds. The pyruvate dehydrogenase subunit E1 acts first, using the cofactor thiamine pyrophosphate (TPP) to help remove a CO_2 from the pyruvate to generate hydroxyethyl-TPP.
>
> This is immediately used as a substrate by E2, resulting in regeneration of TPP and reactivation of pyruvate dehydrogenase, and also making the intermediate acetyl-dihydrolipoamide. Coenzyme A, which is also a substrate for E2, has a sulfhydryl group that attacks the acetyl group of acetyl-dihydrolipoamide. The acetyl group is immediately transferred to Coenzyme A to form the Acetyl-CoA that enters the TCA cycle.
>
> The final step is for the dihydrolipoamide to be oxidized back to lipoamide by E3. It is this oxidation step that generates the NADH from NAD^+.

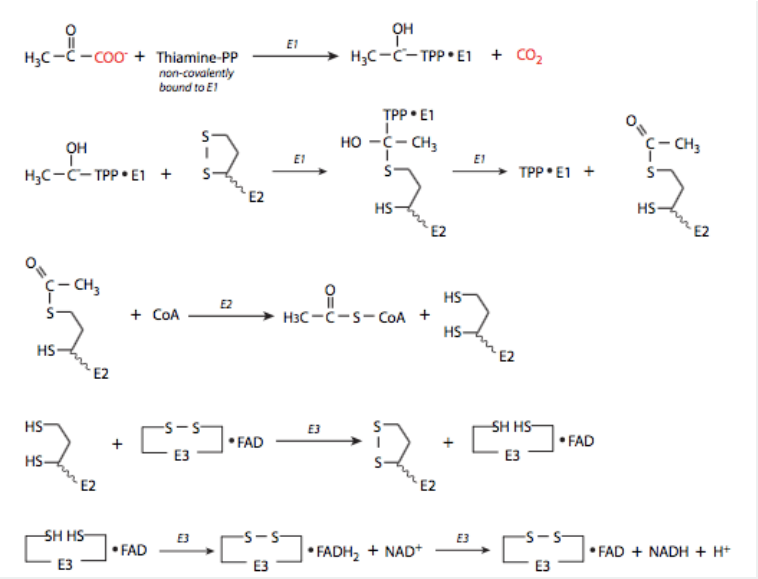


Figure 5.3.4. Pyruvate dehydrogenase is a complex of three enzymatic subunits, E1, E2, and E3, which work in sequence as depicted here.

Now consider the breakdown of glucose. Recall that the complete breakdown of that six-carbon sugar should yield six single-carbon molecules of carbon dioxide. In glycolysis, the glucose is broken down into two molecules of three-carbon pyruvate. As the pyruvate is converted to acetyl-CoA, one CO_2 is generated per molecule of pyruvate. That leaves just four carbons (in two 2-carbon molecules of acetyl-CoA) out of the original glucose 6. The TCA cycle will liberate each of those carbons as CO_2 as well. Knowing the reactions in which the remaining carbons are released is a good way to study the first half of the TCA cycle.

As an integral part of coenzyme A, vitamin B_5, or pantothenic acid, is needed for the TCA cycle, and therefore, for normal efficient generation of ATP. However, unlike some other vitamins, B_5 deficiency is rare, and usually associated with deficiency in other vitamins or general malnourishment. On the other hand, deficiency in another B vitamin involved in pyruvate dehydrogenase activity (Figure 5.3.4), thiamine (B_1), can lead to disease symptoms known as beriberi.

Arsenic, or more specifically arsenic-containing compounds such as arsenite and arsenate, are poisonous to cells by interfering with this reaction. The arsenic compound can interact with dihydrolipoamide, resulting in cyclization by bonding of both sulfhydryl sulfurs to the arsenic atom. This prevents E2 from working, and acetyl-CoA cannot be generated for ATP production via TCA cycle and oxidative phosphorylation. It should be noted that these arsenic compounds also affect other sulfhydryl-containing compounds, and within the context of the TCA cycle, it can also inactivate α-ketoglutarate dehydrogenase, which is similar to the pyruvate dehydrogenase.

Beriberi symptoms are classified in two groups: wet beriberi affects the cardiovascular system with symptoms such as enlarged heart, lung congestion, shortness of breath, swelling of lower legs, congestive heart failure; dry beriberi (also known as Wernicke-Korsakoff syndrome) affects the nervous system. Symptoms include polyneuritis in both central and peripheral nervous system, leading to pain, tingling, loss of sensation in extremities, loss of muscle function or paralysis of the lower legs, vomiting, nystagmus, and eventually mental confusion, speech difficulties, coma and death.

Genetic deficiencies in the pyruvate dehydrogenase complex lead to similar, but more immediately severe problems. The most common mutation is an X-linked dominant mutation in the a subunit of E1. PDC loss-of-function mutations as well as mutations in pyruvate carboxylase and mutations in cytochrome oxidase, are considered causes of Leigh's disease, which is often neonatally fatal, though exceptions have survived a little over a decade. Severe lactic acidosis and the inability to generate sufficient energy, especially in neurons (which would normally be able to metabolize fat - see section of fatty acid catabolism - but cannot in these patients) and muscle cells, is the underlying cause of the symptoms.

2. Acetyl-CoA enters the tricarboxylic acid cycle as a substrate of citrate synthase, which adds it to oxaloacetate to make citrate. This is the reason that this cycle is also called the citric acid cycle. Citrate, having three carboxyl groups, is a tricarboxylic acid, leading to the name that this text will use. The other common name for this is the Krebs cycle, as it was first proposed by Hans Krebs in 1937.

> Citrate synthase is a dimeric enzyme that in its native form has a binding cleft for oxaloacetate. Binding of oxaloacetate causes a conformational shift closing the oxaloacetate binding site, locks it in and simultaneously reveals the acetyl-CoA binding site. The current model for this reaction involves three steps: Acetyl-CoA is converted to an enol intermediate, which attacks the oxaloacetate to form citronyl-CoA (S-citryl-CoA), which is then hydrolyzed to citrate and Coenzyme A.

3. In the next step, aconitase rearranges citrate to make isocitrate.

> Aconitase pushes citrate into a cis-aconitate intermediate, which is then converted to isocitrate. Interestingly, while aconitase contains an Fe-S cluster, it does not appear to participate in redox reactions as is usually the case for such groups. Instead, its purpose is to hold the cis-aconitate in its place within the enzyme as it [the cis-aconitate] undergoes a bizarre molecular flip on its way to isocitrate.

Sodium fluoroacetate, also known as compound 1080, is a common pesticide that is used primarily against rodent and other mammalian pests, and can act in humans if ingested. Once introduced to the organism, it can be converted to fluoroacetyl-CoA and then to fluorocitrate, which then acts as a competitive inhibitor of aconitase. As such, the poisoning most severely and quickly affects tissues with high energy needs. No effective antidotes are recognized for lethal doses of fluoroacetate poisoning.

4. Isocitrate is a substrate for isocitrate dehydrogenase, which transfers a high energy electron from the isocitrate onto NAD^+ to make NADH and α-ketoglutarate. This reaction also liberates one CO_2. For those keeping track at home, that leaves two more carbons from the six in glucose.

> The NAD^+-dependent isocitrate dehydrogenase is actually found in two isoforms in mammals: an NAD^+-utilizing isoform in the mitochondrial matrix, and an isoform that uses $NADP^+$ that is found in the cytosol as well as the mitochondria. The reaction starts with NADH-generating oxidation of isocitrate to oxoalosuccinate, which is then decarboxylated with the help of a Mn^{2+} or Mg^{2+} cofactor to release the carbon dioxide and form α-ketoglutarate.

5. Alpha-ketoglutarate is also oxidized (by α-ketoglutarate dehydrogenase) generating NADH and succinyl-CoA. Like acetyl-CoA, this CoA-associated compound contains a high energy thioester bond. This reaction liberates the final CO_2 from the glucose.

> α-ketoglutarate dehydrogenase is very similar to pyruvate dehydrogenase complex structurally and mechanistically. There are three enzymes: the α-ketoglutarate dehydrogenase, a dihydrolipoyl transsuccinylase, and dihydrolipoyl dehydrogenase. Also similar to pyruvate dehydrogenase complex, the end product is a molecule containing a high energy thioester bond.

6. The CoA is regenerated by succinyl-CoA synthetase, which also forms succinate and GTP or ATP. This GTP is energetically an ATP equivalent, and made in animal cells. Bacterial and plant homologues of this enzyme use ADP and make ATP. Formation of this ATP/GTP is possible because the high-energy thioester bond of succinyl-CoA is broken.

> Succinyl-CoA synthetase first brings together the succinyl-CoA and inorganic phosphate (in solution within the mitochondrial matrix as well as the cytosol) to produce succinyl phosphate and liberate the CoA. Then the phosphate is transferred from the succinyl phosphate, to the enzyme itself temporarily, which then drops the succinate. And finally, the phosphate is transferred to GDP/ADP.

7. Next, the succinate is oxidized. The enzyme that does this, succinate dehydrogenase, is a little different from the other dehydrogenases because this one happens to be embedded in the inner mitochondrial membrane, and instead of transferring the electron to NAD^+, the electron is transferred to FAD, making $FADH_2$, and fumarate. The energy in $FADH_2$ can also be used to

power ATP production similar to the energy in NADH.

> Even though the usual intro-class simplification is that $FADH_2$ is roughly an equivalent to NADH, the situation is actually more complicated. Unlike NAD^+, and for that matter, unlike most occurences of FAD, the FAD is covalently bound to the succinate dehydrogenase. Therefore, it is not a soluble metabolite, nor is it available to be reoxidized quite like NADH. It is, of course, reoxidized. But, this occurs within the context of the electron transport chain (where it is known as Complex II), with the help of Coenzyme Q.

8. Fumarase catalyzes the addition of water to the fumarate to generate malate.

> The carbon double bond of fumarate is attacked by a hydroxyl (OH^-) to form a carbanion transition, which then takes on a proton (H^+) from the enzyme to form the malate. The fumarase enzyme is protonated at the same time that it binds fumarate, and is deprotonated at the end to form the malate.

9. Malate is oxidized by malate dehydrogenase in the final reaction of the TCA cycle to generate more NADH, and oxaloacetate, the latter of which can then be added to acetyl-CoA to start the cycle all over again.

> Malate dehydrogenase is similar in structure to the lactate dehydrogenase and the alcohol dehydrogenase mentioned in the fermentation section. Energetically, the standard free energy change of this reaction is very highly positive (29.7 kJ/mol) but the oxaloacetate is quickly converted to citrate, so that more formation of oxaloactetate is favored over malate formation.

Now that the complete cycle has been described, it should be noted that the regulation of this cycle is primarily through acetyl-CoA and oxaloacetate availability, as well as NADH concentration. As respiration rate increases, NADH levels drop as they are oxidized to make ATP (see next section). This drop in [NADH] then causes an increase in oxaloactetate, which is then used by citrate synthase. [Acetyl-CoA] is regulated by its synthesis by pyruvate dehydrogenase. On the reverse side of regulation, both NADH and succinyl-CoA are strong inhibitors of α-ketoglutarate dehydrogenase. Thus as NADH is used up, the enzyme is disinhibited and increases its production of more NADH.

Having taken the 2 pyruvates created during glycolysis through the TCA cycle to complete oxidation into CO_2, what is our intrepid eukaryotic hero left with? Two ATP-equivalents (GTPs) six NADH, and two $FADH_2$. This hardly seems to be a treasure trove of usable energy worth boasting about. Fortunately, the mitochondrion is not finished. Next, the high energy electrons will take a ride on the electron transport chain, and via the magic of oxidative phosphorylation, produce ATP by the bucket.

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5.4: Oxidative Phosphorylation

Oxidative phosphorylation denotes the phosphorylation of ADP into ATP, utilizing the energy from successive electron transports (hence the “oxidative”). The basic concept is that oxidation of NADH, being highly exergonic, can generate the energy needed to phosphorylate ADP. Since oxidation of NADH by oxygen can potentially release 52 kCal/mol (218 kJ/mol), and the energy needed to phosphorylate ATP is approximately 7.5 kCal/mol (30.5 kJ/mol), we should be able to expect the formation of several ATP per oxidized NADH. Indeed, this is what happens, although not directly. As noted with the breakdown of glucose, a one-step oxidation would generate too much energy for cellular processes to handle, and most would be wasted. So instead of oxidizing NADH directly with O_2, the electrons are transferred to a series of gradually lower-energy carriers until finally reaching oxygen. This sequence is the *electron transport chain*.

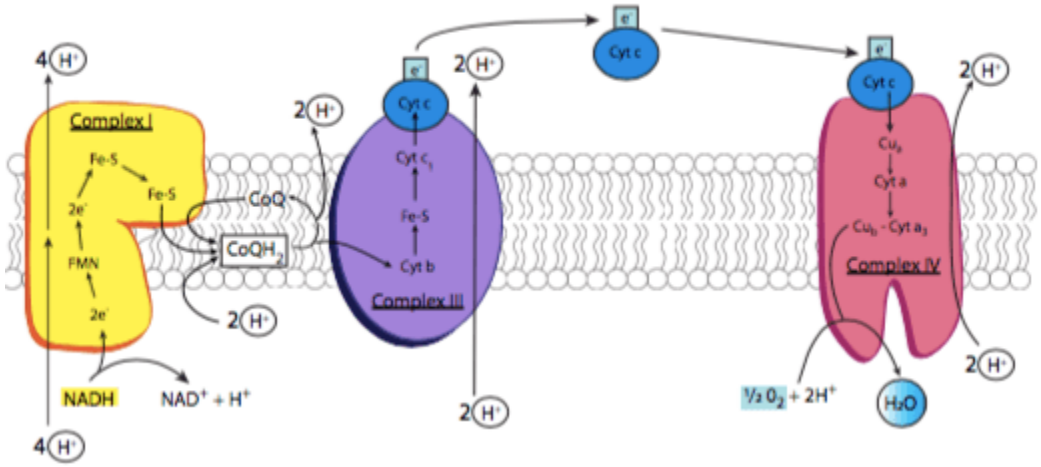


Figure 5.4.6. The primary electron transport pathway in mitochondria. Complexes I, III, and IV are shown. Complex II is pictured in Figure 5.4.10. The complexes are all buried in the inner mitochondrial membrane. Protons are being pumped from the matrix to the intermembrane space utilizing energy sapped from the high energy electrons as they move from a higher-energy carrier to a lower-energy carrier.

The electron transport chain is based on the activity of four major enzyme complexes (conveniently called complexes I-IV) embedded in the inner mitochondrial membrane, along with some small easily diffusible electron carriers to move the electrons from one complex to the next. These complexes are present in extremely high numbers as befits their necessity in generating energy, comprising nearly 75% of the inner membrane mass (in comparison, the plasma membrane of an average eukaryotic cell has a protein concentration closer to 50%). An overview of the process is shown in Figure 5.4.6: as previously noted, electrons are stripped from NADH, and eventually end up on oxygen. As the electrons are moved to lower-energy carriers, energy is released and used to pump protons from the mitochondrial matrix into the intermembrane space.

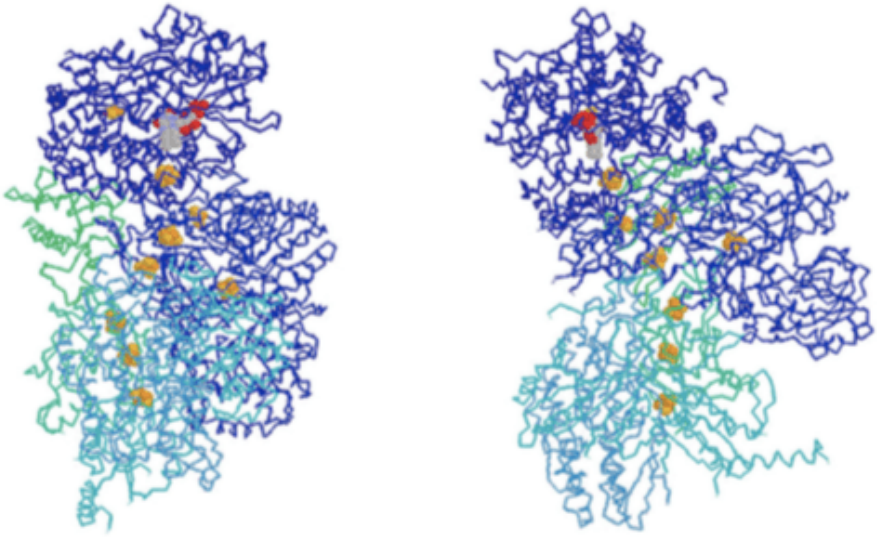


Figure 5.4.7. Although the size of complex I varies somewhat across species, the rough L-shaped three-dimensional conformation is constant. The FMN is located in the larger portion of the complex, while the ubiquinone docking site is located in the short branch. In the Figure above, which depicts two aspects (rotated 90°) of NADH dehydrogenase complex, the FMN is shown in grey and red, while Fe-S centers are shown in orange and yellow. The Figure was generated from data in the RCSB Protein Data Bank.

Complex I is an NADH dehydrogenase. Shown in yellow in Figure 5.4.6, its purpose is to remove a pair of electrons from NADH and transfer them onto ubiquinone (Coenzyme Q or CoQ), a small hydrophobic electron carrier that can then carry the electrons to complex III. This is a multistep process that involves first transferring the electrons onto an associated flavin mononucleotide (FMN) molecule, which then transfers the electrons to a set of iron-sulfur moieties connected to the enzyme complex itself (structure in Figure 5.4.7). Finally, the electrons are moved onto ubiquinone. As these transfers occur, the energy that is released during these transfers powers the pumping of 4 H+ ions across the inner mitochondrial membrane. Complex I is inhibited by rotenone, a pesticide used primarily against insects and shes.

We'll take a mental pass on complex II for now and hit it at the end of this roll call. The reasons will be apparent then.

Complex III is also known as the cytochrome bc_1 complex (Figure 5.4.6, purple). The purpose of this complex is to pass the electrons from ubiquinone onto cytochrome c. The use of ubiquinone is important here, because it is stable with either two, or just one, extra electron. Cytochrome c, on the other hand, can only carry one electron. So, this complex docks ubiquinone, and holds it until it has passed its first electron onto cytochome c, which then moves onto complex IV, and then its second electron onto another cytochrome c. With each transfer, two protons are pumped across the membrane.

Finally, cytochrome c drops the electron off to complex IV, cytochrome c oxidase (Figure 5.4.6, red). Cytochrome c oxidase accomplishes the final step: transferring electrons onto oxygen atoms to make water. The really interesting thing about this process is that the enzyme must hold onto the electrons as they are transferred one at a time from cytochrome c, until it holds four electrons. Then, it can transfer one pair to each of the oxygen atoms in molecular oxygen (O_2). It is very important to do this because transferring any less than all four electrons would lead to the creation of reactive oxygen species (ROS) that could cause damage to the enzymes and membranes of the mitochondria.

> In fact, some well known poisons act at exactly this point. Both cyanide and carbon monoxide can bind with higher affinity than oxygen at the heme in complex IV. Since neither can accept electrons, the effect is just as though no oxygen was available.
>
> Although cytochrome c oxidase is sometimes abbreviated COX, it is *not* the target of the COX-2 inhibitors that are used pharmaceutically in pain management, e.g. Bextra, Celebrex, or Vioxx. That refers to a family of enzymes known as the cyclooxygenases.

Oxygen is absolutely required. If oxygen is not available, there is no place to transfer the electrons, and very quickly, the electron transport chain is halted and carriers such as cytochrome c and CoQ cannot release their electrons and eventually there are no

more available carriers. Similarly, when that happens, NAD^+ is not regenerated, so the TCA cycle is also stuck. This leaves only the anaerobic non-oxygen-requiring glycolysis- fermentation cycle for generating ATP.

We now return to complex II (see Figure 5.4.10). We mentioned complex II as succinate dehydrogenase when discussing the TCA cycle. It also participates in the electron transport chain by passing electrons to ubiquinone. However, rather than transferring electrons that originated from NADH like the other three complexes of the electron transport chain, the electrons originate from the covalently bound electron carrier FADH2 (flavin adenine dinucleotide), which received the electrons from succinate, as described in the TCA cycle section. Once the electrons have been passed to ubiquinone, it then moves on to complex III to drop off those electrons to cytochrome c, and the rest of the electron transport chain continues. FAD, the oxidized form of FADH2, is then ready to participate in the next redox cycle.

The purpose of this electron transport chain, with respect to ATP generation, is the pumping of H+ from the mitochondrial matrix into the intermembranous space. Since the concentration of protons is higher in the intermembrane space, it will take energy to move them against the concentration gradient, which is where our high-energy electrons come into the picture. As they move from one carrier to the next, they are moving from a higher to a lower energy state. This implies that some energy is lost from the electron, and some of that energy is tapped by the enzymes of the electron transport chain to move protons from the matrix to the intermembrane space.

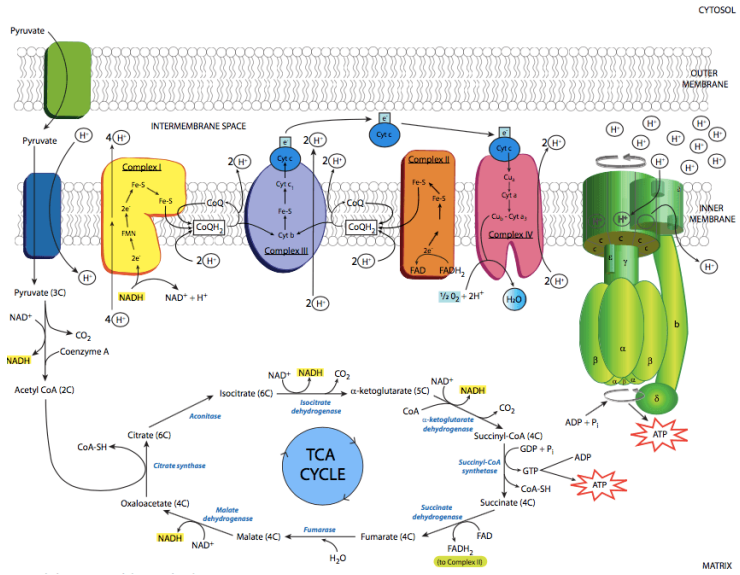


Figure 5.4.10. Catabolic reactions of the mitochondria.

There are two methods by which the protons are moved: the redox loop, and the proton pump. The proton pump, which is the method by which complex IV moves protons, is the easier to understand: H^+ is bound on the matrix side of the enzyme in its reduced state (after it has received an electron), and a conformational shift occurs upon reoxidation to open the enzyme up to the intermembrane side, and the H^+ is released. The redox loop, which occurs in complex I, and in complex III in a variation called the Q cycle, essentially posits that an initial redox center requires the binding of both the high energy electron and a proton from the matrix side of the membrane. When the electron is transferred to the next redox center in the chain, a proton is released to the intermembrane space.

Whatever the mechanism, what is the point of all this proton pumping? As you might suspect, using up energy to pump an ion against its concentration gradient isn't done for the fun of it. Rather, this generates significant potential energy across the inner mitochondrial membrane. And, it so happens that there is an enzyme that can convert that energy into the physiologically useful chemical form of ATP. This enzyme is, not surprisingly, named ATP synthase (Figure 5.4.8). It is also referred to in some texts as the F1F0-ATPase, based on its reverse activity (at the expense of ATP, it can pump protons), and the fact that it can be broken down into two major functional units: F1 which can hydrolyze but not synthesize ATP and is a soluble protein, and F0 which is an insoluble transmembrane protein.

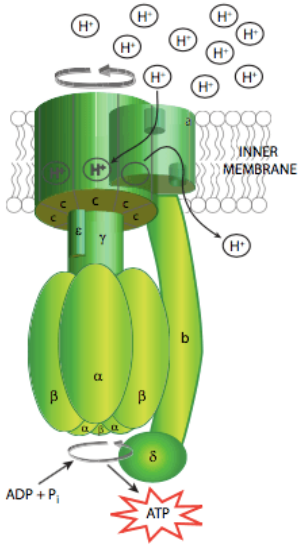


Figure 5.4.8. ATP synthase. As protons pass through the ATP synthase, they release energy by going from high concentration to low. This energy drives the rotational movement of the shaft and the generation of ATP.

The ATP synthase is an extraordinary example of an enzyme that transforms the energy inherent in a concentration gradient across a membrane into mechanical energy, and finally into chemical bond energy. It is descriptively called a “rotary engine” because the very generalized sequence of events is as follows: protons ow down their gradient through a proton channel subunit of the ATP synthase, in owing down the gradient, energy is released, this energy causes rotation of a multisubunit “wheel”-like subunit attached to a spindle/axle (g subunit) which also spins. The spinning of this asymmetrically shaped spindle unit causes conformational changes in the catalytic subunit (made of the a and b subunits) it is attached to, changing an ADP+Pi binding site to a catalytic site that can “squeeze” the molecules together into an ATP, and then finally open up to release the ATP (Figure 5.4.9).

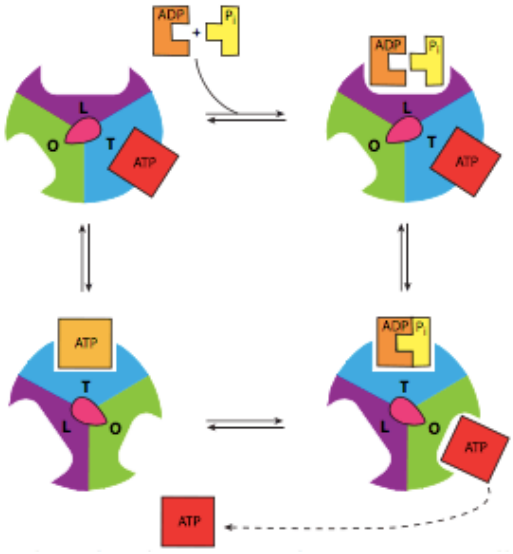


Figure 5.4.9. ATP synthase head rotation. The rotating spindle causes asymmetric changes to the shape of the three potential binding sites, cycling them through the loose (L) conformation that binds ADP and P_i, the tight (T) conformation that literally squeezes the two substrates together into ATP, and the open (O) conformation that allows ATP.

Of course, it isn’t quite that simple (Figure 5.4.8). Starting with the initial movement of pro- tons, as they move from the intermembrane space into the ATP synthase, they enter a small hydrophilic channel (a) and then bind onto one of the c-subunits of the “water wheel” c-ring. Binding of the H^+ to the c-subunit causes it to lose affinity for the a- subunit, allowing it to spin, and simultaneously causes a conformational change that essentially pushes off against the a-subunit, initiating the movement. Once

it has spun around almost a complete turn, the H^+ is positioned by another channel (b), which funnels it from the c-subunit into the matrix. The c-subunit structure is connected to an asymmetric spindle that is itself connected to the catalytic subunits.

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5.5: Uncoupling Electron Transport from ATP Synthesis

So, that is oxidative phosphorylation. It productively utilizes the energy of the proton gradient across the inner mitochondrial membrane (created by oxidation-powered pumps) to drive ATP formation at an approximate rate of 3 protons to 1 ATP. The system is normally highly self-regulated due to impermeability of the inner mitochondrial membrane to H^+. If the ATP is not used up quickly, then its concentration slows the action of ATP synthases, which slow the movement of protons out of the intermembrane space. This buildup of protons will eventually be enough that the free energy needed to transfer a proton into the intermembrane space (from the electron transport chain) will not be sufficient to overcome the concentration gradient. Electron transport is slowed, and working backwards, the chain reaction slows respiration rates in general. As the cell/organism requires more energy and uses up the ATP more quickly, protons flow more quickly and the electron transport chain is disinhibited. Thus there is a direct association between respiration rate and physiological energy need.

Interestingly, there is an exception to this tight coupling of the electron transport chain and formation of ATP. The purpose of brown fat (aka brown adipose tissue), which is most often found in newborn and hibernating mammals, is to generate non-shivering (non-movement-based) heat to keep the animal warm. This is accomplished by uncoupling the electron transport chain from the ATP synthesis. This uncoupling is a hormonally controlled process based on the presence of a mitochondrial proton channel called thermogenin. The hormone norepinephrine increases production of free fatty acids, which open the thermogenin channel. This allows protons to ow from the intermembrane space back into the matrix without having to go through ATP synthase. Because of this, the electron transport chain can keep chugging away, ATP levels do not build up, there is no reduction in respiration rate, and the excess energy not being used in ATP production is released as heat.

> In fact, 2,4-dinitrophenol, which is used in a variety of research and industrial applications today, was at one time used as dieting drug (in the 1930's) because through a different mechanism, it too uncoupled electron transport from ATP synthesis. Its mechanism of action derived from its ability to carry and release protons as it freely diffused through the mitochondrial membrane (since it is a small hydrophobic molecule). As this continues, cells catabolize more and more stores of carbohydrates and fats, which is the reason for the interest by dieters. Unfortunately for some of those dieters, this pharmacological means of uncoupling the electron transport chain from the ATP synthesis had no regulation other than the amount of DNP taken. In cases of overdose, respiration rates could rise dramatically while producing little ATP and a great deal of heat. In fact, overdose illness and death are generally due to the spike in body temperature rather than lowered ATP availability. Unfortunately, there are still some dieters and bodybuilders who self-medicate with DNP despite the dangers.

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5.6: Structure of Electron Carriers

Though they have been mentioned frequently in the earlier parts of this chapter, the structures of the electron transport chain participants, and particularly of the moieties that temporarily hold extra electrons, have not been addressed. So, now is the time to do so. The major players are the flavin mononucleotide (FMN) that plays a role in complex I, ubiquinone (Coenzyme Q), the lipid-soluble electron carrier, the heme groups of the cytochromes, and iron-sulfur clusters, found in complexes I, II, and III.

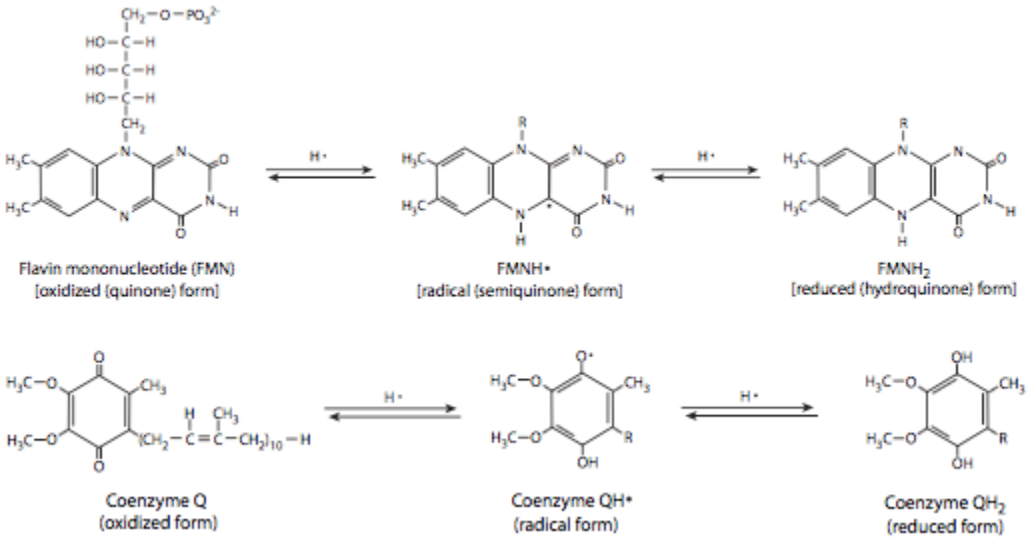


Figure 5.6.11. Flavin mononucleotide and Ubiquinone are electron carriers.

Flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), are pictured in Figure 5.6.11. Note the triple-ring structure and the three possible oxidation states. All three states are stable - the semiquinone state is not merely a transient form. This stability allows the conversion from carriers that can only handle one electron to carriers that can handle two electrons, and vice versa. The same holds true for ubiquinone - stable as ubiquinone (fully oxidized), semiubiquinone (radical state), and ubiquinol (fully reduced). Alternative nomenclature for these molecules is Coenzyme Q, $CoQH^+$, and $CoQH_2$. Note the aromaticity gained by ubiquinone when it is reduced. This enhances its stability and its suitability as a receiver of electrons from NADH.

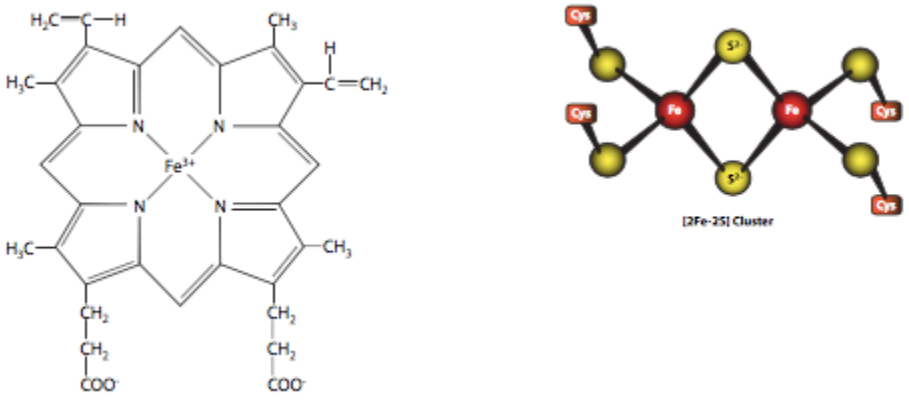


Figure 5.6.12. Left: The heme group shows one of the possible combinations of the groups attached to the outside of the porphyrin ring. Right: The Fe-S centers also have variations, such as 4 Fe and 4 S, or even just 1 Fe surrounded by the S atoms of the four cysteines.

Heme groups (Figure 5.6.12) are considerably larger, encompassing a porphyrin ring with an iron ion held in its center. This iron ion alternates between ferric (Fe^{3+}) and ferrous (Fe^{2+}) states as the heme group is oxidized and reduced, respectively. In the case of complex IV, the iron ion can form a complex with O_2, which can then receive the electrons being held by the ring structure. This large structure is particularly important because it needs to be able to transfer a total of 4 electrons to reduce O_2 to 2 H_2O.

Finally, Fe-S clusters (Figure 5.6.12) can also act as electron carrying moieties. Like in the heme group, the iron atom can readily switch between the ferric and ferrous states.

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5.7: 5.7 Starch and Glycogen Depolymerization

Of course, for many organisms, the food used by cells is not in the form of simple glucose solutions, but made up of various polymeric biomolecules. The breakdown of those molecules is described in the next sections.

Starch and Glycogen Depolymerization

Glycogen and starch are long branched polymers of glucose that provide a rapidly available source of glucose molecules for glycolysis. In omnivores and herbivores, the primary source of carbohydrates (and thus glucose) is dietary starch. The catabolism of the amylose and amylopectin in humans begins in the mouth with salivary a-*amylase*. This enzyme breaks α(1-4) bonds of both starch molecules except at the ends and near branch points (in the case of amylopectin). Though the salivary enzyme is inactivated by the acidity of the stomach, a pancreatic a-amylase goes to work on starch that has reached the small intestine. The product of these digestions includes maltose, maltotriose, and dextrins. These are acted upon by other intestinal enzymes: α-*glucosidase* removes individual glucoses from oligosaccharides, and a-*dextrinase*, also known as debranching enzyme, can break α(1-6) bonds as well as the α(1-4) bonds.

Glycogen breakdown is different since most glycogen breakdown is occurring internal to the cells of an organism rather than in the digestive tract. The primary enzyme is *phosphorylase* (also known as glycogen phosphorylase), which breaks the bond of a terminal glucose to its neighbor by substituting a phosphate group. This generates glucose-1-phosphate, which can be converted to glucose-6-phosphate by *phosphoglucomutase*. The G6P, of course, can enter the glycolytic pathway. A *glycogen debranching enzyme* is also important, as the phosphorylase is unable to work closer than five glucose residues to a branch site.

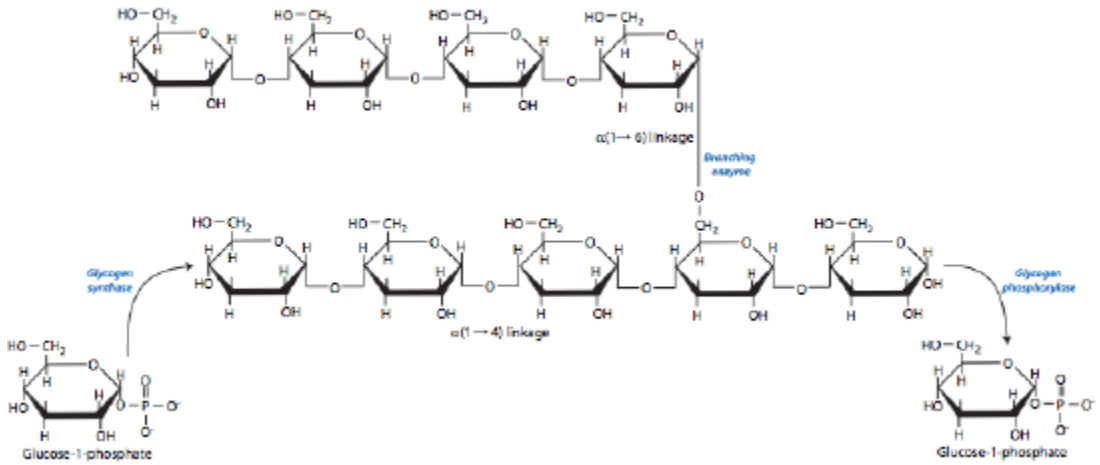


Figure 5.7.13. Glycogen is a storage form of glucose that is broken down by glycogen phosphorylase (linear a(1-4) bonds) and debranching enzyme (branchpoint α*(1-6) bonds)*.

Phosphorylase is a homodimer that is allosterically controlled by glucose, G6P, and ATP negatively, and by AMP positively. In addition to allosteric binding sites for these molecules and a substrate binding site, phosphorylase also binds pyridoxal-5-phosphate as an essential cofactor. P5P is derived from pyridoxine, or vitamin B_6.

Much like the phosphoglycerate mutase in step 8 of glycolysis, phosphoglucomutase is a phosphorylated enzyme that temporarily transfers is phosphate group to the substrate to form a glucose-1,6-bisphosphate intermediate.

Debranching enzyme actually has two functions: it transfers a trisaccharide from a 4-sugar branch on the “1” side of an α(1-6) branching linkage to the end of a branch connected to the “6” side of the branchpoint. It then hydrolyzes the α(1-6) connecting the nal glucose of the branch, leaving an unbranched chain of glucose for phosphorylase to attack.

The use of glycogen presents an interesting question: why use it as an energy storage molecule when fats are more abundant in most animals, and more efficient at packaging potential energy? As described in the next section, fatty acids can only be metabolized aerobically, so they cannot serve as a backup fuel source in anaerobic conditions. Furthermore, even in aerobic conditions, fatty acid catabolism cannot generate glucose, which is not only needed for cellular fuel, but in the bloodstream for feedback control mechanisms regulating organismic metabolism.

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5.8: 5.8 Fatty Acid Breakdown

Hormone-sensitive lipase in adipose tissue hydrolyzes the stored fat in those cells into glycerol and fatty acids. Glycerol can enter the glycolytic cycle via conversion to dihydroxyacetone phosphate (a two-step conversion using glycerol kinase and glycerol-3-phosphate dehydrogenase). The fatty acids are secreted from the adipose cells into the bloodstream where they bind to a carrier protein, albumin. This complex can then be brought inside of other cells by endocytosis, where they can be broken down as an energy source.

The breakdown of fatty acids occurs by β-oxidation inside the mitochondrial matrix (Figure 5.8.14).

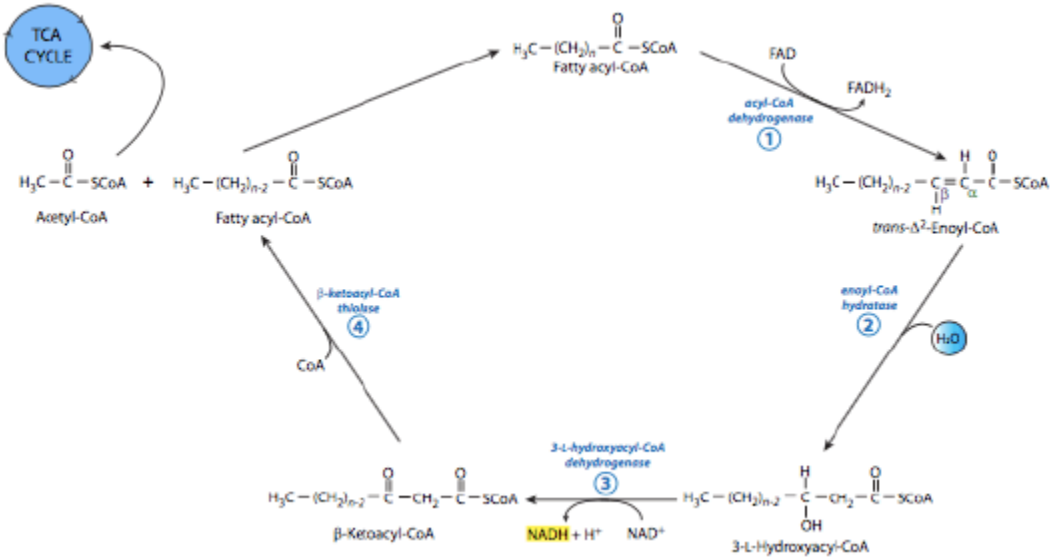


Figure 5.8.14. b oxidation of fatty acids happens in the mitochondrial matrix.

Since the inner mitochondrial membrane is impermeable to long-chain free fatty acids, they must first be activated to fatty acyl-CoA and linked to carnitine, an amino acid derivative synthesized from methionine and lysine (see Figure 5.8.15). The first step is performed by one of a family of enzymes known as acyl-CoA synthetases or thiokinases, and requires Coenzyme A and ATP hydrolysis. These reactions occur either on the cy- toplasmic surface of the mitochondrial outer membrane or the endoplasmic reticulum, where acyl-CoA synthetases are embedded. In the second reaction, carnitine palmitoyl- transferase I on the outside of the inner mitochondrial membrane links the acyl chain to carnitine, releasing CoA. The acyl-carnitine is transported into the mitochondrial matrix where carnitine palmitoyltransferase II releases the fatty acyl chain from the carnitine and reattaches it to an molecule of CoA.

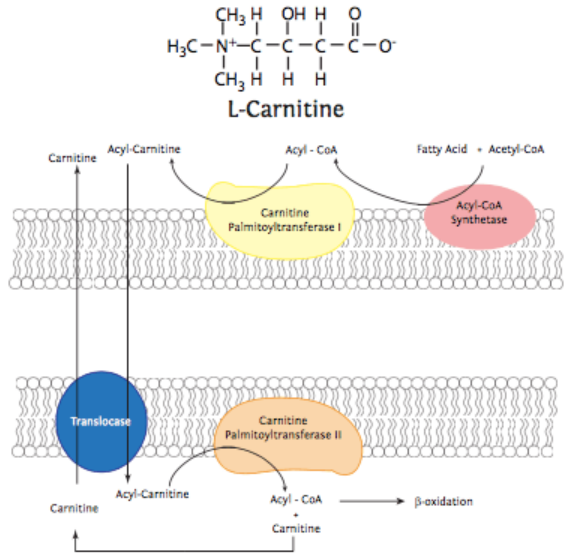


Figure 5.8.15. Fatty acid breakdown by Carnitine Palmitoyl Transferases.

Carnitine deficiency syndromes can occur when there is either a dysfunctional mutation of carnitine palmitoyltransferase or a severe deficiency of intracellular carnitine. Since most of the carnitine in the body is found in cardiac and voluntary muscle, the usual symptoms are muscle weakness and cardiac arrhythmias, as well as hypoketosis. In neonates, the arrythmias can lead to death. Carnitine supplementation is a successful treatment in systemic carnitine deficiency due to either low carnitine intake or defects in the carnitine transporter embedded in the cell membranes. However, if the defect is in the palmitoyltransferase, supplementation will be unsuccessful.

Carnitine is widely sold as a dietary supplement for increasing weight loss by enhancing fat catabolism. The basic idea is obvious: carnitine is needed for long-chain fatty acid breakdown, so more carnitine = more fat burned. However, that only holds true if carnitine levels are below saturation levels for the palmitoyltransferases. Because 75% of the carnitine in the body must be ingested (only 25% is synthesized), this is a mild possibility, depending on diet. Currently, the biomedical community has not reached a consensus on the efficacy of carnitine supplementation on fatty acid oxidation in carnitine-sufficient persons.

In the mitochondrial matrix, β-oxidation occurs in four steps to yield an acyl-CoA chain that is shortened by two carbons, and an acetyl-CoA that can then enter the TCA. . The β refers to the second closest carbon to the one attached to CoA. The bond that will be broken is the bond between the α and β carbons. All even-numbered, fully saturated, fatty acids can thus be completely oxidized. The presence of double bonds in un- saturated fatty acids introduces complications to this process that must be addressed using additional enzymes that either move the double bond or remove it.

Most animals and plants generate even-numbered fatty acids; however, some marine animals (e.g. smelt, mullet) and some plants and bacteria synthesize odd-chain fatty acids as well. The same enzymes responsible for β oxidation of even-numbered fatty acids can handle odd-numbered fatty acids as well, except that the final degradation yields propionyl-CoA instead of acetyl-CoA.

Propionyl-CoA is converted to succinyl-CoA through a series of three enzymes: propionyl-CoA carboxylase, methylmalonyl-CoA racemase, and methylmalonyl-CoA mutase. The succinyl-CoA could theoretically enter the TCA cycle, but recall that the succinyl-CoA is simply recycled and never actually consumed by the TCA cycle. Thus, in order for the succinyl-CoA to contribute to the energy needs of the cell, it must first be converted to malate (steps 6-8 of TCA cycle), which is then converted to pyruvate by malic enzyme, also known as decarboxylating malate dehydrogenase. Pyruvate can then enter and be consumed by the TCA cycle.

Vitamin B_{12}, or 5'-deoxyadenosylcobalamin, is a coenzyme component of methylmalonyl-CoA mutase, but it is not made by either plants or animals. It is only made by certain bacteria, some of which live in the intestinal tracts of herbivores. Herbivores thus absorb the B_{12} for their use, and carnivores obtain their B_{12} from eating herbivores. Defects in methylmalonyl-

CoA mutase or severe deficiency in vitamin B_{12} (most often in vegetarians) can lead to methymalonyl aciduria/acidemia, that can be fatal in untreated infants due to acidosis. However, depending on the cause, it can be treated with high doses of B_{12} and/or by avoiding dietary odd-chain fats and proteins rich in isoleucine, leucine, or methionine, which also catabolize to propionyl-CoA. Pernicious anemia, in which usually elderly patients have very low levels of red blood cells and hemoglobin, as well as neurodegeneration, is also related to B_{12}. However, it is usually not due to a vitamin deficiency, but rather to the insufficient secretion of *intrinsic factor*, which binds B_{12} in the stomach and then is taken into intestinal cells by receptor-mediated endocytosis.

In addition to oxidation in the mitochondria, fatty acids also undergo β-oxidation in peroxisomes. However, generally, the oxidation in peroxisomes is limited, and the purpose is to shorten long fatty acids in preparation for final degradation in the mitochondria.

In addition to the more common single-chain fatty acids, cells will also encounter branched fatty acids, which block β-oxidation is alkyl group is on the β carbon. In these cases, phytanic acid for example, a oxidation is necessary to generate an intermediate with the alkyl group on the a carbon. This is then followed by β-oxidation to completion.

Finally (with respect to fatty acid catabolism), it must be noted that in liver especially, a large part of the acetyl-CoA generated by oxidation of fatty acids does not enter the TCA cycle. Instead, it is converted into acetoacetate or D-β-hydroxybutyrate, which along with acetone, are known, somewhat bizarrely, as ketone bodies. These molecules are water soluble, and transported through the bloodstream as energy sources for a variety of tissues, even including brain, which typically only uses glucose as fuel since fatty acids cannot pass through the blood-brain barrier. However, ketone bodies can penetrate and are used by brain cells under starvation conditions.

Ketoacidosis is a condition in which ketone bodies are being produced much faster than they are used. This leads to a buildup of the molecules in the bloodstream, which lowers the pH, since the molecules are acidic. An easy diagnostic of ketoacidosis is a sweet somewhat fruity smell (of acetone) on the breath. This condition can be an indication of diabetes, but may also occur when a person is consuming a high-fat/low-carb diet. When the body's metabolism is not using glucose/carbohydrates as the primary food source for either reason, fat is used instead, increasing production of ketone bodies. Left untreated, severe ketoacidosis can lead to cell damage as the blood acidifies, and compensation by increased exhalation of carbon dioxide and lead to respiratory failure in susceptible individuals.

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5.9: Amino Acid Degradation

Proteins are broken down by a variety of proteases that hydrolyze the peptide bonds to generate smaller peptides and amino acids. Those amino acids that are not used for building new proteins may be broken down further to enter the metabolic processes discussed in this chapter.

> There is a large variety of proteases, classified into one of six groups (as of 2008): serine proteases, metalloproteases, aspartic acid proteases, cysteine proteases, threonine proteases, and glutamic acid proteases. All of them work by forming a nucleophile at their active site to attack the peptide carbonyl group. They differ in the construction of their active sites, and the specificity of the target sequences to be cleaved. The MEROPS database (http:// merops.sanger.ac.uk/) lists hundreds of enzymes and their specific recognition sites. As with other enzymes, recognition is based on formation of stabilizing hydrogen bonds between enzyme and target. In the case of proteases, many of the important stabilizing bonds must be formed right around the cleavage site, thus leading to specific recognition sequences.
>
> Although cleavage is often thought of as a way of destroying the activity of a protein, in fact, specific cleavage of inhibitory parts of a protein can activate it. A prominent example of this (the caspase cascade) is discussed in the apoptosis section of the cell cycle chapter.
>
> Some proteases are secreted and do their work extracellularly. These include digestive enzymes such as pepsin, trypsin, and chymotrypsin, as well as bloodstream proteases like thrombin and plasmin that help control clotting. The immune system also uses proteases to destroy invading cells and viruses.

In their conversion to metabolic intermediates, the amino acids first undergo deamination. The primary goal of deamination is to excrete excess nitrogen (as urea) and then use or convert (to glucose) the remaining carbon skeleton. This deamination is a two-part process: the first step to deamination is usually a transamination catalyzed by an aminotransferase, in which the amino group of the amino acid is transferred to α-ketoglutarate which then yields a new α-keto acid of the amino acid and glutamate.

The amino group of glutamate could then transferred to oxaloacetate to form α-keto-glutarate and aspartate. That series of transaminations transforms the original amino acid, but does not get rid of the amino group nitrogen. The alternative pathway is deamination of the glutamate by glutamate dehydrogenase, which generates α-ketoglutarate and ammonia, using either NAD^+ or NADP as the oxidizing agent.

The amino acids break down into one of the following seven metabolic intermediates: pyruvate, acetyl-CoA, acetoacetate, a-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate as follows: 1) Ala, Cys, Gly, Ser, Thr, Trp break down to pyruvate; 2) Ile, Leu, Lys, Thr to acetyl-CoA; 3) Leu, Lys, Phe, Trp, Tyr to acetoacetate; 4) Arg, Glu, Gln, His, Pro to α-ketoglutarate; 5) Ile, Met, Val to succinyl-CoA; 6) Asp, Phe, Tyr to fumarate; 7) Asn, Asp to oxaloacetate.

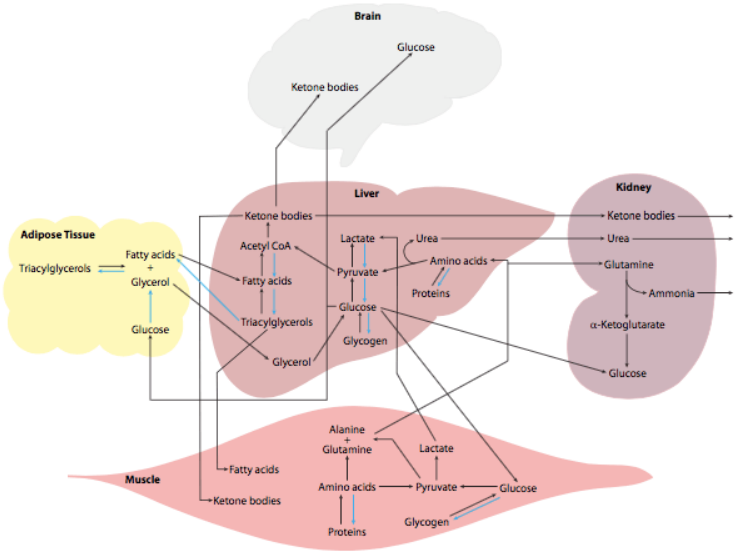


Figure 5.9.16. Overview of human major metabolites. Although most cells in the body carry out many of the metabolic activities described in this chapter and the next, the advantage of multicellular organisms is that certain cell types, tissues, or organs may become specialized to process particular metabolic reactions more e ciently than other cells, and thus take on a lot of that burden for the organism.

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6: METABOLISM II – ANABOLIC REACTIONS

As pointed out at the beginning of this book, most of the energy for life on this planet originates from the sun. In the last chapter, the discussion was on the breakdown of complex molecules such as sugars and fats that hold great, but difficult to access, potential energy to produce molecules like ATP that can act as more readily accessible sources of cellular energy. This energy is then used to synthesize the more complex biomolecules necessary to build living cells. That synthesis, the formation of sugars, fatty acids, and amino acids, is the focus of this chapter. Although technically the polymerization of nucleic acids and proteins are anabolic processes, they are not included in this chapter and are examined in detail separately.

6.1: Photosynthesis

6.2: The Calvin Cycle

6.3: The Pentose Phosphate Pathway

6.4: Gluconeogenesis

6.5: Glycogen Synthesis

6.6: Fatty Acid Synthesis

6.7: Amino Acid Synthesis

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6.1: Photosynthesis

In one way or another, the energy of sugar and fat fuel molecules is derived from photosynthesis - the conversion of solar light energy into chemical bond energy, whether directly in photosynthetic plant cells and certain photosynthetic bacteria, or indirectly by the ingestion of those plants and bacteria. Photosynthesis is a simple idea: atmospheric carbon dioxide molecules are joined with water molecules to form sugars and oxygen:

$$\mathrm{CO_2 + H_2O \xrightarrow{light} CH_2O + O_2} \tag{6.1.1}$$

The production of usable energy from sunlight and the fixation of atmospheric carbon dioxide are two separate sets of reactions. In plants, photosynthesis takes place only in cells containing chloroplasts. Chloroplasts are organelles with an evolutionary origin suspected to be similar to that of mitochondria, and like mitochondria, chloroplasts generate ATP and use a nicotinamide-based high-energy electron carrier. There are further similarities: they both have highly folded inner membranes, though in chloroplasts, there are three membranes in all, while mitochondria only have two. Finally, an electron transport chain is embedded in the thylakoid membrane of chloroplasts, functioning very similarly to electron transport in the mitochondria. In addition to the electron transport components and ATP synthase (structurally and functionally almost identical to mitochondrial ATP synthase), the thylakoid membrane is also rich in a set of molecules that are not found in the inner mitochondrial membrane: light-absorbing pigment molecules.

In plants, these pigment molecules fall into two classes: the chlorophylls and the carotenoids (Figure 6.1.1) but only the chlorophylls can mediate photosynthesis. Photosynthetic bacteria do not contain chlorophyll, but do have carotenoid pigments that can carry out photosynthesis. Both are hydrophobic hydrocarbons that are held in place within the plane of the membrane by transmembrane proteins. Chlorophylls are easily recognizable by the very large Mg^{2+}-containing porphyrin ring, while the carotenoids are long hydrocarbon chains that may or may not have small ring structures on the ends (e.g. β-carotene). While there is variation in the chlorophyll family, they all impart a green color to the leaf. Carotenoids, on the other hand have a much wider range of colors from yellows to reds. Both chlorophylls and carotenoids are able to absorb light energy of a particular wavelength/energy range and enter an unstable excited state. When the molecule returns to its ground state, the energy would be emitted as heat or light in an isolated situation. However within the context of the pigment arrays (antenna complex) in a living cell, most of the energy is shuttled to another pigment molecule of lower energy by resonance transfer. As described below, only one pair of chlorophyll molecules in an antenna complex will actually eject an electron as it drops from an excited state back to ground state. It is the transfer of that high-energy electron that powers photosynthesis.

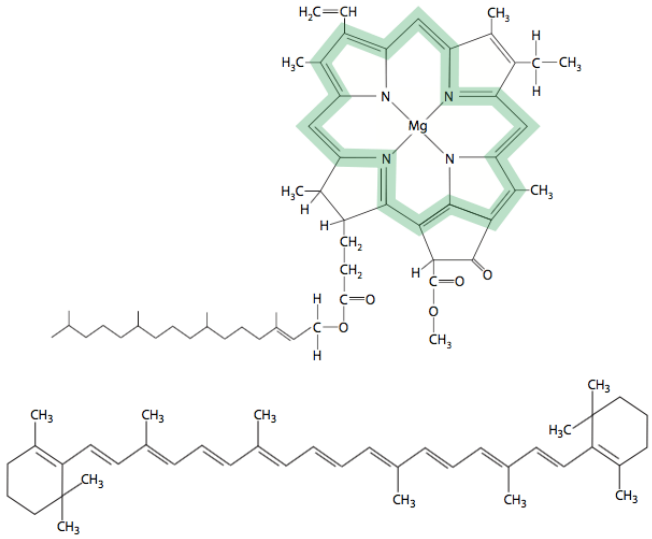


Figure 6.1.1. Chlorophyll (top) and β-*carotene (bottom)*

Chlorophyll molecules are made up of a phytol hydrocarbon tail that anchors the molecule within a membrane, and an electron- carrying porphyrin ring containing a magnesium cation. Note that the phytol tail is not drawn to scale with the porphyrin ring in Figure 6.1.1. Among different types of chlorophyll, the chemical groups attached to the ring may vary, and this variation is responsible for differences in the absorption spectrum from one type of chlorophyll to the next. For example, chlorophyll a has absorption peaks at approximately 430 and 662 nm, whereas chlorophyll b has peaks at 453 and 642 nm. The difference between the two is small: at C7, there is a $—CH_3$ group on chlorophyll a, but a —CHO group on chlorophyll b. Presently, there are five known chlorophylls: chlorophyll a is found in all photosynthetic organisms, chlorophyll b is only found in plants, chlorophylls c1 and c2 are found in photosynthetic algae, and chlorophyll d is found in cyanobacteria.

Carotenoids have two functions. As noted in the primary text at left, they can participate in energy transfer in toward the reaction center chlorophylls. They are also a protectant molecule, preventing reaction center auto-oxidation. Carotenoids can be highly efficient free radical scavengers due to the conjugation of alternating single-double carbon bond structures.

Photosynthesis can be divided into two mechanisms: the *light reactions*, which use light energy to excite the electrons of certain chlorophylls, and participate in the electron transport chain to generate ATP and NADPH, and the *dark reactions*, which use that ATP and NADPH to fix carbon from CO_2 into organic molecules (carbohydrates). As the name implies, the light reactions require light energy to excite the chlorophyll and begin electron transport. Dark reactions, however, do not require darkness. They are technically light-independent, but in some plants, the dark reactions run better in the light for reasons to be discussed.

The light reactions are intimately tied to the anatomy of the thylakoid membrane; specifically, the arrangement of light-absorbing pigment molecules in antenna complexes, also called light-harvesting complexes (sometimes abbreviated LHC, not to be confused with the Large Hadron Collider). These pigments are held by proteins in ordered three-dimensional groups so that the pigments that absorb the highest energy light are toward the periphery, and the lowest-energy-absorbing chlorophylls are in the center (Figure 6.1.2). Sunlight is composed of a broad range of wavelengths, some of which are transiently absorbed by the pigments. After a pigment molecule absorbs a photon, the energy is released and passed on to a pigment tuned to a slightly lower energy level (longer wavelength), and from there to an even lower-energy pigment, and so on until it reaches the reaction center chlorophylls. In this way, energy from a wide range of light wavelengths/energies can all contribute to the ATP and NADPH production by the light reactions. The antenna complex is crucial because it allows the use of a greater portion of the solar light spectrum. And, as a tightly-packed three-dimensional array, photons that pass by one pigment molecule may well hit another one on its way through the array. All of these characteristics combine to increase the efficiency of light use for photosynthesis. The reaction center chlorophylls (P680 for photosystem II, P700 for photosystem I) are the only chlorophylls that actually send excited electrons into the electron transport chain. The other chlorophylls and pigments only act to transfer the energy to the reaction center.

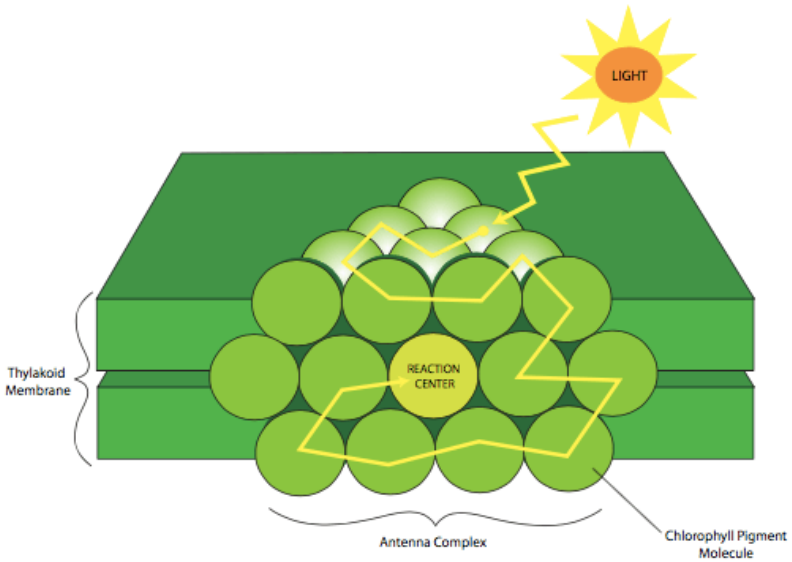


Figure 6.1.2. Pigment molecules are arranged in an antenna complex in the thylakoid membrane.

When excited, the reaction center chlorophyll of photosystem II (Figure 6.1.3) begins the process of electron transport. This

chlorophyll is part of a protein complex that also includes a Mn-based oxygen-evolving complex (OEC), pheophytin, and a docking site for plastoquinone. Although the chlorophyll electron is the one excited by the solar energy, the origin of the electrons to keep the chlorophyll replenished comes from the splitting (oxidation) of water to O_2 and 4 H^+.

> The OEC, or oxygen-evolving complex (also WOC, water oxidizing complex) is a metalloenzyme with a Mn4OXCa catalytic cluster, where X is the number of m-oxo-bridges connecting the metal atoms, with surrounding amino acids, especially crucial tyrosines, also playing a role in the coordination sphere of the active site. The overall complex undergoes a series of 4 oxidation state changes as the P680 chlorophylls are excited by the light energy and transfer electrons, but at present it is not known what the exact oxidation state of any given Mn atom is through this series of state changes. The crucial reaction is the formation of the O-O bond to form O_2. There are two proposed models for this mechanism. One is that the O-O bond is formed when the OEC has reached its fully oxidized state, and an oxygen in a m-oxo-bridge radical state interacts with a water molecule. The other proposed mechanism is that the O-O bond forms earlier as a complexed peroxide held by the OEC center.

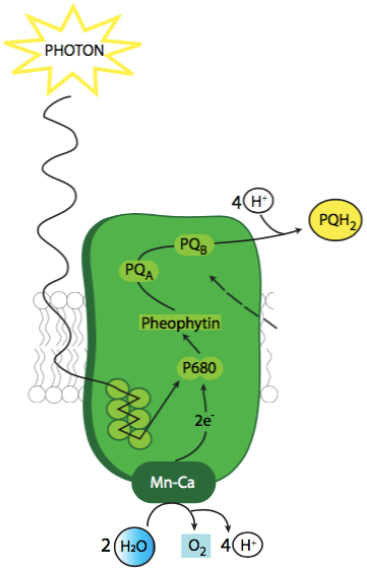


Figure 6.1.3. Photosystem II (which feeds electrons into photosystem I).

The question of how a cell could generate the energy needed to split water was long a thorny issue because water is an exceptionally stable molecule. The current model suggests that the energy comes from an extremely strong oxidizer in the form of $P680^+$. After P680 is energized by light, an excited electron has enough energy to break away from the chlorophyll and jumps to pheophytin. Pheophytin becomes Pheo- temporarily, and the charge separation in the complex between $P680^+$ and $Pheo^-$ helps to enhance the oxidative power of $P680^+$. That extraordinarily strong attraction for electrons is what allows the P680 chlorophyll to tear them away from H_2O and split the water. In fact, $P680^+$ is one of the strongest biological oxidizers known. Since four electrons must be taken to fully oxidize two water molecules and generate molecular oxygen, four photoexcitation events are needed. While the exact mechanism is still to be elucidated, it appears that the OEC helps to stabilize the water molecule during this process as well as holding onto each electron as it comes off.

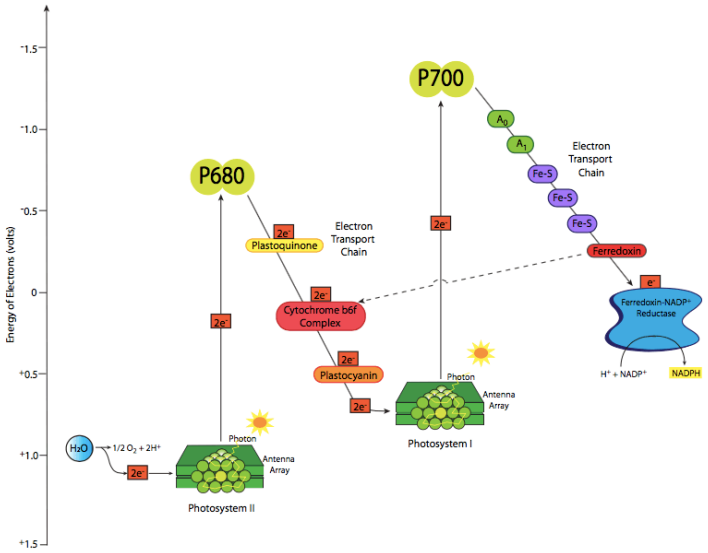


Figure 6.1.4. Change in electron energy moving through photosystems II and I. Light energy is needed in both photosystems to boost the electron energy high enough to move to the next electron carrier.

The excited electrons, moving from the OEC to P680$^+$ to pheophytin, next move to the lipid-soluble carrier, plastoquinone. The similarity of the name with the mitochondrial carrier ubiquinone is not a coincidence. They function similarly, and as the plastoquinone takes on the electrons, it also takes on protons from the stromal side of the thylakoid membrane. The PQ moves within the membrane from pheophytin to cytochrome b_6f. As the electrons are transferred to cytochrome b_6f, the protons are then dropped off on the lumenal side of the membrane, increasing their concentration in the chloroplast lumen, and building a proton gradient to power ATP synthase. Cytochrome b_6f passes the electrons on to plastocyanin, an aqueous-phase carrier, which shuttles the electrons to the P700 reaction center chlorophyll of photosystem I. However, after all the transfers, the energy level of the electrons is now fairly low (Figure 6.1.4) and unable to power the upcoming reactions. Since it is now on a reaction center chlorophyll, the obvious answer is to re-energize it with a bit of sunlight. This raises the electron energy sufficiently to reduce ferredoxin. Now things get a little complicated.

This part of photosynthesis can take one of two directions, the linear pathway, which generates both NADPH and ATP, and the cyclic pathway which mostly generates ATP. Most of the time, the linear pathway is taken, with the electrons on ferredoxin transferred via ferredoxin-NADPH reductase (FNR) onto NADPH. However, sometimes the cell requires significantly more ATP than NADPH, in which case, the electrons from ferredoxin are transferred back to plastoquinone via ferredoxin-plastoquinone reductase. This acts just as described above, and pumps more protons across the membrane to power the ATP synthase. ATP synthesis goes up and NADPH synthesis goes down.

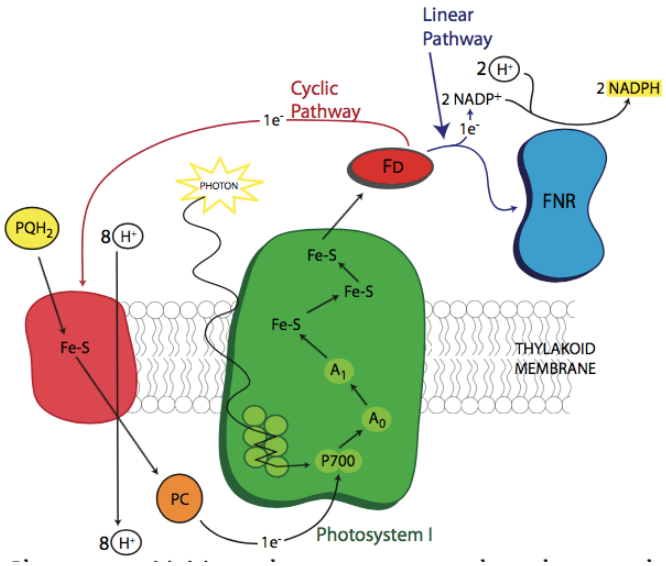


Figure 6.1.5. Photosystem I initiates electron movement through two pathways.

The ATP and NADPH generated by the chloroplast are almost exclusively used by the chloroplast itself (and not distributed to the rest of the cell) to power the dark reactions, which are energetically expensive. In fact, when the light reactions are not running due to darkness, some plant cells have mechanisms to prevent the dark reactions from using the limited resources of cellular, non-chloroplastic, respiration. The simplest method of such limitation is the pH sensitivity of *rubisco* (ribulose bis-phosphate carboxylase), at least in C3 plants (see below). Rubisco has a very sharp pH optimum at about pH 8.0, so while the light reactions are running and the protons are being pumped, the pH rises to about 8 and rubisco works, but in the dark, the pH drops back to its basal level close to 7.0, inhibiting rubisco activity.

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6.2: The Calvin Cycle

The dark (carbon fixation) reactions vary depending on the type of plant. The most common set of carbon fixation reactions is found in C3-type plants, which are so named because the major stable intermediate is the 3-carbon molecule, glyceraldehyde-3-phosphate. These reactions, best known as the Calvin cycle (Figure 6.2.6), fix CO_2 onto the pentose, ribulose 1,5-bis-phosphate (RuBP).

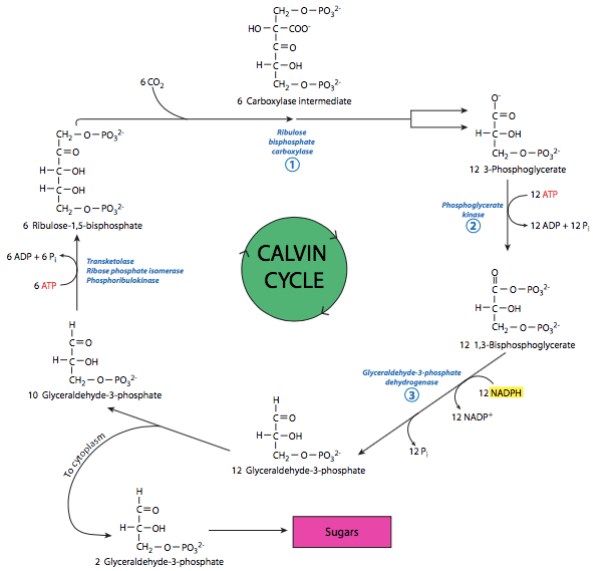


Figure 6.2.6. The Calvin cycle fixes atmospheric carbon to ribulose 1,5-bisphosphate to form the organic 3-carbon intermediate 3-phosphoglycerate for the formation of sugars.

The production part of the cycle begins with formation of RuBP from glyceraldehyde-3-phosphate. Then, the rate-limiting step occurs:

Ribulose 1,5-bisphosphate and CO2 are joined together by rubisco. Carboxylases are relatively slow enzymes as a family, and rubisco is one of the slowest. A 6-carbon intermediate is formed but it is unstable, and quickly breaks down to yield two molecules of 3-phosphoglycerate. Some familiar enzymes (from glycolysis, although this is happening in the stroma, not the cytoplasm) now come into play.

Phosphoglycerate kinase phosphorylates 3-PG to 1,3-bisphosphoglycerate. 1,3-BPG is then reduced by glyceraldehyde-3-phosphate dehydrogenase to form glyceraldehyde-3-P. This step requires the energy released from oxidation of NADPH. A small portion ($1/6^{th}$) of the GAP that is made is then exported from the chloroplast and will be used to form more complex carbohydrates. However, the majority is recycled through the recovery phase of the Calvin cycle to regenerate NADP.

As if having a central enzyme that moves at a snail's pace and needing to recycle the majority of its potential product was not bad enough, C3 plants also have to contend with the hijacking of rubisco for a competing, and energy-wasting, set of reactions known as *photorespiration*. Under conditions of low CO_2 and high O_2 in the local atmosphere, oxygen, instead of carbon dioxide, binds to rubisco and forms 3-PG and 2-phosphoglycolate from its reaction with RuBP. As detailed in Figure 6.2.7, the 2-phosphoglycolate is dephosphorylated to glycolate and transported out of the chloroplast. From there, it undergoes a series of reactions in the peroxisomes and mitochondria to transform it to 3-PG, which can then go in the chloroplast and participate in the Calvin cycle. Unfortunately for the cell, in the course of these reactions, NADH and ATP are used, thus lowering the energy availability inside the cell.

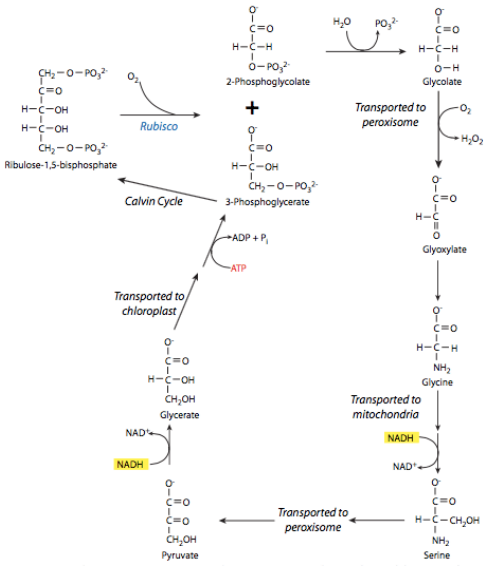


Figure 6.2.7. Photorespiration. Rubisco can catalyze the addition of O_2 to RuBP, producing 3PG, which can be used by the Calvin cycle, and 2-phosphoglycolate, which is converted to glycolate, transported out of the chloroplast, converted to glycerate over several steps in the peroxisome and mitochondria, and shipped back to the chloroplast. This is an energetically expensive process (note NADH and ATP used).

This is a particular problem in hot climates, because the oxygenase activity of rubisco increases more than the carboxylase activity as the temperature increases. This leads to an interesting side effect: in C3 plants, as the temperature rises and CO_2 is outcompeted by O_2 for rubisco binding, the stomata of the leaves need to remain open for longer in order to allow for acquisition of enough CO_2 from the atmosphere. This in turn allows more water vapor from inside the cell to escape, leading to dehydration. C3 plants are thus at a competitive disadvantage in hot dry climates in comparison to plants that do not use rubisco for carbon fixation.

What about plants adapted to such climates? C4 plants, which include some grasses, corn, sugarcane, and weeds, utilize PEP carboxylase (which does not have the annoying photorespiratory capabilities of rubisco and a higher affinity for CO_2) to fix carbon dioxide to PEP, making oxaloacetate. In an interesting twist, the oxaloactetate, after conversion to malate, is decarboxylated to yield CO_2 again, which is fed to rubisco and the Calvin cycle.

> PEP carboxylase actually fixes $HCO3^-$ to PEP rather than CO_2 directly. The atmospheric CO_2 is converted to the bicarbonate by carbonic anhydrase.

The C4 mechanism, also called the Hatch-Slack pathway, utilizes two sets of cells, an outer layer (mesophyll) that takes in air and fixes the CO_2 to PEP and produces malate, and an inner layer of cells (bundle sheath) that takes the malate, and decarboxylates it for its rubisco enzyme. The two cells are connected via plasmodesmata (see Cell-cell Interactions chapter). Although energetically more expensive than carbon fixation by C3 plants in cooler climates, the C4 pathway overtakes C3 in efficiency as temperatures rise and photorespiration increases.

> Variations of this pathway have been found in which aspartate is transported to the bundle-sheath cells instead of malate. After decarboxylation of the malate by malic enzyme (NAD-dependent in some species, NADP-dependent in others) to release the CO_2 for rubisco, the resulting pyruvate is shuttled back to the mesophyll cell where it is phosphorylated by pyruvate-phosphate dikinase to generate PEP for re-entry into the C4 cycle.

Desert plants go one step further than C4 plants. Living in environments that are extremely hot and dry during the day, but relatively cool at night, many desert succulents (like cacti) are diurnal, and only open their stomata at night (when temperatures are significantly lower and water evaporates far more slowly) for CO_2 gathering, which is then fixed via the CAM pathway to malate.

Then in the daylight hours, CO_2 is released from the malate and used in the Calvin cycle to generate carbohydrates.

The crassulacean acid metabolism (CAM) pathway

The crassulacean acid metabolism (CAM) pathway is named for a carbon fixation pathway discovered in the Crassulaceae family of succulent plants including pineapples as well as various cactus species. It utilizes a similar biochemical mechanism as the C4 pathway, but occurs within a single photosynthetic cell. The major difference is that the CO_2 is only taken in at night, and it quickly turned into malate, which is stored in vacuoles until daytime. The malate is then released and decarboxylated to provide the RuBP carboxylase (rubisco) with a steady stream of CO_2 for fixation. Because there is such a rush of PEP carboxylase activity at night to fix the atmospheric CO_2 to PEP, there is a high rate of starch breakdown to provide the glucose for glycolytic generation of PEP. Interestingly, as the malate is decarboxylated in the day, its product, pyruvate, can then be used to re-synthesize glucose (see gluconeogenesis section) and then starch.

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6.3: The Pentose Phosphate Pathway

NADPH is found not only in plants, but in animal cells as well. Although our first discussion of NADPH was in the context of photosynthesis, it is also a general reducing agent in any cell. It is also crucial to note that though introductory texts often consider NAD^+/NADH and NADP/NADPH similarly as high energy electron carriers, and although they are structurally differentiated only by a phosphate group (on the 2'-OH of adenosine), they are *not* interchangeable in the metabolic pathways of a cell. NADP/NADPH is used in reductive metabolic pathways, whereas NAD^+/NADH is used in oxidative pathways. With such an important role in biosynthesis, it is no surprise that its production is part of a major metabolic pathway, the pentose phosphate pathway (Figure 6.3.7), also called the phosphogluconate pathway, and the hexose monophosphate shunt.

- In step 1 of this pathway, glucose-6-phosphate and $NADP^+$ are bound to glucose-6-phosphate dehydrogenase, which transfers a hydride ion from glucose-6-phosphate to $NADP^+$ to form 6-phosphoglucono-d-lactone and NADPH.
- In step 2, the 6-phosphoglucono-d-lactone is hydrolyzed to 6-phosphogluconate using 6-phosphogluconolactonase. This reaction actually proceeds fairly quickly even without the enzyme.
- In step 3, the 6-phosphogluconate is decarboxylated by 6-phosphogluconate dehydrogenase, in the process producing more NADPH, as well as the five-carbon sugar, ribulose-5-phosphate. This metabolite is used by the cell as the basis for nucleotide synthesis. This concludes the NADPH-producing portion of the pentose phosphate pathway.

However, it is useful, in the context of this chapter, to also consider the fate the Ru5P, which is converted to ribose-5-P by ribulose-5-P isomerase or it is converted to xylulose-5-phosphate using ribulose-5-P epimerase. The ribose-5-phosphate is used in nucleotide synthesis, so plays an important role in not only nucleic acid production, but general metabolism (e.g. for ATP).

Ribulose-5-phosphate and NADPH are the most significant products of this pathway. As mentioned earlier, NADPH is important as a general reducing agent. The mechanism for this involves glutathione and glutathione reductase. Glutathione is the primary scavenger of reactive oxygen species such as oxides and peroxides, and the key regulator of cellular oxidative stress. The reduced form of the glutathione tripeptide (Glu-Cys-Gly) dimerizes with another glutathione via disulfide bond as they donate electrons to oxidizers, and is regenerated by glutathione reductase. NADPH is a necessary cofactor for glutathione reductase activity, providing the electrons to reduce the G-S-S-G dimer.

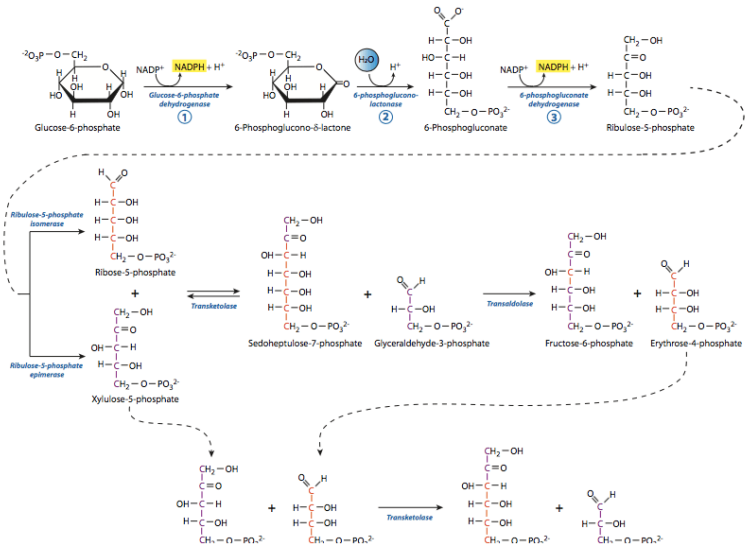


Figure 6.3.7: The pentose phosphate pathway. The first three reactions generate the energy carrier NADPH in the process of converting glucose-6-phosphate to ribulose-5-phosphate. The Ru5P is important as a precursor to nucleotide synthesis, as well as for production of other sugars and important metabolic intermediates, such as fructose-6-phosphate and glyceraldehyde-3-phosphate. Transketolase then transfers the terminal two carbons of ribulose-5-P to xylulose-5-P, making sedoheptulose-7-phosphate and G3P. Transaldolase comes up next. It transfers a 3-carbon unit from sedoheptulose-7-P to the G3P, forming erythrose-4-phosphate and fructose-6-phosphate.Transketolase is used again at this point, transferring a 2-carbon unit from xylulose-5-phosphate - to erythrose-4-phosphate and generating more G3P and fructose-6-P.

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6.4: Gluconeogenesis

Having considered the initial anabolic reaction of life - carbon fixation by photosynthesis, we now turn our attention to utilizing the smaller metabolites to generate glucose and other sugars and carbohydrates. Glucose is the most important fuel for most organisms, and the only fuel for some cell types, such as brain neurons. Potential building blocks of glucose include many of the products and intermediates of glycolysis and the TCA cycle, as well as most amino acids. The key reaction is conversion of any of these compounds into oxaloactetate before using them to make glucose. In animals, the amino acids leucine and isoleucine, as well as any fatty acids, cannot be used to build glucose because they convert first to acetyl-CoA, and animals have no pathway for acetyl-CoA to oxaloacetate conversion. Plants, on the other hand, can push acetyl-CoA to oxaloacetate through the glyoxylate cycle, which will be discussed shortly.

The process of gluconeogenesis is in many ways the simple opposite of glycolysis, so it is not surprising that some of the enzymes used in glycolysis are the same as those used for gluconeogenesis. However, there are a few exceptions. These arose (and have probably evolved) for two major reasons -

1. the thermodynamics of the reaction are prohibitive, and
2. the need for independent control of the catabolic and anabolic processes.

Since there is this parallel, we will explore gluconeogenesis first by starting with one of the major products of glycolysis, pyruvate. Pyruvate can be converted to oxaloacetate by pyruvate carboxylase, in a reaction requiring ATP hydrolysis. The oaxaloacetate is then converted to phosphoenolpyruvate (PEP) by PEP carboxykinase, which also uses nucleotide triphosphate hydrolysis for energy, though this time it is GTP.

> Interestingly, PEP carboxykinase (PEPCK) is unregulated at the protein level. There are no known activators or inhibitors of its activity. The only regulation of PEPCK appears to be at the level of transcription: glucagon can stimulate it (as can gluocorticoids and thyroid hormone), while insulin can inhibit it. The other gluconeogenic enzymes, though, do have direct activators and inhibitors. They are allosteric modulators, binding away from, but influencing the shape and efficacy of the substrate binding site. In examining the regulation of these enzymes, one important regulator stands out because it is not a metabolite of either glycolysis or gluconeogenesis. Fructose-2,6-bisphosphate (F2,6P) is an activator of phosphofructokinase, and an inhibitor of fructose bis-phosphatase. F2,6P levels are controlled by fructose-bis-phosphatase-2 and phosphofructokinase-2, which are themselves controlled by levels of fructose-6-phosphate, as well as through a hormone-driven signaling cascade shown in the Figure on the next page.

As shown in the summary/comparison (Figure $6.4.8$), from the formation of PEP to the formation of fructose-1,6-bisphosphate the enzymes used in gluconeogenesis are exactly the same enzymes used in glycolysis. This works because the free energy change in those reactions is relatively small. However, in the dephosphorylation of fructose-1,6-bisphosphate to to fructose-6-phosphate, and subsequently in the dephosphorylation of glucose-6-phosphate to glucose, there is a large free energy change that works against the gluconeogenic reactions. Thus, the enzymes that drive these reactions are different from the enzymes that drive the reverse reactions in glycolysis (i.e. hexokinase, phosphofructokinase). These two hydrolytic reactions are catalyzed by *fructose bis-phosphatase* and *glucose-6-phosphatase*, respectively. Full reversal of glycolysis in animals is limited, however, to liver and kidney, since they are the only tissues that express glucose-6-phosphatase. Other tissues use different mechanisms for generating glucose (e.g. glycogenolysis).

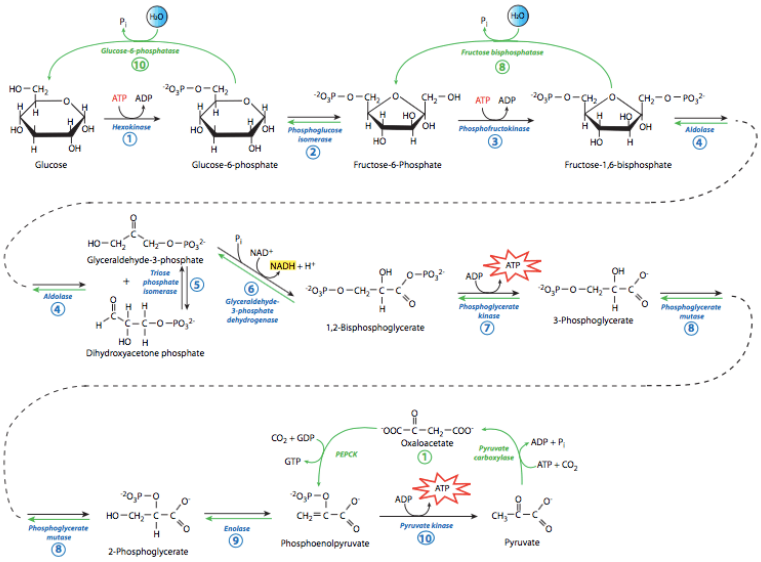


Figure 6.4.8. Gluconeogenesis (shown in green arrows) shares some, but not all enzymes with the reverse process, glycolysis (black arrows).

The glyoxylate cycle provides a mechanism for plants to convert acetyl-CoA into oxaloacetate, and therefore contribute to gluconeogenesis. This allows them to convert fatty acids and the hydrophobic amino acids leucine and isoleucine into glucose when necessary. The ability to do this comes from a plant-specific organelle called the glyoxysome, as well as some mitochondrial enzymes. The glyoxysomal part of the cycle consists of ve steps, of which the first three contribute to the conversion, while the last two steps regenerate the glyoxysomal oxaloacetate (Figure 6.4.9).

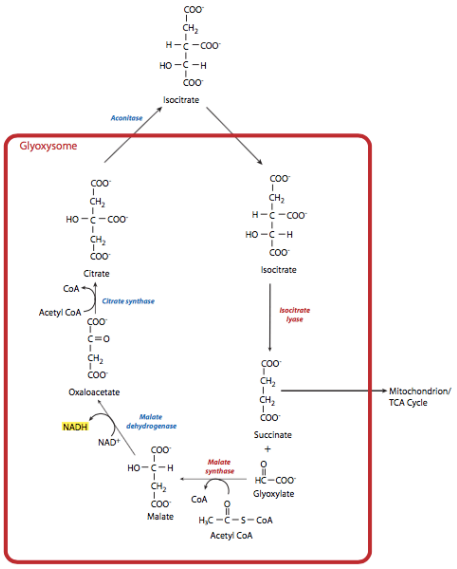


Figure 6.4.9. The glyoxylate cycle.

- Once the macromolecules have been broken down to acetyl-CoA, they enter the glyoxysome and combine with oxaloacetate to make citrate. This is catalyzed by citrate synthase just as in the mitochondrial TCA cycle. The next reaction also uses a familiar enzyme: aconitase catalyzes the conversion of citrate to isocitrate. However, the aconitase is a cytosolic enzyme, so the citrate is transported out of the glyoxysome and then the isocitrate transported back in.
- At this point, the glyoxysomal-specific enzyme, isocitrate lyase, hydrolyzes isocitrate to yield succinate and glyoxylate. The succinate is transported to the mitochondrion, where TCA cycle enzymes convert it to fumarate and then malate, which is transported out to the cytosol. In the cytosol, the malate is converted to oxaloactetate through malate dehydrogenase, and gluconeogenesis can proceed.
- The glyoxylate is acted upon by another glyoxysomal enzyme, malate synthase, which adds it to acetyl-CoA to form malate.
- The final step of the glyoxysomal portion of the glyoxylate cycle is oxidation of the malate to oxaloacetate by glyoxysomal malate dehydrogenase.

So, to summarize, the pool of oxaloacetate within the glyoxysome is used and regenerated within the glyoxysome. Acetyl-CoA is converted to succinate within the glyoxysome, but then goes to the mitochondrion for conversion to malate, and finally the cytosol for conversion to a separate pool of oxaloacetate that is then used in gluconeogenesis.

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6.5: Glycogen Synthesis

Although glucose is the primary fuel for cells, it is not an efficient molecule for long-term storage in complex (i.e. greater than single-celled) organisms. Therefore, in both plants and animals, the glucose molecules are linked together to form polysaccharides known as glucans. In animals, the glucan formed is glycogen, which consists of glucose molecules linked by α(1->4) glycosidic bonds, and branching α(1->6) bonds approximately between 8 to 14 residues apart. The average size of a glycogen unit is a cytoplasmic granule containing over 100000 glucose molecules. The addition of a glucose-1-phosphate to another (or to a glycogen chain) is energetically unfavorable, so it must be coupled with a sufficiently exergonic reaction to proceed.

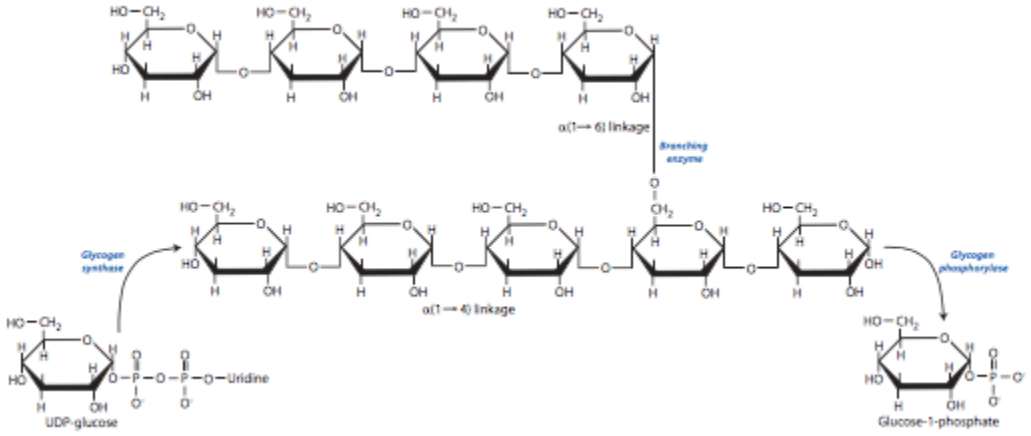


Figure 6.5.10. Glycogen synthesis

Glycogen synthesis begins with UDP-glucose phosphorylase, which combines the nucleotide uridine triphosphate (UTP) with glucose-1-phosphate to release pyrophosphate (PP_i) and form UDP-glucose.

The phosphoanhydride exchange reaction catalyzed by UDP-glucose phosphorylase is minimally exergonic. However, the pyrophosphate released is quickly hydrolyzed by inorganic pyrophosphatase, a ubiquitous cytosolic enzyme, in a highly exergonic reaction. This pyrophosphate hydrolysis is a mechanism utilized in many biosynthetic pathways to provide energy for otherwise endergonic reactions.

In the next step, glycogen synthase attaches the UDP-glucose to the pre-existing glycogen chain with an α(1->4) linkage. It cannot join two individual glucoses together, only add to a pre-existing chain. This means that there must be some workaround for the first two glucoses: glycogenin is an enzyme that catalyzes the addition of UDP-glucose to itself, and can do so for up to seven UDP-glucose molecules, thus forming a short primer for glycogen synthase to work with. Furthermore, glycogen synthase can only add glucoses with an α(1->4) link. For branching to occur, a branching enzyme (specifically, amylo-(1,4->1,6)-transglycosylase is needed. This enzyme can transfer terminal chain segments to the 6-carbon hydroxyl of any glucose in a glycogen chain. However, the branches can only be added if there are at least 4 glucose residues between them, and if the originating chain was at least 11 residues in length.

Oligosaccharide Synthesis

Like glycogen synthesis, oligosaccharide synthesis also requires the initial step of coupling the sugar with a nucleotide. In mammals, a major disaccharide is lactose, which is the linkage of a galactose and a glucose, and the formation is catalyzed by lactose synthase. However, before the lactose synthase is able to act, the galactose must first be in the form of a UDP-galactose. Similarly, in plants, the major disaccharide is sucrose, formed by the linkage of UDP-glucose and fructose-6-phosphate. This results in sucrose-6-phosphate, which is then readily dephosphorylated to sucrose. These kinds of mechanisms are also used in the glycosylation of proteins and lipids, which will be discussed primarily in the protein processing and trafficking chapter.

> Mutation of galactose-1-phosphate uridylyltransferase or mutations of other enzymes in this pathway (uridylyl transferase mutations are most common and usually most severe) can lead to galactosemia, a human genetic disease whose symptoms begin in infancy and may include mental retardation, liver damage, jaundice, vomiting, and lethargy. The cause of these symptoms is generally a buildup of galactose-1-phosphate, especially in the liver and nervous tissue. Fortunately, with early diagnosis, the symptoms can be prevented by avoiding milk products (lactose).

The major hexose species besides glucose are fructose, mannose, and galactose. Interconversion between these hexoses can occur via intermediates, as demonstrated in glycolysis (glucose-6-P to fructose-6-P). Mannose-6-P can be converted to fructose-6-P by phosphomannose isomerase. Galactose can be converted similarly, to galactose-1-P and then to glucose-1-P. The galactose to glucose conversion can also take place by epimerization of UDP-Glucose to UDP-galactose via intermediate redox using NAD^+/ NADH.

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6.6: Fatty Acid Synthesis

This anabolic process is accomplished using a different set of enzymes than the catabolism of fatty acids discussed earlier. Fatty acid synthesis (Figure 6.6.11) starts with the formation of palmitic acid (C16) from acetyl-CoA and malonyl-CoA (which is itself a 3-carbon molecule formed from acetyl-CoA). Another difference between the catabolic and anabolic reactions for fatty acids is the location: whereas we saw that catabolism occurs largely in the mitochondria, fatty acid synthesis is run from a single large cytoplasmic enzyme complex. The fatty acid synthase system is comprised of seven enzymes linked together with an acyl carrier protein (ACP). As mentioned, this complex is found in the cytoplasm, so its substrates must be as well. The acetyl-CoA in the cytoplasm is primarily derived from the mitochondrial acetyl-CoA via a citrate-malate shuttle that couples deacetylation in the mitochondrion with acetylation in the cytosol.

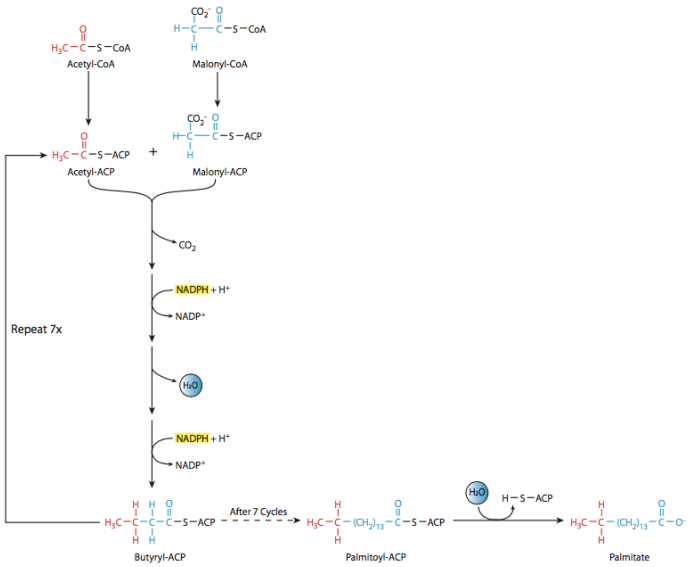


Figure 6.6.11. Fatty acid synthesis

The acetyl-CoA and malonyl-CoA are linked to the synthase and ACP, then there is a sequence of acetyl group transfers that runs a total of seven times to form palmitoyl-ACP, from which the palmitic acid is finally released. Palmitic acid is the precursor for variety of long-chain fatty acids such as stearic acid, palmitoleic acid, and oleic acid. Generally, there is either an elongation or sometimes a desaturation step. However, desaturation is a tricky process for vertebrates. The desaturation at C9 to form oleic acid from stearic acid can occur; however, other desaturations such as desaturation at C-12 to generate linoleic acid are not possible in vertebrates. Interestingly, they can be carried out in plant species. Furthermore, even though linoleic acid cannot be synthesized by vertebrates, it is nevertheless needed by vertebrates, which build arachidonic acid, prostaglandins, and other molecules from it. Linoleic acid is therefore considered an *essential* fatty acid, since it must be ingested by the animal.

These fatty acids are then used to form the triacylglycerols that form the bulk of the energy storage molecules in most animals. Triacylglycerols are synthesized by the reaction of fatty acyl-CoA chains with glycerol-3-phosphate. Two rounds of this reaction yields diacylglygerol-3-phosphate (phosphatidic acid). After the action of phosphatidate phosphatase, the phosphatidic acid is converted to 1,2-diacylglycerol. This reacts with fatty acyl-CoA to form the final triacyglycerol.

Each of the fatty acyl chain additions generates an ester bond, which requires a significant energy input: that energy comes from a linked ATP hydrolysis reaction for each chain addition.

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6.7: Amino Acid Synthesis

In humans, only half of the standard amino acids (Glu, Gln, Pro, Asp, Asn, Ala, Gly, Ser, Tyr, Cys) can be synthesized (Figure 6.7.12 and 13), and are thus classified the nonessential amino acids. Within this group, the first three, glutamate, glutamine, and proline, have a shared anabolic pathway. It begins with glutamate dehydrogenase, which adds ammonia to α-ketoglutarate in the presence of NADPH to form glutamate. This is a key reaction for all amino acid synthesis: glutamate is a nitrogen (amino group) donor for the production of all the other amino acids.

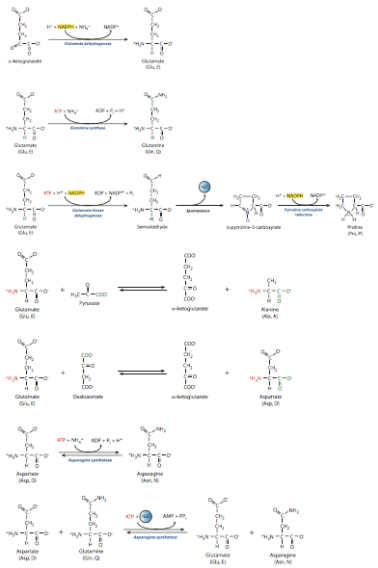


Figure 6.7.12. Synthetic reaction for amino acids: Glutamate, Glutamine, Proline, Ala- nine, Aspartate, Asparagine.

Glutamine synthetase catalyzes the formation of glutamine from glutamate and ammonia. This is an important biochemical reaction for a completely different reason: it is the primary route for ammonia detoxification.

Proline is synthesized from glutamate in a two-step process that begins with the reduction of glutamate to a semialdehyde form that spontaneously cyclizes to D-pyrroline-5-carboxylate. This is reduced by pyrroline carboxylate reductase to proline.

Alanine and Aspartate are the products of glutamate-based transamination of pyruvate and oxaloacetate, respectively.

Asparagine is synthesized through one of two known pathways. In bacteria, an asparagine synthetase combines aspartate and ammonia. However, in mammals, the aspartate gets its amino group from glutamine.

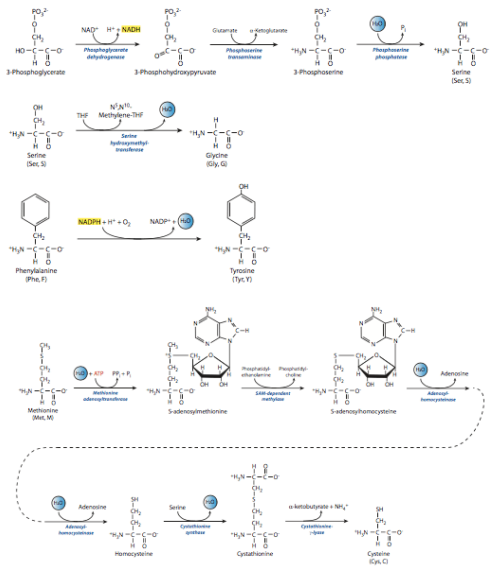


Figure 6.7.13. Synthetic reactions for amino acids: Serine, Glycine, Tyrosine, and Cysteine.

The synthesis of serine begins with the metabolic intermediate 3-phosphoglycerate (glycolysis). Phosphoglycerate dehydrogenase oxidizes it to 3-phosphohydroxypyruvate. An amino group is donated by glutamate in a reaction catalyzed by phosphoserine transaminase, forming 3-phosphoserine, and finally the phosphate is removed by phosphoserine phosphatase to produce serine.

Serine is the immediate precursor to glycine, which is formed by serine hydroxymethyltransferase. This enzyme requires the coenzyme tetrahydrofolate (THF), which is a derivative of vitamin B_9 (folic acid).

Serine is also a precursor for cysteine, although the synthesis of cysteine actually begins with the essential amino acid methionine. Methionine is converted to S-adenosylmethionine by methionine adenosyltransferase. This is then converted to S-adenosylhomocysteine by a member of the SAM-dependent methylase family. The sugar is removed by adenosylhomocysteinase, and the resultant homocysteine is connected by cystathionine synthase to the serine molecule to form cystathionine. Finally, cystathionine-g-lyase catalyzes the production of cysteine.

Tyrosine is another amino acid that depends on an essential amino acid as a precursor. In this case, phenylalanine oxygenase reduces phenylalanine to produce the tyrosine.

In general, the synthesis of essential amino acids, usually in microorganisms, is much more complex than for the nonessential amino acids and is best left to a full-fledged biochemistry course.

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7: DNA

DNA: the stuff of life. Well, not really, despite the hype. DNA does contain the instructions to make a lot of the stuff of life (proteins), although again, not all the stuff of life. At least not directly. Deoxyribonucleic acid (and its very close cousin ribonucleic acid, or RNA) is a very long chain polymer. You may recall that a polymer is just a really big molecule made by connecting many small similar molecules together). In this polymer, the small (monomer) molecules are known as nucleotides, and are composed of a pentose (5-carbon sugar either deoxyribose or ribose), a nitrogenous base, and a phosphate group.

7.1: DNA Structure

7.2: Semi-Conservative DNA Replication

7.3: Prokaryotic Replication

7.4: Eukaryotic Replication

7.5: DNA Lesions

7.6: DNA Repair

7.7: Telomeres

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7.1: DNA Structure

As you can see in Figure1, the nucleotides only vary slightly, and only in the nitrogenous base. In the case of DNA, those bases are adenine, guanine, cytosine, and thymine. Note the similarity of the shapes of adenine and guanine, and also the similarity between cytosine and thymine. A and G are classified as purines, while C and T are classified as pyrimidines. As long as we're naming things, notice "deoxyribose" and "ribose". As the name implies, deoxyribose is just a ribose without an oxygen. More specifically, where there is a hydroxyl group attached to the 2-carbon of ribose, there is only a hydrogen attached to the 2-carbon of deoxyribose. That is the only difference between the two sugars.

In randomly constructing a single strand of nucleic acid *in vitro*, there are no particular rules regarding the ordering of the nucleotides with respect to their bases. The identities of their nitrogenous bases are irrelevant because the nucleotides are attached by phosphodiester bonds through the phosphate group and the pentose. It is therefore often referred to as the sugar-phosphate backbone. If we break down the word "phosphodiester", we see that it quite handily describes the connection: the sugars are connected by two ester bonds (—O—) with a phosphorous in between. One of the ideas that often confuses students is the directionality of this bond, and therefore, of nucleic acids in general. For example, when we talk about DNA polymerase, the enzyme that catalyzes the addition of nucleotides in living cells, we say that it works in a 5-prime (5') to 3-prime (3') direction. This may seem like arcane molecular-biologist-speak, but it is actually very simple. Take another look at two of the nucleotides joined together by the phosphodiester bond (Figure 7.1.1, bottom left). An adenine nucleotide is joined to a cytosine nucleotide. The phosphodiester bond will always link the 5-carbon of one deoxyribose (or ribose in RNA) to the 3-carbon of the next sugar. This also means that on one end of a chain of linked nucleotides, there will be a free 5' phosphate (-PO_4) group, and on the other end, a free 3' hydroxyl (-OH). These define the directionality of a strand of DNA or RNA.

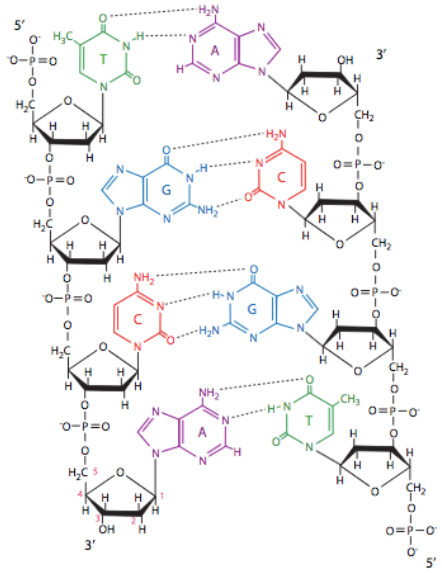


Figure 7.1.1. DNA. Deoxyribonucleic acid is a polymer chain of nucleotides connected by 5' to 3' phosphodiester bonds. DNA normally exists as a two antiparallel complementary strands held together by hydrogen bonds between adenines (A) and thymines (T), and between guanines (G) and cytosines (C).

DNA is normally found as a double-stranded molecule in the cell whereas RNA is mostly single-stranded. It is important to understand though, that under the appropriate conditions, DNA could be made single-stranded, and RNA can be double-stranded. In fact, the molecules are so similar that it is even possible to create double-stranded hybrid molecules with one strand of DNA and one of RNA. Interestingly, RNA-RNA double helices and RNA-DNA double helices are actually slightly more stable than the more conventional DNA-DNA double helix.

The basis of the double-stranded nature of DNA, and in fact the basis of nucleic acids as the medium for storage and transfer of genetic information, is *base-pairing*. Base-pairing refers to the formation of hydrogen bonds between adenines and thymines, and between guanines and cytosines. These pairs are significantly more stable than any association formed with the other possible bases. Furthermore, when these base-pair associations form in the context of two strands of nucleic acids, their spacing is also uniform and highly stable. You may recall that hydrogen bonds are relatively weak bonds. However, in the context of DNA, the

hydrogen bonding is what makes DNA extremely stable and therefore well suited as a long-term storage medium for genetic information. Since even in simple prokaryotes, DNA double helices are at least thousands of nucleotides long, this means that there are several thousand hydrogen bonds holding the two strands together. Although any individual nucleotide-to-nucleotide hydrogen bonding interaction could easily be temporarily disrupted by a slight increase in temperature, or a miniscule change in the ionic strength of the solution, a full double-helix of DNA requires very high temperatures (generally over 90°C) to completely denature the double helix into individual strands.

Because there is an exact one-to-one pairing of nucleotides, it turns out that the two strands are essentially backup copies of each other - a safety net in the event that nucleotides are lost from one strand. In fact, even if parts of both strands are damaged, as long as the other strand is intact in the area of damage, then the essential information is still there in the complementary sequence of the opposite strand and can be written into place. Keep in mind though, that while one strand of DNA can thus act as a “backup” of the other, the two strands are not identical - they are complementary. An interesting consequence of this system of complementary and antiparallel strands is that the two strands can each carry unique information.

> Bi-directional gene pairs are two genes on opposite strands of DNA, but sharing a promoter, which lies in between them. Since DNA can only be made in one direction, 5’ to 3’, this bi-directional promoter, often a CpG island (see next chapter), thus sends the RNA polymerase for each gene in opposite physical directions. This has been shown for a number of genes involved in cancers (breast, ovarian), and is a mechanism for coordinating the expression of networks of gene products.

The strands of a DNA double-helix are antiparallel. This means that if we looked at a double-helix of DNA from left to right, one strand would be constructed in the 5’ to 3’ direction, while the complementary strand is constructed in the 3’ to 5’ direction. This is important to the function of enzymes that create and repair DNA, as we will be discussing soon. In Figure 7.1.1, the left strand is 5’ to 3’ from top to bottom, and the other is 5’ to 3’ from bottom to top.

From a physical standpoint, DNA molecules are negatively charged (all those phosphates), and normally a double-helix with a right-handed twist. In this normal (also called the “B” conformation) state, one full twist of the molecule encompasses 11 base pairs, with 0.34 nm between each nucleotide base. Each of the nitrogenous bases are planar, and when paired with the complementary base, forms a at planar “rung” on the “ladder” of DNA. These are perpendicular to the longitudinal axis of the DNA. Most of the free-floating DNA in a cell, and most DNA in any aqueous solution of near-physiological osmolarity and pH, is found in this B conformation. However, other conformations have been found, usually under very specific environmental circumstances. A compressed conformation, A-DNA, was observed as an artifact of *in vitro* crystallization, with slightly more bases per turn, shorter turn length, and base-pairs that are not perpendicular to the longitudinal axis. Another, Z-DNA, appears to form transiently in GC-rich stretches of DNA in which, interestingly, the DNA twists the opposite direction.

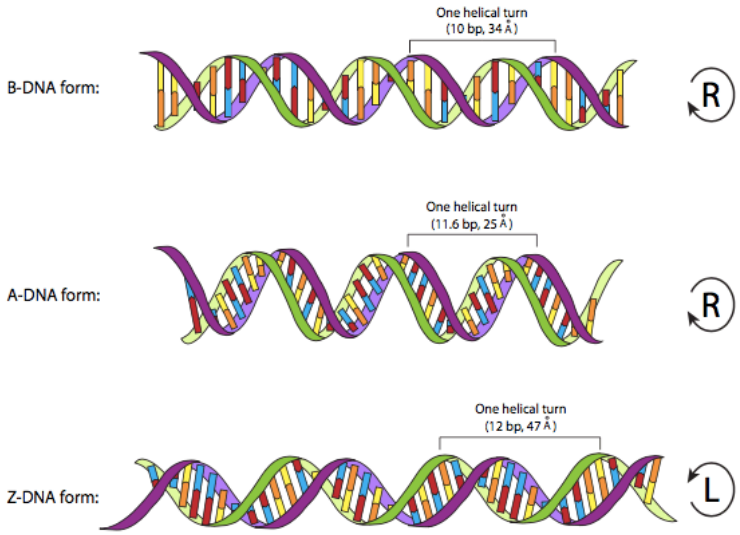


Figure 7.1.2. Three conformations of DNA. B-DNA is most common, A-DNA is likely an artifact of crystallization in vitro, and Z-DNA may form transiently in parts of the chromosome.

It has been suggested that both the A and Z forms of DNA are, in fact, physiologically relevant. There is evidence to suggest that the A form may occur in RNA-DNA hybrid double helices as well as when DNA is complexed to some enzymes. The Z conformation may occur in response to methylation of the DNA. Furthermore, the “normal” B-DNA conformation is something of a idealized structure based on being fully hydrated, as is certainly very likely inside a cell. However, that hydration state is constantly changing, albeit minutely, so the DNA conformation will often vary slightly from the B-conformation parameters in Figure 7.1.2.

In prokaryotes, the DNA is found in the cytoplasm (rather obvious since there is no other choice in those simple organisms), while in eukaryotes, the DNA is found inside the nucleus. Despite the differences in their locations, the level of protection from external forces, and most of all, their sizes, both prokaryotic and eukaryotic DNA is packaged with proteins that help to organize and stabilize the overall chromosome structure. Relatively little is understood with regard to prokaryotic chromosomal packaging although there are structural similarities between some of the proteins found in prokaryotic and eukaryotic chromosomes. Therefore, most introductory cell biology courses stick to eukaryotic chromosomal packaging.

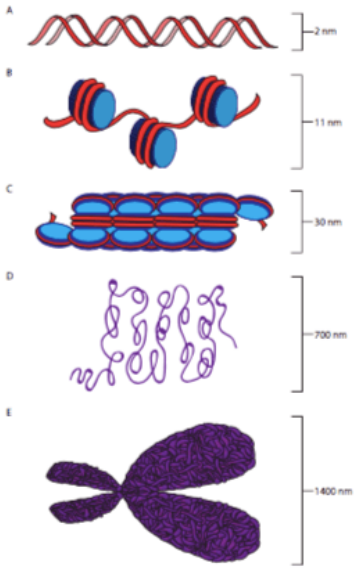


Figure 7.1.3. DNA packaging. (A) A naked strand of DNA is approximately 2 nm in diameter. (B) Histones, which are octameric proteins depicted here as a roughly cylindrical protein, have positive charges distributed on the outer surface to interact with the negatively-charged DNA backbone. (C) Even the organization afforded by histone binding can leave an unmanageable tangle of DNA, especially with longer eukaryotic genomes, and therefore the histone-bound DNA is packaged into the “30-nm strand”. This is held together, in part, by histone interactions. (D) The 30-nm fibers are looped into 700-nm fibers, which are themselves formed into the typical eukaryotic chromosome (E).

Naked DNA, whether prokaryotic or eukaryotic, is an extremely thin strand of material, roughly 11 nm in diameter. However, given the size of eukaryotic genomes, if the DNA was stored that way inside the nucleus, it would become unmanageably tangled. Picture a bucket into which you have tossed a hundred meters of yarn without any attempt whatsoever to organize it by coiling it or bunching it. Now consider whether you would be able to reach into that bucket pull on one strand, and expect to pull up only one strand, or if instead you are likely to pull up at least a small tangle of yarn. The cell does essentially what you would do with the yarn to keep it organized: it is packaged neatly into smaller, manageable skeins. In the case of DNA, each chromosome is looped around a **histone complex** to form the first order of chromosomal organization: the nucleosome.

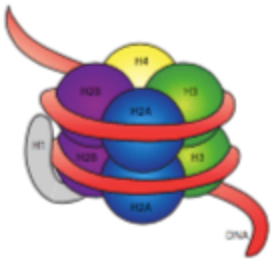


Figure 7.1.4. The nucleosome is composed of slightly over two turns of DNA around a histone core containing two copies each of H2A, H2B, H3, and H4 histones. The H1 histone is not part of the core unit and functions in coor- dinating interaction between nucleosomes.

> The 30-nm fiber is held together by two sets of interactions. First, the linker histone, H1, brings the nucleosomes together into an approximate 30-nm structure. This structure is then stabilized by disulfide bonds that form between the H2A histone of one nucleosome and the H4 histone of its neighbor.

Histones are a family of basic (positively-charged) proteins. They all function primarily in organizing DNA, and the nucleosome is formed when DNA wraps (a little over 2 times) around a core of eight histones - two each of H2A, H2B, H3, and H4. The number and position of the positive charges (mostly from lysines and arginines) are crucial to their ability to tightly bind DNA, which as previously pointed out, is very negatively charged. That “opposites attract” idea is not just a dating tip from the advice columns.

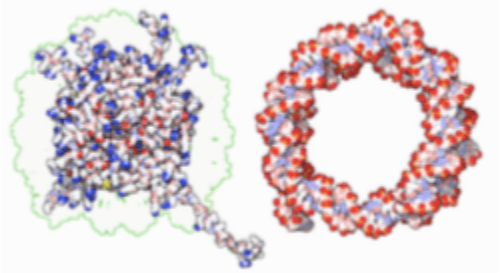


Figure from RCSB Protein Data Bank (http://www.rcsb.org).

Upon examination of the 3D structure of the histone core complex, we see that while relatively uncharged protein interaction domains hold the histones together in the center, the positively charged residues are found around the outside of the complex, available to interact with the negatively charged phosphates of DNA.

In a later chapter, we will discuss how enzymes read the DNA to transcribe its information onto smaller, more manageable pieces of RNA. For now, we only need to be aware that at any given time, much of the DNA is packaged tightly away, while some parts of the DNA are not. Because the parts that are available for use can vary depending on what is happening to/in the cell at any given time, the packaging of DNA must be dynamic. There must be a mechanism to quickly loosen the binding of DNA to histones when that DNA is needed for gene expression, and to tighten the binding when it is not. As it turns out, this process involves *acetylation* and *deacetylation* of the histones.

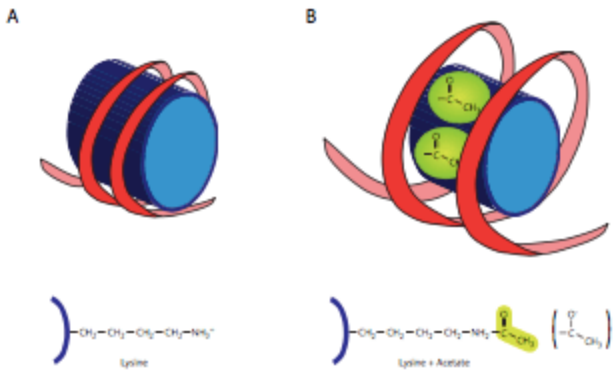


Figure 7.1.6. (A) Deacetylated histone allows interaction between the negatively charged phosphates of the DNA and the positively charged lysines of the histone. (B) When the histone is acetylated, not only is the positive charge on the lysine lost, the acetyl group also imparts a negative charge, repelling the DNA phosphates.

Histone Acetyltransferases (HATs) are enzymes that place an acetyl group on a lysine of a histone protein. The acetyl groups are negatively charged, and the acetylation not only adds a negatively charged group, it also removes the positive charge from the lysine. This has the effect of not only neutralizing a point of attraction between the protein and the DNA, but even slightly repelling it (with like charges). On the other side of the mechanism, Histone Deactylases (HDACs) are enzymes that remove the acetylation, and thereby restore the interaction between histone protein and DNA. Since these are such important enzymes, it stands to reason that they are not allowed to operate willy-nilly on any available histone, and in fact, they are often found in a complex with other proteins that control and coordinate their activation with other processes such as activation of transcription.

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7.2: Semi-Conservative DNA Replication

DNA replication is similar to transcription in its most general idea: a polymerase enzyme reads a strand of DNA one nucleotide at a time, it takes a random nucleotide from the nucleoplasm, and if it is complementary to the nucleotide in the DNA, the polymerase adds it to the new strand it is creating. Of course, there are significant differences between replication and transcription too, not the least of which is that both strands of DNA are being read simultaneously in order to create two new complementary strands that will eventually result in a complete and nearly perfect copy of an entire organismal genome.

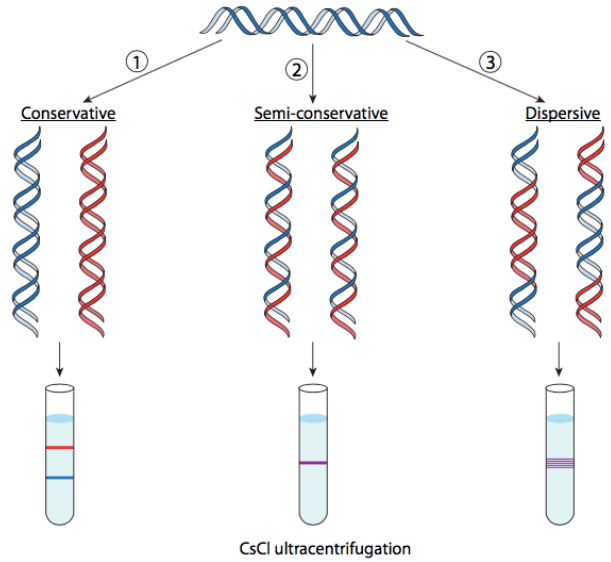


Figure 7.2.7. DNA replication. Prior to the discovery of the enzymes involved in replication, three general mechanisms were proposed. In conservative replication, the original DNA strands stay associated with each other, while the newly made DNA forms its own double-helix. Semi-conservative replication posits the creation of hybrid old-new double helices. Dispersive replication proposed molecules composed of randomized fragments of double-old and double-new DNA.

One of the most important concepts of DNA replication is that it is a *semi-conservative* process (Figure 7.2.7). This means that every double helix in the new generation of an organism consists of one complete "old" strand and one complete "new" strand wrapped around each other. This is in contrast to the two other possible models of DNA replication, the conservative model, and the dispersive model. A conservative mechanism of replication proposes that the old DNA is used as a template only and is not incorporated into the new double-helix. Thus the new cell has one completely new double-helix and one completely old double-helix. The dispersive model of replication posits a final product in which each double helix of DNA is a mixture of fragments of old and new DNA. In light of current knowledge, it is difficult to imagine a dispersive mechanism, but at the time, there were no mechanistic models at all. The Meselson-Stahl experiments (1958) clearly demonstrated that the mechanism must be semi-conservative, and this was confirmed once the key enzymes were discovered and their mechanisms elucidated.

In the Meselson-Stahl experiments, E. coli were first incubated with ^{15}N, a heavy isotope of nitrogen. Although it is only a difference in mass of one neutron per atom, there is a great enough difference in mass between heavy nitrogen-containing DNA (in the purine and pyrimidine bases) and light/normal nitrogen-containing DNA that they can be separated from one another by ultracentrifugation through a CsCl concentration gradient (Figure 7.2.7).

Over 14 generations, this led to a population of *E. coli* that had heavy nitrogen incorporated into all of the DNA (shown in blue). Then, the bacteria are grown for one or two divisions in "light" nitrogen, ^{14}N. When the DNA from the bacterial populations was examined by centrifugation, it was found that instead of light DNA and heavy DNA, as would be expected if DNA replications was conservative, there was a single band in and intermediate position on the gradient. This supports a semi-conservative model in which each strand of original DNA not only acts as a template for making new DNA, it is itself incorporated into the new double-helix.

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7.3: Prokaryotic Replication

DNA replication begins at an origin of replication. There is only one origin in prokaryotes (in *E. coli*, oriC) and it is characterized by arrays of repeated sequences. These sequences wrap around a DNA-binding protein, and in doing so, exert pressure on the H-bonds between the strands of DNA, and the chromosome begins to unzip in an AT-rich area wrapped around this protein. Remember that A-T pairs are 33% weaker than G-C pairs due to fewer hydrogen bonds. The use of AT-rich stretches of DNA as points of strand separation is a recurring theme through a variety of DNA operations. The separation of the two strands is bidirectional, and DNA polymerases will act in both directions in order to finish the process as quickly as possible. Speed is important here because while replication is happening, the DNA is vulnerable to breakage, and most metabolic processes are shut down to devote the energy to the replication. Even in prokaryotes, where DNA molecules are orders of magnitude smaller than in eukaryotes, the size of the DNA molecule when it is unraveled from protective packaging proteins makes it highly susceptible to physical damage just from movements of the cell.

The first OriC binding protein, DnaA, binds to DnaA boxes, which are 9 base pair segments with a consensus sequence of TTATCCACA. OriC has five of these repeats, and one DnaA protein binds to each of them. HU and IHF are histone-like proteins that associate with DnaA and together bend that part of the DNA into a circular loop, situating it just over the other major feature of oriC, the 13-bp AT-rich repeats (GATCTNTTNTTTT). DnaA hydrolyzes ATP and breaks the H-bonds between strands in the 13mer repeats, also known as melting the DNA. This allows complexes of DnaB [and DnaC, which is a loading protein that helps attach DnaB(6) to the strand with accompanying hydrolysis of ATP. Also, five more DnaA are recruited to stabilize the loop.] to bind to each single-stranded region of the DNA on opposite sides of the newly opened replication bubble.

DnaB is a **helicase**; its enzymatic activity is to unzip/unwind the DNA ahead of the DNA polymerase, to give it single-stranded DNA to read and copy. It does so in association with single-stranded-DNA binding proteins (SSBs), and DNA gyrase. The function of SSB is nearly self-explanatory: single-stranded DNA is like RNA in its ability to form complex secondary structures by internal base-pairing, so SSB prevents that. DNA gyrase is a type II topoisomerase, and is tasked with introducing negative supercoiling to the DNA. This is necessary because the unzipping of the DNA by helicase also unwinds it (since it is a double helix) and causes the introduction of positive supercoiling. This means that the entire circular molecule twists on itself: imagine holding a rubber band in two hands and twisting it. As the supercoiling accumulates, the DNA becomes more tightly coiled, to the point that it would be impossible for helicase to unzip it. DnaB/gyrase can relieve this stress by temporarily cutting the double-stranded DNA, passing a loop of the molecule through the gap, and resealing it.

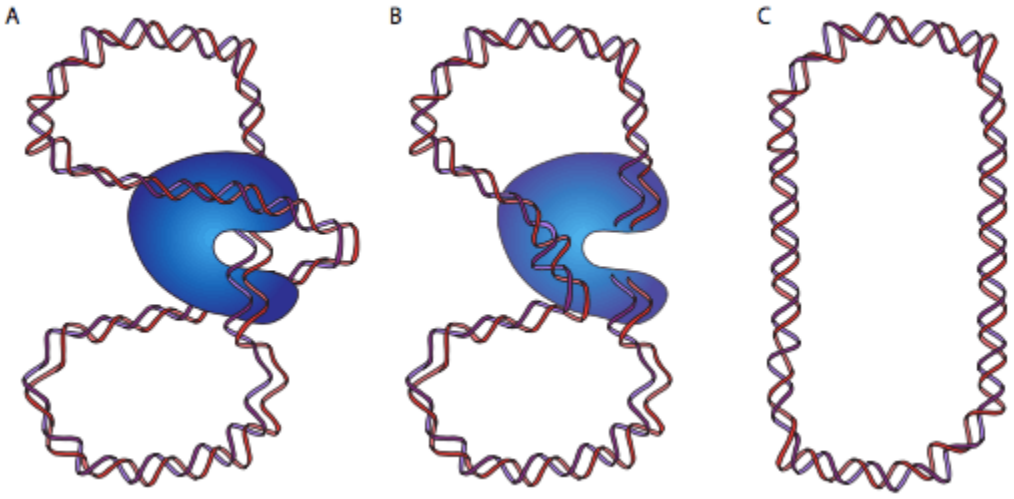


Figure 7.3.8. Type II DNA topoisomerases like DNA gyrase relieve supercoiling by making temporary double-strand cut.

This (hopefully) makes a lot more sense looking at the diagram. Or, going back to our rubber band, give the rubber band a twist or two, then tape down the two ends. If you snip the rubber band, and pass an adjacent portion of the rubber band through that snip, then reconnect the cut ends, you will find that there is one less twist. Nifty, eh? At this point, some of you are going to say, but if you twist a free-floating rubber band, as one might imagine a free-floating circular DNA chromosome in *E. coli*, you would expect it to naturally untwist. Technically, yes, but due to the large mass of the chromosome, its association with various proteins and the cell membrane, and the viscosity of its environment, it does not behave as though it were completely free.

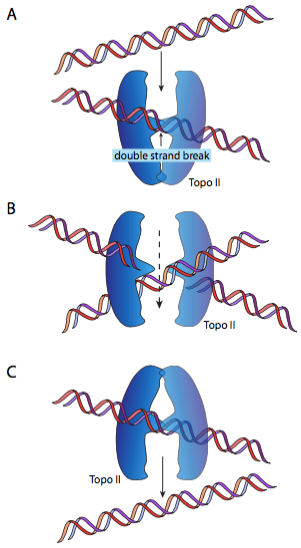


Figure 7.3.9. Detail of DNA topoisomerase type II action. (A) First, the enzyme binds to the DNA and initiates an endonuclease activity, cutting both strands of the DNA at that point. (B) This complete transection of the DNA (as opposed to the single-strand cut by type I topoisomerases) allows another part of the same DNA molecule to slip through the gap. (C) Finally, the two temporarily broken ends of the DNA, which had been held closely in place by the enzyme.

Once oriC has been opened and the helicases have attached to the two sides of the replication fork, the replication machine, aka the replisome can begin to form. However, before the DNA polymerases take positions, they need to be primed. *DNA polymerases are unable to join two individual free nucleotides* together to begin forming a nucleic acid; they can only add onto a pre-existing strand of at least two nucleotides. Therefore, a specialized RNA polymerase (RNAP's do not have this limitation) known as primase is a part of the replisome, and reads creates a short RNA strand termed the *primer* for the DNA polymerase to add onto. Although only a few nucleotides are needed, the prokaryotic primers may be as long as 60 nt depending on the species.

At least five prokaryotic DNA polymerases have been discovered to date. The primary DNA polymerase for replication in *E. coli* is DNA Polymerase III (Pol III). Pol I is also involved in the basic mechanism of DNA replication, primarily to ll in gaps created during lagging strand synthesis (defined 3 pages ahead) or through error-correcting mechanisms. DNA polymerase II and the recently discovered Pol IV and Pol V do not participate in chromosomal replication, but rather are used to synthesize DNA when certain types of repair is needed at other times in the cellular life cycle.

DNA polymerase III is a multi-subunit holoenzyme, with α, ε, and θ subunits comprising the core polymerase, and τ, γ, δ, δ', χ, Ψ, and β coming together to form the complete holoenzyme. The core polymerase has two activities: the α subunit is the polymerase function, reading a strand of DNA and synthesizing a complementary strand with great speed, around 150 nt/sec; the ε subunit is a 3'-5' "proofreading" exonuclease and acts as an immediate proofreader, removing the last nucleotide if it is incorrect. This proof-reading does not reach any further back: it only acts on the most recently added nucleotide to correct misincorporation. Other mechanisms and enzymes are used to correct DNA lesions that arise at other times. [As a matter of nomenclature, *exo*nucleases only cut off nucleotides from DNA or RNA from either end, but not in the middle. *Endo*nucleases cleave phosphodiester bonds located deeper within a nucleic acid strand.] The θ subunit has no enzymatic activity and regulates the exonuclease function. Although it has polymerase activity, the Pol III core polymerase has poor processivity - that is, it can only add up to 15 nucleotides before dissociating from the template DNA. Since genomes of *E. coli* strains average near 5 million base pairs, replication in little 15 nt segments would be extraordinarily inefficient.

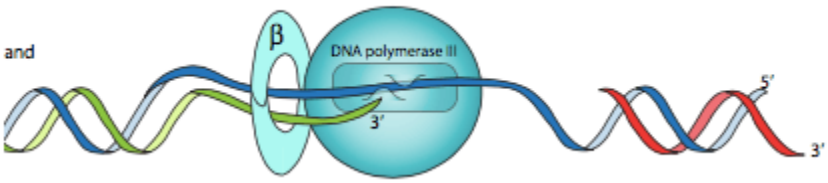


Figure 7.3.10. The dimeric β *clamp holds DNA Polymerase III on the template, allowing it polymerize more nucleotides before dissociating.*

The clamp loader complex is an ATPase assembly that binds to the β-clamp unit upon binding of ATP (but the ATPase activity is not turned on). When the complex then binds to DNA, it activates the ATPase, and the resulting hydrolysis of ATP leads to conformational changes that open up the clamp temporarily (to encircle or to move off of the DNA strand), and then dissociation of the clamp loader from the clamp assembly.

This is where the β subunit is needed. Also known as the β clamp, it is a dimer of semicircular subunits that has a central hole through which the DNA is threaded. The core polymerase, via an α-β interaction, is attached to this β clamp so that it stays on the DNA longer, increasing the processivity of Pol III to over 50000nt. The β clamp is loaded onto (and unloaded off of) the DNA by a clamp loader complex (also called γ complex) consisting of γ (x3), δ, δ', χ, and Ψ subunits.

The replication bubble has two replication forks - once the DNA is opened up (unzipped) at the origin, a replication machine can form on each end, with the helicases heading in opposite directions. For simplification, we will consider just one fork — opening left to right — in this discussion with the understanding that the same thing is happening with the other fork, but in the opposite direction.

The first thing to notice when looking at a diagram of a replication fork (Figure 7.3.11) is that the two single-stranded portions of template DNA are anti-parallel. This should come as no surprise at this point in the course, but it does introduce an interesting mechanical problem. Helicase opens up the double stranded DNA and leads the rest of the replication machine along. So, in the single-stranded region trailing the helicase, if we look left to right, one template strand is 3' to 5' (in blue), while the other is 5' to 3' (in red). Since we know that nucleic acids are polymerized by adding the 5' phosphate of a new nucleotide to the 3' hydroxyl of the previous nucleotide (5' to 3', in green), this means that one of the strands, called the *leading strand*, is being synthesized in the same direction that the replication machine moves. No problem there.

The other strand is problematic: looked at linearly, the newly synthesized strand would be going 3' to 5' from left to right, but DNA polymerases cannot add nucleotides that way. How do cells resolve this problem? A number of possibilities have been proposed, but the current model is depicted here. The replication machine consists of the helicase, primases, and two DNA polymerase III holoenzymes moving in the same physical direction (following the helicase). In fact, the pol III complexes are physically linked through τ subunits.

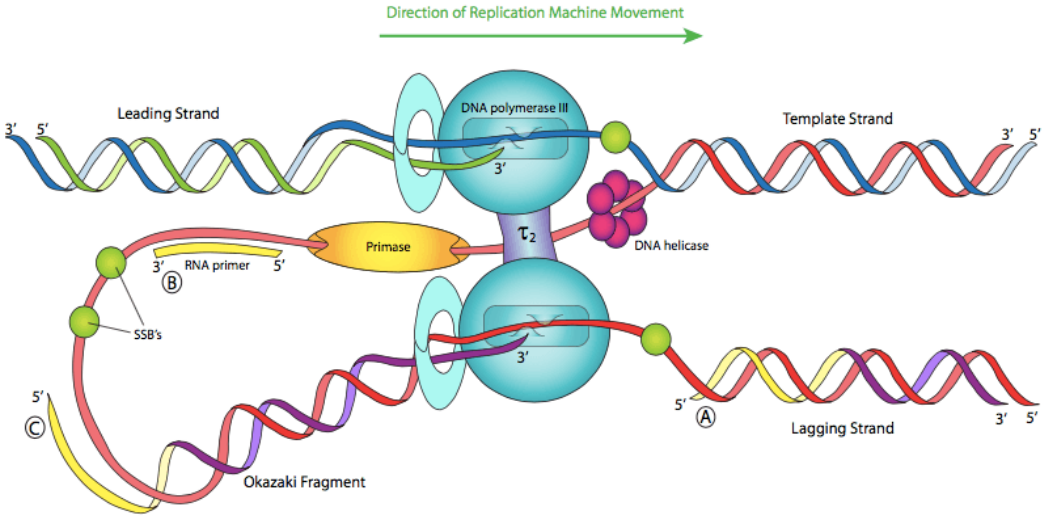


Figure 7.3.11. DNA Replication in prokaryotes.

In order for the template strand that is 5' to 3' from left to right to be replicated, the strand must be fed into the polymerase backwards. This can be accomplished either by turning the polymerase around or by looping the DNA around. As the Figure shows, the current model is that the primase is also moving along left to right, so it has just a short time to quickly synthesize a short primer before having to move forward with the replisome and starting up again, leaving intermittent primers in its wake. Because of this, Pol III is forced to synthesize only short fragments of the chromosome at a time, called Okazaki fragments after their discoverer. Pol III begins synthesizing by adding nucleotides onto the 3' end of a primer and continues until it hits the 5' end of

the next primer. It does not (and *can not*) connect the strand it is synthesizing with the 5' primer end.

DNA replication is called a semi-discontinuous process because while the leading strand is being synthesized continuously, the lagging strand is synthesized in fragments. This leads to two major problems: first, there are little bits of RNA left behind in the newly made strands (just at the 5' end for the leading strand, in many places for the lagging); and second, Pol III can only add free nucleotides to a fragment of single stranded DNA; it cannot connect another fragment. Therefore, the new "strand" is not whole, but riddled with missing phosphodiester bonds.

The first problem is resolved by DNA polymerase I. Unlike Pol III, Pol I is a monomeric protein and acts alone, without additional proteins. There are also 10-20 times as many Pol I molecules as there are Pol III molecules, since they are needed for so many Okazaki fragments. DNA Polymerase I has three activities: (1) like Pol III, it can synthesize a DNA strand based on a DNA template, (2) also like Pol III, it is a 3'-5' proofreading exonuclease, but unlike Pol III, (3) it is also a 5'-3' exonuclease. The 5'-3' exonuclease activity is crucial in removing the RNA primer (Figure 7.3.12). The 5'-3' exonuclease binds to double- stranded DNA that has a single-stranded break in the phosphodiester backbone such as what happens after Okazaki fragments have been synthesized from one primer to the next, but cannot be connected. This 5'-3' exonuclease then removes the RNA primer. The polymerase activity then adds new DNA nucleotides to the upstream Okazaki fragment, filling in the gap created by the removal of the RNA primer. The proofreading exonuclease acts just like it does for Pol III, immediately removing a newly incorporated incorrect nucleotide. After proofreading, the overall error rate of nucleotide incorporation is approximately 1 in 10^7.

> Technically, the 5'-3' exonuclease cleaves the DNA at a double-stranded region downstream of the nick, and may then remove anywhere from 1-10nt at a time. Experimentally, the 5'-3' exonuclease activity can be cleaved from the rest of Pol I by the protease trypsin. This generates the "Klenow fragment" containing the polymerase and 3'-5' proofreading exonuclease.

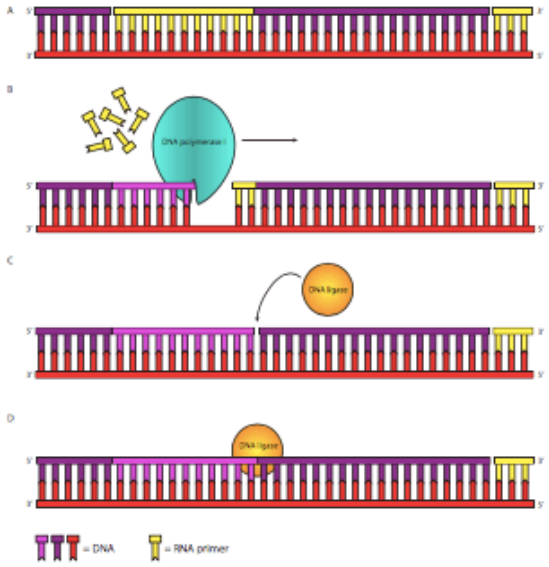


Figure 7.3.12. Lagging Strand Synthesis. After DNA polymerase III has extended the primers (yellow), DNA polymerase I removes the primer and replaces it by adding onto the previous fragment. When it finishes removing RNA, and replacing it with DNA, it leaves the DNA with a missing phosphodiester bond between the pol III-synthesized DNA downstream and the pol I-synthesized DNA upstream. This break in the sugar-phosphate backbone is repaired by DNA ligase.

Even though the RNA has been replaced with DNA, this still leaves a fragmented strand. The last major player in the DNA replication story finally appears: DNA ligase. This enzyme has one simple but crucial task: it catalyzes the attack of the 3'-OH from one fragment on the 5' phosphate of the next fragment, generating a phosphodiester bond. This reaction requires energy in the form of hydrolysis of either ATP or NAD^+ depending on the species (*E. coli* uses NAD^+) generating AMP and either PP_i or NMN^+.

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7.4: Eukaryotic Replication

Given the crucial nature of chromosomal replication for life to exist, it is not surprising to find that eukaryotic DNA replication is very similar to the prokaryotic process. This section will highlight some of the differences, which are generally elaborations on the prokaryotic version.

Unlike prokaryotes, eukaryotic chromosomes often have multiple origins of replication. Considering the size of eukaryotic chromosomes, this is necessary to finish complete replication in a timely manner. Each of these origins defines a *replicon*, or the stretch of the DNA that is replicated from a particular origin. The replicons do not replicate at exactly the same time (although all within the same phase of the cell cycle, see chapter 15), so it is important to make sure that replicons are used only once during a cell cycle.

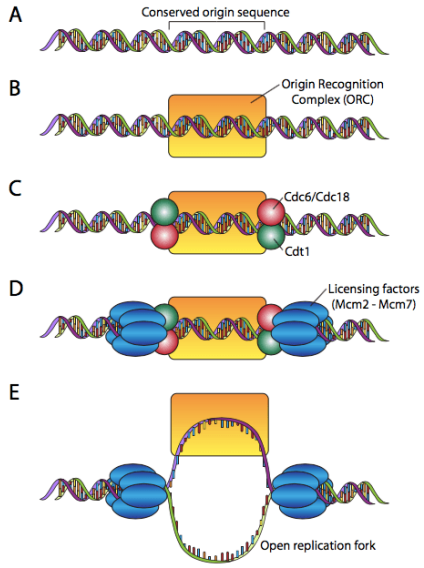


Figure 7.4.13. Eukaryotic Origin of Replication

This requires a “licensing” mechanism. During the cell cycle phase before DNA replication is initiated, a pre-replicative complex is assembled at each origin (Figure 7.4.13). The origins are highly variable in composition and length, ranging from ~100 to well over 10000 base pairs. The pre-RC proteins, on the other hand, are very highly conserved. The pre-RC begins by making the ORC (origin recognition complex, not a creature battling Frodo and Aragorn), which is comprised of six subunits (Orc1-Orc6). Although there is not significant sequence homology, the ORC approximates the function of the DnaA protein in *E. coli*. To complete the pre-RC, the ORC recruits a pair of proteins, Cdc6 and Cdt1 to each side, and they bind the MCM complex (a hexamer of Mcm2-Mcm6 that has an inactive helicase activity), leading to the fully licensed pre-RC. The origin is now ready for activation.

Activation of the pre-RC at an origin of replication requires first Mcm10, which facilitates protein kinases that phosphorylate the MCM complex, activating the helicase activity, and making the replication fork ready to accept the replication machine.

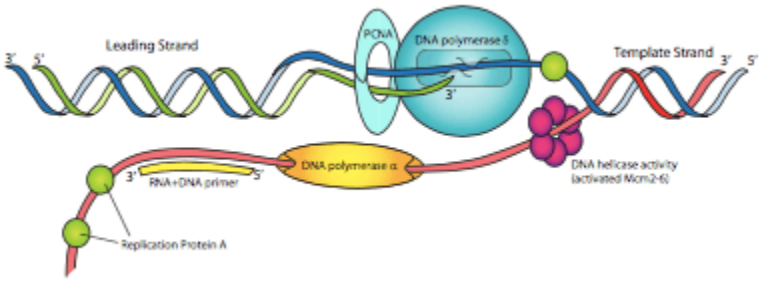


Figure 7.4.14. The eukaryotic replication machine is very similar to the prokaryotic machine.

The components of the eukaryotic replication machine may have different names from the prokaryotic, but the functions should be very familiar (Figure 7.4.14). There is a primase to make RNA primers, and tightly associated with the primase is DNA polymerase α. Pol α has neither a 5'-3' exonuclease nor a 3'-5' proofreading exonuclease, but it can synthesize DNA. However, this is not the primary DNA polymerase. The primase/pol a complex is essentially a fancy primase that begins with a short <10nt RNA primer and then adds another short ~15nt DNA fragment, making a *hybrid primer*.

Replication factor C (RFC), acting like the prokaryotic clamp loader complex, then replaces pol α with PCNA, the eukaryotic version of the β clamp. This then recruits DNA polymerase δ, which is the primary replicative DNA polymerase, equivalent to prokaryotic Pol III in function, and necessary for both leading and lagging strand synthesis. Finally, instead of SSB, eukaryotic cells use replication protein A (RPA) to organize and control single-stranded DNA as it is generated during the replicative process.

You may have noticed that none of the eukaryotic DNA polymerases discussed so far have a 5' to 3' exonuclease activity as used by prokaryotic DNA polymerase I to remove primers. In place of that, RNaseH1 and and FEN-1 remove the RNA primers (all but one ribonucleotide, and the last ribonucleotide, respectively). Interestingly, FEN-1 also excises chunks of lagging strand DNA within about 15 base pairs of the RNA if they contain mistakes. This seems to help alleviate the problem of lower fidelity of replication by pol α, which has no proofreading capability. After RNaseH1 and FEN-1 have removed primers and near-primer mistakes, pol δ fills in the missing nucleotides, and a ligase enzyme joins the fragments. Pol ε does have a 3'-5' exonuclease activity that chops single-stranded DNA into small oligonucleotide fragments and is also associated with the replication machine. The function of Pol ε is not clearly understood.

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7.5: DNA Lesions

The robust nature of DNA due to its complementary double strands has been noted several times already. We now consider in more detail the repair processes that rescue damaged DNA. DNA is not nearly as robust as popular media makes it out to be. In fact, to take the blockbuster book and film, *Jurassic Park*, as an example, Although there is unquestionably some DNA to be found either embedded in amber-bound parasites, or perhaps in preserved soft tissue (found deep in a fossilized femur, Schweitzer et al, 2007). It is likely to be heavily degraded, and accurate reproduction is impossible without many samples to work from.

The most common insult to the DNA of living organisms is depurination, in which the β-N-glycosidic bond between an adenine or guanine and the deoxyribose is hydrolyzed. In mammalian cells, it is estimated at nearly 10000 purines per cell generation, and generally, the average rate of loss at physiological pH and ionic strength, and at 37°C, is approximately 3 x 10^{-11} /sec. Depyrimidination of cytosine and thymine residues can also occur, but do so at a much slower rate than depurination. Despite the high rate of loss of these bases, they are generally remediated easily by base excision repair (BER), which is discussed later in this section. Therefore it is rare for depurination or depyrimidination to lead to mutation.

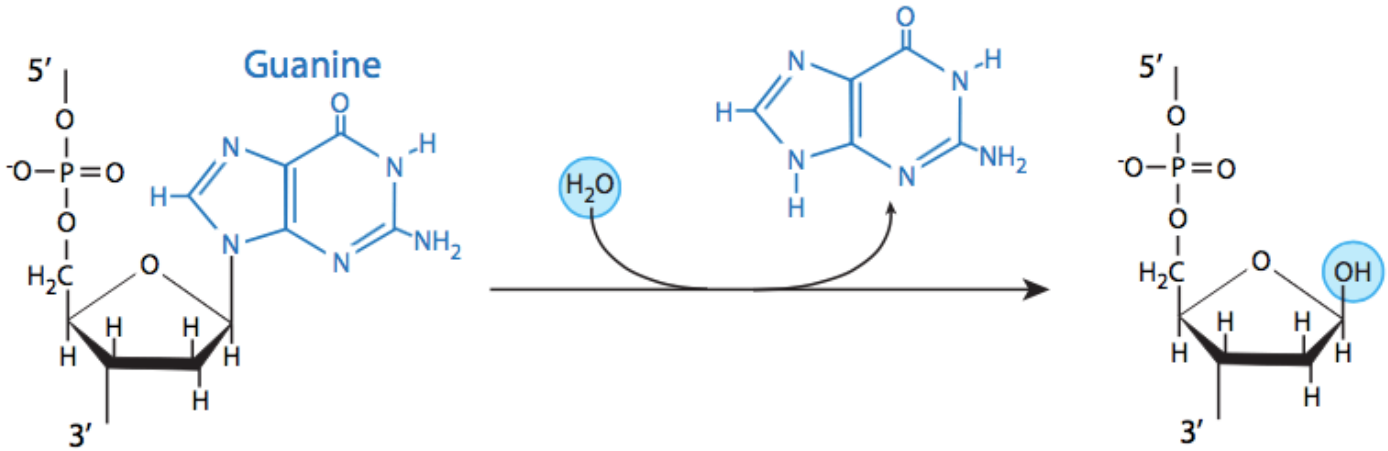


Figure 7.5.15. Depurination of guanines (or adenines) is a common DNA lesion.

Three of the four DNA bases, adenine, guanine, and cytosine, contain amine groups that can be lost in a variety of pH and temperature-dependent reactions that convert the bases to hypoxanthine, xanthine, and uracil, respectively. This can sometimes lead to permanent mutations since during replication, they serve as a template for the synthesis of a complementary strand, and where a guanine should go, for example (complementary to cytosine), an adenine may be inserted (because it complements uracil, the deamination product of cytosine).

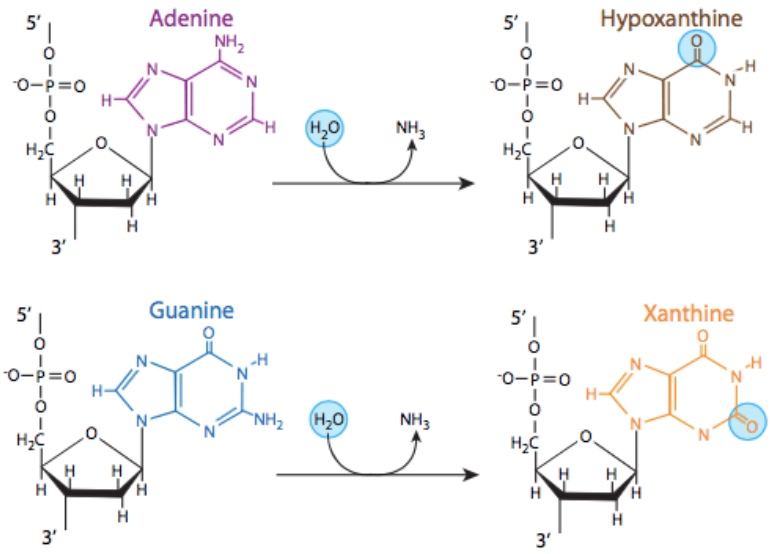


Figure 7.5.16A. Deamination of adenine and guanine can lead to mutations upon replication if unrepaired.

Another deamination, of the modified base methylcytosine, can also lead to a mutation upon replication. Some cytosines may be methylated as part of a regulatory process to inactivate certain genes in eukaryotes, or in prokaryotes as protection against restriction endonucleases. When the methylated cytosine is deaminated, it produces a thymine, which changes the complementary nucleotide (upon replication) from a guanine to an adenine. Deamination of cytosines occurs at nearly the same rate as depurination, but deamination of other bases are not as pervasive: deamination of adenines, for example, is 50 times less likely than deamination of cytosine.

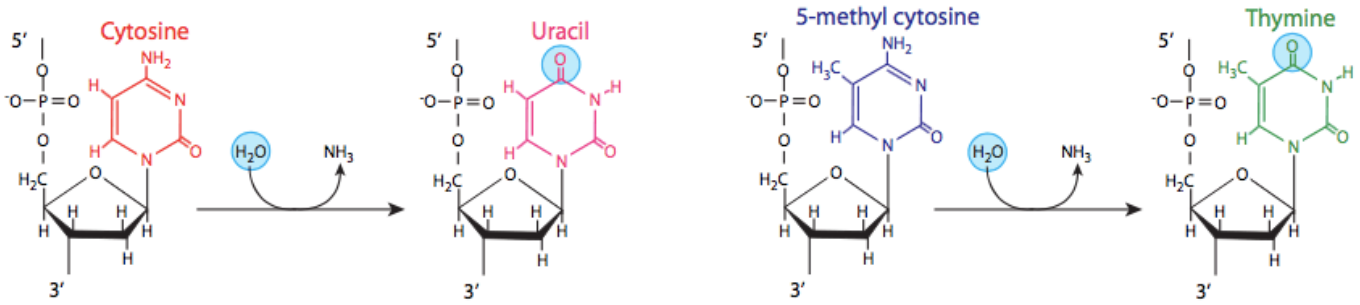


Figure 7.5.16B. Deamination of cytosine, and methylcytosine can lead to mutations upon replication if unrepaired.

> Thymine good, Uracil bad. Why is thymine found in DNA rather than uracil? It turns out that the frequency of cytosine deamination may yield a clue as to why cells have gone the extra step (literally, since uracil is a precursor in thymine biosynthesis) to make a new “standard” nucleotide for DNA when uracil worked just fine for RNA, presumably the older genetic molecule. Consider this: if uracil was standard for DNA, then the very frequent deamination conversions of C to U would not be caught by error-checking for non-DNA bases, and the mutation rate would skyrocket. Fortunately, since T has evolved to be the standard base-pairing partner of adenine in DNA, uracil is quickly recognized and removed by multiple uracil DNA glycosylases (more on that later in this chapter), and the integrity of our DNA sequences is much safer.

All DNA bases can spontaneously shift to a tautomeric isomer (amino to imino, keto to enol, etc), although equilibrium leans heavily toward one than the other. When a rare tautomer occurs, it base-pairs differently than its more common structural form: guanines with thymines and adenines with cytosines. Here again, a mutation can be propagated during replication of the DNA.

DNA inside a cell must also contend with reactive oxidative species (ROS) generated by the cell’s metabolic processes. These include singlet oxygen, peroxide and peroxide radicals, as well as hydroxyl radicals. although it is thought that the hydrogen peroxide and peroxide radicals do not directly attack the DNA but rather generate hydroxyl radicals that do. Most of these ROS are generated in the mitochondria during oxidative phosphorylation and leak out, although some may be generated in peroxisomes,

or in some cytosolic reactions. Depending on what part of the DNA is targeted, ROS can cause a range of lesions including strand breaks and removal of bases.

Ionizing radiation (e.g. X-rays) and ultraviolet radiation can each cause DNA lesions. Ionizing radiation is often a cause for double-stranded breaks of the DNA. As described later in the chapter, the repair process for double-stranded breaks necessarily leads to some loss of information, and could potentially knock out a gene. Ultraviolet radiation that hits adjacent thymines can cause them to react and form a cyclobutyl (four carbons bonded in closed loop) thymine dimer. The dimer pulls each thymine towards the other, out of the normal alignment. Depending on the structural form of the dimer, this is sufficient to stymie the replication machine and halt replication. However, some data suggests that normal base-pairing to adenine may be possible under some conditions, although, it is likely only one base-pair would result, and the missing base could lead to either random substitution or a deletion in the newly synthesized strand.

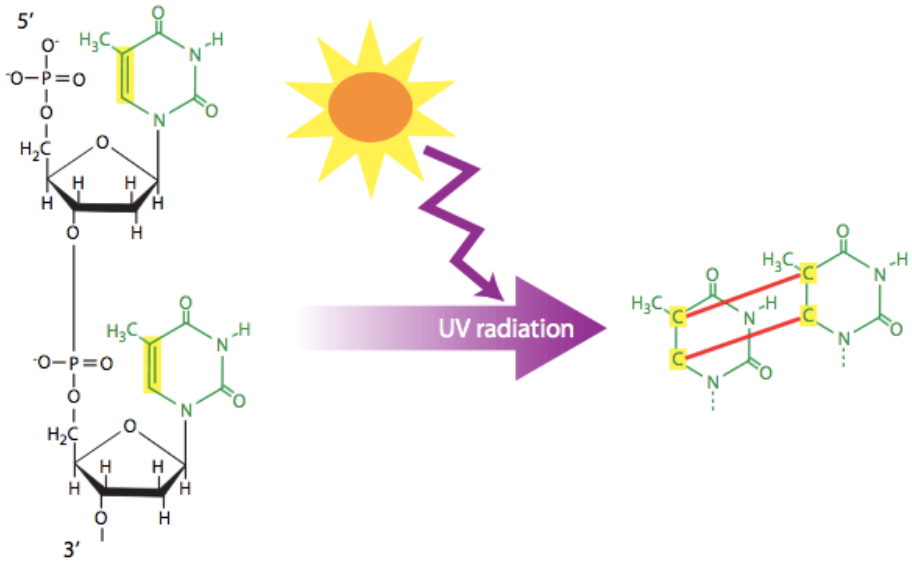


Figure 7.5.17. Ultraviolet radiation can be absorbed by some DNA and commonly causes pyrimidine cyclobutyl dimers connecting adjacent nucleotide bases.

Finally, we consider the formation of chemical adducts (covalently attached groups) on DNA. They may come from a variety of sources, including lipid oxidation, cigarette smoke, and fungal toxins. These adducts attach to the DNA in different ways, so there are a variety of different effects from the adducts as well. Some may be very small adducts - many environmental carcinogens are alkylating agents, transferring methyl groups or other small alkyl groups to the DNA. Other adducts are larger, but also attach covalently to a nitrogenous base of DNA. Common examples are benzo(a)pyrene, a major mutagenic component of cigarette smoke, and aflatoxin B1, produced by a variety of Aspergillus-family fungi. Benzo(a)pyrene is converted to benzo(a)pyrene diol epoxide, which can then attack the DNA. When this happens, the at pyrene ring intercalates between bases, causing steric changes that lead to local deformation of the DNA and disruption of normal DNA replication.

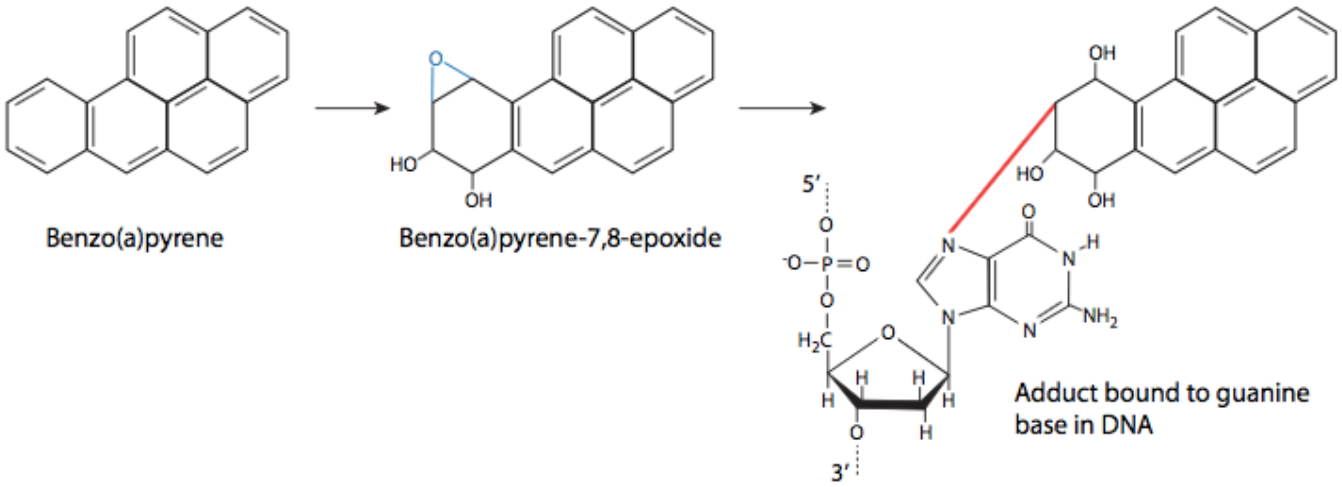


Figure 7.5.18. Benzo(a)pyrene is converted to an epoxide form by the cell. The epoxide can form an adduct on DNA.

Aflatoxin B1 is the primary aflatoxin produced by some species (esp. *flavus, parasiticus*) of Aspergillus, a very common mold that grows on stored grain (as well as detritus and other dead or dying plant matter). In addition to infecting grain, it is a common problem with stored peanuts. At high levels, aflatoxin is acutely toxic, but at lower levels, it has the insidious property of being unnoticeably toxic but mutagenic. Like benzo(a) pyrene, it is metabolized into an epoxide and will then react with DNA to form an adduct that can disrupt replication.

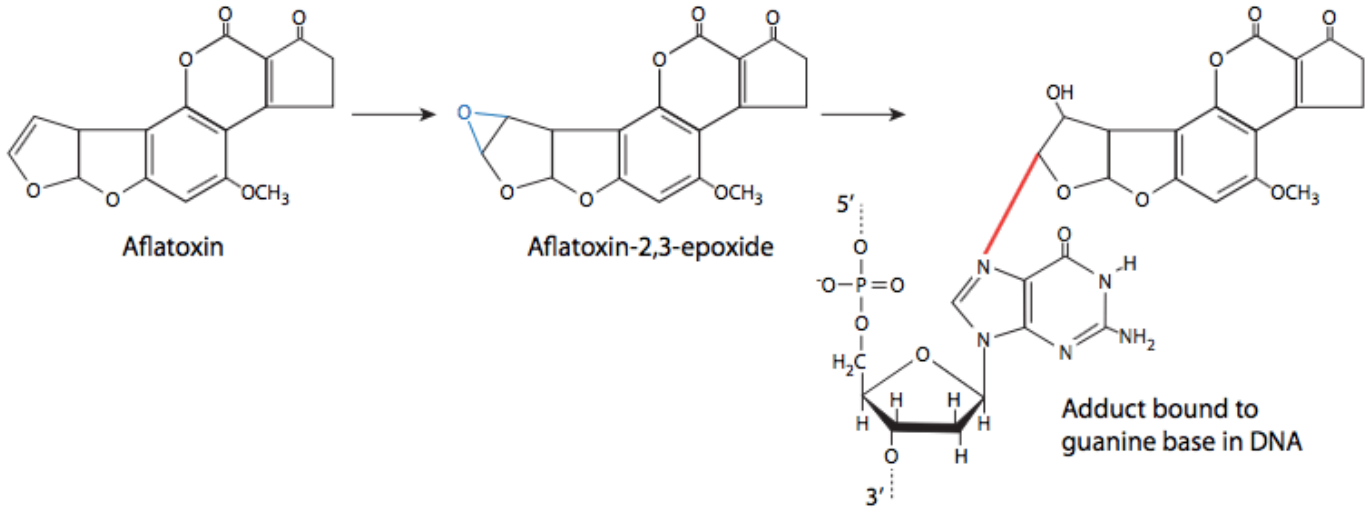


Figure 7.5.19. The epoxide form of aflatoxin also forms adducts on DNA.

Some alkylating agents, particularly N-nitroso compounds, are formed in the acidic conditions of the stomach from nitrosation of naturally occurring nitrites produced from food (reduction of nitrates), or environmental nitrites in drinking water. Ironically, while some alkylating agents can cause cancers, others are used therapeutically as anticancer treatments, e.g. mitomycin, melphalan. The idea, as with many cancer treatments, is that although such drugs cause DNA damage to non-cancerous cells as well as cancer cells, the high rate of cancer cell proliferation gives them fewer chances for repair of damaged DNA, and thus greater likelihood that the damage might halt replication and lead to cell death.

In a similar vein, crosslinking chemotherapeutic agents such as cisplatin (a platinum atom bonded to two chloride groups and two amino groups) also bind to DNA. The chloride groups are displaced first by water and then by other groups including sites on DNA. Although sometimes classified as an alkylating agent, it obviously is not, but it acts similarly. Cisplatin goes

a step further than a simple alkylating agent though, because it has another reactive site and can thus crosslink (covalently bond) another nucleotide, possibly on another strand of DNA, making a strong obstruction to DNA replication. Cisplatin can also crosslink proteins to DNA.

Benzo(a)pyrene and aflatoxin B1 are not themselves mutagens. Once they are in the cell, the normal metabolism of these compounds leads to diol epoxide formation, which can then attack the DNA. Although the 7-nitrogen (N7) of guanine is more nucleophilic, and is a target for aflatoxin, most benzo(a)pyrene diol epoxide adducts attach to the 2-nitrogen of guanine residues.

There are federal standards (20-300 parts per billion depending on usage) for aflatoxin in various forms of grain-based animal feed, especially corn-based feeds, because the toxin can pass through the animal into milk, as well as linger in the meat. In addition to feed, there are federal maximums for peanuts and peanut products, brazil nuts, pistachios, and other foodstuffs (actionable at 20 ppb).

Well then, what's a poor cell to do when its DNA is being constantly ravaged? As it turns out, there are some very good repair processes that are constantly at work on the DNA, scanning it for defects, and where possible, making repairs. Often the repairs are perfect, if the complementary strand is intact, sometimes mutations must be introduced, and finally there are occasions when repair is impossible, and apoptosis is triggered to kill the cell and prevent propagation of damaged DNA.

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7.6: DNA Repair

Strictly defined, the simplest repair mechanism does not use an enzyme. Dealkylation, or removal of alkyl groups (like $—CH_3$ or $—C_2H_5$) involves only the transfer of an alkyl group from an O6-methylguanine or O6-ethylguanine onto O6-alkylguanyl-DNA alkyltransferase. Despite the name, the alkyltransferase is not really an enzyme, since it is permanently altered and inactivated by the reaction and therefore does not fit the definition of a catalyst. Note that this does not remediate alkylation at N7 or other sites, just the O6-linked ones.

The next simplest repair mechanism is the uncoupling of pyrimidine cyclobutyl dimers. This can be accomplished through the activity of DNA *photolyases*, also known as photoreactivating enzymes. These are named not just because the formation of the pyrimidine cyclobutyl dimers is usually due to UV light exposure, but because the repair enzymes themselves require exposure to light (300-500 nm, near UV to visible blue) to catalyze the dimer-breaking reaction.

> More specifically, DNA photolyases (a ~60kD protein), are non-covalently associated with a chromophore (N^5,N^{10}-methylenyltetrahydrofolate or 5-deazaflavin) and an FADH—. The photolyase binds to the pyrimidine cyclobutyl dimer of either single-stranded or double-stranded DNA in a light-independent and sequence-independent manner. However it does not catalyze any change in the bond until light is absorbed by the chromophore, which then transfers the energy to FADH—, causing it to eject and electron to the dimer, thus breaking it apart.

While dealkylation and dimer lysis are relatively simple processes that make only a subtle change to the DNA, excision repair mechanisms are more complicated and require multiple enzymatic steps to complete. When a small (not sterically bulky) lesion is limited to a single base, whether missing from depurination or incorrectly formed due to deamination or misincorporation, the process known as *base excision repair* (BER) is engaged. As illustrated in Figure 7.6.20, if a non-conventional base is recognized, it is then removed by an appropriate *DNA glycosylase*. At present (Genbank search, July 2009), there are at least 8 specific genes encoding human DNA glycosylases, although three encode glycosylases that recognize uracil in various situations. Once the base has been removed by the glycosylase, an endonuclease is enlisted to break the phosphodiester bonds than hold the now-empty phosphodeoxyribose. The resulting gap in the DNA is filled in by a DNA polymerase and finally the strand is reconnected by DNA ligase.

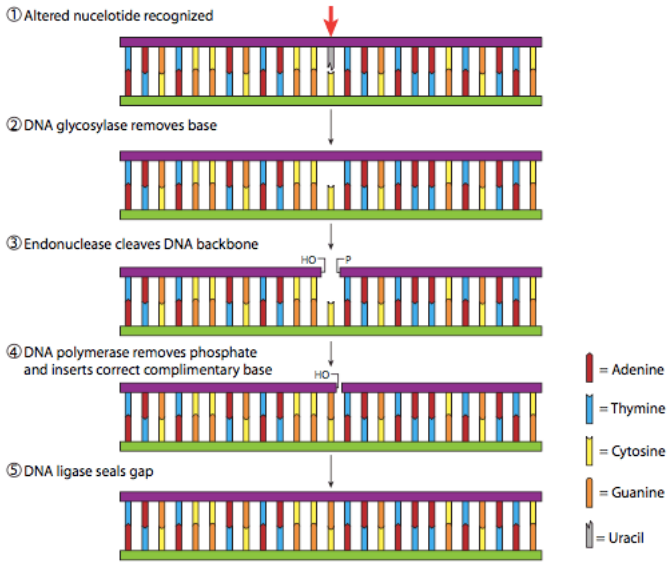


Figure 7.6.20. Base Excision 1 Repair requires five separate steps.

In the case of bulky lesions that significantly alter the physical presentation of the DNA to the polymerases and other enzymes that process DNA, a different type of repair process is involved. Nucleotide excision repair (NER), perhaps better named *poly*nucleotide excision repair, involves the removal of the lesion as well as some of the nucleotides in the immediate vicinity.

There are two major initiators of NER: either a non-transcriptionally active portion of the DNA is scanned by XPC (Figure 7.6.21 A), which recognizes a bulky lesion and recruits the repair complex, or as a gene is being transcribed, RNA polymerase runs into a lesion, and then recruits the repair complex via CSA and CSB (Figure 7.6.21F and G). If the detection is through XPC, one of the early repair factors recruited to the site is Transcription Factor IIH/XPB/XPD, which is a DNA helicase (Figure 7.6.21B). This type of global genome detection is inefficient and relatively slow, but provides a basal level of error-checking for all DNA. In the case of DNA being transcribed, the RNA polymerase complex already includes TFIIH, of which XPB and XPD are a part. This transcriptionally-directed detection is more efficient and targets those parts of the DNA in greatest use in a given cell. In the next step (Figure 7.6.21C), XPG, associated with BRCA1/2, and XPF, associated with ERCC1, excise a portion of the affected strand, including but not limited to the lesion itself. DNA polymerase δ or ε can then add onto the free 3'OH to ll in the gap based on the complementary strand sequence (Figure 7.6.21D). Finally, the repair is connected on its 3' end to the rest of the strand by DNA ligase (Figure 7.6.21E).

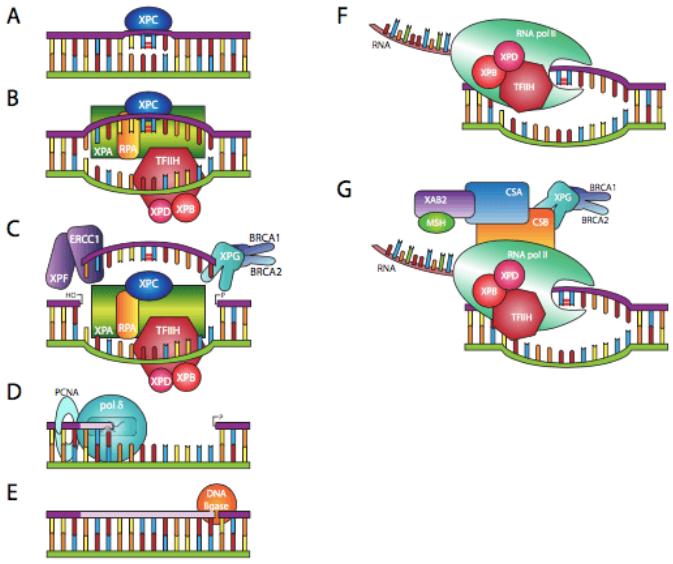


Figure 7.6.21. Nucleotide Excision Repair in Eukaryotic Systems

The "XP" in XPC, XPB, XPD, and the others in Figure 7.6.21 refers to xeroderma pigmentosa, another autosomal recessive disease, of which the primary characteristic is the formation of skin carcinomas at a young age. Because NER is a major form of pyrimidine dimer repair (in addition to photolyases), its disruption by mutations to one or more of the XP genes leads to extreme sensitivity to UV-induced lesions. Affected individuals must minimize exposure to the sun. The name of the disease comes from the characteristic pigmented lesions (keratoses) that often form on the skin when exposed to sun.

CSA and CSB are named for Cockayne syndrome, an autosomal recessive aging disorder. Mutations in either gene can cause the disorder, which is characterized by premature aging, stunted growth, photosensitivity, and developmental defects of the nervous system. Presumably, knocking out the DNA repair capability of CSA or CSB leads to fast accumulation of damage, inability to transcribe needed genes, and eventually cell death.

A sort of variation on NER is the **mismatch repair (MMR) system**. This is best understood in prokaryotes: in *E. coli*, MutS is a small protein that forms homodimers at mismatch sites. The MutS dimers recruit two MutL proteins, each of which interacts with one of the MutS units. Each MutS/MutL complex pushes DNA through inwardly, forming a loop with the mismatch in the center of the loop. This continues until one of the MutS/MutL complexes encounters a *hemimethylated* GATC sequence. This causes recruitment of MutH, a highly specialized endonuclease that makes a single-stranded nick in the backbone of the non-methylated strand. This provides an opening for the 3'-5' exonuclease I or the 5'-3' exonuclease VII (or RecJ) to degrade the strand from the nick to the point of mismatch. This is then, as you may have guessed, filled in by DNA polymerase and the backbone connected by ligase. In eukaryotes, multiple homologues to the MutS and MutL proteins have been discovered and the process is similar, but not clearly understood yet, as no homologue to MutH has yet been discovered.

Recall that in *E. coli*, Dam methyltransferases eventually methylate the DNA as a method of protecting its genome, but newly synthesized DNA is not methylated. Thus, the assumption is that the methylated strand contains the original and correct base, while the mismatch is due to misincorporation in the newer strand.

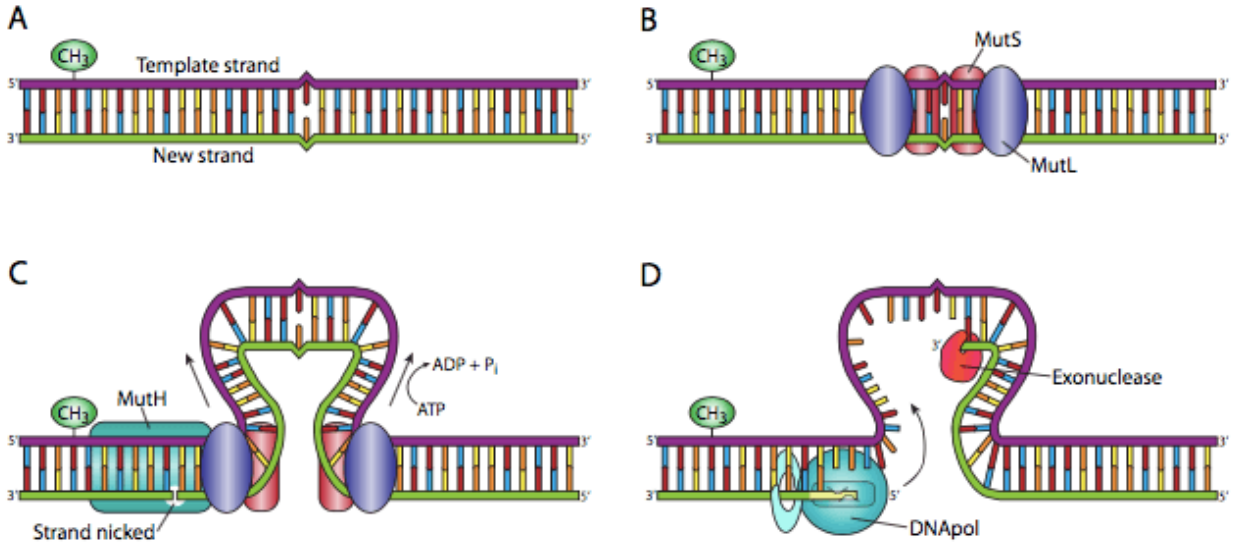


Figure 7.6.22. Mismatch Repair System. Mismatched nucleotides do not for normal H-bonds, and cause a minor disturbance (A) in the DNA structure. (B) MutS dimerizes at mismatch sites and each recruits a MutL protein. (C) The MutS/L complex pushes DNA through to form a loop with the mismatch in the center. When a methylated GATC is encountered, the MutS/L stop moving DNA and recruit the MutH endonuclease. An exonuclease then digests the DNA strand to the mismatch. (D) Finally, a DNA polymerase fills in the missing segment and the backbone is sealed by ligase.

Another prokaryotic DNA repair system is the SOS response. As depicted in Figure 7.6.23 below, if there is no damage, RecA is inactive, so LexA protein can repress the production of more SOS repair proteins. However, if there is damage, RecA proteins bind to the single stranded DNA and are activated. They in turn cleave the LexA repressor allowing production from a number of DNA repair genes.

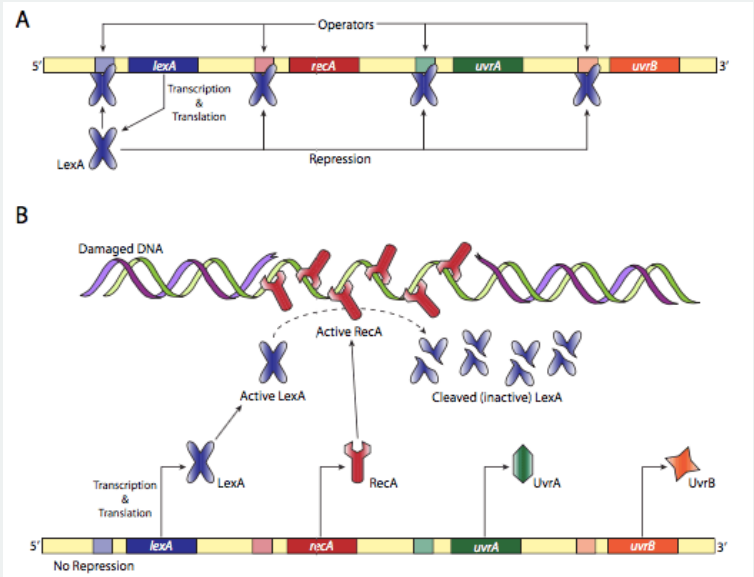


Figure 7.6.23. The prokaryotic SOS operon. (A) repressed state when there is no DNA damage, and (B) the activated state in which DNA damage has been detected.

So far, the repairs have been based on the assumption that a lesion affects only one strand, and the other strand can provide a reliable template for effecting repairs without the loss of information. Unfortunately, that is not always the case, and some lesions and repair processes necessarily lead to sequence loss. When a double-strand break occurs, perhaps as the product of ionizing radiation, the most common repair mechanism is known as **non-homologous end joining (NHEJ)**. The double-stranded ends are first recognized by Ku, a heterodimeric circular protein that binds the DNA ends. Ku then recruits the kinase DNA-PK_{CS}. The DNA-PK_{CS} acts as a bridge to bring the two ends together, and a DNA ligase can then join the ends together. If the strands were broken in different places, resulting in complementary single-stranded overhangs at each end (like those generated by some restriction endonucleases) then the repair is often perfect, since the complementary sequences align the two ends correctly in their original positions. However, if the strand ends have already been acted upon by nucleases and are no longer complementary, then the rejoining of the ends will likely lead to loss of information. In some parts of the DNA, this would have little effect, but if it happened within a gene, the mutated gene product could have abnormal or compromised function.

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7.7: Telomeres

If there is a mechanism for recognizing loose ends of DNA, what about the ends of every eukaryotic chromosome? They are linear chromosomes, so they have ends, right? What prevents the double-strand-break repair systems from mis-recognizing them all as broken DNA and concatenating all of the chromosomes together? Interestingly, the answer to this question is intimately tied up with the answer to the problem of end- replication, which was very briefly alluded to in our description of replication.

The end-replication problem is one that affects all linear chromosomes. It boils down to one simple fact: an RNA primer is needed to start any DNA replication. So on the 5' end of each strand is an RNA primer (Figure 7.7.24 in yellow) that gets removed by the error-correction process. Thus with every round of replication, information is lost from the 5' end of each strand of each chromosome.

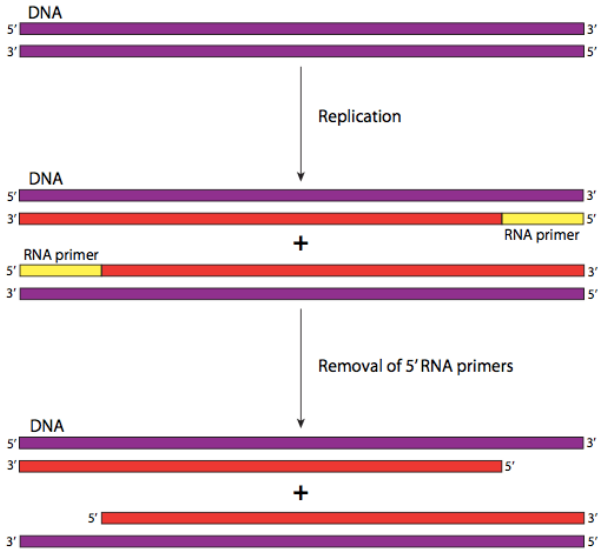


Figure 7.7.24. End-replication problem.

Eventually, crucial genes are lost and the cell will die; most likely many cellular functions will be compromised long before that happens. The solution to the end-replication problem might be considered more a treatment of symptoms than a cure, to use an analogy to medicine. In short, during the very early stages of an organism's life, a lot of non-coding DNA is added onto the ends of the DNA so that as the cell and its progeny continue to reproduce, the nucleotides do not affect any functional genes. This process is catalyzed by the enzyme telomerase.

Telomerase is a large holoenzyme that acts as a reverse transcriptase, reading a self-contained RNA template to add on telomere sequence to the 3' ends of linear chromosomes. Note that this does not add onto the 5' ends - as mentioned above, there is nothing to be done about the 5' ends directly. However, while telomerases are active, the 3' ends can be extended, and thus when the 5' end primer is removed, the sequence lost (forever) from that strand of DNA is only a telomeric repeat and not something more useful. The repeats are well conserved across eukaryotes, and almost completely conserved across mammalian species (shown in Figure 7.7.25).

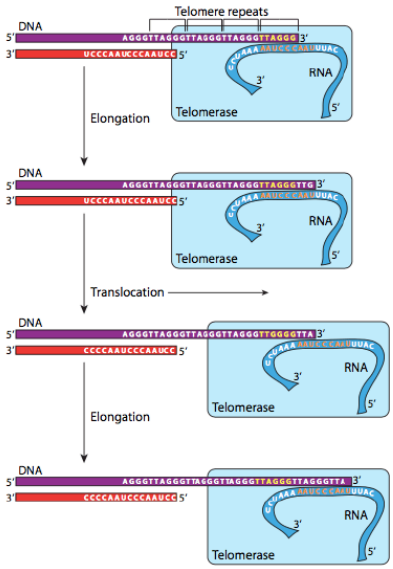


Figure 7.7.25. Telomerases are reverse transcriptases that generate DNA repeats from a built-in RNA template.

In metazoans, telomerase activity is high in the embryonic stages of life, but is virtually non-existent in adults except in cell types that must constantly proliferate (e.g. blood and epithelial cells). The activity of telomerase is primarily regulated by expression of the TERT gene (telomerase reverse transcriptase) although construction of the full telomerase also requires the expression of the TERC gene (the telomerase RNA, also abbreviated TR), and dyskerin. Roughly speaking, the number of telomeric repeats that are placed on a chromosome in early development determines the number of DNA replications and cell divisions that the cell can undergo before succumbing to apoptosis (programmed cell death). Experiments on cells in culture demonstrate a strong correlation between telomere length and longevity, and it is known that cells taken from people with the premature aging disease, progeria, have relatively short telomeres.

Conversely, cancer cells almost universally have unregulated expression of telomerase. Given that a defining characteristic of cancer cells is the ability to proliferate rapidly and indefinitely, turning telomerase back on is, not surprisingly, an important aspect of carcinogenesis. It is therefore a target for anti-cancer treatments; however, to date no telomerase-targeting therapies have proven effective.

Now that we know about telomeres, the question that started this section becomes even more problematic: with these repeated sequence overhangs, how are chromosomes prevented from connecting end-to-end through a double-strand repair-like process? In part due to their repeated sequences, telomeres are able to form end-caps and protect chromosomal ends. The telomeres protect the ends of each chromosome by binding to protective proteins and by forming complex structures. Telomere end binding proteins (TEBP) bind to the 3' overhanging end of the telomere. Other capping proteins, such as the mammalian TRF1 and TRF2 (telomere repeat binding factors) not only bind the telomere, but help to organize it into large looped structures known as T-loops (Figure 7.7.26).

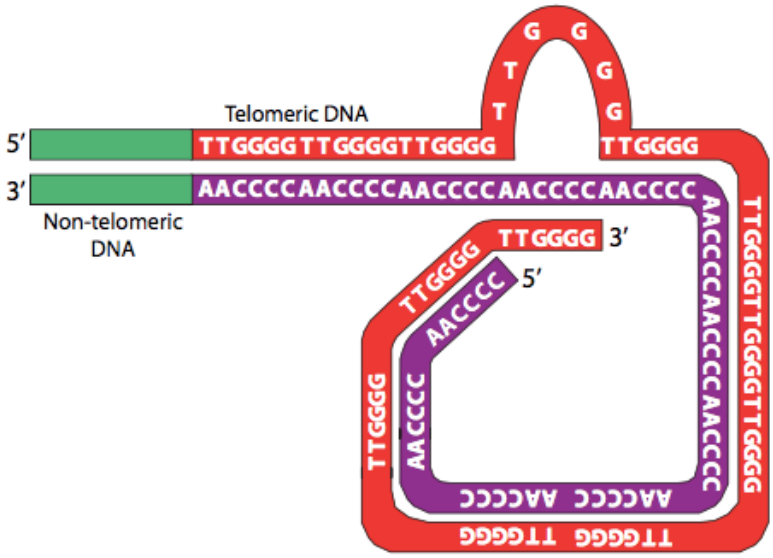


Figure 7.7.26. Telomeres form protective T-loop structures on the ends of linear chromosomes.

Finally, the T-loop ends are further stabilized by the formation of G-quartets (Figure 7.7.27). G-quartets are a cyclic tetramers that can form in sequences with four consecutive guanine residues, which hydrogen-bond to each other to make a linked square shape stabilized by a metal ion in the center. Furthermore in cases like the telomere, in which such sequences are repeated, the G-quartets can stack and associate three-dimensionally, increasing their stability.

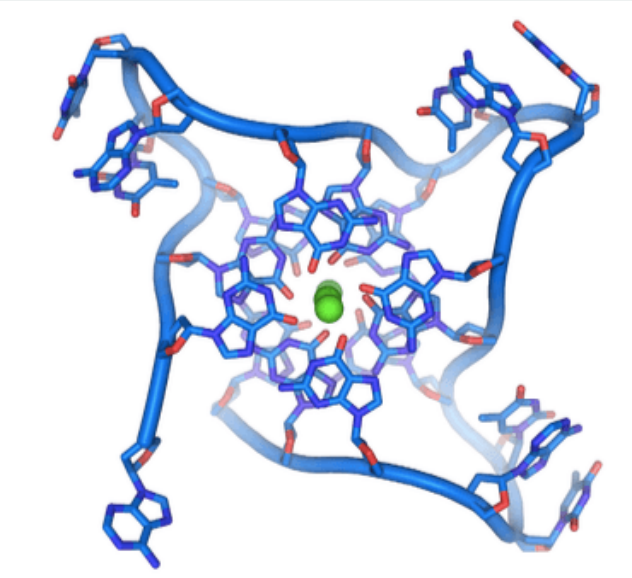


Figure 7.7.27. G-quartets form by hydrogen bonding of fours consecutive guanine residues stabilized by a metal ion (green), and can form three-dimensional stacks due to telomeric repeats. Produced from Protein DataBank data by T. Splettstoesser.

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8: TRANSCRIPTION

Although DNA is an excellent medium for the storage of information, the very characteristic that makes it so stable and inherently self-correcting - being double-stranded - also makes it unwieldy for using that genetic information to make cell components. Since the informational parts of the molecule (the nitrogenous bases) are locked inside the ladder, reading it requires the energetically expensive task of breaking all the hydrogen bonds holding the two strands together. To do so for every single copy of each protein needed by the cell would not only take a lot of energy, but a lot of time. Instead, there must be a mechanism to take the information from DNA once (or a few times), and then make many copies of a protein from that single piece of information. That mechanism is transcription.

8.1: Introduction to Transcription

8.2: Prokaryotic Transcription

8.3: Eukaryotic Transcription

8.4: Post-Transcriptional Processing of RNA

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8.1: Introduction to Transcription

To obtain the genetic information in a form that is easily read and then used to synthesize functioning proteins, the DNA must first be transcribed into RNA (ribonucleic acid). As we saw in chapter 1, RNA is extremely similar to DNA, using some of the same nitrogenous bases (adenine, guanine, cytosine) as well as one unique to RNA, uracil. Notice that uracil is very similar to thymine (chapter 7, Figure1), particularly in the placement and spacing of the hydrogen-bonding atoms. Since it is the hydrogen-bonding interaction of these bases (i.e. base-pairing of guanine to cytosine, adenine to thymine/uracil) that forms the basis of information transfer from original DNA to daughter cell DNA, it is logical to expect that the same kind of base-pairing mechanism is used to move the information from a storage state in the double-stranded nucleic acid (DNA) to a more useful/usable state in the form of a single-stranded nucleic acid (RNA).

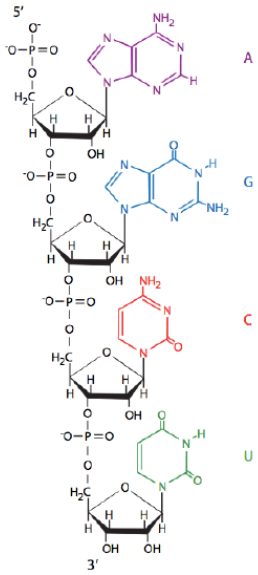


Figure 8.1.1. Ribonucleic Acid (RNA) is a polymer of the nucleotides adenine, guanine, cytosine, and uracil, connected by 3'-5' phosphodiester bonds.

In contrast to its cellular role as a transient and disposable carrier of genetic information, RNA is thought to have been the primary molecule responsible for making life possible on earth. It has long been postulated that it served dual roles as both a repository of genetic information and as a rudimentary enzyme to act upon that information. Unfortunately, prebiotic chemists have been stymied for decades in coming up with a reasonable synthetic pathway by which RNA could arise from the simple molecules of the earth's primordial "soup". The key problem was that ribose could be synthesized, though not particularly efficiently, and bases could be synthesized, but there was no way to connect them together. The chemistry would not allow a condensation reaction between the bases and sugars. In 2009, by leaving behind the conventional idea that ribonucleotides must have been synthesized from ribose and purines/pyrimidines, Powner, Gerland, and Sutherland (*Nature* 459:239-242, 2009) showed that in fact, ribonucleotides could be synthesized from the chemical conditions of a newly formed earth. Rather than attempt to make each "part" and put them together, Powner et al synthesized a molecule that contained parts of what would eventually be both the ribose and a pyrimidine, 2-aminooxazole. Through a series of reactions that utilized phosphate as a catalyst and scavenger, all of which were clearly plausible in the current model of primordial earth, both ribocytidine and ribouridine were created. Of course, this is only the beginning, since this does not extend directly to the formation of purine nucleotides, but it is a very significant step in prebiotic chemistry, and an excellent example of the virtues of "stepping outside of the box" sometimes.

The process of copying DNA into RNA is called transcription. In both prokaryotes and eukaryotes, transcription requires certain control elements (sequences of nucleotides within the DNA) to proceed properly. These elements are a promoter, a start site, and a stop site. The need for a recognizable point to begin and a point to end the process is fairly obvious. The promoter is somewhat different. The promoter controls the frequency of transcription. If you imagine the needs of a cell at any given time, clearly not all gene products are needed in the same quantity at the same time. There must be a way to control when or if transcription occurs,

and at what speed.

The bare-bones version of the process goes something like this: (1) special docking proteins recognize the promoter sequence and bind to it, unzipping a small section around the “start” site; (2) RNA polymerase binds to those special proteins and to the little bit of single-stranded DNA that has just opened up; (3) a helicase enzyme (part of, or attached to the polymerase) unzips the DNA; (4) the RNA polymerase follows behind the helicase, “reading” the DNA sequence, taking ribonucleotides from the environment, matching them against the DNA template, and if they match, adding them to the previous ribonucleotide or RNA chain. This continues until the polymerase reaches the stop site, at which point, it detaches from the template DNA, also releasing the newly made RNA copy of that DNA. Of course, if that was all there was to it, there wouldn’t be entire journals dedicated to studying RNA, its transcription, and the control of that transcription.

The sequence of the promoter is directly related to its function. There may be promoters for housekeeping genes (needed constantly, but at low copy number), “normal” genes (needed as the cell’s situation dictates, rate of transcription also varies), stress response genes (needed rarely), and a variety of other categories. Even within a category, the sequence of the promoter determines its strength. This is based upon what is known as the “consensus sequence”. The consensus sequence is a theoretical “best” promoter based on a survey of all genes in a particular category. The Figure below shows an alignment of the promoter sequences of a variety of different genes, all of which are regulated by the same type of promoter and promoter-binding-protein. The highlighted boxes show areas centered around -35 (35 nucleotides upstream of the start site) and -10.

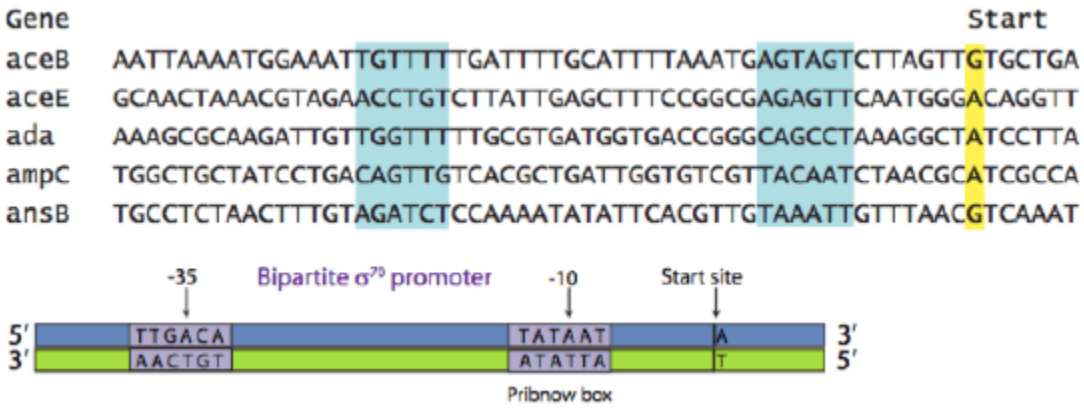


Figure 8.1.2. Upstream control sequences of various E. coli genes. Below it is the consensus sequence for for σ^{70} *promoters*.

The consensus sequence in Figure 8.1.2 shows the most common nucleotide found at each position within those areas of similarity. In this example, the most common prokaryotic promoter is shown: the σ^{70} promoter, so called because it is recognized and bound by the σ^{70} transcription factor. [Here, and by universally accepted convention, recognition sequences of DNA are written as the nucleotides would occur from 5’ to 3’ on the sense, or non-template, strand.] It is a two-part promoter, with a region centered around -35 (consensus TTGACA), and a region (sometimes called Pribnow box, consensus TATAAT) centered around -10. The (-) sign indicates that the nucleotide is “upstream” of the start site. Upstream means “to the left” when the nucleotides are written as a string of letters, and it means “on the 5’ side of” with respect to the 5’-3’ directionality of a DNA strand. Notice the relationship between the various individual promoters and the consensus sequence. In general, those promoters with more matches to the consensus sequence are stronger promoters.

A few paragraphs ago, the task of the promoter was defined as controlling the frequency of transcription. How does it do that? What does it mean to be a stronger (or weaker) promoter? First, keep in mind that the expression of any given gene is not automatic, or 100%. At any point in time, many of a cell’s genes will be near 0%, or shut off. However, even genes that are turned on are transcribed at different rates. One of the governing factors is the recognition of the promoter site by the RNA Polymerase. For stronger promoters, the RNA polymerase is more likely to recognize the site, dock properly, open up the double helix, and begin transcribing. On the other hand, the RNA polymerase can potentially recognize weaker promoters, but it is less likely to do so, instead passing it by as just another unimportant stretch of DNA. While this is partially a matter of recognition by the polymerase, keep in mind that it is actually governed by recognition of the promoter sequence by the general transcription factors (to be discussed shortly) such as sigma factors in prokaryotes that are recognized by the polymerase.

Notice that there is a high proportion of (A)denines and (T)hymines in the σ^{70} promoter sequences. This is true for many promoters in both prokaryotic and eukaryotic genes. As you probably suspected, this is advantageous because there are only two H-bonds between A-T pairs (as opposed to 3 H-bonds between G-C pairs), which means that it is 33% easier to unzip.

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8.2: Prokaryotic Transcription

In *E. coli*, as with other prokaryotes, there is only one true RNA polymerase (not including the specialty RNA polymerase, primase, which makes short RNA primers for DNA replication). The polymerase is a multi-subunit holoenzyme comprised primarily of two α subunits, a β subunit, a β' subunit, an ω subunit, and a σ subunit. The α subunits are primarily structural, assembling the holoenzyme and associated regulatory factors. The β subunit contains the polymerase activity that catalyzes the synthesis of RNA, while the β' subunit is used to nonspecifically bind to DNA. The ω subunit is involved in assembly of the holoenzyme and may also play a role in maintaining the structural integrity of the RNA polymerase. Finally, there is the σ subunit, which does not stay closely associated with the core enzyme (αββ'ω) except when helping to initiate transcription, and is used to recognize the promoter by simultaneously decreasing the affinity of RNAP to DNA in general, but increasing the affinity of RNAP for specific DNA promoter sequences. Why decrease the affinity for non-specific DNA? When the RNAP is not in use, it does not just float about in the nucleoplasm: it is bound quite tightly along the DNA. When the sigma is bound, the decreased affinity allows the RNAP holoenzyme to move along the DNA and scan for promoter sequences. There are multiple isoforms of the σsubunit (such as the sigma-70 mentioned above), each of which recognizes different promoter sequences. All isoforms perform the same basic function of properly locating the RNAP to the start of a gene, and all isoforms only stay attached to the holoenzyme for that one transient purpose, after which they are released (usually after transcribing about ten nucleotides).

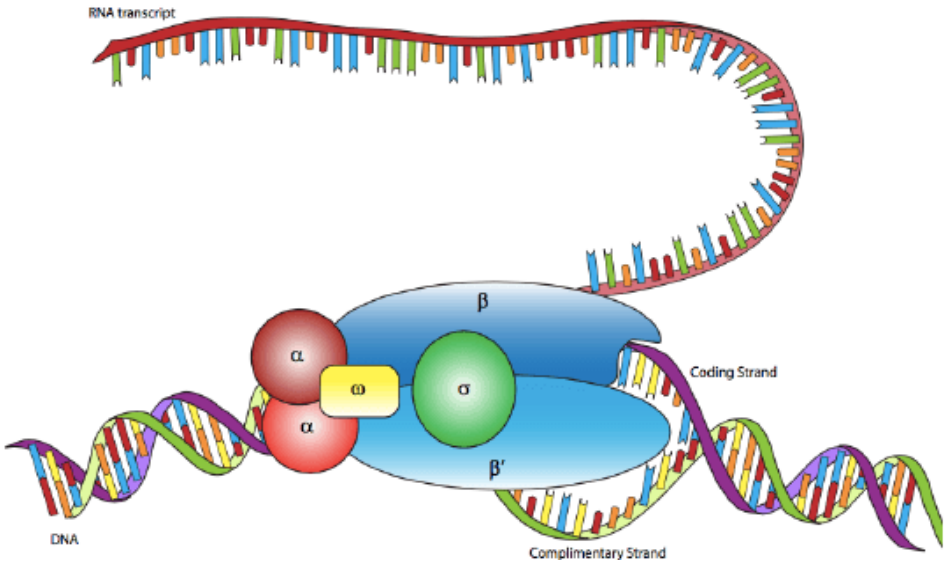


Figure 8.2.3. Prokaryotic RNA Polymerases consist of two α *subunits, a* β, β', ω, *and* σ *subunits.*

Although RNA polymerase was discovered in 1960, the *E. coli* RNAP has not yet been successfully mapped by x-ray crystallography. However, it is very similar to the RNAP of the archaean species, *Thermophilus aquaticus*, which is highly stable (= easier to crystallize) and for which an x-ray crystallographic structure has been elucidated. The data from the Taq RNAP structure and electron microscopic analyses of *E. coli* RNAP produce a lobster-claw-shaped holoenzyme. The inner surface of the claw is lined with positively charged amino acids that can interact with the negatively charged DNA, and when the holoenzyme binds a sigma subunit, the two halves of the claw (formed mostly by the beta and beta' subunits) move closer together to interact with the DNA.

Rifamycins are a class of antibiotics that include rifamycin B, made by the bacteria *Streptomyces mediterranei* (incidentally just one of many antibiotics derived from the Streptomyces genus), and rifampicin, its synthetic cousin. They work by binding within the DNA-RNA channel near the active site of RNA polymerase, which sterically prevents the addition of nucleotides to the RNA strand. If the organism cannot transcribe RNA, it cannot use the RNA to make the enzymes and other proteins necessary for life either, and dies. The rifamycin binding site is highly conserved in most prokaryotes but not in eukaryotes, so the antibiotic kills bacteria specifically with little chance of harm to eukaryotes.

Once the holoenzyme has recognized and bound tightly to the DNA at the promoter site, the next step is to "melt" the DNA

(breaking the H-bonds and separating the strands of the double helix) in that area so that the RNAP can proceed downstream, read the template DNA strand, and produce the new RNA. Often many RNA transcripts of a gene are needed to produce a large number of active proteins in a short span of time. Highly transcriptionally active genes therefore often have multiple RNA polymerases reading them, one right after another. Generally, an RNA polymerase only needs to process about 15 nucleotides before there is room for another RNAP can bind the promoter and start another transcript.

> Strand separation is an energetically difficult process due to the strength of the combined H-bonds between the strands, and often an RNAP may make several short-lived abortive attempts before finally prying open the double helix long and far enough to allow the RNAP to stabilize and transcribe continuously to the stop site.

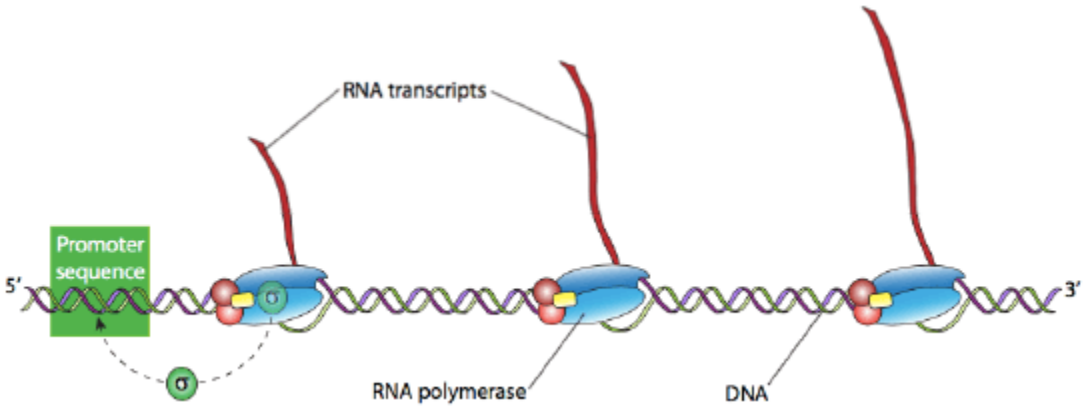


Figure 8.2.4. A new RNA polymerase can begin transcribing a gene before the previous one has finished.

The elongation phase of transcription proceeds in a 5' to 3' direction, which is to say that new nucleotides are added to the 3'-OH of the growing strand. Elongation is a stochastic process in which one of the plentiful free- floating ribonucleotides drops into the active site of RNAP opposite the DNA template. If it is the correct nucleotide (complementary to the template), then H-bonds will temporarily form, stabilizing the new nucleotide in place long enough for the RNAP to catalyze the formation of a phosphodiester bond between the 3'-OH of the RNA-in-progress and the 5'-phosphate of the nucleotide. However, if it is the incorrect nucleotide, the proper H-bonds do not form, and the nucleotide usually dissociates from the active site before the RNAP has a chance to bind it to the growing RNA strand. Obviously, this is not a perfect system, and in fact, the error rate for transcription is quite high at approximately 1 in 10000 nucleotides. Fortunately, the cell generally churns out many copies of RNA from any given gene very quickly (approximately 80 nucleotides per second), most of which are either error-free or have errors that do not affect the function of the end-product protein. Furthermore, unlike DNA, in which errors of replication get carried along from one generation of cells to the next, RNA is not a storage medium, and its transient nature means that even mutations that severely impact the protein function only affect the few proteins translated from that one RNA, not the proteins generated from other RNAs made from the same template gene, much less subsequent generations. In other words, to misappropriate a phrase from the movie Meatballs, "It just doesn't matter."

Eventually, the RNA polymerase reaches the end of the gene and stops transcribing. The termination site is usually marked by a sequence of 4-10 adenine (A) residues on the template strand, and some have a palindromic G-C-rich region that forms a hairpin loop just upstream of the series of adenines. In the first case, it is thought that the resulting string of A-U base pairs is unstable and may lead to the RNAP and the new RNA strand falling off the template DNA while at the same time, the hairpin structure may cause the RNAP to stop or pause, and this can also lead to it dissociating from the DNA. Only about half of all transcription termination sites are marked in this way though, and the others have no significant hairpin loops or easily recognizable sequences other than a series of G-C-rich regions. In this type of termination site, the enzymatic co-factor, rho, is required for termination, and so this is known as rho-dependent termination. Rho is an RNA-binding protein with helicase activity, so it is postulated that it effects termination by forcing the RNA strand off of the DNA template.

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8.3: Eukaryotic Transcription

Transcription in eukaryotes is more complicated, but follows the same general ideas. The promoter sequences are much more varied both in placement (with respect to the start site) and size. As we will see in the next chapter, eukaryotic genes have many more control elements regulating their expression than do prokaryotic genes. Not only are there more control elements, there are also more RNA polymerases, which serve different specific cellular functions. Obviously, the broad function and location of all the RNA polymerases is the same: read a DNA template and transcribe an RNA copy of it; and since the DNA is found only in the nucleus, so are the polymerases. However, the polymerases differ in exactly what kinds of RNA they produce. RNA Poly- merase I is specialized for producing pre-rRNA (rRNA = ribosomal RNA). The pre-rRNA is cleaved post-transcriptionally and incorporated into the ribosomes. Since ribosomes are assembled in the the nucleolus, that is the part of the nucleus in which most RNA Polymerase I is concentrated. RNA Polymerase III also makes an RNA (5S) that is incorporated into the ribosome. It also makes other *untranslated* RNAs such as tRNAs and a variety of small nuclear RNAs. The only RNA polymerase that makes the translatable RNA (mRNA, or messenger RNA) that most people think of when RNA is referred to generically, is RNA polymerase II. This is the RNA polymerase that produces pre-mRNA, which after some processing, becomes mRNA, is transported out of the nucleus, and finally translated into proteins. All of the eukaryotic RNA polymerases are composed of two large subunits, roughly analogous to the β and β' subunits of prokaryotic RNAP, but instead of just three or four other subunits, there are over a dozen smaller subunits to the eukaryotic RNA polymerase holoenzymes.

Initiation of transcription is also much more complicated. Not only is there great variety in promoters recognized by RNAP II, both RNAP I and RNAP III recognize promoters with particular structural characteristics.

> The eukaryotic RNA polymerases were named I, II, and III based on their elution order from ion-exchange chromatography purification. They are also partially distinguishable by their sensitivity to α-amanitin and related amatoxin-family mushroom poisons. RNAP I (and prokaryotic RNAP) is insensitive to these toxins, RNAP III is somewhat sensitive (K_d ~10^{-6} M), and RNAP II is highly sensitive (K_d ~10^{-8} M). These toxins act by binding to a site in the RNA-DNA cleft and interfering with translocation of the RNA. That is, there is no problem with importing a nucleotide or with attaching it to the new RNA, but the RNA strand cannot move through the active site and allow the next nucleotide to be added.

One of the most common eukaryotic RNAP II promoters is the TATA box, named for the highly conserved motif that defines it. Although it appears similar to the Pribnow box in prokaryotes, it is generally located further upstream from the start site, and its position is far more variable. Whereas the Pribnow box is located at -10, the TATA box may be located closer to -30 +/- 4. Also, rather than just a sigma factor to recognize the promoter in conjunction with the polymerase core enzyme, the eukaryotic promoter is recognized by a multi-subunit complex called transcription factor IID (TFIID). TFIID is comprised of TATA-binding protein (TBP) and several TBP-associated factors (TAFs).

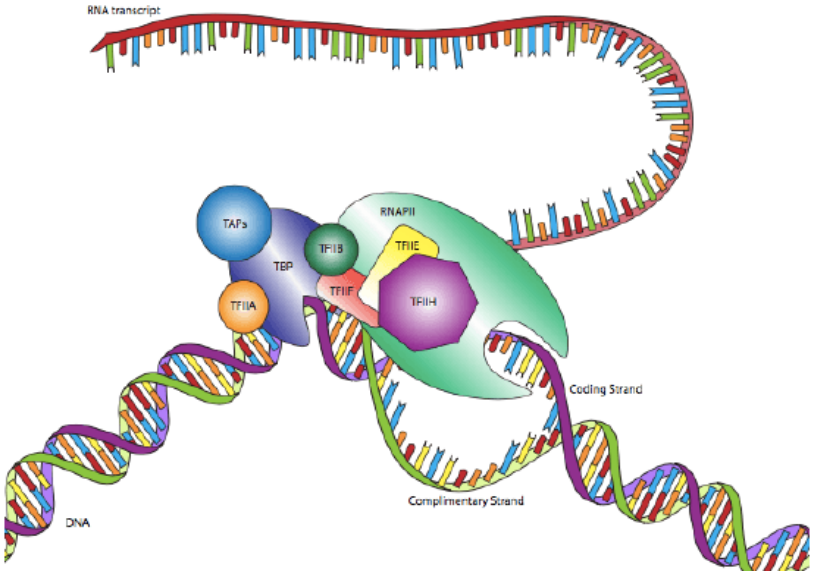


Figure 8.3.5. Eukaryotic Transcription. An initiation complex of several transcription factors is needed to dock the RNA Polymerase II in position to begin transcription.

This binding of the promoter by TFIID occurs independently of RNA Polymerase II, and in fact, RNAP II will not attach to TFIID at this time. After TFIID has bound the TATA box, two more transcription factors, TFIIA and TFIIB, attach to the TFIID as well as the nearby DNA, stabilizing the complex. TFIIF attaches to TFIID and TFIIB to allow docking of the RNA Polymerase II. The complex is still not ready to begin transcription: two more factors are required. TFIIE binds TFIIF and RNAP II, and finally, TFIIH attaches to RNAP II, providing a helicase activity needed to pry apart the two strands of DNA and allow the polymerase to read one of them. TFIIH also has another important enzymatic activity: it is also a serine kinase that phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II. There are several serines in the CTD, and as they are sequentially phosphorylated, the CTD extends like a (negatively charged) tail and helps to promote separation between the RNAP II and the TFIID/promoter.

Elongation of the RNA strand in eukaryotes is very similar to that in prokaryotes with the obvious difference that transcription occurs in the nucleus rather than in the cytoplasm. Thus, in prokaryotes, the RNA can be used for translation of proteins even as it is still being transcribed from the DNA! In eukaryotes, the situation is significantly more complex: there are a number of post-transcriptional events (5' end-capping, 3' polyadenylation, and often RNA splicing) that must occur before the RNA is ready to be transported out of the nucleus and made available for translation in the cytoplasm.

Termination of eukaryotic transcription is not well-described at this writing. RNAP I appears to require a DNA-binding termination factor, which is not analogous to the prokaryotic Rho factor, which is an RNA binding protein. RNAP III terminates transcription without any external factor, and this termination usually occurs after adding a series of uridine residues. However, it does not appear to use the hairpin loop structure found in rho-independent bacterial transcription. The termination of protein-coding RNAP II transcripts is linked to an enzyme complex that also cleaves part of the 3' end of the RNA off, and adds a poly-A tail. However, it is not clear how the polyadenylation complex is involved in determining the point of transcription termination, which can be over 1000 nucleotides beyond the poly-A site (e.g. the β-globin gene in *Mus musculus*). Upon termination and release from the RNAP II and template DNA, the RNA is known as the primary transcript, but must undergo post-transcriptional processing before it is a mature messenger RNA (mRNA) ready to be exported to the cytoplasm and used to direct translation.

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8.4: Post-Transcriptional Processing of RNA

The first of the post-transcriptional events is 5’ end capping. Once the 5’ end of a nascent RNA extends free of the RNAP II approximately 20-30 nt, it is ready to be capped by a 7-methylguanosine structure. This 5’ “cap” serves as a recognition site for transport of the completed mRNA out of the nucleus and into the cytoplasm.

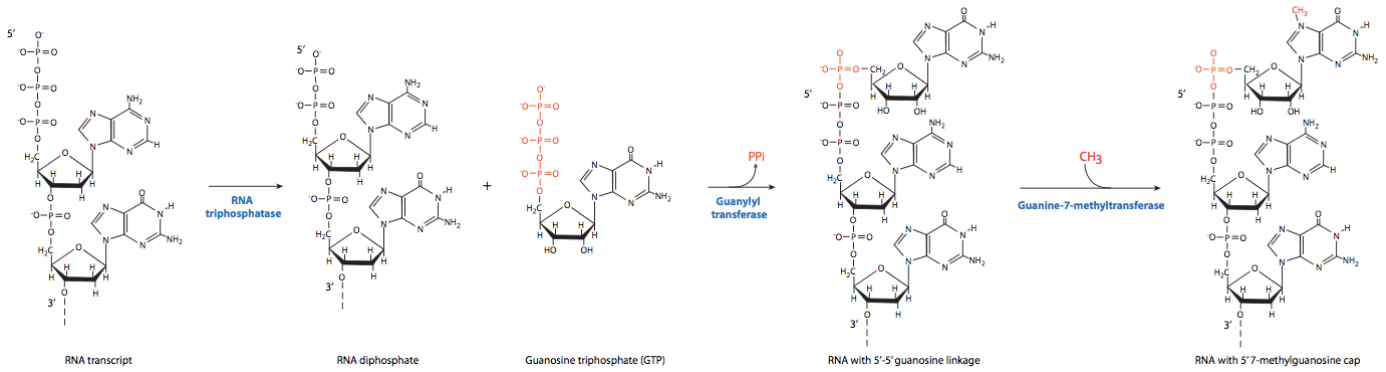


Figure 8.4.6. Capping the 5’ end of eukaryotic transcripts.

The process actually involves three steps. First, RNA triphosphatase removes the 5’-terminal triphosphate group. Guanylation by GTP is catalyzed by capping enzyme, forming an unusual 5’-5’ “backward” bond between the new guanine and the first nucleotide of the RNA transcript. Finally, guanine-7-methyltransferase methylates the newly attached guanine.

On the opposite end of the RNA, on the free 3’-OH, *polyadenylation* occurs. As noted previously, an enzyme complex that docks to a site on the CTD tail of RNAP II cleaves a portion of the 3’ end near an AAUAAA recognition sequence and then serially adds a large number of adenine residues. The poly(A) tail is not required for translation, but it has an effect on the stability of transcripts in the cytoplasm. As mRNA molecules stay in the cytoplasm longer, the poly(A) tail is gradually removed. Once the poly(A) tail is gone, the mRNA will soon be destroyed. mRNA molecules with longer poly(A) tails are generally longer-lived in the cytoplasm than those with shorter tails, but there is currently no evidence for a directly proportional effect.

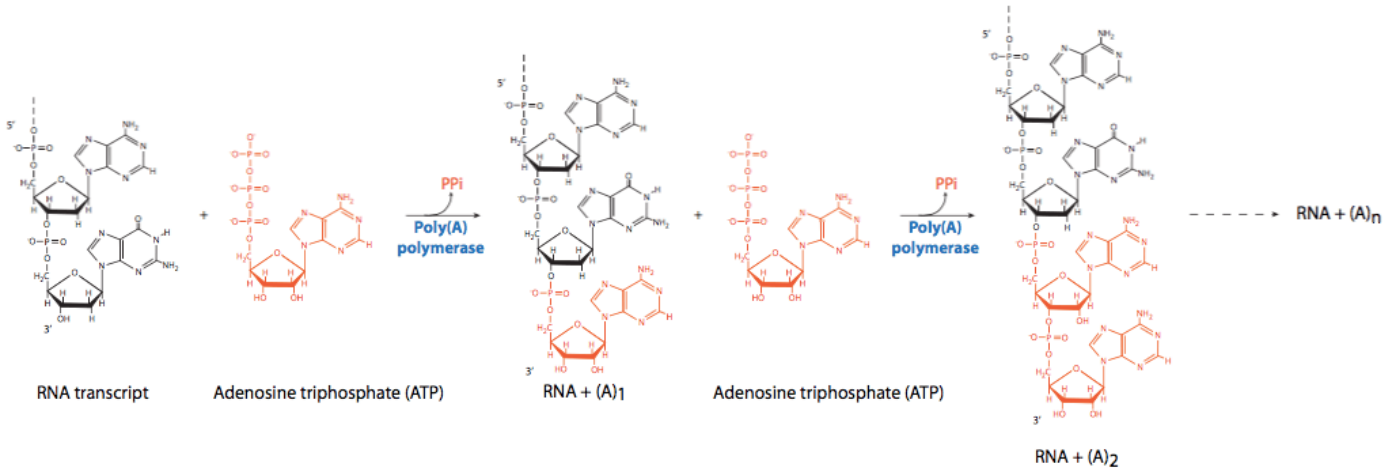


Figure 8.4.7. Polyadenylation of the primary transcript.

Although the enzyme that cleaves the primary transcript in preparation for polyadenylation has not been identified, two nonenzymatic factors, the excitingly-named cleavage factor I (CFI) and cleavage factor II (CFII) have been implicated. The serial adenylation comes from the activity of poly(A) polymerase (PAP) in conjunction with CPSF (cleavage and polyadenylation speci city factor), which binds to the RNA. PAP itself has relatively poor affinity for RNA. As with other nucleic acid polymerases, it adds new nucleotides onto the free 3'-OH of the pre-existing chain. To encourage processivity (continuous polymerization) poly(A) binding protein II (PABII) joins the polyadenylation complex, and is involved in controlling the final length of the poly(A) tail. It should be noted that PABII is a nuclear protein and should not be confused with PABP (poly(A) binding protein) which binds to mRNA molecules in the cytoplasm and plays a role in protecting them from nuclease attack.

The third and most complicated modification to newly-transcribed eukaryotic RNA is *splicing*. Unlike prokaryotic RNA, which is a continuously translatable coding region immediately as it comes out of the RNA polymerase, most eukaryotic RNAs have interrupted coding regions. Splicing is the process by which the non-coding regions, known as *introns*, are removed, and the coding regions, known as *exons*, are connected together. In some RNAs, this can happen autonomously, with part of the RNA acting as an enzymatic catalyst for the process. This requires that the RNA have a specific secondary and tertiary structure, bringing the two exons close together while looping out the intron. It was the study of this phenomenon that led to the discovery of ribozymes, which are enzymes made of RNA.

Until the discovery of ribozymes, it had been assumed that only the enzymes could only be generated with the diversity of structures possible with the amino acids in proteins.

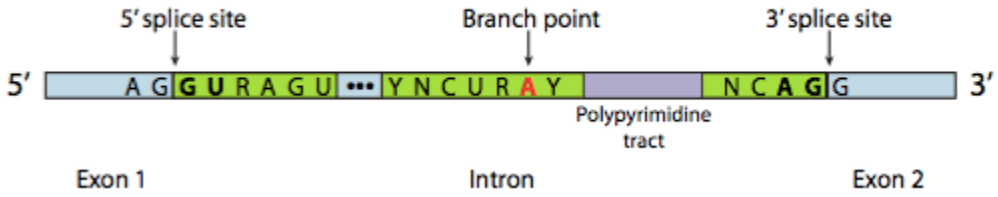


Figure 8.4.8. Consensus sequences for splicing.

In most cases, however, splicing is carried out by a multi-subunit protein complex known as the spliceosome. Whether it is self-spliced or by spliceosome, there are three main sequence components needed to define an intron that is going to be spliced out (Figure 8.4.8). There is a *5' splice site* with the consensus sequence AG|GUAAGU. There is a *3' splice site* that starts with an 11-nucleotide polypyrimidine tract followed by NCAG|G. And somewhere in between the two, there is a branchpoint adenine, typically within a YNCURAY sequence (Y is a pyrimidine, N is any nucleotide, R is a purine). Splicing is actually a set of two sequential transesterification reactions, and requires physical proximity of the reactive sites by bending and looping of the RNA, either autonomously or around protein factors know as snRNPs (pronounced "snurps"). SnRNPs is an acronym for small nuclear ribonucleoproteins. They contain both a protein and a small nuclear RNA (snRNA) component; the latter helps with sequence recognition. Examination of the structure of the snRNA part of these spliceosome snRNPs shows that they are very similar to the shapes taken by the RNA transcript itself in cases of self-splicing. Keeping that in mind, much of the following description of spliceosome-mediated splicing happens in self-splicing as well.

Although the snRNPs are the primary components of the spliceosome, a variety of other splicing factors also play a role. The most prominent are U2AF (U2-associated factor, which binds to the polypyrimidine tract, and SF1 (splicing factor 1, aka branch- point protein BPP) which binds to consensus sequence near the branchpoint. Together they help to properly position the U2 snRNP. There are also a variety of other less-studied splicing factors from the SR protein family (C-terminal Serine-Arginine binding motif) and the hnRNP (heterogenous nuclear ribonucleo- protein) families that act to recruit the primary members of the spliceosome to their proper locations.

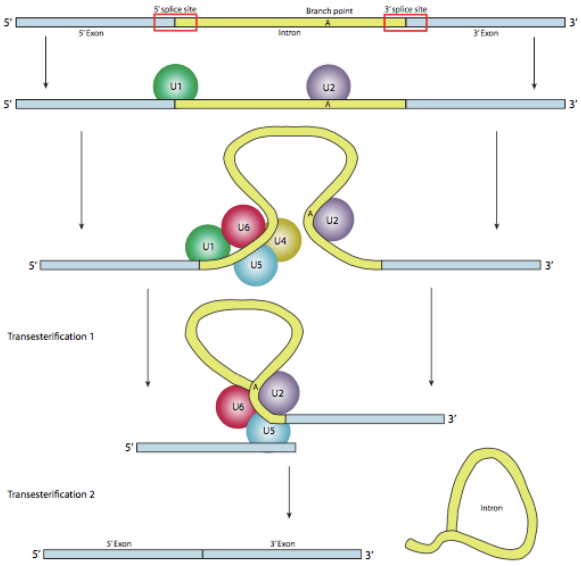


Figure 8.4.9. RNA splicing by spliceosome. Description in text below.

In the first step, the U1 snRNP binds to the intronic portion of the 5' splice site. Next, the U2 snRNP binds to the consensus site around the branchpoint, but importantly, there is no base-pairing to the branchpoint A itself. Instead, due to basepairing of U2 with the surrounding sequence, the branchpoint A is forced to bulge out from the rest of the RNA in that region. U4, U5, and U6 join the spliceosome together, but while U5 binds to the 5' exon, and U6 displaces U1 at the 5' splice site, U4 is only transiently attached and also falls off the spliceosome before the first transesterification reaction. As the Figure shows, in this reaction, the 2'-OH of the branchpoint A nucleophilically attacks the 5'-phosphate of the first intron nucleotide to form a lariat structure in which the 5' end of the intron is connected to the branchpoint via a 2',5'-phosphodiester bond. This releases the 5' exon (and the whole 5' half of the RNA for that matter), but it is kept in close proximity to the 3' exon (and the rest of the RNA) by U5, which attaches to both exons. This allows the second transesterification to take place, in which the 3'- OH of the first exon attacks the 5' phosphate at the beginning of the second exon, thus simultaneously breaking the bond between the intron and the second exon, and also connecting the two exons via a conventional 3',5'-phosphodiester bond. The intron, in the shape of a lariat, is thus released and will be quickly degraded.

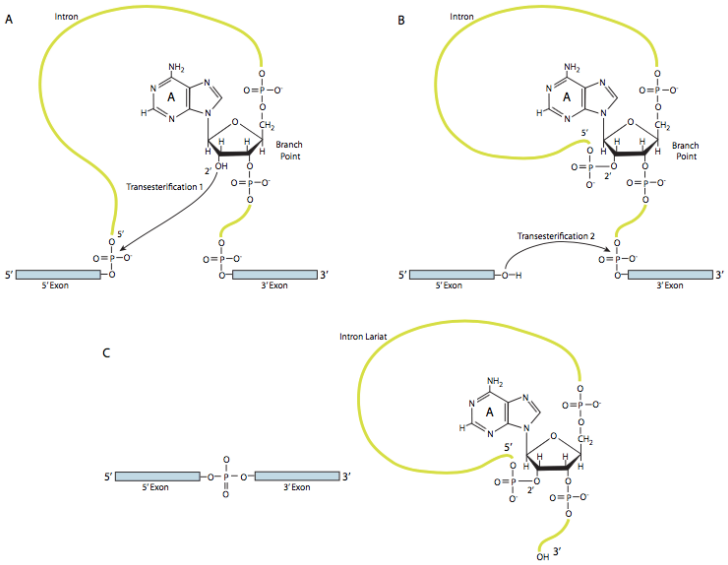


Figure 8.4.10. (A) Transesterification reaction 1 connects the 2'-OH of the branch- point ribose to the 5'-phos- phate of the 3' end of exon 1. (B) Transesterification 2 is an attack by the remaining -OH on the 3' end of exon 1 on the 5' phosphate of exon 2. (C) This simultaneously releases the lariat and con- nects the exons.

Splicing is an efficient (with respect to genome size) way to generate protein diversity. In alternative splicing, some potential introns may be spliced out under certain circumstances but remain as coding sequence under other circumstances. Recall that

the splice sites are recognized by base-pairing and therefore, there can be stronger and weaker splice sites depending on how close they are to the consensus and the complementary sequence on the snRNPs. Therefore, a gene with several potential introns may have all introns spliced out 80% of the time, but the other 20% of the time, perhaps only one or two introns are spliced out. Adding variability, there are splicing factors that may bind near splice sites and can either make them more easily recognizable, or nearly hidden.

The classic example of alternative splicing is the gene encoding α-tropomyosin (Figure 8.4.11). By splicing in/out different combinations of exons, a single gene can generate seven different proteins, depending on the tissue type. In these cases, particular types of cells or tissues contain specific combinations of splicing factors, and therefore control the recognition of specific splice sites, leading to the different splicing patterns.

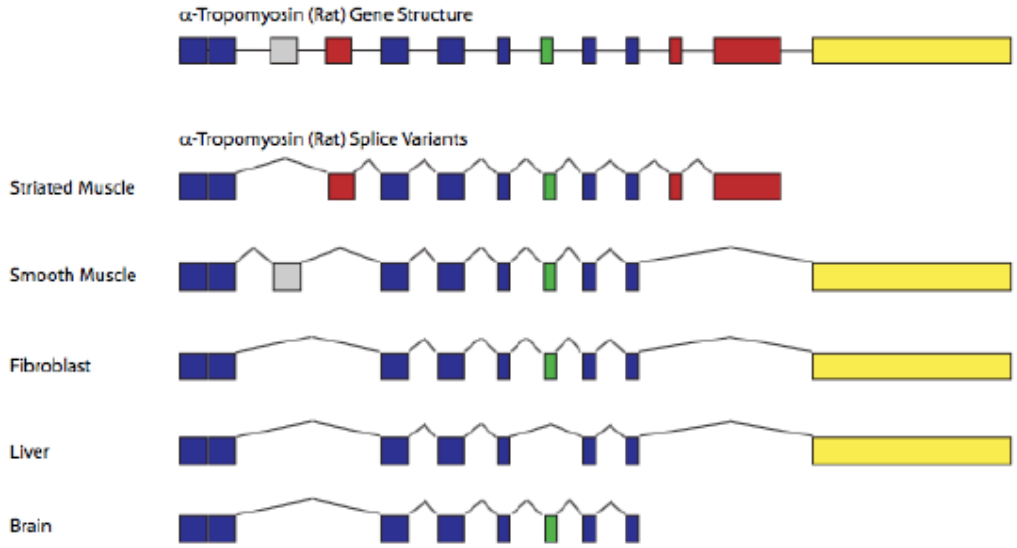


Figure 8.4.11. Alternative splicing of the α-*tropomyosin gene leads to different forms of the mRNA and protein in different cell types.*

Although this concludes the discussion of basic mechanisms of transcription, the next chapter is really a continuation of this one: control of gene expression in its simplest form is regulating the recognition of a promoter sequence by an RNA polymerase.

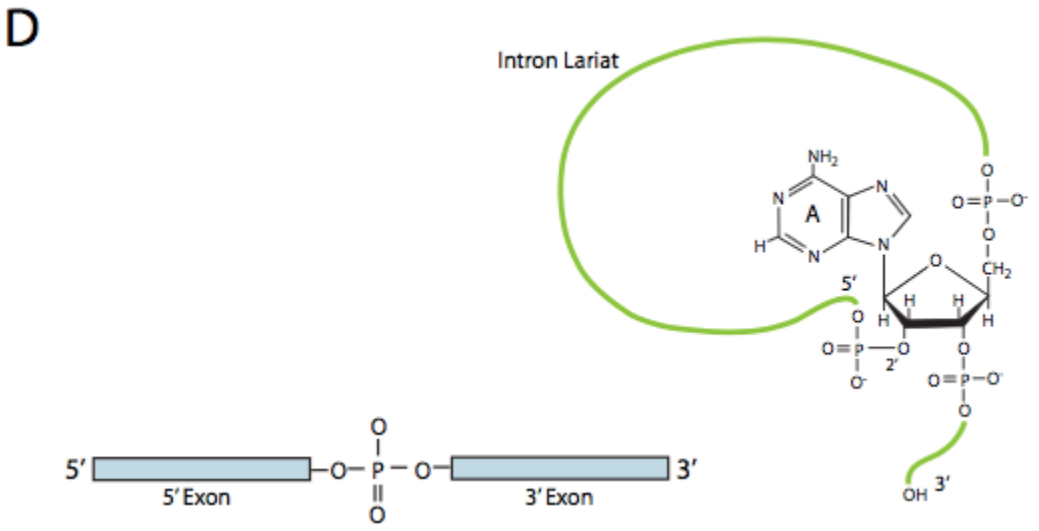


Figure 8.4.12. Splicing of RNA. Splicing removes some portions of a primary transcript (introns) while combining the remaining RNA (exons) to form the final mRNA sequence. The chemical mechanism is a series of two transesterifications. The first accomplishes the looping of the intron, while the second releases that intron as a “lariat” while simultaneously joining the two exons together. Splicing speci city relies on three landmarks on the RNA, most important of which is the branchpoint, an adenine residue that is the connection point for the intron loop formation. The other two landmarks are the 3’ and the 5’ splice sites, each of which are sequences that bind to snRNPs to bring together the spliceosome.

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9: GENE REGULATION

To define a gene, a stretch of DNA must have a promoter, a start site, and and a stop site. In a prokaryote, these are necessary and often sufficient, but in a eukaryote, they are still necessary, but seldom sufficient. This chapter discusses the other elements, both positive and negative, that are used to regulate the expression (i.e. transcription) of a gene. It is primarily a story of transcription factors and the recognition elements to which they bind.

9.1: Prokaryotic Transcriptional Regulation
9.2: Eukaryotic Transcriptional Regulation

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9.1: Prokaryotic Transcriptional Regulation

Unlike multicellular organisms, in which most cells are in a tightly regulated internal environment, most prokaryotic cells are constantly responding to changing conditions in their immediate environment, such as changes in salt concentration, temperature, acidity, or nutrient availability. Because these organisms must respond quickly, the lifetime of an RNA is kept short, on the order of several minutes - so gene products that are not useful in the new conditions do not waste resources. For the same reason, initiation of new transcription must also occur very quickly - so that gene products that are needed to stabilize the cell in the new conditions are rapidly available. A fast and efficient control system is needed, and in prokaryotes, this means that the controls on transcription are simple activators and repressors. For some genes, both may be used for regulation, while for others, only one is needed to change from a default state of expression or non-expression.

A classic example of repressor control of gene expression, the lac operon, also illustrates another method by which bacteria may control the expression of genes. An *operon* is a group of genes whose products participate in the same metabolic pathway, and are transcribed under the control of a single promoter. The lac operon consists of three genes (lacZ, lacY, lacA) that participate in the catabolism of the disaccharide, lactose. LacZ is β-galactosidase, an enzyme that cleaves lactose into galactose and glucose. LacY is β-galactoside permease, which transports lactose from the extracellular environment into the cell. Both are required for lactose catabolism. Oddly, lacA is not absolutely required for lactose metabolism, but its function is related to the other two: it is a β-galactoside transacetylase that transfers acetyl groups from acetyl-CoA to lactose. All three are translated (they retain their individual start and stop codons for translation, not to be confused with the start and stop of transcription) from a single transcript. Of particular interest with respect to the regulation of this transcription is the structure of the promoter region. Note that in addition to the expected σ^{70} promoter upstream of the start site, there is another control sequence on each side of the start site (Figure 9.1.1A).

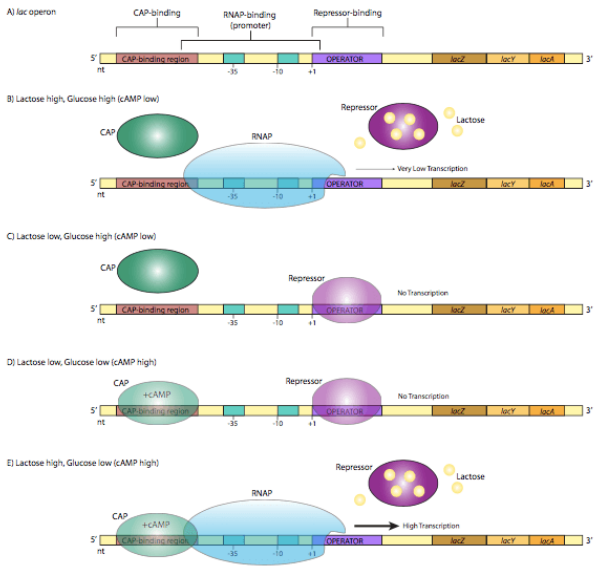


Figure 9.1.1. The *lac* operon.

> Note that the helix-turn-helix (HTH) motif, which is common in bacterial DNA-binding proteins, is not the same thing as the helix-loop-helix DNA-binding proteins that are used in many eukaryotic systems. An elaboration of the basic HTH motif, known as the winged helix motif, is also found in a variety of prokaryotic DNA- binding proteins.

The operator is a sequence of DNA that lies between the promoter and the start site. It is recognized by the lac repressor, a DNA binding protein with a helix-turn-helix motif. In the absence of lactose (Figure 9.1.1C), the lac repressor has a high affinity for the operator sequence and binds tightly, obstructing the start site and forming a physical “roadblock” to transcription by preventing the RNA polymerase from moving forward from the promoter. This makes sense physiologically because the cell is more efficient metabolizing glucose, and if there is no lactose around, then it is a waste of resources to make enzymes that

metabolize it. However, what if there is suddenly an abundance of lactose in the environment? As the lactose is taken into the cell, intracellular levels rise, and now enzymes are needed to utilize this new food source. The lactose actually turns on the expression of enzymes that will metabolize it! Specifically, the lactose binds to the lac repressor protein (4 lactose binding sites), which causes a conformational change that releases it from the operator sequence (Figure 9.1.1B). Now an RNA polymerase that attaches at the lac operon promoter can proceed to transcribe the message unhindered, producing RNA and subsequently proteins that are used to break down the lactose. This continues as long as there is abundant lactose in the cell. As the lactose levels drop, repressor proteins are no longer bound by lactose, and can once again bind the operator and inhibit expression of the operon once again. For now, ignore the CAP protein in Figure 9.1.1, and parts D and E. We'll come back to that. The lac operon is an example of an *inducible* operon, in which the native state is "off" and the introduction of and inducer (in this case lactose) will bind the repressor and turn the operon "on".

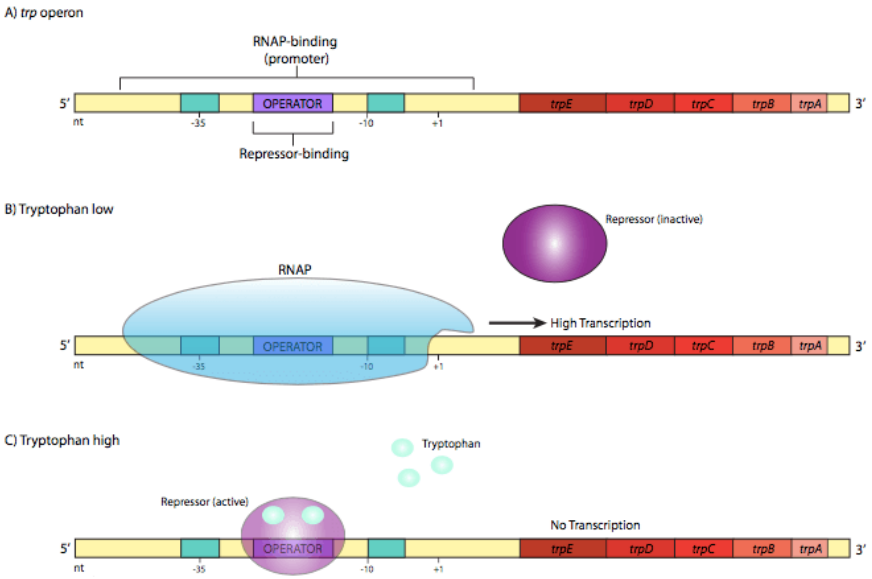


Figure 9.1.2. The *trp* operon.

In contrast, there are also operons with the reverse mechanism. An example of one such *repressible* operon is the trp operon (Figure 9.1.2). This operon contains ve genes that are involved in the synthesis of the amino acid tryptophan: trpE and trpD, which together encode the subunits of anthranilate synthetase, trpC, which encodes N-(5'- phosphoribosyl)-anthranilate isomerase, and trpB and trpA, which each encode sub-units of tryptophan synthetase. The trp repressor is larger and more complex than the lac repressor, but it also utilizes a helix-turn-helix DNA-binding motif.

However, it differs in a crucial aspect. In its native form, it does not bind to the opera- tor sequence. It only binds to the operator after it has first bound tryptophan (two molecules of trp bind to one repressor). This is the opposite of the lac repressor, but when considering the physiological function of these genes, this should make perfect sense. As long as there is no tryptophan, the operator is unbound, allowing the RNA polymerase to transcribe the genes needed to make tryptophan (Figure 9.1.2B). When enough tryptophan has accumulated in the cell, some of the "extra" tryptophan binds to the trp repressor, which activates it and allows it to bind to the operator (Figure 9.1.2C). When this happens, the RNAP cannot reach the start site, and resources are not wasted transcribing genes for enzymes that make something the cell already has a lot of.

Let us now return to the lac operon in Figure 9.1.1. It turns out that even when the operon is induced by the presence of lactose, the rate of transcription is low. The limitation is not from the repressor - that has been removed as described above (Figure 9.1.1B). Instead, the low expression is due to a low-affinity promoter. This is true not just of the lac operon, but also other non-glucose-pathway sugar-catabolism genes. There is a simple explanation: even if there are abundant alternate sugars available (e.g. lactose), if there is glucose available, it is the cell's most efficient and preferred pathway for energy production, and the production of enzymes for other pathways would be an inefficient use of resources. So, when and how is the lac operon really turned on?

The answer lies in a CAP, catabolite gene activator protein, also known as CRP, or cAMP receptor protein. It is a small homodimeric DNA binding protein that binds to a sequence that overlaps the 5' side of the promoter. In the presence of cAMP, which binds to the protein, CAP has a high affinity for the DNA recognition sequence, and binds to it (Figure 9.1.1E). The protein then helps to recruit the RNAP to the promoter site, binding directly to the C-terminal domain of the RNAP a subunit to increase the affinity of the polymerase for the promoter sequence to overcome a weak promoter.

What does cAMP have to do with this? When there is abundant extracellular glucose, there is little cAMP. The enzyme that synthesizes cAMP, adenylate cyclase, is negatively regulated by glucose transport. However, when there is little environmental glucose, adenylate cyclase is more active, makes cAMP, which binds CAP, and leads to robust production of lactose catabolism enzymes. CAP is an example of an activator that can control gene expression in a positive direction.

In *E. coli*, cAMP levels are not directly tied to intracellular glucose levels or glucose metabolism. Rather, cAMP levels are altered by glucose transport through a phosphoenolpyruvate-dependent phosphotransferase system (PTS), part of which is de-phosphorylated (the crr gene product, also known as EIIA) when glucose is moved inward. The phosphorylated EIIA~P is an activator of adenylate cyclase. So, as glucose moves into the cell, cAMP levels drop due to inactive adenylate cyclase.

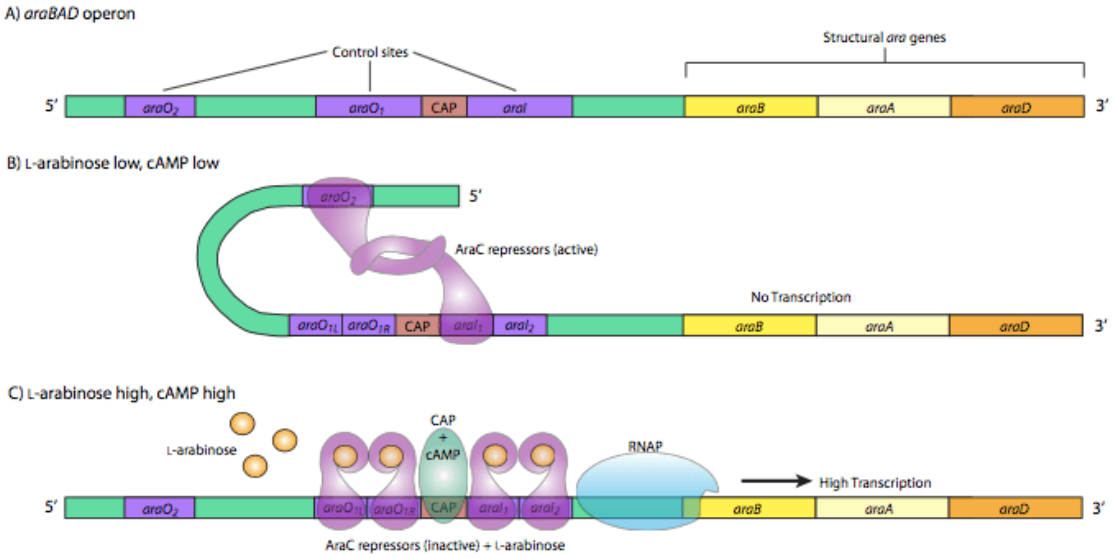


Figure 9.1.3. The araBAD operon.

The last, and most complicated example of prokaryotic metabolic gene control is the araBAD operon. This operon produces enzymes used for the catabolism of the 5-carbon sugar, L-arabinose. The interesting thing about this operon is the presence of both positive and negative control elements that are used by the same control protein, araC. When there is little or no arabinose, the araC binds to the operator sequences araO2 and araI1. The two araC proteins then interact, which causes the DNA to loop around preventing RNAP from binding to the promoter and transcribing araBAD. Furthermore, this operon is also under the control of CAP, and the double araC loop structure also prevents CAP from binding. However, when there is plentiful arabinose, araC repres- sors bind the arabinose and then interact differently, still forming dimers, but now in a different conformation that leads to binding of araO1L and araO1R together as well as araI1 and araI2. The arabinose-bound araC at the araI sites interact with RNAP and together with CAP promote strong activation of araBAD expression.

The SOS repair system

Not all operons are concerned with coordinating metabolic activities. An important non-metabolic operon in E. coli is the LexA/ RecA SOS response operon, which contains genes that are involved in DNA repair. The SOS repair system is invoked to allow DNA replication to continue through areas of damaged DNA, but with the penalty of low fidelity. One of the gene products of this operon, RecA, is important in recognizing and repairing damage caused by UV light. It also functions as a regulator of the LexA repressor protein. LexA is actually a repressor for multiple SOS operons, binding to a common operator sequence upstream of each gene/operon. It is activated when RecA, upon detecting DNA damage, undergoes a conformational shift and activates protease activity, which then cleaves LexA, allowing transcription from the SOS genes/ operons.

SOS repair is error-prone because when the replisome encounters bulky damage, it undergoes “replication fork collapse” in which the DNA polymerase III units are released. The replacement, or bypass, polymerases, Pol IV (dinB), and Pol V (umuDC), do not have 3’—5’ proofreading exonuclease activity. Misincorporation of G opposite thymine dimers occurs at about half the rate of proper A incorporation, and generally, the bypass polymerases are about 1000 times more error-prone than Pol II or Pol I.

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9.2: Eukaryotic Transcriptional Regulation

As with almost every comparison with prokaryotic systems, regulation of eukaryotic transcription is much more complex than prokaryotic gene control, although still based on similar mechanisms of activators and repressors. There is no close eukaryotic equivalent to operons, though: eukaryotic genes are always transcribed one per mRNA. The previous chapter described the formation of a preinitiation complex of transcription factors for RNA polymerase II. These transcription factors (e.g. TFIID, TFIIH, etc.) are known as **general transcription factors**, and are required for transcription of any gene at any level. However, there are also **specific transcription factors**, usually referred to simply as transcription factors (TF), that modulate the frequency of transcription of particular genes. Some upstream elements and their associated TFs are fairly common, while others are gene or gene-family specific. An example of the former is the upstream element AACCAAT and its associated transcription factor, CP1. Another transcription factor, Sp1, is similarly common, and binds to a consensus sequence of ACGCCC. Both are used in the control of the beta-globin gene, along with more specific transcription factors, such as GATA-1, which binds a consensus AAGTATCACT and is primarily produced in blood cells. This illustrates another option found in eukaryotic control that is not found in prokaryotes: tissue-specific gene expression. Genes, being in the DNA, are technically available to any and every cell, but obviously the needs of a blood cell differ a great deal from the needs of a liver cell, or a neuron. Therefore, each cell may produce transcription factors that are specific to its cell or tissue type. These transcription factors can then allow or repress expression of multiple genes that help de ne this particular cell type, assuming they all have the recognition sequences for the TFs. These recognition sequences are also known as **response elements** (RE).

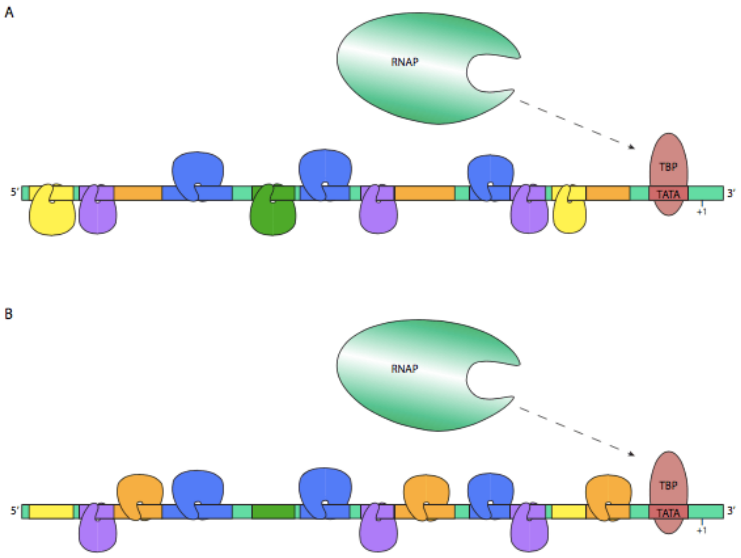


Figure 9.2.5. Eukaryotic transcription factors can work in complex combinations. In this Figure, the transcription factors hanging downward are representative of inhibitory TFs, while those riding upright on the DNA are considered enhancers. Thus, the RNA polymerase in (A) has a lower probability of transcribing this gene, while the RNAP in (B) is more likely to, perhaps because the TF nearest the promoter interacts with the RNAP to stabilize its interactions with TFIID. In this way, the same gene may be expressed in very different amounts and at different times depending on the transcription factors expressed in a particular cell type.

Very often, a combination of many transcription factors, both enhancers and silencers, is responsible for the ultimate expression rate of a given eukaryotic gene. This can be done in a graded fashion, in which expression becomes stronger or weaker as more enhancers or silencers are bound, respectively, or it can be a binary mode of control, in which a well-defined group of TFs are required to turn on transcription, and missing just one can effectively shut down transcription entirely. In the first case, activating TFs generally bind to the GTFs or RNA Polymerase II directly to help them recognize the promoter more efficiently or stably, while repressing TFs may bind to the activating TFs, or to the GTFs or RNAP II, in preventing recognition of the promoter, or destabilizing the RNAP II preinitiation complex. In the second case, activation hinges on the building of an enhanceosome, in which transcription factors and protein scaffolding elements and coactivators come together to position and stabilize the preinitiation complex and RNAP II on the promoter. The most prominent and nearly ubiquitous coactivator is named *Mediator*, and binds to the CTD of the β' subunit of RNA polymerase II and also to a variety of transcription factors.

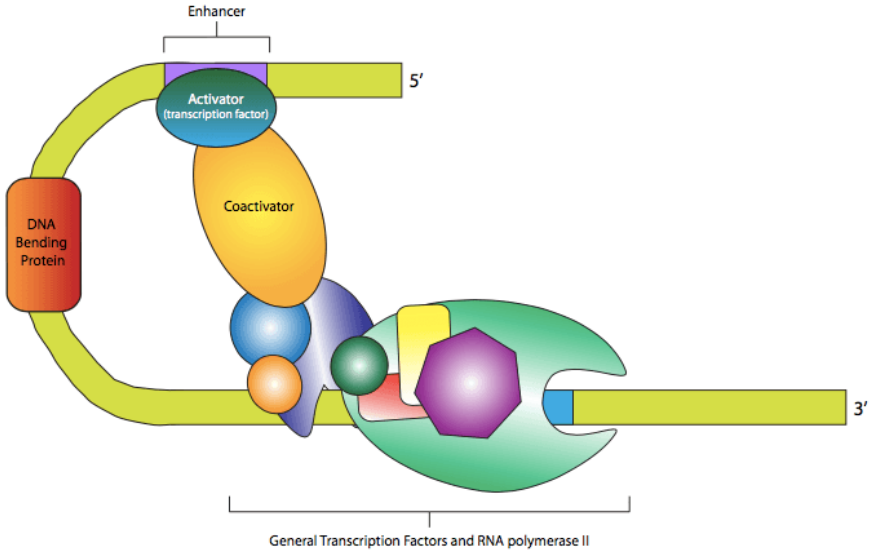


Figure 9.2.6. An enhancer can stabilize or recruit components of the transcription machine through a coactivator protein.

Eukaryotic transcription factors, while varied, usually contain at least one of the following transcription factor motifs: zinc fingers, leucine zippers, basic helix-loop-helix domains, Rel homology region domains, or a variation thereof.

The zinc finger motif was the first DNA-binding domain to be discovered, and was found in a general transcription factor associated with RNA polymerase III. The initial structure found was a repeating ~30-amino acid motif with two invariant Cys and two invariant His residues that together bind a Zn^{2+} ion and thus bring a tight loop or " finger" of basic potentially DNA-binding residues together. The basic finger binds to the major groove of the DNA, with the exact sequence-matching characteristics determined by the topology of the particular residues that make up the finger. Although most DNA binding motifs insert a positively-charged α-helical domain into the major groove of DNA, the zinc-finger proteins are the only ones that combine several such motifs to interact with the DNA in several sequential sites.

In addition to the first type of Zn^{2+}-binding site described with two Cys and two His (Cys_2-His_2), there are two major variations to note. The first is the Cys_2-Cys_2 type, which is characteristic of steroid receptor transcription factors such as the glucocorticoid receptor or estrogen receptor. We will consider them in more detail later with the discussion of intracellular signal transduction, but for now, the general idea is that unactivated steroid hormone receptors are found in the cytoplasm, where they come in contact with and bind their cognate hormone molecule. They then translocate to the nucleus, where they dimerize and are able to act as transcription factors. The second major variation of the zinc finger is the binuclear Cys_6, which carries six Cys residues to create a slightly larger "basket" in which two Zn^{2+} ions are held, rather than just one. The best-studied example of this type of zinc-finger protein is GAL4, a yeast metabolic transcription factor.

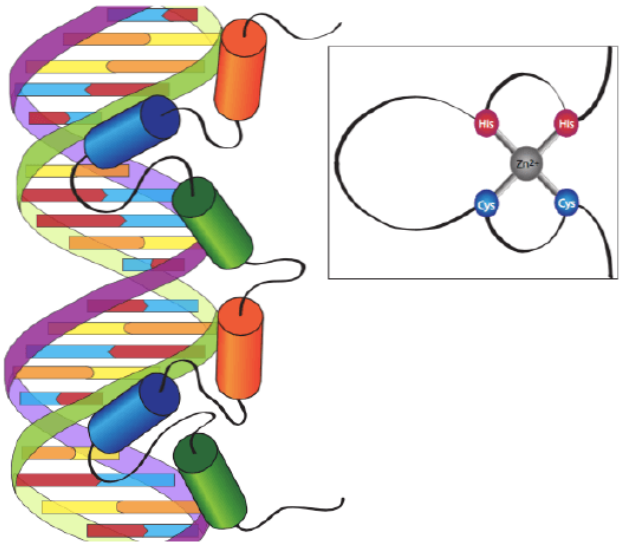


Figure 9.2.7. Zinc-finger family transcription factor (left) and close-up of Zn^{2+} binding site (right). Each cylinder represents an α-*helical domain*.

The next motif is the leucine zipper. Although this is a common motif for transcription factors, it is important to note that unlike the zinc-finger, the leucine zipper itself is not a DNA-binding motif. Rather, it is a protein dimerization motif, and determines the way in which two protein subunits interact. However, the leucine zipper is a common structural motif in transcription factors. It works through opposing domains of regularly spaced hydrophobic amino acids, particularly leucines, which are very effective at holding the two subunits together in the aqueous environment of the cell. The leucines are found in every 7th residue position of an α-helical domain, leading to a coiled-coil superstructure when two subunits interact. The (+) charged DNA-binding domains of these proteins are usually N-terminal to the leucine zippers, as in the case of the bZIP category of leucine zipper proteins (the name stands for basic region leucine zipper).

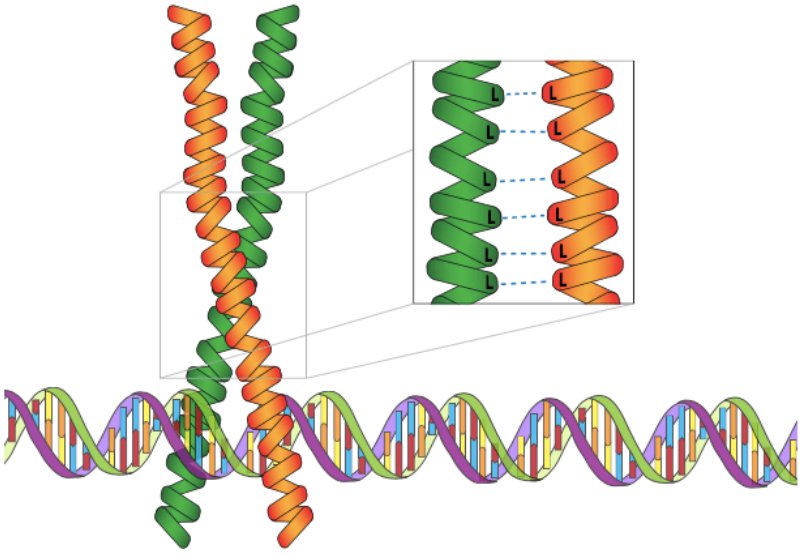


Figure 9.2.8. Leucine zipper.

The bHLH, or basic helix-loop-helix domains appear to be elaborations on the leucine zipper theme. In this case, the N-terminal region is highly basic, making it ideal for interacting with DNA, and this basic domain, which is also helical, leads into the first helix (H1) of the motif, which is then connected by a non-helical loop of amino acids, leading into a second helical region (H2). Beyond the bHLH, these transcription factors may merge into a leucine zipper motif or other protein interaction domain for dimerization. Though the primary binding domain is N-terminal to H1, the H1 domains also appear to play a role in binding the major groove of the DNA. [Example myc]

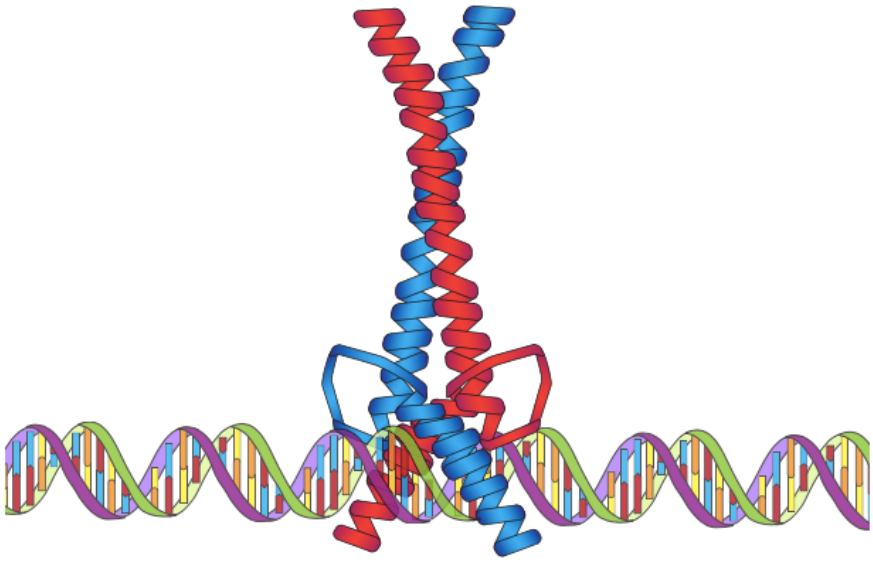


Figure 9.2.9. Binding of a basic helix-loop-helix (bHLH) class transcription factor.

NF-kB (nuclear factor kB) is a ubiquitous transcription factor discovered (and most noticeable) in the immune system. When active, it is a heterodimer, with both subunits containing a Rel homology region (RHR). Rel is an oncogene, and the RHR are named for their similarity to the previously-sequenced rel. The RHR domains bind to DNA with extraordinary affinity, due in part to having ve loops for DNA contact per subunit. Just as with the other types of transcription factors, some RHR-containing proteins are repressors, while others are activators.

The regulation of NF-kB is rather interesting: once it is in the nucleus, it is generally active. However, it is, as almost all cellular proteins, made in the cytoplasm. Inhibitors of NF-kB (IkB) also reside in the cytoplasm, and they act by binding the NF-kB and covering the nuclear localization signal that allows its import into the nucleus. Thus sequestered, the NF-kB must remain in the cytoplasm inactive until some stimulus activates IkB kinase, which phosphorylates the IkB and leads to ubiquitination and degradation, finally releasing the NF-kB from its bonds.

Because it can be mobilized quickly (compared to synthesizing new protein), NF-kB is consider a rapid-response transcription factor that is often used to begin expression of a gene needed soon after it has been “ordered” by a signal, either extracellular or intracellular. Not surprisingly for a factor discovered in the immune system, it is activated in response to bacterial and viral antigens, as well as other types of cellular stress or insult.

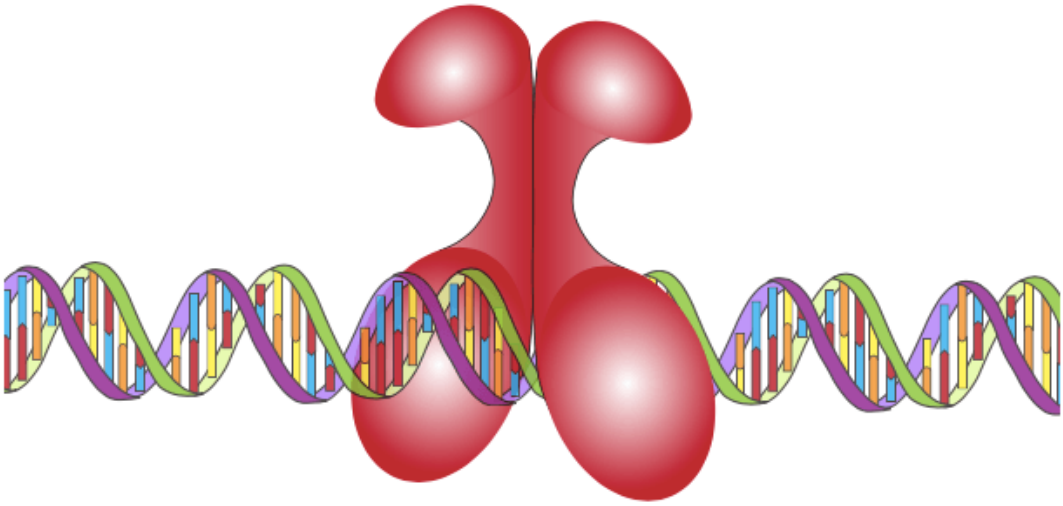


Figure 9.2.10. RHR domains are a DNA-binding domain found in the NF-kB family of transcription factors.

In addition to the relatively short-term regulation of gene expression controlled by binding transcription factors to regulatory elements, there are also stronger methods of locking away a gene to prevent its expression. In chapter 7, acetylation and deacetylation of histones was discussed as a method for decreasing and increasing their affinity for DNA. This can be controlled (Figure 9.2.11B) by the recruitment of histone deacetylase (HDAC) to particular genes via repressor/co-repressor complexes. The deacetylase forces tight winding of the targeted DNA to the histones, precluding access by RNA polymerases or general transcription factors.

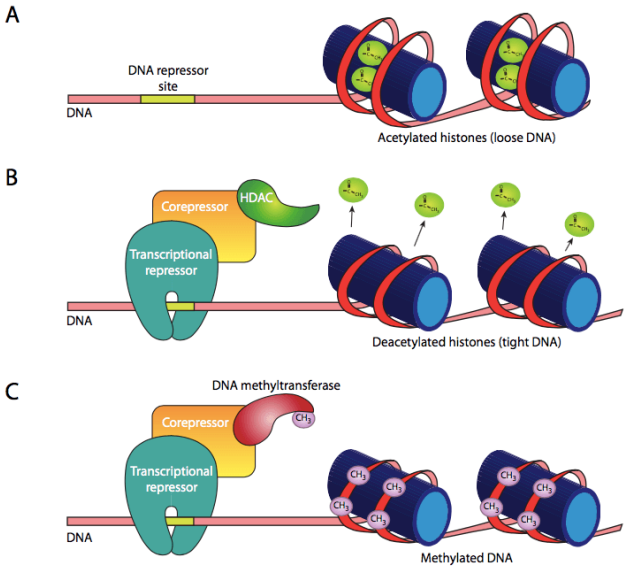


Figure 9.2.11. Long-term gene repression. (A) Acetylation of histones allows DNA to move off, potentially freeing the genes in that region for expression. (B) Specific regions can be wrapped more tightly around histones through the action of HDAC, removing the acetyl groups. (C) Methylation of the DNA can also prevent expression, either by physically blocking access, or by recruiting HDAC.

Another recruiter of HDAC are MBD proteins, which bind to methylated DNA. DNA methylation in mammals usually occurs on CpG dinucleotide sequences. This methylation appears to have the effect of blocking access of transcription factors and enzymes to the DNA. It can do so directly, or by recruiting MBD (methyl-CpG-binding domain) proteins. In either case, methylation is a long-term method of locking up genes, and is the mechanism for turning off genes that would never be used in a particular cell type (e.g. hemoglobin in neurons).

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10: TRANSLATION

The RNA polymerase has done its job (or in the case of prokaryotes, may still be in the process of doing its job), so now what happens to the RNA? For RNA that is destined to provide instructions for making a protein, then it needs to be translated, which is a job for Superman™! Oops, actually it's a job for ribosomes.

10.1: Introduction to Ribosomes

10.2: Prokaryotic Ribosomes

10.3: Eukaryotic Ribosomes

10.4: The Genetic Code

10.5: tRNA are Rather Odd Ducks

10.6: Prokaryotic Translation

10.7: Eukaryotic Translation

10.8: Regulation of Translation

Thumbanil: Diagram of RNA translation. (CC BY 3.0 - unported; Kelvinsong).

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10.1: Introduction to Ribosomes

Ribosomes are a complex of RNA and protein that bind to and processively move down (from 5' to 3' end) a strand of mRNA, picking up aminoacyl-tRNAs, checking to see if they are complementary to the RNA tri-nucleotide being "read" at the moment, and adding them to the new polypeptide chain if they are. The RNA part of the ribosomes are generated by the organism's general purpose RNA polymerase in prokaryotes, and generated by the RNA polymerases I and III in eukaryotes. Recall that RNA Pol II is used by eukaryotes to generate protein-coding mRNA's. Although the numbers of RNA strands and protein subunits differ between the prokaryote and eukaryote, the mechanism for translation is remarkably well conserved.

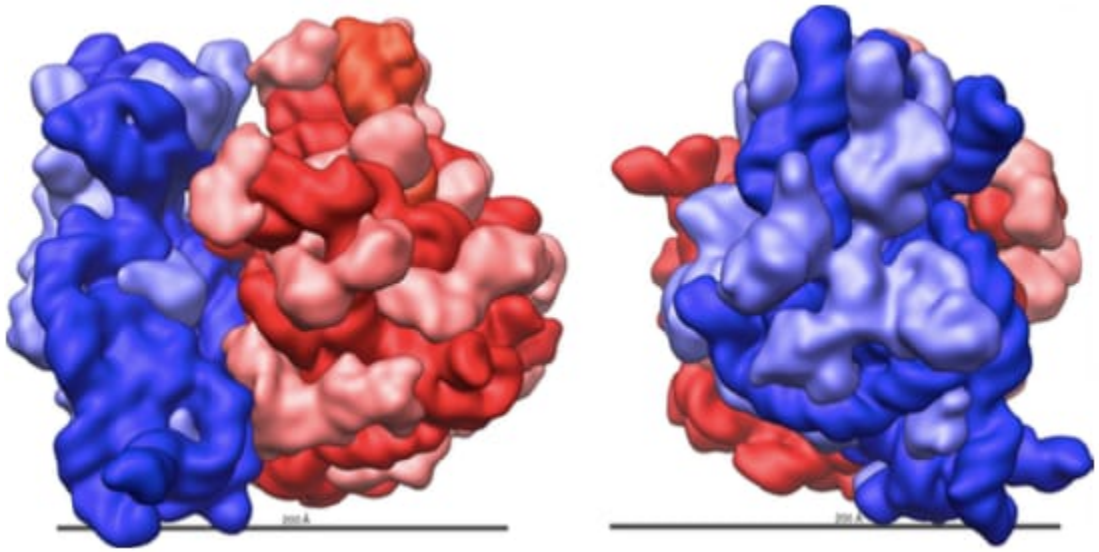


Figure 10.1.1. Two views of a prokaryotic ribosome. The large ribosomal subunit (50S) is shown in red, while the small ribosomal subunit (30S) is shown in blue. 3D images generated from data in the RCSB Protein Data Bank.

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10.2: Prokaryotic Ribosomes

The prokaryotic ribosomes contain 3 RNA strands and 52 protein subunits which can be divided into 1 RNA and 21 proteins in the small ribosomal subunit (aka the 30S subunit) and 2 RNA and 31 proteins in the large ribosomal subunit (50S subunit). The small subunit locates the start site and moves along the RNA. The large ribosomal subunit contains the aminoacyl transferase enzyme activity that connects amino acids to make a protein. Neither subunit is sufficient to carry out translation by itself. They must come together to form the full 70S ribosome for translation to occur. If you aren't already familiar with the nomenclature, you're probably thinking that it's obvious why I went into biology rather than math. My competence at basic computation aside, there is a method to the madness. The "S" in 30S or 50S indicates Svedberg units, or a measurement of the sedimentation rate when the molecules in question are centrifuged under standard conditions. Because the rate of sedimentation depends on both the mass *and the shape* of a molecule, numbers do not always add up.

The genes for the prokaryotic rRNA molecules are arranged in an operon and thus come from a single transcript. Depending on the organism, there may be several such operons in the genome to ensure steady production of this crucial enzymatic complex. However, these RNAs are not translated, so instead of having multiple translation start codons to signal the beginning of each gene, the single transcript is cleaved post-transcriptionally by Ribonuclease III (RNase III) into 25S, 18S, and 5S segments, and these are then further trimmed by RNase III and RNase M into the final 23S, 16S, and 5S rRNAs found in the ribosomes. Although the 5S does not appreciably differ in sedimentation rate, it is in fact slightly "shaved" in post-transcriptional editing.

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10.3: Eukaryotic Ribosomes

Like the RNA molecules in prokaryotic ribosomes, the eukaryotic rRNA molecules are also post-transcriptionally cleaved from larger transcripts. This processing, and the subsequent assembly of the large and small ribosomal subunits are carried out in the nucleolus, a region of the nucleus specialized for ribosome production, and containing not only high concentrations of rRNA and ribsomal proteins, but also RNA polymerase I and RNA polymerase III. In contrast, RNA polymerase II, as befits its broader purpose, is found throughout the nucleus.

> Because of the density of material in the nucleolus needed for constant ribosome production, it is often readily visible under various types of microscopy despite not being bounded by a membrane.

The **40S** small ribosomal subunit in eukaryotes also has just 1 rRNA, and has 33 proteins. The **60S**, or large ribosomal subunit in eukaryotes has three rRNA molecules, two of which are roughly analogous to the prokaryote (28S and 5S eukaryotic, 23S and 5S prokaryotic), and one, the 5.8S, that binds with complementary sequence on part of the 28S rRNA. It also contains 50 proteins. These ribosomal subunits have roughly the same function as the prokaryotic versions: the small subunit in conjunction with various initiation factors is responsible for finding the start site and positioning the ribosome on the mRNA, while the large subunit houses the docking sites for incoming and spent aminoacyl-tRNAs and contains the catalytic component to attach amino acids via peptide bonds.

The ribosomal RNA precursors (**pre-rRNA**) are remarkably conserved in eukaryotes, with the 28S, 5.8S, and 18S rRNAs encoded within a single transcript. This transcript, synthesized by RNA polymerase I, always has the same 5' to 3' order: 18S, 5.8S, 28S. After the 45S pre-rRNA is transcribed, it is immediately bound by nucleolar proteins in preparation for cleavage and base modification. However, it is primarily the **small nuclelolar RNAs (snoRNAs)**, not the proteins, that determine the position of the modifications. The primary modifications are 2'-hydroxylmethylation and transformation of some uridines into pseudouridines. These snoRNAs are sometimes transcribed independently by RNA polymerase III or II, but often are formed from the introns of pre-mRNA transcripts. Oddly, some of these introns come from pre-mRNAs that form unused mRNAs!

For the nucleolus to be the site of ribosome assembly, the ribosomal proteins must be available to interact with the rRNAs. By mechanisms discussed in the next chapter, the mRNAs for the ribosomal proteins are translated (as are all proteins) in the cytoplasm, but the resulting proteins are then imported into the nucleus for assembly into either the large or small ribosomal subunit. The subunits are then exported back out to the cytoplasm, where they can carry out their function.

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10.4: The Genetic Code

We have blithely described the purpose of the DNA chromosomes as carrying the information for building the proteins of the cell, and the RNA as the intermediary for doing so. Exactly how is it, though, that a molecule made up of just four different nucleotides joined together (albeit thousands and even thousands of thousands of them), can tell the cell which of twenty-odd amino acids to string together to form a functional protein? The obvious solution was that since there are not enough individual unique nucleotides to code for each amino acid, there must be combinations of nucleotides that designate particular amino acids. A doublet code, would allow for only 16 different combinations (4 possible nucleotides in the first position x 4 possible nucleotides in the second position = 16 combinations) and would not be enough to encode the 20 amino acids. However, a triplet code would yield 64 combinations, easily enough to encode 20 amino acids. So would a quadruplet or quintuplet code, for that matter, but those would be wasteful of resources, and thus less likely. Further investigation proved the existence of a triplet code as described in the table below.

With so many combinations and only 20 amino acids, what does the cell do with the other possibilities? The genetic code is a *degenerate code*, which means that there is redundancy so that most amino acids are encoded by more than one triplet combination (codon). Although it is a redundant code, it is not an ambiguous code: under normal circumstances, a given codon encodes one and only one amino acid. In addition to the 20 amino acids, there are also three “stop codons” dedicated to ending translation. The three stop codons also have colloquial names: UAA (ochre), UAG (amber), UGA (opal), with UAA being the most common in prokaryotic genes.

> The colloquial names were started when the discoverers of UAG decided to name the codon after a friend whose last name translated into “amber”. Opal and ochre were named to continue the idea of giving stop codons color names.
>
> The stop codons are sometimes also used to encode what are now considered the 21st and 22nd amino acids, selenocysteine (UGA) and pyrrolysine (UAG). These amino acids have been discovered to be consistently encoded in some species of prokarya and archaea.

Note that there are no dedicated start codons: instead, AUG codes for both methionine and the start of translation, depending on the circumstance, as explained forthwith. The initial Met is a methionine, but in prokaryotes, it is a specially modified formyl-methionine (f-Met). The tRNA is also specialized and is different from the tRNA that carries methionine to the ribosome for addition to a growing polypeptide. Therefore, in referring to a loaded initiator tRNA, the usual nomenclature is fMet-$tRNA_i$ or fMet-$tRNA_f$. There also seems to be a little more leeway in defining the start site in prokaryotes than in eukaryotes, as some bacteria use GUG or UUG. Though these codons normally encode valine and leucine, respectively, when they are used as start codons, the initiator tRNA brings in f-Met.

Although the genetic code as described is nearly universal, there are some situations in which it has been modified, and the modifications retained in evolutionarily stable environments. The mitochondria in a broad range of organisms demonstrate stable changes to the genetic code including converting the AGA from encoding arginine into a stop codon and changing AAA from encoding lysine to encoding asparagine. Rarely, a change is found in translation of an organismic (nuclear) genome, but most of those rare alterations are conversions to or from stop codons.

> Other minor alterations to the genetic code exist as well, but the universality of the code in general remains. Some mitochondrial DNAs can use different start codons: human mitochondrial ribosomes can use AUA and AUU. In some yeast species, the CGA and CGC codons for arginine are unused. Many of these changes have been cataloged by the National Center for Biotechnology Information (NCBI) based on work by Jukes and Osawa at the University of California at Berkeley (USA) and the University of Nagoya (Japan), respectively.

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10.5: tRNA are Rather Odd Ducks

In prokaryotes, tRNA can be found either as single genes or as parts of operons that can also contain combinations of mRNAs or rRNAs. In any case, whether from a single gene, or after the initial cleavage to separate the tRNA transcript from the rest of the transcript, the resulting pre-tRNA has an N-terminal leader (41 nt in *E. coli*) that is excised by RNase P. That cleavage is universal for any prokaryotic tRNA. After that, there are variations in the minor excisions carried out by a variety of nucleases that produce the tRNA in its final length though not its mature sequence, as we will see in a few paragraphs.

Eukaryotic pre-tRNA (transcribed by RNA polymerase III) similarly has an N-terminal leader removed by RNase P. Unlike the prokaryote though, the length can vary between different tRNAs of the same species. Some eukaryotic pre-tRNA transcripts also contain introns, especially in the anticodon loop, that must be spliced out for the tRNA to function normally. These introns are different from the self-splicing or spliceosome- spliced transcripts discussed in the transcription chapter. Here, the splicing function is carried out not by ribozymes, but by conventional (protein) enzymes. Interestingly, RNaseP also removes a 3' sequence from the pre-tRNA, but then another 3' sequence is added back on. This new 3' end is always CCA, and is added by three successive rounds with tRNA nucleotidyl transferase.

When RNA was introduced, it was noted that although extremely similar to DNA on many counts, it is normally single stranded, and that property, combined with the opportunity for complementary base pairing within a strand, allows it to do something far different than double-stranded DNA: it can form highly complex secondary structures. One of the simplest and clearest examples of this is tRNA, which depends on its conformation to accomplish its cellular function. The prototypical cloverleaf-form tRNA diagram is shown in Figure 10.5.2 on the left, with a 3D model derived from x-ray crystallographic data on the right. As you can see, the fully-splayed-out shape has four stem-loop "arms" with the amino acid attached to the acceptor arm, which is on the opposite side of the tRNA from the anticodon arm, which is where the tRNA must match with the mRNA codon during translation. Roughly perpendicular to the acceptor-anticodon axis are the D arm and the TyC arm. In some tRNAs, there are actually five total arms with a very short loop between the TyC and anticodon arms. The arm-like stem and loop structures are formed by two areas of strong complementarity (the stems, base-paired together) interrupted by a short non-complementary sequence (the loop). In general terms, the arms are used to properly position the tRNA within the ribosome as well as recognizing the mRNA codon and bringing in the correct amino acid.

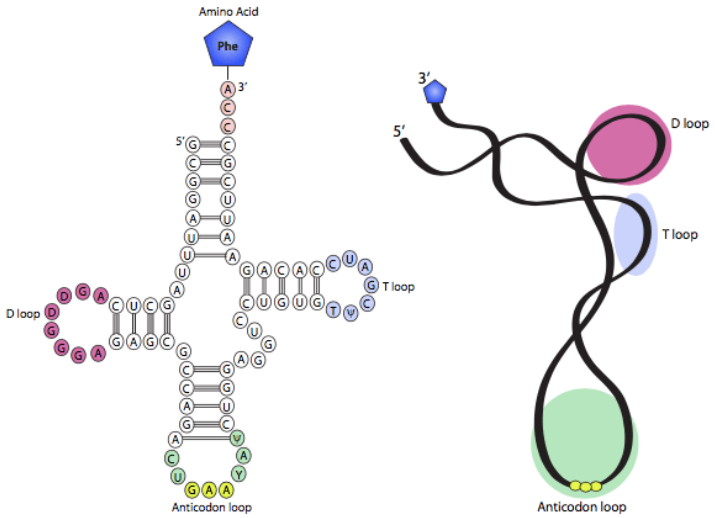


Figure 10.5.2. tRNA structure. At left, the classic clover-leaf, splayed out for simplicity. At right, a more accurate representation of the tRNA in pseudo-3D.

When it comes time for the tRNA to match its anticodon with the codon on the mRNA, the code is not followed "to the letter" if you will pardon the pun. There is a phenomenon called "wobble" in which a codon-anticodon match is allowed and stabilized for translation even if the nucleotide in the third position is not complementary. Wobble can occur because the conformation of the tRNA allows a little flexibility to that position of the anticodon, permitting H-bonds to form where they normally would not. This is not a universal phenomenon though: it only applies to situations where a U or a G is in the first position of the anticodon

(matching the third position of codon). Following the convention of nucleic acid sequences, the sequence is always written 5' to 3', even though in the case of codon-anticodon matching, the strands of mRNA and tRNA are antiparallel:

In addition to being allowed a bit of wobble in complementary base-pairing, tRNA molecules have another peculiarity. After being initially incorporated into a tRNA through conventional transcription, there is extensive modification of some of the bases of the tRNA. This affects both purines and pyrimidines, and can range from simple additions such as methylation or extensive restructuring of the sugar skeleton itself, as in the conversion of guanosine to wyosine (W). Over 50 different modifications have been catalogued to date. These modifications can be nearly universal, such as the dihydrouridine (D) found in the tRNA D loop, or more specific, such as the G to W conversion found primarily in tRNAPhe of certain species (examples have been identified in both prokaryotic and eukaryotic species). As many as 10% of the bases in a tRNA may be modified. Naturally, alterations to the sugar base of the nucleotides can also alter the base-pairing characteristics. For example, one common modified base, inosine, can complement U, C, or A. This aberrant complementary base pairing can be equal among the suitor bases, or it may be biased, as in the case of 5-methoxyuridine, which can recognize A, G, or U, but the recognition of U is poor.

Charging the tRNA

The knowledge of the genetic code begs the question: how is the correct amino acid attached to any given tRNA? A class of enzymes called the *aminoacyl tRNA synthetases* are responsible for recognizing both a specific tRNA and a specific amino acid, binding an ATP for energy and then joining them together (sometimes called "charging the tRNA") with hydrolysis of the ATP. Specificity is a difficult task for the synthetase since amino acids are built from the same backbone and are so similar in mass. Distinguishing between tRNA molecules is easier, since they are larger and their secondary structures also allow for greater variation and therefore greater ease of discrimination. There is also a built-in pre-attachment proofreading mechanism in that tRNA molecules that fit the synthetase well (i.e. the correct ones) maintain contact longer and allow the reaction to proceed whereas ill-fitting and incorrect tRNA molecules are likely to disassociate from the synthetase before it tries to attach the amino acid.

> Charging an aminoacyl tRNA synthetase with its amino acid requires energy. The synthetase first binds a molecule of ATP and the appropriate amino acid, which react resulting in the formation of aminoacyl-adenylate and pyrophosphate. The PP_i is released and the synthetase now binds to the proper tRNA. Finally, the amino acid is transferred to the tRNA. Depending on the class of synthetase, the amino acid may attach to the 2'-OH of the terminal A (class I) or to the 3'-OH of the terminal A (class II) of the tRNA. Phe-tRNA synthetase is the exception: it is structurally a class II enzyme but transfers the Phe onto the 2'-OH. Note that amino acids transferred onto the 2'-OH are soon moved to the 3'-OH anyway due to a transesterification reaction.

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10.6: Prokaryotic Translation

As soon as the RNA has emerged from the RNAP and there is sufficient space to accommodate a ribosome, translation can begin in prokaryotes. In fact, for highly expressed genes, it would not be unusual to see multiple RNA polymerases transcribing the DNA and multiple ribosomes on each of the transcripts translating the mRNA to protein! The process begins with the small ribosomal subunit (and only the small subunit - if it is attached to the large subunit, it is unable to bind the mRNA), which binds to the mRNA loosely and starts to scan it for a recognition sequence called the Shine-Dalgarno sequence, after its discoverers. Once this is recognized by the small ribosomal subunit rRNA, the small subunit is positioned around the start codon (AUG). This process is facilitated by initiation factors as follows.

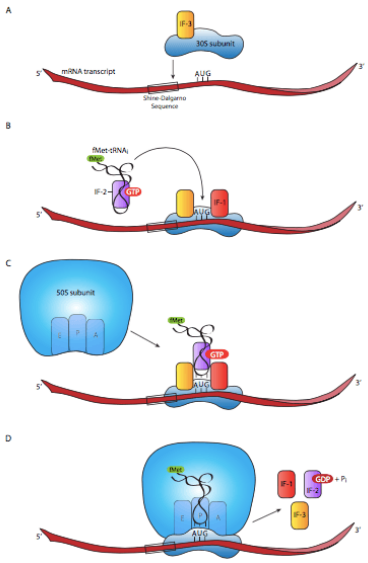


Figure 10.6.3. Initiation of Translation in Prokaryotes. (A) 30S subunit binds to Shine-Dalgarno sequence. (B) fMet-tRNA$_i$ is loaded into the middle slot of the small ribosomal subunit. Initiation factors occupy the other two slots. (C) The large ribosomal subunit docks with the small subunit. (D) The initiation factors are released and the ribosome is ready to start translation.

The 30S ribosomal subunit dissociates from the 50S ribosomal subunit if it was associated with one, and binds to intiation factors IF-1 and IF-3. IF-1 binds to the A site, where it prevents new aminoacyl-tRNA molecules from entering before the full ribosome is assembled. It also facilitates the assembly and stabilization of the initiation complex. IF-3 is required to allow the 30S subunit to bind to mRNA. Once this has occurred, IF-2-GTP arrives on scene, carrying with it the initiator aminoacyl-tRNA. This settles into the P site, which is positioned so that the anticodon of the tRNA settles over the AUG start codon of the mRNA. Hydrolysis of the GTP attached to IF-2 and release of all the initiation factors is needed to allow the 50S subunit to bind to the 30S subunit to form the full and fully functional ribosome. Because GTP hydrolysis was required, the joining of the subunits is irreversible spontaneously, and requires expenditure of energy upon termination of translation. Once the 50S subunit joins with the 30S subunit, the A site is ready to accept the next aminoacyl-tRNA.

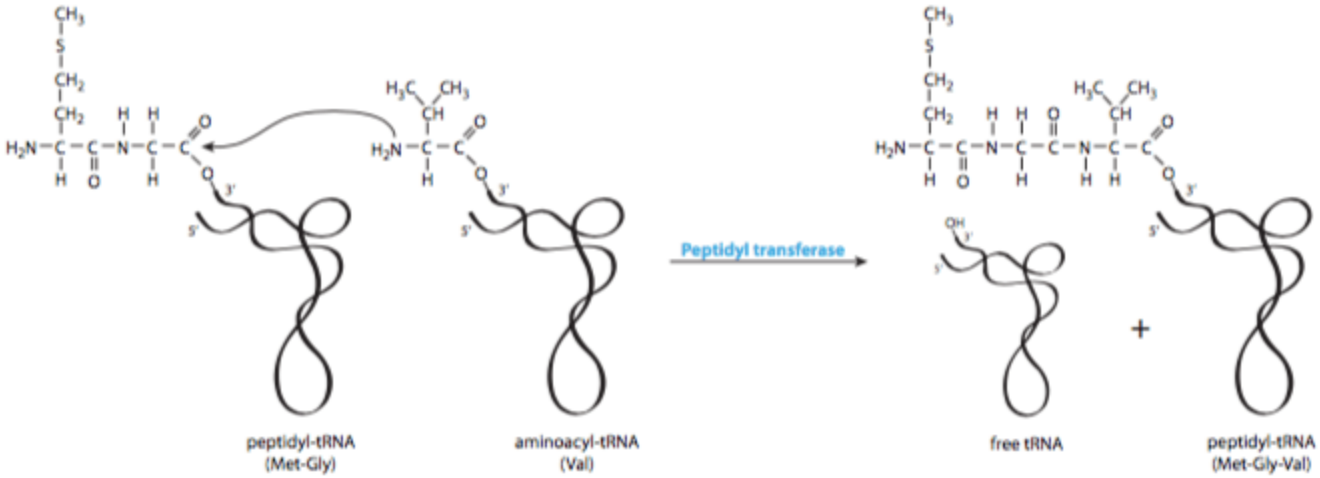


Figure 10.6.4. Peptide bond formation at the addition of the third amino acid. The previous two amino acids are peptide bonded together as well as attached to the tRNA from the second amino acid. The aminoacyl- tRNA bond is broken and transferred/transformed to the peptide bond connecting the initial dipeptide to the third amino acid.

A common and understandable misconception is that the new amino acid brought to the ribosome is added onto the growing polypeptide chain. In fact, the mechanism is exactly the opposite: the polypeptide is added onto the new amino acid (Figure 10.6.4). This begins from the second amino acid to be added to a new protein (Figure 10.6.5). The rst amino acid, a methionine, you should recall, came in along with IF-2 and the initiator tRNA. The new aminoacyl-tRNA is escorted by EF-Tu, an elongation factor that carries a GTP. Once the aa-tRNA is in place, EF-Tu hydrolyzes the GTP and dissociates from the aminoacyl-tRNA and ribosome.

> For a long time, there was a bit of mystery surrounding the simultaneous docking of two tRNA molecules on immediately adjacent codons of mRNA. Under normal conditions, there should not be enough room, since the tRNAs are fairly bulky and one should obstruct the other from reaching the mRNA to make a codon-anticodon match. The matter was finally cleared up in 2001 with x-ray crystallographic examinations showing a bend in the mRNA between the codon in the P slot and the codon in the A slot. The bend puts the two associated tRNAs at slightly different angles and thus creates just enough room for both to maintain basepair- ing hydrogen bonds with the mRNA. See Yusupov et al, *Science* 292 (5518): 883-896, 2001.

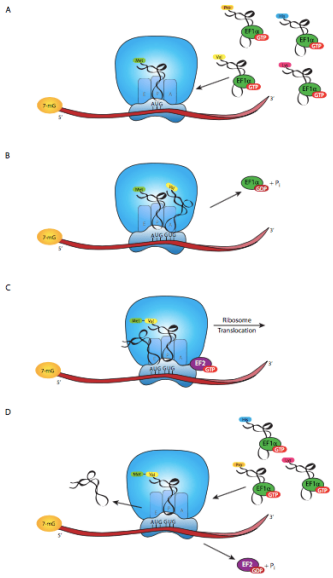


Figure 10.6.5. Elongation of the polypeptide chain. (A) a new aminoacyl-tRNA drops into the A slot of the ribosome. (B) the fMet is moved off its $tRNA_i$ and peptide-bonded to the new amino acid, which is still attached to its tRNA. (C) Ribosome shifts over to the right. (D) empty $tRNA_i$ is ejected.

When a new aminoacyl-tRNA drops into the A slot of the ribosome, the anticodon is lined up with the codon of the mRNA. If there is no complementarity, the aminoacyl-tRNA soon floats back out of the slot to be replaced by another candidate. However, if there is complementarity (or something close enough, recalling the idea of wobble) then H-bonds form between the codon and anti-codon, the tRNA changes conformation, which shifts the conformation of EF-Tu, causing hydrolysis of GTP to GDP + P_i, and release from the aa-tRNA. The codon-anticodon interaction is stable long enough for the catalytic activity of the ribosome to hydrolyze the bond between fMet and the tRNAf in the P slot, and attach the fMet to the new amino acid with a peptide bond in the A slot. The new amino acid is still attached to its tRNA, and as this process occurs, the ribosome shifts position with respect to the mRNA and tRNAs. This puts the now- empty (no amino acid attached) tRNAf in the E slot, the tRNAaa in the P slot, attached to that aa which is bonded to Met, and the A slot is again open for a new tRNA to come in. The elongation factor EF-G binds near the A slot as soon as EF-Tu leaves, and is required for ribosomal translocation, providing energy for the process by hydrolyzing a GTP that it carries with it to the ribosome. From my students' experiences, the best way to learn this seems to be to study the diagrams and see the movements of the molecules, filling in the mechanistic details in your mind. This process continues until the ribosome brings the A slot in line with a stop codon.

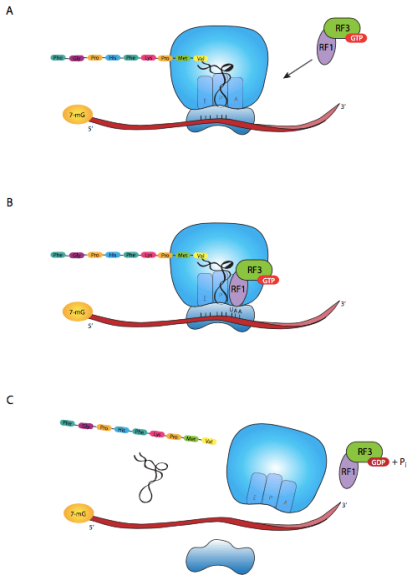


Figure 10.6.6. Termination of translation.

There is no tRNA with an anticodon for the stop codon. Instead, there is a set of release factors that t into the A site of the ribosome, bind to the stop codon, and activate the ribosome to cut the bond between the polypeptide chain and the last tRNA (Figure 10.6.6). Depending on which stop codon is present either RF1 (recognizes UAA or UAG) or RF2 (for UAA or UGA) first enters the A slot. The RF1 or RF2 is complexed with RF3, which is involved in subsequent releasing of the RF complex from the A slot. This is necessary because once the polypeptide has been released from the ribosome, the mRNA must be released. Ribosome releasing factor (RRF) also binds in the A slot, which causes a conformational change in the ribosome releasing the previous and now empty tRNA. Finally, EF-G binds to RRF, and with an accompanying hydrolysis of GTP, causes dis- sociation of the ribosome into separate large and small subunits. Note that it is the combination of EF-G/RRF that causes dissociation; EF-G alone plays a different role in ribosome movement when it is not at the stop codon.

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10.7: Eukaryotic Translation

Eukaryotic translation, as with transcription, is satisfyingly similar (from a student studying point of view, or from an evolutionary conservation one) to the prokaryotic case. The initiation process is slightly more complicated, but the elongation and termination processes are the same, but with eukaryotic homologues of the appropriate elongation and release factors.

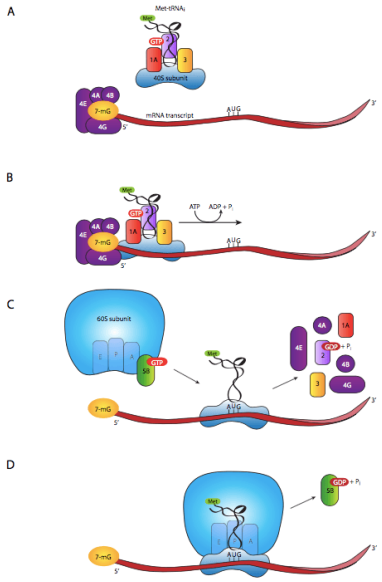


Figure 10.7.7. Initiation of Translation in Eukaryotes.

For eukaryotes, each mRNA encodes one and only one gene (as opposed to multi-gene transcripts such as operons), so there isn't much question of which AUG is a start codon, and which are just regular methionines. Therefore, there is no requirement for a Shine-Dalgarno sequence in eukaryotes. The small ribosomal subunit, accompanied by eukaryotic initiation factors eIF-3, eIF-2, and Met-tRNA$_i$, together known as the terneary complex, binds to eIF-1A. Meanwhile, eIF-4A, -4B, -4E, and -4G bind to the 5' (7-methyguanosine) cap of the mRNA (g. 7A). The small subunit complex and the eIF4/ mRNA cap-binding complex interact to form the 43S complex, which then begins scanning the mRNA from 5' to 3' looking for the first AUG.

> Usually, but not always, the first AUG is the start codon for eukaryotic genes. However, the context of the AUG matters, and it is a much stronger (i.e. more frequently recognized and used) start codon if there is a purine residue (A or G) at -3 and a G at +4. See Kozak, M., *Biochimie* **76**: 815-821, 1994.

Once the 43S scanning complex has found the start codon, the initiation factors drop off, and the large ribosomal subunit arrives. The large ribosomal subunit has bound eIF-6, which prevents it from reassociating with small subunits, and its removal is required first. Another factor, eIF-5 enters the scene during the coupling process between the large and small ribosomal subunits, and hydrolysis of an eIF-5-attached GTP is required to complete the docking of the subunits and the formation of a complete functional ribosome on the mRNA.

Elongation is functionally the same as in prokaryotes except that the functions of EF-Tu is taken care of by EF-1a, also with hydrolysis of GTP. EF-2 is the eukaryotic analog of EF-G, and utilizes GTP hydrolysis for translocation of the ribosome. Termination uses eukaryotic homologues of the release factors, though eRF-1 takes the place of both prokaryotic RF-1 and RF-2.

Although polyribosomes (aka polysomes) can form on both prokaryotic and eukaryotic mRNAs, eukaryotic polysomes have an additional twist. Technically, a polysome is simply an mRNA with multiple ribosomes translating it simultaneously, but in eukaryotes, the polysome also has a unique morphology because it utilizes PABPI, or poly-A binding protein. This protein not only binds to the 3' poly-A tail of an mRNA, it also interacts with the eIF-4 initiation factors, which thus loops the mRNA into a circular shape. That way, once the ribosome reaches the end of the gene and releases from the mRNA, it is physically near the beginning of the mRNA to start translating again.

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10.8: Regulation of Translation

Gene expression is primarily regulated at the pre-transcriptional level, but there are a number of mechanisms for regulation of translation as well. One well-studied animal system is the iron-sensitive RNA-binding protein, which regulates the expression of genes involved in regulating intracellular levels of iron ions. Two of these genes, *ferritin*, which safely sequesters iron ions inside cells, and *transferrin*, which transports iron from the blood into the cell, both utilize this translational regulation system in a feedback loop to respond to intracellular iron concentration, but they react in opposite ways. The key interaction is between the iron response elements (IRE), which are sequences of mRNA that form short stem-loop structures, and IRE-BP, the protein that recognizes and binds to the IREs. In the case of the ferritin gene, the IRE sequences are situated upstream of the start codon. When there is high iron, the IRE-BP is inactive, and the stem-loop structures are melted and overrun by the ribosome, allowing translation of ferritin, which is an iron-binding protein. As the iron concentration drops, the IRE-BP is activated and binds around the IRE stem-loop structures, stabilizing them and preventing the ribosome from proceeding. This prevents the production of ferritin when there is little iron to bind.

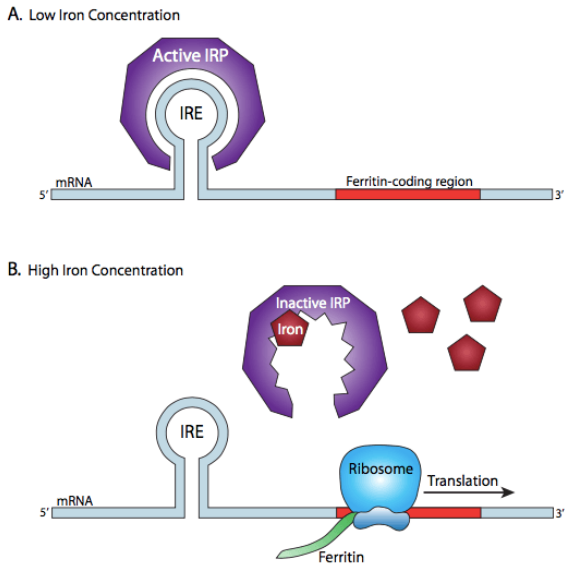


Figure 10.8.8. Pre-translational control of gene expression by iron-response protein (IRP), which binds to either the iron-response element (IRE), unless it has bound iron.

Transferrin also uses iron response elements and IRE-binding proteins, but in a very different mechanism. The IRE sequences of the transferrin gene are located downstream of the *stop* codon, and play no direct role in allowing or preventing translation.

However, when there is low intracellular iron and there is a need for more transferrin to bring iron into the cell, the IRE-BP is activated as in the previous case, and it binds to the IREs to stabilize the stem-loop structures. In this case; however, it prevents the 3' poly-A tail degradation that would normally occur over time. Once the poly-A tail is degraded, the rest of the mRNA is destroyed soon thereafter. As mentioned in the transcription chapter, the longer poly-A tails are associated with greater persistence in the cytoplasm, allowing more translation before they are destroyed. The IRE-BP system in this case externally prolongs the lifetime of the mRNA when that gene product is needed in higher amounts.

Since mRNA is a single-stranded nucleic acid and thus able to bind complementary sequence, it is not too surprising to find that one of the ways that a cell can regulate translation is using another piece of RNA. Micro RNAs (miRNAs) were discovered as very short (~20 nucleotides) non-protein-coding genes in the nematode, *C. elegans*. Since their initial discovery (Lee et al, *Cell* **75**: 843-54, 1993), hundreds have been found in various eukaryotes, including humans. The expression pattern of the miRNA genes is highly specific to tissue and developmental stage. Many are predicted to form stem-loop structures, and appear to hybridize to 3'-untranslated sequences of mRNA thus blocking initiation of translation on those mRNA molecules. They may also work through a mechanism similar to the siRNA discussed below, but there is clear evidence that mRNA levels are not necessarily altered by miRNA-directed translational control.

MicroRNAs are currently under investigation for their roles as either oncogenes or tumor suppressors (reviewed in Garzon et al, *Ann. Rev. Med.* **60**: 167-79, 2009). Approximately half of known human miRNAs are located at fragile sites, breakpoints, and other regions associated with cancers (Calin et al, *Proc. Nat. Acad. Sci. (USA)* **101**: 2999-3004, 2004). For example, miR-21 is not only upregulated in a number of tumors, its overexpression blocks apoptosis - a necessary step to allow abnormal cells to continue to live and divide rather than die out. Conversely, miR-15a is significantly depressed in some tumor cells, and overexpression can slow or stop the cell cycle, even inducing apoptosis.

Another mechanism for translational control that uses small RNA molecules is RNA interference (RNA_i). This was first discovered as an experimentally induced repression of translation when short *double-stranded* RNA molecules, a few hundred nucleotides in length and containing the same sequence as a target mRNA, were introduced into cells. The effect was dramatic: most of the mRNA with the target sequence was quickly destroyed. The current mechanistic model of RNA_i repression is that first, the double-stranded molecules are cleaved by an endonuclease called *Dicer*, which cleaves with over-hanging single-stranded 3' ends. This allows the short fragments (siRNA, ~20nt long) to form a complex with several proteins (RISC, RNA-induced silencing complex). The RISC splits the double-stranded fragments into single strands, one of which is an exact complement to the mRNA. Because of the complementarity, this is a stable interaction, and the double-stranded region appears to signal an endonuclease to destroy the mRNA/siRNA hybrid.

The final method of controlling levels of gene expression is control after the fact, i.e., by targeted destruction of the gene product protein. While some proteins keep working until they fall apart, others are only meant for short-term use (e.g. to signal a short phase in the cell cycle) and need to be removed for the cell to function properly. Removal, in this sense, would be a euphemism for chopped up and recycled. The ubiquitin-proteasome system is a tag-and-destroy mechanism in which proteins that have outlived their usefulness are polyubiquitinated. Ubiquitin is a small (76 amino acids, ~5.6 kDa), highly conserved (96% between human and yeast sequences) eukaryotic protein (Figure 10.8.9) that can be attached to other proteins through the action of three sequential enzymatic steps, each catalyzed by a different enzyme.

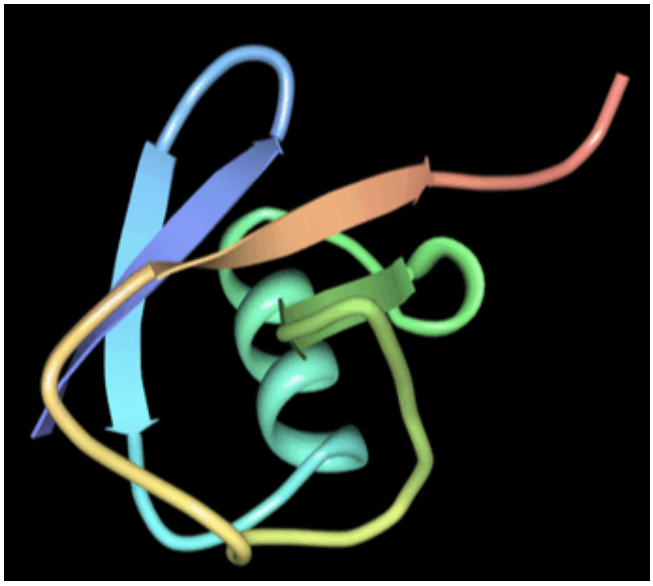


Figure 10.8.9. Ubiquitin. This 3D representation was generated from the file 1ubi (synthetic human ubiquitin) in the RCSB Protein Data Bank

E1 activates the ubiquitin by combining it with ATP to make ubiquitin-adenylate, and then transfers the ubiquitin to itself via a cysteine thioester bond. Through a trans(thio) esterification reaction, the ubiquitin is then transferred to a cysteine in the E2 enzyme, also known as ubiquitin-conjugating enzyme. Finally, E3, or ubiquitin ligase, interacts with both E2-ubiquitin and the protein designated for destruction, transferring the ubiquitin to the target protein. After several rounds, the polyubiquitinated protein is send to the proteasome for destruction.

Mutations in E3 genes can cause a variety of human medical disorders such as the neurodevelopmental disorders Angelman syndrome, Hippel-Lindau syndrome, or the general growth disorder known as 3-M syndrome. Mechanisms linking malfunction in ubiquitination pathways and symptoms of these disorders are not currently known.

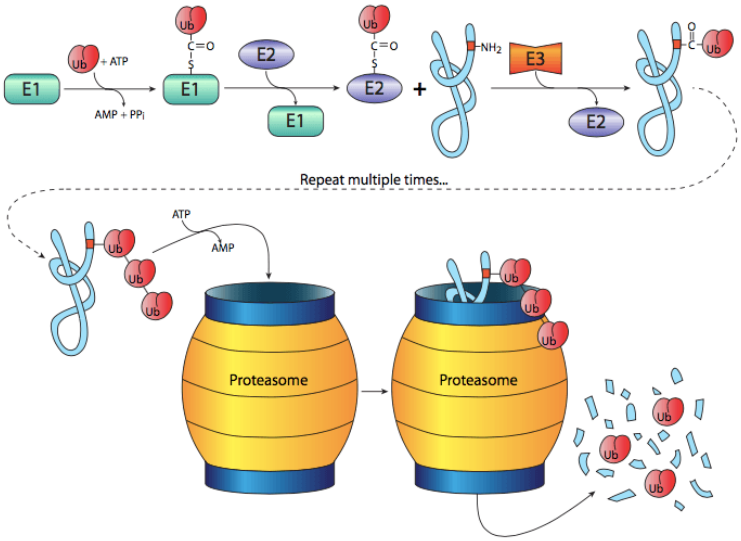


Figure 10.8.10. Polyubiquitination of a targeted protein (blue) requires three ubiquitinating enzymes, E1, E2, and E3. Once tagged, the protein is positioned in the proteasome by binding of the polyubiquitin tail to the outer surface of the proteasome. The proteasome then cleaves the protein into small polypeptides.

Proteasomes are very large protein complexes arranged as a four-layered barrel (the 20S subunit) capped by a regulatory subunit (19S) on each end. The two outer rings are each composed of 7 α subunits that function as entry gates to the central rings, each of which is composed of 7 β subunits, and which contain along the interior surface, 6 proteolytic sites. The 19S regulatory units control the opening and closing of the gates into the 20S catalytic barrel. The entire proteasome is sometimes referred to as a 26S particle.

A polyubiquitinated protein is first bound to the 19S regulatory unit in an ATP-dependent reaction (the 19S contains ATPase activity). 19S unit opens the gates of the 20S unit, possibly involving ATP hydrolysis, and guides the protein into the central proteolytic chamber. The protease activity of proteasomes is unique in that it is a threonine protease, and it cuts most proteins into regular 8-9 residue polypeptides, although this can vary.

As we will see in the cell cycle chapter, proteasomes are a crucial component to precise regulation of protein functions.

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11: PROTEIN MODIFICATION AND TRAFFICKING

Once a polypeptide has been translated and released from the ribosome, it may be ready for use, but often it must undergo post-translational processing to become fully functional. While many of these processes are carried out in both prokaryotes and eukaryotes, the presence of organelles provides the need as well as some of the mechanisms for eukaryote-specific modifications such as glycosylation and targeting.

11.1: Proteolytic Cleavage

11.2: Protein Trafficking

11.3: Protein Folding in the Endoplasmic Reticulum

11.4: N-linked Protein Glycosylation Begins in the ER

11.5: O-linked Protein Glycosylation Takes Place Entirely in the Golgi

11.6: Vesicular Transport

11.7: Receptor-mediated Endocytosis

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11.1: Proteolytic Cleavage

The most common modification is proteolytic cleavage. Some of the pre-cleavage polypeptides are immediately cleaved, while others are stored as inactive precursors to form a pool of enzymes (or other kinds of proteins) that can be activated very quickly, on a timescale of seconds to minutes, as compared to having to go through transcription and translation, or even just translation. Interestingly, though methionine (Met) is universally the first amino acid of a newly synthesized polypeptide, many proteins have that methionine cleaved off (also true for some prokaryotic f-Met).

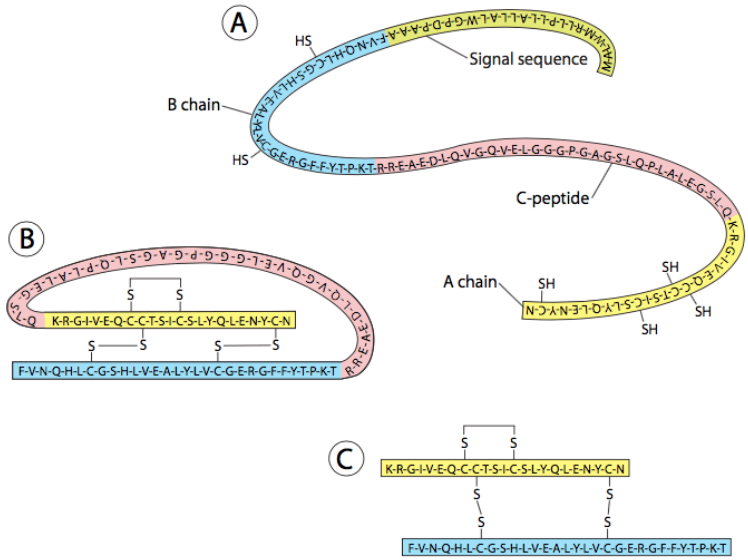


Figure 11.1.1. Proteolytic processing is necessary to make biologically active insulin. (A) The linear protein contains a signal sequence, which is cleaved after the protein enters the ER, an A chain, a B chain, and a C-peptide. (B) Inside the ER, the proinsulin (insulin precursor) folds and disulfide bonds form between cysteines. (C) Finally, two cleavages release the C peptide, which leaves the A and B chains attached by the disulfide bonds. This is now active insulin.

Activation of proteins by cleavage of precursors is a common theme: the precursor protein is termed a proprotein, and the peptide that is cleaved off of it to activate the protein is called the *propeptide*. Among the better known examples of proteins that are derived from proproteins are the hormone insulin, the cell death protein family of caspases, and the Alzheimer-associated neural protein β-amyloid. Insulin is an interesting example (Figure 11.1.1) in mammals: preproinsulin (inactive as a hormone) is first translated from the insulin mRNA. After a cleavage that removes an N-terminal sequence, proinsulin (still inactive) is generated. The proinsulin forms some internal disulfide bonds, and when the final proteolytic action occurs, a substantial chunk (called the C-peptide) is taken out of the middle of the proinsulin. Since the protein was internally disulfide bonded though, the two end pieces remain connected to become the active insulin hormone.

Another interesting protein processing example is that of collagen assembly (Figure 11.1.2). As you will read in chapter 13, collagen is a very large secreted protein that provides structure and shock absorbance for the extracellular matrix in animals. You can find it in skin, hooves, cartilage, and various connective tissues. An individual collagen protein is actually a twisted triple-helix of three subunits. The collagen subunits are made as procollagen, and propeptides are lopped off of both N- and C- termini to generate the final protein. However, they are not cleaved off until after the three subunits assemble around one another. In fact, collagen subunits that have already been processed do not assemble into triple-helical proteins. The propeptide sequences are clearly necessary for efficient assembly of the final protein complex.

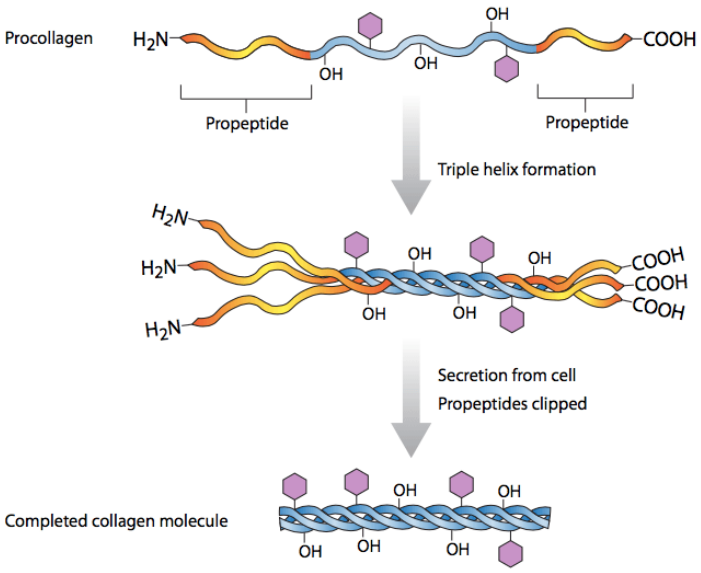


Figure 11.1.2. Processing and assembly of procollagen into collagen.

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11.2: Protein Trafficking

The idea that propeptide sequences have important functions in protein maturation beyond just keeping them from being active is not exclusive to assembly. A major class of cleaved peptide sequences is *signal peptides*. Signal peptides direct the protein from the cytoplasm into a particular cellular compartment. In the case of prokaryotes, this essentially means the cell membrane, but for eukaryotes, there are specific signal peptides that can direct the protein to the nucleus, to the mitochondria, to the endoplasmic reticulum, and other intracellular organelles. The peptides are specifically recognized by receptors on the membranes of particular compartments, which then help to guide the insertion of the protein into or through the membrane. Almost all protein synthesis in eukaryotes is carried out in the cytoplasm (with the exception of a few proteins in the chloroplasts and mitochondria), so proteins found in any other compartment or embedded in any membrane must have been targeted and transported into that compartment by its signal sequence.

> Although this is primarily considered a eukaryotic process given that there are so many potential targets, prokaryotes do have membrane proteins (in fact, some 800 different ones in *E. coli* comprising ~20% of total protein), and they are positioned there with the aid of insertase enzymes such as YidC and complexes such as Sec translocase. The Sec translocase uses a signal recognition particle (SRP) much like that in eukaryotes, and will be discussed later in this chapter when the SRP is introduced. YidC, which has eukaryotic homologues (e.g. Oxa1 in mitochondria), is a 61 kDa transmembrane protein that is placed in the membrane through an SRP-Sec translocase mechanism. Once there, YidC interacts with nascent polypeptides (once they reach ~70 amino acids long) that have begun to interact with the lipids of the cell membrane, and pushes the protein into/through the membrane.

The nucleus is one such compartment, and examples of the proteins found within include DNA and RNA polymerases, transcription factors, and histones. These and other nuclear proteins have an N-terminal signal sequence known as the NLS, or nuclear localization signal. This is a well-studied pathway that involves a set of importin adapter proteins and the nuclear pore complex (Figure 11.2.3). Transport into the nucleus is particularly challenging because it has a double membrane (remember that it is contiguous with the endoplasmic reticulum membrane. Although there are other mechanisms for making proteins that are embedded in the nuclear membrane, the primary mechanism for import and export of large molecules into and out of the nucleus itself is the *nuclear pore complex*. The complex is very large and can be made of over 50 different proteins (nucleoporins, sometimes called nups). The nucleoporins are assembled into a large open octagonal pore through the nuclear membranes. As Figure 11.2.3 indicates, there are antenna-like fibrils on the cytoplasmic face, and these help to guide proteins from their origin in the cytoplasm to the nuclear pore, and on the nuclear side there is a basket structure. Of course, not all proteins are allowed into the nucleus, and the mechanism for distinguishing appropriate targets is straightforward. The protein must bear a nuclear localization signal (NLS). While in the cytoplasm, an importin-α protein binds to the NLS of a nuclear protein, and also binds to an importin-β. The importin-β is recognized and bound by the nuclear pore complex. The details of the transport mechanism are murky, but phenylalanine-glycine repeats in the nucleoporin subunits (FG-nups) are thought to be involved.

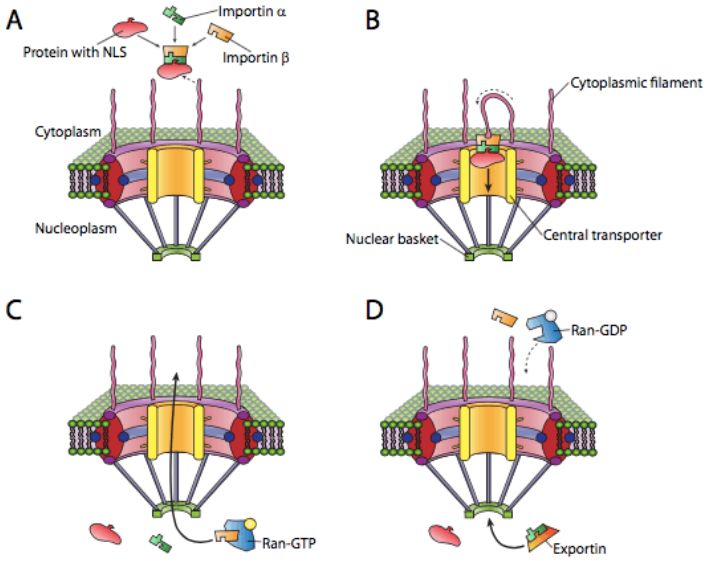


Figure 11.2.3. Transport through nuclear pore

Once the nucleoprotein-importin aggregate is moved into the nucleus, Ran-GTP, a small GTPase, causes the aggregate to dissociate (Figure 11.2.3c). The imported protein is released in the nucleus. The importins are also released in the nucleus, but they are exported back out again to be reused with another protein targeted for the nucleus.

> The mechanisms of small GTPase activation of other processes will be discussed again in more detail in later chapters (cytoskel- eton, signaling). The key to understanding the mechanism is to remember that the GTPase hydrolyzes GTP to GDP, but still holds onto the GDP. Although the GTPase will hydrolyze GTP spontaneously, the GTPase-activating protein, GAP (or Ran-GAP in this case) greatly speeds the rate of hydrolysis. In order to cycle the system back to GTP, the GDP is not re-phosphorylated: it is *exchanged* for a new GTP. The exchange is greatly facilitated by the action of an accessory protein, the guanine nucleotide exchange factor (GEF), in this particular case, a Ran-GEF.

Export from the nucleus to the cytoplasm also occurs through the nuclear pore. The Ran-GTP is also a part of the export complex (Figure 11.2.3d), and in conjunction with an *exportin* protein and whatever is to be exported, is moved out of the nucleus via the nuclear pore. Once in the cytoplasm, the hydrolysis of GTP to GDP by Ran (activated by Ran-GAP, a cytoplasmic protein) provides the energy to dissociate the cargo (e.g. mRNA) from the exporting transport molecules. The Ran-GDP then binds to importins, re-enters the nucleus, and the GDP is exchanged for GTP.

The nuclear pore is the only transport complex that spans dual membrane layers, although there are coordinated pairs of transport complexes in double-membraned organelles such as mitochondria. The transport proteins in the outer mitochondrial membrane link with transport proteins in the inner mitochondrial membrane to move matrix-bound proteins (e.g. those involved in the TCA cycle) in from the cytoplasm. The complexes that move proteins across the outer membrane are made up of Tom (translocator outer membrane) family of proteins. Some of the proteins will stay embedded in the outer membrane: they are processed by a SAM (sorting and assembly machinery) complex also embedded in the outer membrane). Meanwhile, others continue to the Tim (translocator inner membrane) proteins that move them across the inner membrane. As with the nuclear proteins, there is a consensus signal sequence on mitochondrial proteins that is bound by cytosolic chaperones that bring them to the Tom transporters. As shown in the table below, there are signal sequences/propeptides that target proteins to several other compartments.

Of particular importance for the rest of this chapter, is the sequence targeting proteins to the endoplasmic reticulum, and by extension, any proteins destined for the ER, the Golgi apparatus, the cell membrane, vesicles and vesicularly-derived compartments, and secretion out of the cell. Here, in addition to an N-terminal signal sequence, the position of secondary internal signal sequences (sometimes called signal patches) helps to determine the disposition of the protein as it enters the ER.

The initial insertion requires recognition of the signal sequence by *SRP*, the signal recognition protein. The SRP is a G-protein and exchanges its bound GDP for a GTP upon binding to a protein's signal sequence. The SRP with its attached protein then docks to

a receptor (called the SRP receptor, astoundingly enough) embedded in the ER membrane and extending into the cytoplasm. The SRP usually binds as soon as the signal sequence is available, and when it does so, it arrests translation until it is docked to the ER membrane. Incidentally, this is the origin of the "rough" endoplasmic reticulum: the ribosomes studding the ER are attached to the ER cytoplasmic surface by the nascent polypeptide it is producing and an SRP. The SRP receptor can exist on its own or in association with a *translocon*, which is a bipartite translocation channel. The SRP receptor (SR) is also a GTPase, and is usually carrying a GDP molecule when unassociated. However, upon association with the translocon it exchanges its GDP for a GTP. These GTPs are important because when the SRP binds to the SR, both GTPase activities are activated and the resulting release of energy dissociates both from the translocon and the nascent polypeptide. This relieves the block on translation imposed by the SRP, and the new protein is pushed on through the translocon as it is being synthesized. Once the signal sequence has completely entered the lumen of the ER, it reveals a recognition site for *signal peptidase*, a hydrolytic enzyme that resides in the ER lumen and whose purpose is to snip off the signal peptide.

Prokaryotes also use an SRP homolog. In *E. coli*, the SRP is simple, made up of one protein subunit (Ffh) and a small 4.5S RNA. By comparison, some higher eukaryotes have an SRP comprised of six different proteins subunits and a 7S RNA. Similarly, there is a simple prokaryotic homologue to the SRP receptor, FtsY. An interesting difference is that FtsY generally does not interact with exported proteins, and appears to be necessary only for membrane-embedded proteins. Otherwise, there are many similarities in mechanism for SRP-based insertion of membrane proteins in eukaryotic and prokaryotic species, including GTP dependence, and completion of the mechanism by a translocase (SecYEG in *E. coli*).

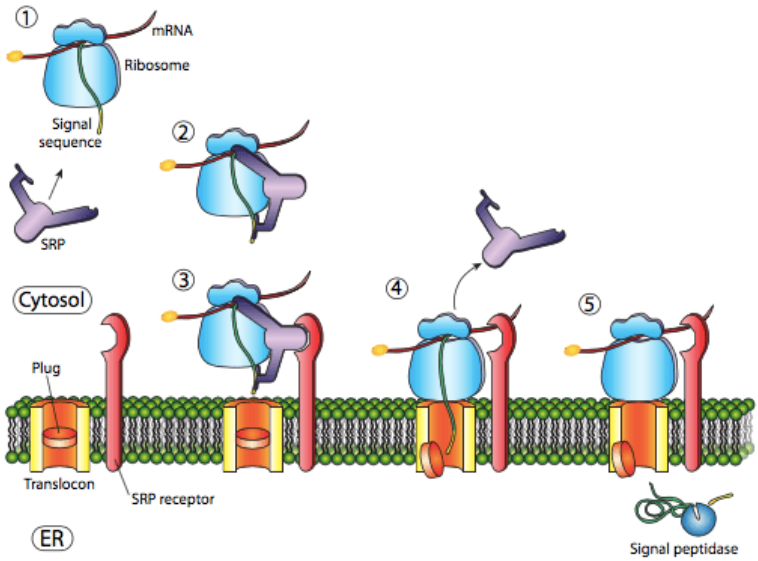


Figure 11.2.4. SRP and its receptor SR mediate movement of proteins through the ER membrane. The SRP recognizes the signal sequence and binds to it and the ribosome, temporarily arresting translation. The SRP-polypeptide-ribosome complex is bound by its receptor, SR, which positions the complex on a translocon. Once the ribosome and polypeptide are docked on the translocon, the SRP dissociates, and translation resumes, with the polypeptide moving through the translocon as it is being synthesized.

If that was the only signal sequence in the protein, the remainder of the protein is synthesized and pushed through the translocon and a soluble protein is deposited in the ER lumen, as shown in Figure 11.2.4. What about proteins that are embedded in a membrane? Transmembrane proteins have internal signal sequences (sometimes called signal patches). Depending on their relative locations, they may be considered either *start*-transfer or *stop*-transfer sequence, where "transfer" refers to translocation of the peptide through the translocon. This is easiest to understand by referring to Figure 11.2.5.

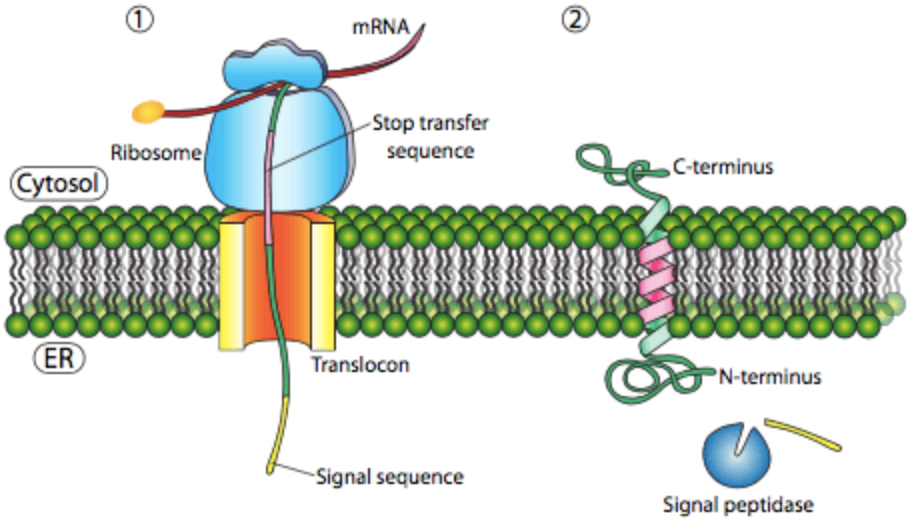


Figure 11.2.5. Single-pass transmembrane protein insertion. (1) the signal sequence has allowed the ribosome to dock on a translocon and newly made polypeptide is threaded through until the stop-transfer sequence. (2) The hydrophobic stop transfer sequence gets "stuck" in the membrane, forcing the rest of the polypeptide to stay in the cytoplasm as it is translated.

If there is a significant stretch of mostly-uninterrupted hydrophobic residues, it would be considered a stop-transfer signal, as that part of the protein can get stuck in the translocon (and subsequently the ER membrane) forcing the remainder of the protein to remain outside the ER. This would generate a protein that inserts into the membrane once, with its N-terminus in the ER lumen and the C-terminus in the cytoplasm. In a multi-pass transmembrane protein, there could be several start- and stop- transfer hydrophobic signal patches.

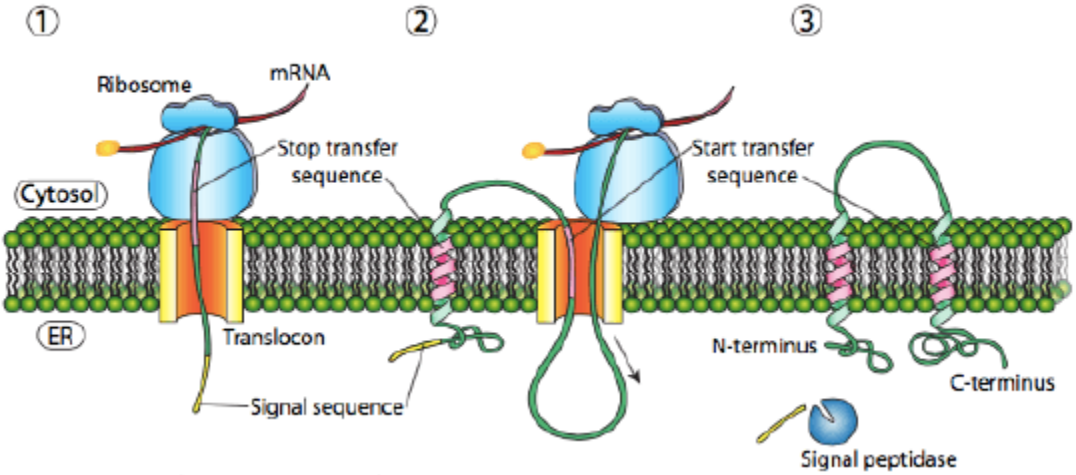


Figure 11.2.6. Insertion of 2-pass transmembrane protein.

Building on the single-pass example, if there was another signal patch after the stop-transfer sequence, it would act as a start-transfer sequence, attaching to a translocon and allowing the remainder of the protein to be moved into the ER. This results in a protein with both N- and C- termini in the ER lumen, passing through the ER membrane twice, and with a cytoplasmic loop sticking out. Of course, the N-terminus could be on the other side. For a cytoplasmic N-terminus, the protein cannot have an N-terminal signal sequence (Figure 11.2.7). It has an internal signal patch instead. It plays essentially the same role, but the orientation of the patch means that the N-terminal stays cytoplasmic. The polypeptide translated after the patch is fed into the ER. And just as in the last example, multiple stop- and start- sequences can reinsert the protein in the membrane and change the facing of the next portion.

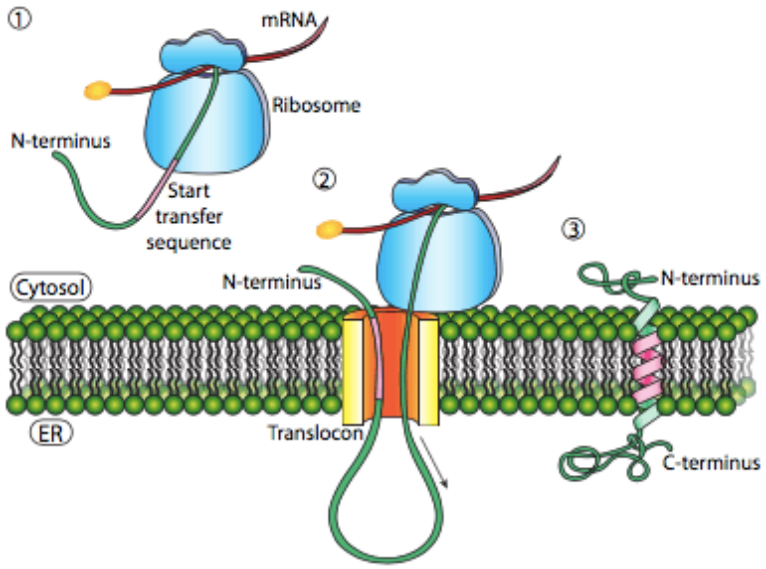


Figure 11.2.7. Insertion of a single-pass protein with N-terminus in cytoplasm uses a signal patch but no N-terminal signal.

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11.3: Protein Folding in the Endoplasmic Reticulum

The endoplasmic reticulum (ER) lumen plays four major protein processing roles:

1. folding/refolding of the polypeptide,
2. glycosylation of the protein,
3. assembly of multi-subunit proteins, and
4. packaging of proteins into vesicles.

Refolding of proteins is an important process because the initial folding patterns as the polypeptide is still being translated and unfinished may not be the optimal folding pattern once the entire protein is available. This is true not just of H-bonds, but of the more permanent (i.e. covalent) disulfide bonds as well. Looking at the hypothetical example polypeptide, the secondary structure of the N-terminal half may lead to the formation of a stable disulfide bond between the first cysteine and the second cysteine, but in the context of the whole protein, a more stable disulfide bond might be formed between cysteine 1 and cysteine 4. The exchange of disulfide bonding targets is catalyzed by **protein disulfide isomerase** (PDI).

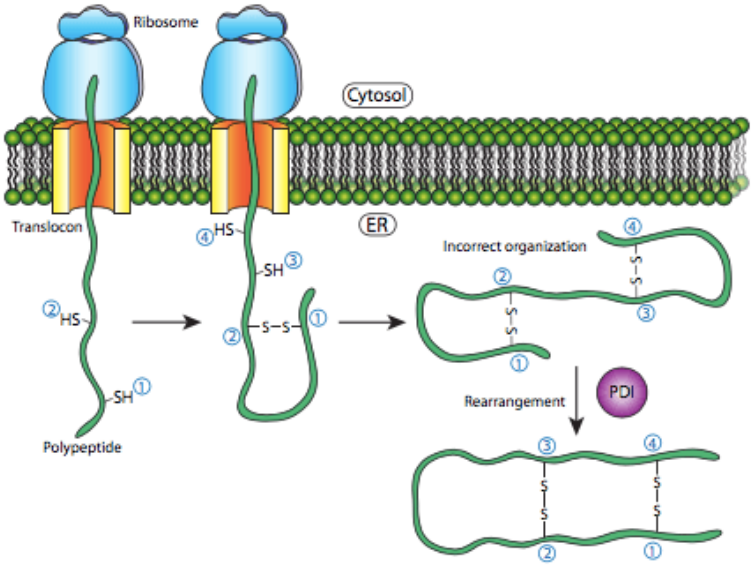


Figure 11.3.8. Protein Disulfide Isomerase rearranges disulfide bonds.

The internal redox environment of the endoplasmic reticulum, is significantly more oxidative than that in the cytoplasm. This is largely determined by glutathione, which is found in a 30:1 GSH:GSSG ratio or higher in the cytoplasm but at nearly 1:1 ratio in the ER lumen. This oxidative environment is also conducive to the disulfide remodeling. It should be noted that PDI does not choose the “correct” bonding partners. It simply moves the existing disulfide bonds to a more energetically stable arrangement. As the rest of the polypeptide continues to refold, breaking and making H-bonds quickly, new potential disulfide bond partners may move near one another and PDI can again attempt to rearrange the disulfide bonding pattern if the resulting pattern is more thermodynamically stable.

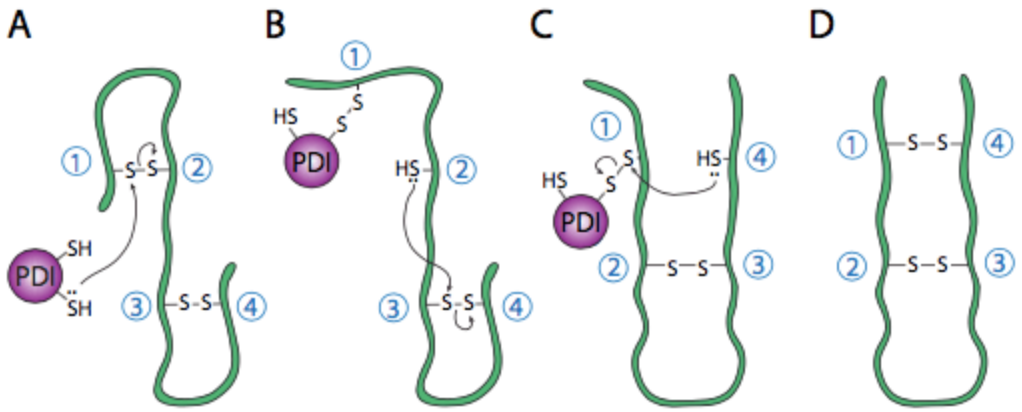


Figure 11.3.9. Protein Disulfide Isomerase. This enzyme uses a sulfhydryl group of a cysteine residue as temporary bonding partner in order to break disulfide bonds on the target protein and allow for new ones to form. Note that the formation of a new bond is not directed by PDI, but is instead a stochastic process in which a stronger binding partner displaces the PDI —SH.

The assembly of multisubunit proteins and the refolding of polypeptides are similar in their use of chaperone proteins that help prevent premature folding, sequestering parts of the protein from H-bonding interaction until the full protein is in the ER lumen.

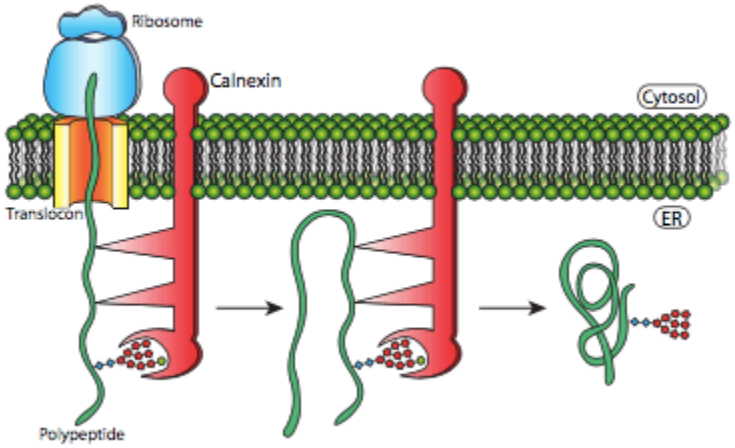


Figure 11.3.10. Protein folding is optimized in the ER. Proteins such as calnexin can temporarily bind to nascent polypeptides, preventing them from forming secondary structures from incomplete information, releasing the protein for folding once the entire polypeptide has been translated.

This mechanism simply makes finding the thermodynamically optimal conformation easier by preventing the formation of some potential suboptimal conformations. These chaperone proteins bind to the new proteins as they enter the lumen through the translocon and in addition to simply preventing incorrect bonds that would have to be broken, they also prevent premature interaction of multiple polypeptides with one another. This can be a problem because prior to the proper folding that would normally hide such domains within the protein, the immature polypeptides may have interaction domains exposed, leading to indiscriminate binding, and potentially precipitation of insoluble protein aggregates.

Chaperones

Chaperone proteins can also be found in prokaryotes, archaea, and in the cytoplasm of eukaryotes. These are somewhat similar to each other, and function somewhat differently than the types of folding proteins found in the ER lumen. They are referred to generally as chaperonins, and the best characterized is the GroEL/ GroES complex in *E. coli*. As the structure in Figure 11.3.11 indicates, it is similar in shape to the proteasome, although with a completely different function. GroEL is made up of two stacked rings, each composed of 7 subunits, with a large central cavity and a large area of hydrophobic residues at its opening. GroES is also composed of 7 subunits, and acts as a cap on one end of the GroEL. However, GroES only caps

GroEL in the presence of ATP. Upon hydrolysis of the ATP, the chaperonins undergo major concerted conformational changes that impinge on the protein inside, causing refolding, and then the GroES dissociates and the protein is released back into the cytosol.

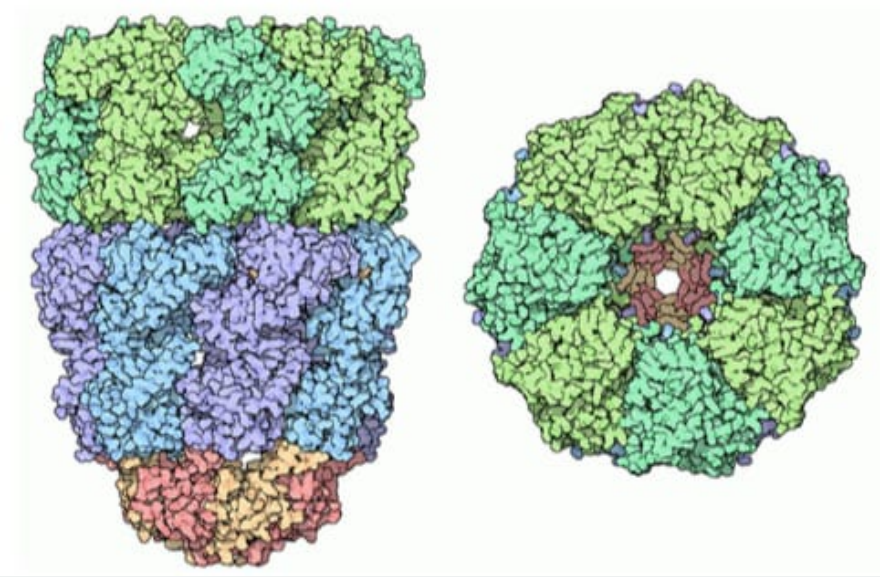


Figure 11.3.11. GroEL/GroES complex. The two heptameric rings of GroEL are shown in green and blue/purple. The GroES heptamer (red/yellow) caps the GroEL complex in the presence of ATP. Illustration by D.S. Goodsell, 2002.

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11.4: N-linked Protein Glycosylation Begins in the ER

Glycosylation is an important modification to eukaryotic proteins because the added sugar residues are often used as molecular flags or recognition signals to other cells than come in contact with them. There are two types of protein glycosylation, both of which require import of the target polypeptide into the ER. N-linked glycosylation actually begins in the endoplasmic reticulum, but O-linked glycosylation does not occur until the polypeptide has been transported into the Golgi apparatus. Therefore, it is also the case that N-linked glycosylation can (and is) usually beginning as a co-translational mechanism, whereas O-linked glycosylation must be occurring post-translationally. Other major differences in the two types of glycosylation are (1) N-linked glycosylation occurs on asparagine (N) residues within an N-X-S or N-X-T sequence (X is any amino acid other than P or D) while O-linked glycosylation occurs on the side chain hydroxyl oxygen of either serine or threonine residues determined not by surrounding sequence, but by secondary and tertiary structure; (2) N-linked glycosylation begins with a “tree” of 14 specific sugar residues that is then pruned and remodeled, but remains fairly large, while O-linked glycosylation is based on sequential addition of individual sugars, and does not usually extend beyond a few residues.

Technically, N-glycosylation begins before a protein is even being translated, as the dolichol pyrophosphate oligosaccharide (i.e. the sugar “tree” - not an official term, by the way) is synthesized in the ER (Figure 11.4.12) without being triggered by translation or protein entry.

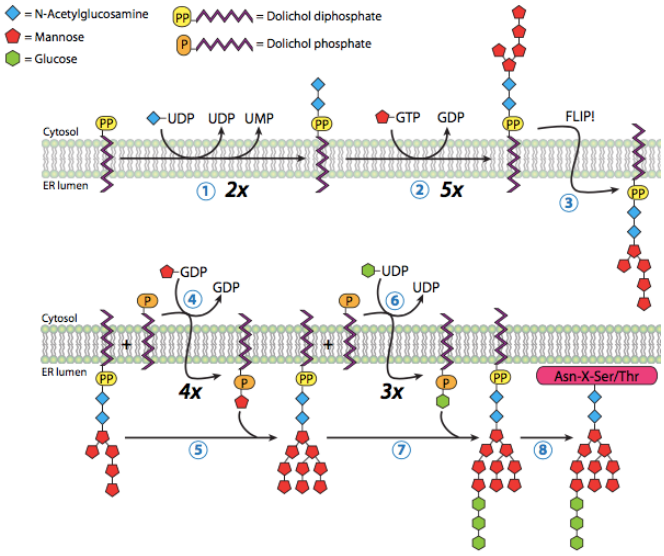


Figure 11.4.12. Formation of N-glycosylation “sugar tree” and attachment to protein. Each step is catalyzed by a glycosyltransferase. Note that the sugar substrates are sugar nucleotides, not isolated sugar molecules.

Dolichol is a long-chain hydrocarbon [between 14-24 isoprene units of 4+1 carbons] found primarily in the ER membrane, and serves as a temporary anchor for the N-glycosylation oligosaccharide as it is being synthesized and as it waits for an appropriate protein to glycosylate. The oligosaccharide synthesis begins with the addition of two N-acetylglucosamine residues to the pyrophosphate linker, followed by a mannose. From this mannose, the oligosaccharide branches, with one branch receiving three more mannose residues and the other receiving one. So far, all of these additions to the oligosaccharide have been taking place in the cytoplasm. Now the glycolipid is *flipped* inwards to the ER lumen! Once in the lumen, four more mannoses are added, and finally three glucose residues top off the structure.

> Not all nucleosides are used for this process: sugars have only been found linked to UDP, GDP, and CMP. UDP is the most versatile, binding N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), N-acetylmuramic acid, galactose, glucose, glucuronic acid, and xylose. GDP is used for mannose and fucose, while CMP is only used for sialic acid.

The enzymes that accomplish the glycosylation are *glycosyltransferases* specific for both the added sugar residue and the target oligosaccharide. The sugars used by the enzymes are not simply the sugar, but nucleotide sugars - usually a sugar linked to a

nucleoside diphosphate, for example, uracil diphosphate glucose (UDP-glucose) or GDP- mannose.

The N-linked oligosaccharide has two physiological roles: it acts as the base for further glycosylation, and it is used as a marker for error-checking of protein folding by the calnexin-calreticulin system (Figure 11.4.13). Once the oligosaccharide is attached to the new polypeptide, the process of further glycosylation begins with the action of a glucosidase and that removes two of the glucoses. The last glucose is necessary to help the glycoprotein dock with either calnexin or calreticulin (Figure13, step 1 or 4), which are very similar proteins that have a slow glucosidase activity and associate with a protein disulfide isomerase-like activity.

> The protein disulfide isomerase-like activity comes from ERp57, which is technically a thiol oxidoreductase, but is functionally similar to PDI.

The major difference is that calreticulin is soluble in the ER lumen while calnexin is bound to the ER membrane. Both temporarily hold onto the glycoprotein giving it time to (re)fold and possibly rearrange disulfide bonds, then it removes the glucose, allowing the glycoprotein to continue on its way. Importantly, if the glycoprotein has not been completely folded (step 2a), the enzyme UDP-glucose:glycoprotein glucosyltransferase (GT) recognizes it and adds back the glucose residue (step 3), forcing it to go through the calreticulin/calnexin cycle again in hopes of folding correctly this time. If it has been folded correctly (step 2b), it can be recognized by ER-α-1,2-mannosidase, which removes a mannose, completing the glycosylation modifications in the ER.

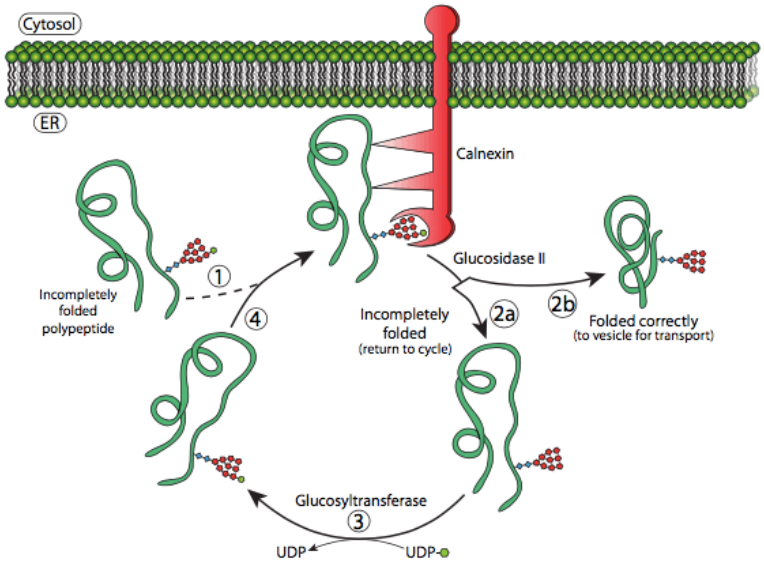


Figure 11.4.13. N-glycosylation can be used in error-checking.

Most glycoproteins continue with oligosaccharide remodeling once they have been moved from the ER to the Golgi apparatus by vesicular transport. There, a variety of glycosidases and glycosyltransferases prune and add to the oligosaccharide. Although the glycosylation is consistent and stereotyped for a given protein, it is still unclear exactly how the glycosylation patterns are determined.

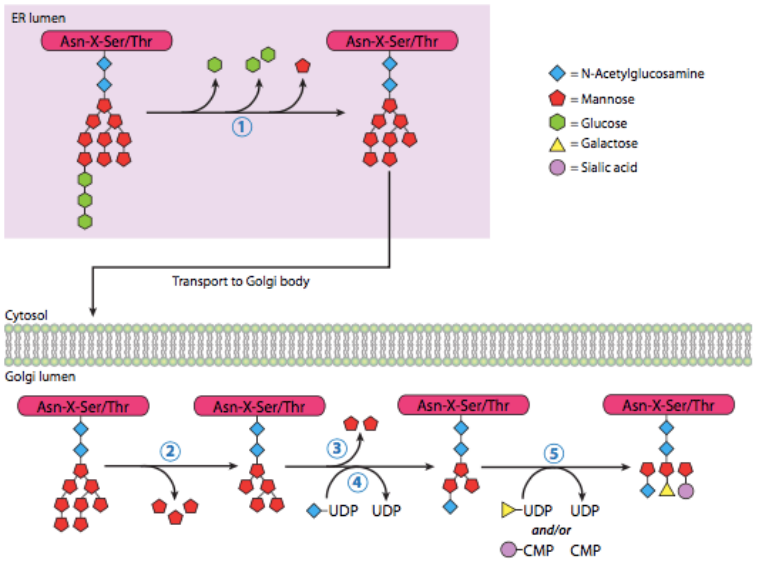


Figure 11.4.14. N-linked glycosylation can continue in the Golgi. Sugars may be added and removed in different patterns by glycosyltransferases resident in the Golgi.

Two common antibiotics, tunicamycin and bacitracin, can target N-linked glycosylation, although their antibiotic properties come from disrupting formation of bacterial cell walls. Tunicamycin is an analogue of UDP-GlcNAc, and inside eukaryotic cells can disrupt the initial oligosaccharide formation by blocking the initial GlcNAc addition to the dolichol-phosphate. Since it can be transported into eukaryotic cells, tunicamycin is not clinically useful due to its toxicity. Bacitracin, on the other hand, is a small cyclic polypeptide that binds to dolichol-PP preventing its dephosphorylation to dolichol-P, which is needed to build the oligosaccharide. Bacitracin is not cell-permeable, so even though it has similar activity to tunicamycin on bacteria by disrupting extracellular glycolipid synthesis needed for cell wall formation, it is harmless to eukaryotes and thus is a useful therapeutic antibiotic.

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11.5: O-linked Protein Glycosylation Takes Place Entirely in the Golgi

O-linked glycoproteins begin their glycosylation with the action of the Golgi-specific enzyme, GalNAc transferase, which attaches an N-acetylgalactosamine to the hydroxyl group of a serine or threonine. The determination of which residue to glycosylate appears to be directed by secondary and tertiary structure as previously mentioned, and often occurs in dense clusters of glycosylation. Despite being fairly small additions (usu. <5 residues), the combined oligosaccharide chains attached to an O-linked glycoprotein can contribute over 50% of the mass of a glycoprotein. Two of the better known O-linked glycoproteins are mucin, a component of saliva, and ZP3, a component of the zona pellucida (which protects egg cells). These two examples also illustrate a key property of glycoproteins and glycolipids in general: the sugars are highly hydrophilic and hold water molecules to them, greatly expanding the volume of the protein.

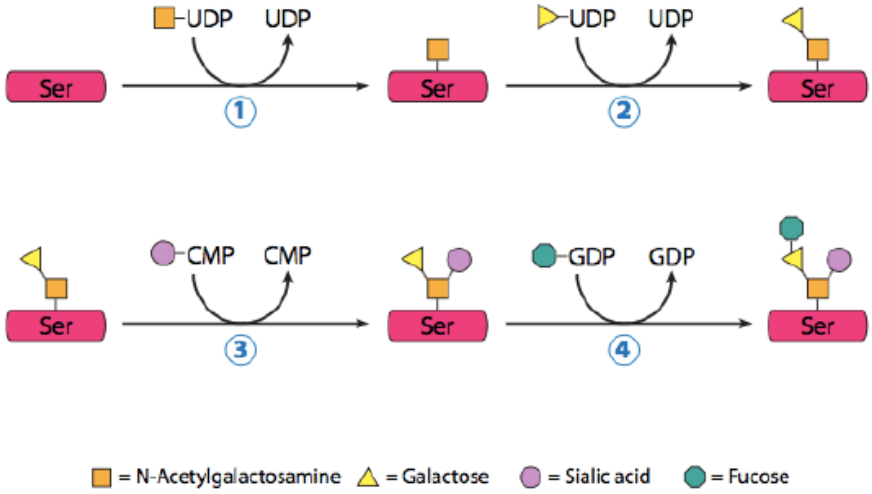


Figure 11.5.15. O-linked glycosylation in the Golgi involves attachment of only a few sugars to serine or threonine.

Interestingly, this protective waterlogged shell can mask parts of the protein core. In the case of the cell adhesion molecule, NCAM, which is a highly polysialylated glyco- protein at certain developmental stages and locations, and unglycosylated in others, the naked protein can be recognized as an adhesive substrate while the glycosylated protein can be recognized as a repulsive substrate to other cells. Even in highly glycosylated proteins though, the sugar residues often acts as recognition sites for other cells. For instance, the zona pellucida is very important as a physical barrier that protects the egg, but glycosylated ZP3 also acts as a sperm receptor.

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11.6: Vesicular Transport

In addition to protein processing, the ER and Golgi also take care of some types of protein transport. Vesicles (membrane-bound bubbles, essentially) pinch off from the ER, Golgi, and other membranous organelles, carrying with them whatever soluble molecules were inside the fluid that was enclosed as well as any molecules embedded in that section of membrane. These vesicles then catch a ride on a molecular motor such as kinesin or myosin, and travel along the cytoskeleton until they dock at the appropriate destination and fuse with the target membrane or organelle. In general, vesicles move from the ER to the cis Golgi, from the cis to the medial Golgi, from the medial to the trans Golgi, and from the trans Golgi to the plasma membrane or other compartments. Although most movement is in this direction, there are also vesicles that move back from the Golgi to the ER, carrying proteins that were supposed to stay in the ER (e.g. PDI) and were accidentally scooped up within a vesicle.

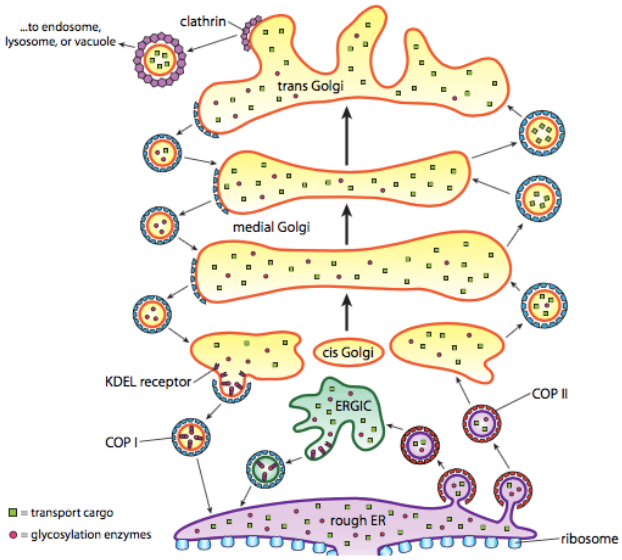


Figure 11.6.16. Vesicles bud from the endoplasmic reticulum and merge to form ERGIC, which matures into the cis Golgi, then the medial Golgi, and finally the trans Golgi. Vesicles may also bud from any of these other compartments to other organelles or to the plasma membrane.

The formation of vesicles is dependent on coat proteins that will, under proper conditions, self-assemble into spherical cages. When associated with transmembrane proteins, they can pull the attached membrane along into a spherical shape also. The major types of coat proteins used in vesicle formation are COPII, COPI, and clathrin.

COPII coat proteins form the vesicles that move from ER to Golgi. COPI coat proteins are used between parts of the Golgi apparatus as well as to form vesicles going from the Golgi back to the ER. Finally, clathrin is used to form vesicles leaving the Golgi for the plasma membrane as well as for vesicles formed from the plasma membrane for endocytosis.

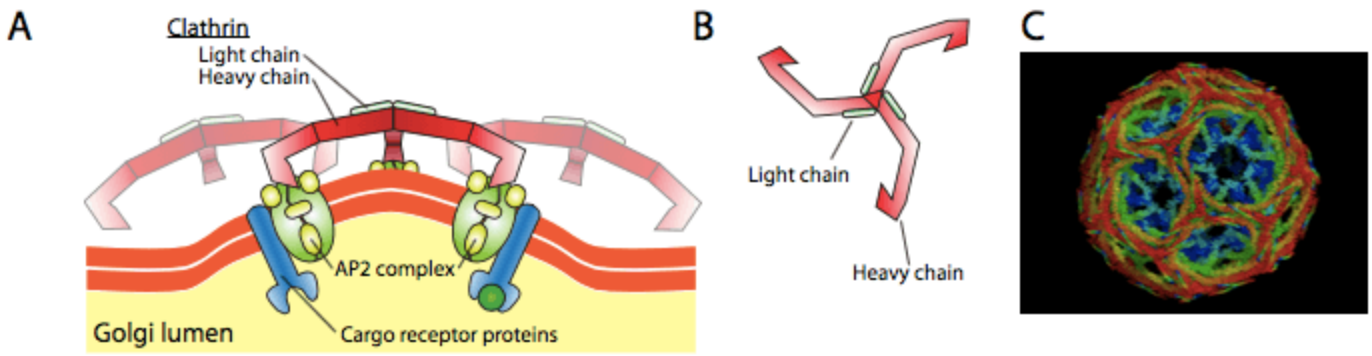


Figure 11.6.17. Clathrin. (A) clathrin binds to adapter proteins which are bound to transmembrane cargo receptors, linking the membrane with the clathrin. (B) A single clathrin triskelion is composed of three heavy chains and three light chains. (c) The triskelions self-assemble into a roughly spherical construct without the need for any additional energy or enzymes.

Clathrin (Figure 11.6.17) is the best described of the three, and the vesicular coats are made from arrangements of clathrin triskelions (from Greek, meaning three-legged). Each triskelion is composed of three heavy chains joined together at the C-terminus, and three light chains, one associated with each heavy chain. The heavy chains of different triskelions interact along the length of their heavy chain "legs" to create a very sturdy construct. The light chains are unnecessary for vesicle formation, and are thought to help prevent accidental interactions of clathrin molecules in the cytoplasm.

There is significant similarity between the vesicle formation mechanisms using these different coat proteins, beginning with the recruitment of ARF1 (ARF stands for ADP ribosylation factor, which has nothing to do with its function here) to the membrane. This requires the ARNO-facilitated exchange of a GTP for GDP (ARNO is ARF nucleotide binding site opener). Once ARF1 has bound GTP, the conformational change reveals an N-terminal myristoyl group which inserts into the membrane. Both COPI and clathrin-coated vesicles use ARF1 and ARNO, but COPII uses similar proteins called Sar1p and Sec12p.

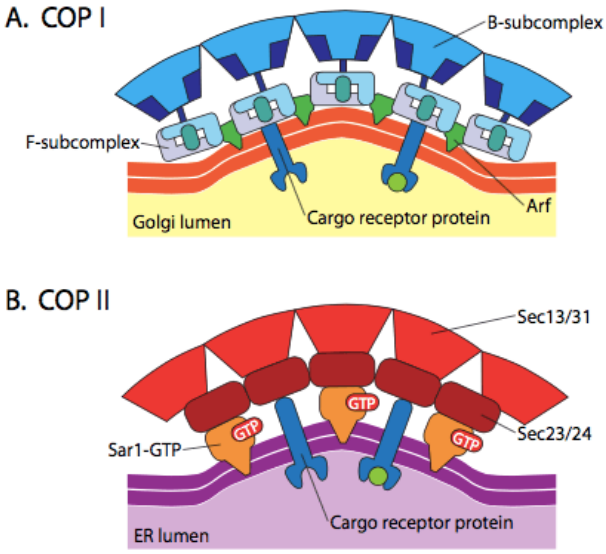


Figure 11.6.18. COP-coated vesicles

The ARF1 (or Sar1p) is used to recruit adapter proteins that bind to the "tail" end of membrane-bound receptor proteins. The business end of these receptors binds to car- go molecules that need to be packaged into the vesicle. The adapter proteins act as the link between the membrane (through the receptors) and the coat proteins. For clathrin, the adapter proteins are AP1 for trans-Golgi-derived vesicles and AP2 for endocytic vesicles. For COPI vesicles, the approximate homologues are the β-, γ-, δ-, and ζ- COPs while the COPII system uses Sec23p and Sec24p.

Finally, the adapters link to the actual coat proteins: clathrin, α- or ε- COP, Sec13p and Sec31p. What these proteins all

have in common is that spontaneously (i.e. without any requirement for energy expenditure), they self-assemble into cage-like spherical structures. Under the electron microscope, the clathrin-coated vesicles are more sharply defined and the hexagonal and pentagonal shapes bounded by the clathrin subunits give the vesicle a “soccer ball” look. COP coatamer-coated vesicles are much fuzzier in appearance under EM.

All three types of vesicle coat proteins have the ability to spontaneously associate into a spherical construct, but only the COPI and COPII coated vesicle also spontaneously “pinch off” the membrane to release the vesicle from its originating membrane. Clathrin-coated vesicles require an external mechanism to release the vesicle (Figure 11.6.19).

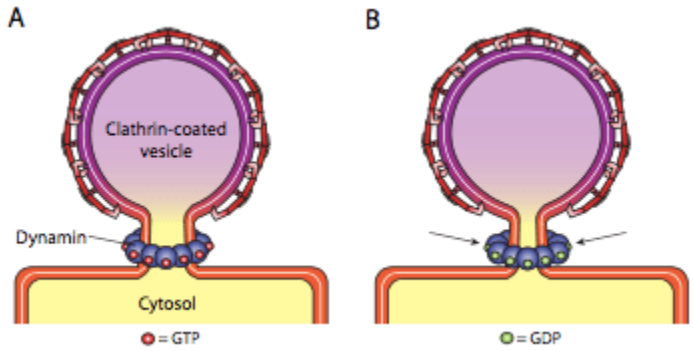


Figure 11.6.19. Dynamin monomers, each of which is a GTPase, polymerizes around the neck of the vesicle. When the GTP is hydrolyzed, the dynamin “noose” tightens and pinches off the vesicle.

Once the vesicle has almost completed, there is still a small stalk or neck of membrane that connects the vesicle to the membrane. Around this stalk, dynamic GTP molecules aggregate in a ring/spiral construction. Dynamin molecules are globular GTPases that contract upon hydrolysis of GTP. When they associate around the vesicle stalk, each dynamin protein contracts, with the combined effect of constricting the stalk enough that the membrane pinches together, sealing off and releasing the vesicle from the originating membrane.

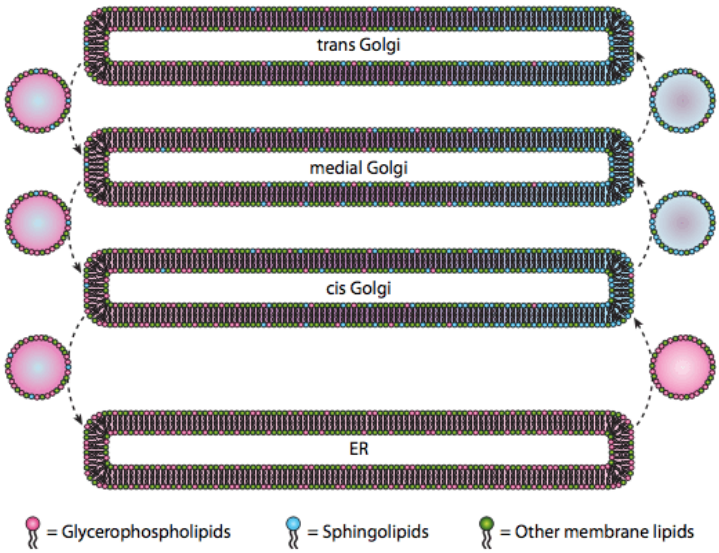


Figure 11.6.20. Glycerophospholipids are made primarily in the ER. Although the ER also makes the ceramide precursors for sphingolipids, the sphingolipids are made only in the Golgi.

Although lipids and membranes were discussed in chapter 4, we neglected to discuss the location of their syntheses in eukaryotes. As Figure 11.6.20 indicates, the synthesis of certain types of lipids is segregated and exclusive. Glycerophospholipids are primarily formed in the endoplasmic reticulum, although they are also made in mitochondria and peroxisomes. In contrast, sphingolipids are not made in the ER (though their ceramide precursors are) in mammals, the

> necessary enzymes are found in the lumen of the cis and medial Golgi. There is evidence of anterograde and retrograde vesicular traffic between the various Golgi and ER compartments, which would theoretically indicate a redistribution of lipid types. However, the sphingolipids tend to aggregate into lipid rafts and seem to be more concentrated in anterograde-moving vesicles.

The coat proteins come off shortly after vesicular release. For clathrin, the process involves Hsc70, an ATPase. However, for COPI or COPII coated vesicles, hydrolysis of the GTP on ARF/Sar1p appears to weaken the coat protein affinity for the adapters and initiates uncoating. The GTPase activator is ARF GAP (or Sec23p) and is an integral part of the COP I (or II) coat.

The vesicles carry two categories of cargo: soluble proteins and transmembrane proteins. Of the soluble proteins, some are taken up in the vesicle by virtue of being bound to a receptor. Other proteins just happen to be in the vicinity and are scooped up as the vesicle forms. Occasionally, a protein is taken up that was not supposed to be; for example, PDI may be enclosed in a vesicle forming from the ER. It has little function in the Golgi, and is needed in the ER, so what happens to it? Fortunately, PDI and many other ER proteins have a C-terminal signal sequence, KDEL (Lysine-Aspartic Acid-Glutamic Acid-Leucine), that screams "I belong in the ER." This sequence is recognized by KDEL receptors inside the Golgi, and binding of the KDEL proteins to the receptors triggers vesicle formation to send them back to the ER.

Secretory vesicles have a special problem with soluble cargo. If the vesicle was to rely simply on enclosing proteins within it during the formation process, it would be difficult to get high concentrations of those proteins. Many secreted proteins are needed by the organism quickly and in significant amounts, so there is a mechanism in the trans Golgi for aggregating secretory proteins. The mechanism uses aggregating proteins such as secretogranin II and chromogranin B that bring together the target proteins in large concentrated granules. These granins work best in the trans Golgi milieu of low pH and high Ca^{2+}, so when the vesicle releases its contents outside of the cell, the higher pH and lower Ca^{2+} breaks apart the aggregates to release the individual proteins.

> There is a consistent pH change during the maturation of the Golgi, so that as we go from ER to Golgi, each compartment has a progressively lower (more acidic) lumenal pH.

Finally, there is the question of targeting the vesicles. The vesicles are much less useful if they are tossed on a molecular freight train and dropped off at random. Therefore, there is a docking mechanism that requires a matching of the v-SNARE protein on the vesicle's cytoplasmic surface and the t-SNARE on the cytoplasmic surface of the target membrane. Fusion of the vesicle to the membrane only proceeds if there is a match. Otherwise, the vesicle cannot fuse, and will attach to another molecular motor to head to another, hopefully correct, destination. This process is aided by tethering proteins which initially make contact with an incoming vesicle and draw it close enough to the target to test for SNARE protein interaction. Other proteins on the vesicle and target membranes then interact and if the SNAREs match, can help to "winch" the vesicle into the target membrane, whereupon the membranes fuse. An important rule of thumb to understanding vesicular fusion and also the directionality of membrane proteins and lipids, is that the cytoplasmic-facing side of a membrane is always going to be facing the cytoplasm. Therefore a protein that is eventually found on the outer surface of the cell membrane will have been inserted into the lumenal surface of the ER membrane to begin with.

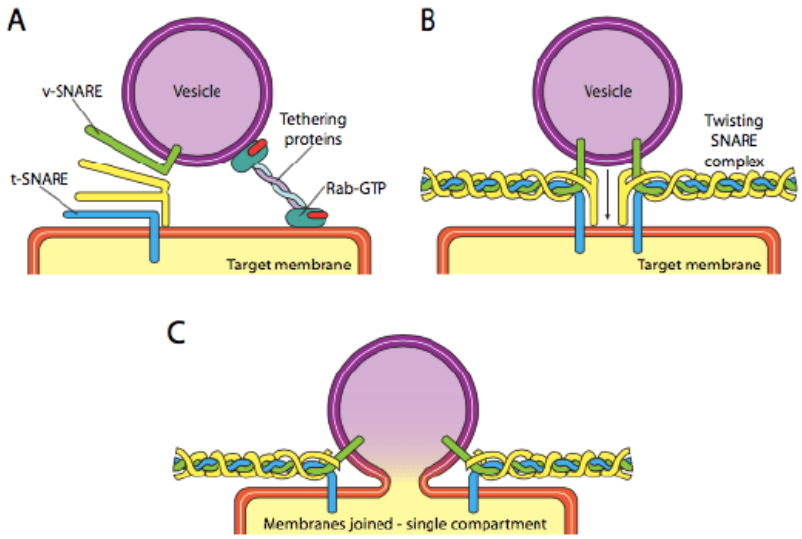


Figure 11.6.21. Vesicles first interact with tethering proteins (A), which help bring the vesicle and target membranes close. SNAREs can then interact, and if they match, then they will begin to twist around each other, ratcheting the two membranes closer as they twist.

More specifically, as a vesicle approaches the target membrane, the tethering protein Rab-GTP, which is linked to the target membrane via a double geranylgeranyl lipid tail, loosely associates with the vesicle and holds it in the vicinity of the target membrane to give the SNARES a chance to work. The v-SNAREs and t-SNAREs now have the opportunity to interact and test for a match. Recently, the SNAREs have been renamed R-SNAREs and Q-SNAREs, respectively, based on conserved arginine and glutamine residues. In addition to these two primary SNAREs, at least one other SNARE is involved, together forming a bundle of four α-helices (four, not three, because at least in the best studied example, one of the SNAREs is bent around so that two of its alpha-helical domains participate in the interaction. The four helices wrap around each other and it is thought that as they do so, they pull the vesicle and the target membrane together.

> The tetanus toxin, tetanospasmin, which is released by *Clostridium tetani* bacteria, causes spasms by acting on nerve cells, and preventing neurotransmitter release. The mechanism for this is that it cleaves synaptobrevin, a SNARE protein, so that the synaptic vesicles cannot fuse with the cell membrane. Botulinum toxin, from *Clostridium botulinum*, also acts on SNAREs to prevent vesicle fusion and neurotransmitter release, although it targets different neurons and so has the opposite effect: tetanus is caused by preventing the release of inhibitory neurotransmitters, while botulism is caused by preventing release of excitatory neurotransmitters.

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11.7: Receptor-mediated Endocytosis

Just as there is vesicular traffic towards the plasma membrane, either for secretion or for incorporation of membrane lipids or proteins, there can also be vesicular traffic from the plasma membrane. Endocytosis is the process by which a coat protein (usually clathrin) on the cytoplasmic side of the plasma membrane, begins to polymerize a coat that draws the membrane with it into a vesicle. However, instead of capturing a bit of ER or Golgi lumen with it, the vesicle contains a little material from outside of the cell. Sometimes endocytosis is initiated internally, perhaps to remove a particular protein from the cell surface (for an example, see trailing edge dynamics in cell motility in the next chapter), but often, the endocytosis is the result of a ligand binding to an extracellular receptor molecule, leading to its activation and subsequent nucleation of a clathrin assembly and vesicle formation.

There are many types of ligands: a nutrient molecule (usually on a carrier protein, as in the examples below) or even an attacking virus which has co-opted the endocytic mechanism to facilitate entry into the cell. The example depicted here is a classic example: endocytosis of cholesterol (via low-density lipoprotein). This illustrates one potential pathway that the receptors and their cargo may take. In the case of cholesterol, the carrier protein is broken down fully, although in the case of transferrin, a serum protein that carries iron in the blood, the carrier protein is just recycled after releasing its transferrin cargo. It is packaged into an exocytic vesicle headed back to the cell surface.

Serum cholesterol is usually esterified and bound by LDL (low density lipoprotein), which then floats about in the bloodstream until it meets up with an LDL receptor on the surface of a cell. When the LDL binds to its receptor, the receptor is activated, and a clathrin-coated vesicle forms around the LDL/receptor complex. LDL receptors tend to aggregate in what are known as clathrin-coated pits — crater-like partial vesicles that already have a small number of polymerized clathrin molecules. The vesicle forms exactly as described previously for Golgi-derived clathrin vesicles: the clathrin self- assembles into a spherical vesicle, and dynamin pinches the vesicle off the cell membrane. This vesicle then fuses with an early endosome, which carries proton pumps in its membrane, causing the environment inside the vesicle to acidify (~pH 6). This acidification can cause conformational shifts in proteins that could, for example, lead to a receptor releasing its ligand, as is the case here with LDL and LDL receptor. The early endosome also functions as a sorting station: the receptor is re-vesicularized and transported back to the plasma membrane. Meanwhile, the LDL is packaged into a different vesicle and heads off for further processing.

> The endosomal proton pumps are ATP-driven, Mg^{2+}-dependent V-type pump (as opposed to the F-type pump in the mitochondrial inner membrane). Structurally, the two are similar though, and ATP hydrolysis drives the rotary unit, which then powers the movement of protons across the membrane from cytoplasm into endosome.

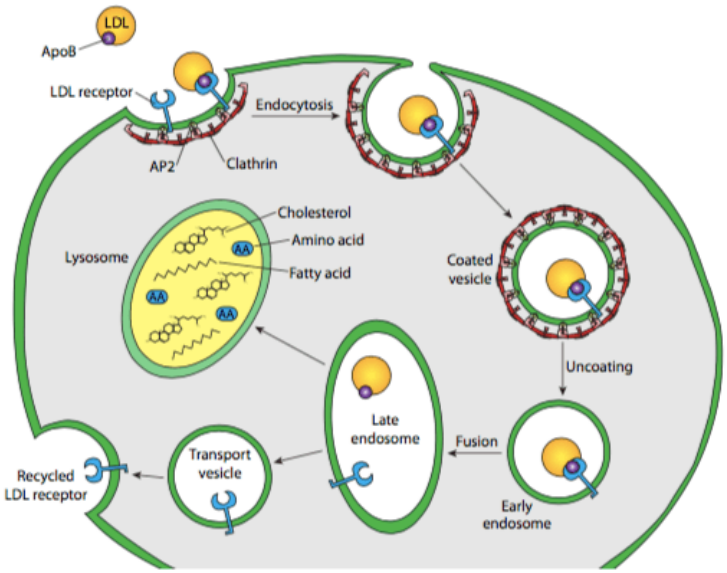


Figure 11.7.22. Receptor-mediated endocytosis of cholesterol via low-density lipoprotein.

The endosomal vesicle with the LDL in it next fuses with another acidic, membrane-bound compartment. The lysosome, at pH

~5.0, is even more acidic than the endosome, and it also contains a large complement of acid hydrolases — hydrolytic enzymes ranging across substrates (including proteases, lipases, glycosidases, nucleases) that operate optimally in acidic conditions, and minimally in the neutral or slightly basic conditions in the cytoplasm. In part, this is a safety mechanism — leakage of digestive enzymes from the lysosome will not result in wholesale digestion of the cell because the enzymes have little or no activity in the cytoplasm. The lysosomal membrane, in addition to having proton pumps to acidify the internal environment, also incorporates many transporter proteins to aid in moving the digestion products of the acid hydrolases out of the lysosome so that the cell can make use of the amino acids, sugars, nucleotides, and lipids that result. Back to our example, that means that the cholesterol esters are broken apart into individual cholesterol molecules, and the lipoprotein is broken down into lipids and amino acids. Interestingly, these transporter proteins are not digested by the lysosomal proteases because they are very heavily glycosylated, which shields potential proteolytic sites from the proteases.

Lysosomal enzymes are specifically tagged by a mannose-6-phosphate that is added in the cis Golgi. This is a two-step process in which N-acetylglucosamine phosphotransferase adds a phospho-GlcNAc to a mannose residue, connecting via the phosphate group, then a phosphodiesterase removes the GlcNAc, leaving the mannose-6-P. This specifically targets lysosomal enzymes because they all have specific protein recognition sequences that the phosphotransferase binds to before transferring the P-GlcNAc. Although the lysosomal enzymes are tagged in the cis Golgi, they do not sort until the trans Golgi, when mannose-6-P receptors bind to the lysosomal enzymes and form lysosomal vesicles that will bud off and travel to late endosomes and lysosomes to deliver their acid hydrolase payload. Again, the pH change is important: in the somewhat acidic (pH 6.5) environment of the trans Golgi, the receptor binds the mannose-6-P-tagged enzymes, but in the more acidic lysosome, the acid hydrolases are released to do their work.

When one or more acid hydrolases do not function properly or do not make it into the lysosome due to improper sorting, the result is incomplete digestion of the lysosomal contents. This in turn leads to the formation of large inclusions of partially digested material inside the lysosomes. This accumulation of material can be cytotoxic, and genetic disorders that affect the expression or sorting of lysosomal hydrolases are collectively referred to as lysosomal storage diseases. These fall into several categories depending on the types of molecules accumulated.

A common and easily treatable disease of glycosaminoglycan accumulation is Hurler's disease, which can be effectively treated and non-neurological effects even reversed by enzyme replacement therapy. Hurler's others in its class affect a wide variety of tissues because glycosaminoglycans are ubiquitous. On the other hand, because the brain is enriched in gangliosides, lysosomal storage diseases like Gaucher's disease show defects primarily in the CNS. Many lysosomal storage diseases have similar presentation: developmental abnormalities, especially stunted bone growth, lack of fine facial features, and neuromuscular weakness.

Since it depends greatly on the contents of the endosome(s) that fused with it, the size and contents of lysosomes can vary greatly. In fact, the lysosome may also degrade internal cellular components through the process of *autophagy*. Usually, this is initiated under starvation conditions which lead to inhibition of mTor, and subsequent expression of autophagic genes. These then interact with mitochondria and other cellular components, and promote the formation of a double-membraned autophagosome around them. The origin of the membranes is unclear, although the ER is suspected. Finally, the autophagosome fuses with a lysosome, and the acid hydrolases break down the cell parts for energy. A variation on this called microautophagy can also occur, in which the lysosome itself invaginates a bit of cytoplasmic material and internalizes an intralysosomal vesicle that is then broken down.

The most severe, I-cell disease (mucolipidosis type II) occurs when nearly all lysosomal enzymes are missing in the fibroblasts of the affected individual. There is severe developmental delay and early growth failure, neuromuscular problems, and malformations in early skeletal development. The severity of this disorder is due to the almost complete lack of lysosomal enzymes, which is caused by a deficiency of GlcNAc phosphotransferase. Without it, no enzymes are tagged for sorting to the lysosome.

Other relatively common disorders include Tay-Sachs and Niemann-Pick diseases. Tay-Sachs is caused by an accumulation of gangliosides in the brain and is usually fatal by 5 years of age. Niemann-Pick, on the other hand, may manifest as Type A with an even shorter life expectancy, or as Type B, in which symptoms are relatively minor. The major difference is that Type A patients have very little (<5%) of their sphingomyelinase activity, while Type B patients have only slightly less than normal (~90%) activity.

Finally, it should be noted that the large vacuoles of plant cells are in fact specialized lysosomes. Recall that vacuoles help to maintain the turgor, or outward water pressure on the cell walls that lead to a rigid plant part rather than a limp, wilted one. One of the ways in which this occurs is that the acid hydrolases inside the vacuole alter the osmotic pressure inside the vacuole to regulate the movement of water either in or out.

Another example of receptor-mediated endocytosis is the import of iron into a mammalian cell. As with serum cholesterol, iron is not generally imported into the cell by itself. Instead, it is bound to apotransferrin, a serum protein that binds two Fe^{3+} ions. Once it has bound the iron ions, the apotransferrin is now referred to as transferrin, and it can be recognized and bound by transferrin receptors (TfR) located on the extracellular surface of cell membranes. This initiates receptor-mediated endocytosis just as described above. However, in this case, the lysosome is not involved. As the transferrin and transferrin receptor reach the early endosome, they do not dissociate, but rather the Fe^{2+} releases from the transferrin, and then exits the endosome via DMT1, a divalent metal transport protein to be used in heme groups or other complexes. This leaves the apotransferrin-TfR complex, which is recycled back to the cell membrane via vesicle. Once the vesicle fuses with the extracellular space, the acidity of the endosome is dissipated and the apotransferrin no longer binds to TfR. Apotransferrin can thus go back to its duty of finding iron ions and bringing them back to the cell.

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12: CYTOSKELETON

When a eukaryotic cell is taken out of its physiological context and placed in a plastic or glass Petri dish, it is generally seen to flatten out to some extent. On a precipice, it would behave like a Salvador Dali watch, oozing over the edge. The immediate assumption, particularly in light of the fact that the cell is known to be mostly water by mass and volume, is that the cell is simply a bag of fluid. However, the cell actually has an intricate microstructure within it, framed internally by the components of the cytoskeleton.

12.1: Introduction to the Cytoskeleton

12.2: Intermediate Filaments

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12.4: Microtubules

12.5: Microtubule Organizing Centers

12.6: Transport on the Cytoskeleton

12.7: Actin - Myosin Structures in Muscle

12.8: Cytoskeletal Dynamics

12.9: Cell Motility

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12.1: Introduction to the Cytoskeleton

When a eukaryotic cell is taken out of its physiological context and placed in a plastic or glass Petri dish, it is generally seen to flatten out to some extent. On a precipice, it would behave like a Salvador Dali watch, oozing over the edge. The immediate assumption, particularly in light of the fact that the cell is known to be mostly water by mass and volume, is that the cell is simply a bag of fluid. However, the cell actually has an intricate microstructure within it, framed internally by the components of the cytoskeleton.

> Although the genes are not particularly well conserved, a combination of genetic similarity and protein structure have confirmed the presence of prokaryotic proteins that are related to eukaryotic cytoskeletal proteins in both form and function. Compared to the eukaryotic cytoskeleton, study of prokaryotic proteins is very recent, and for a long time, there was an assumption that prokaryotes did not have or need cytoskeletal architecture. FtsZ, the bacterial equivalent of tubulin, was discovered in 1980 but most of the work on it has occurred in the last decade. MreB is an actin-like protein, first compared to actin in 1992, and crescentin, an intermediate filament class protein, was only described in 2003. For comprehensive review of prokaryotic cytoskeleton proteins, see Graumann, P.L., *Ann. Rev. Microbiology* **61**:589-618, 2007.

As the name implies, the cytoskeleton acts much like our own skeletons in supporting the general shape of a cell. Unlike our skeletons though, the cytoskeleton is highly dynamic and internally motile, shifting and rearranging in response to the needs of the cell. It also has a variety of purposes beyond simply providing the shape of the cell. Generally, these can be categorized as structural and transport. While all three major components of the cytoskeleton perform each of these functions, they do not do so equally, as their biophysical characteristics are quite different. With respect to structure, at some point in the life of every cell, it must change shape, whether simply increasing or decreasing in size, or a more drastic alteration like the super-elongated form of neurons with axons, the cytoskeleton must be able to respond by dynamically increasing and decreasing the size of the internal structures as needed. Structure also applies to the relative position of internal cellular elements, such as organelles or proteins, to one another. In many highly specialized cells, the segregation of particular structures within certain parts of the cell is crucial for it to function. Transport refers to the movement of molecules and organelles within the cell as well as movement of the cell as a whole. We just discussed intracellular movement of proteins and lipids by way of vesicles in the last chapter. Those vesicles, as we will see in this chapter, are not just floating from one place to another; they are moved purposefully and directionally along the cytoskeleton like cargo on highways or railroad tracks. With respect to whole cell movement, this can range from paddling or swimming by single-celled organisms to the stereotyped and highly coordinated crawling of many cells from their point of origin to their eventual destination during the development of a metazoan organism or the movement of fibroblasts to heal a cut in your skin.

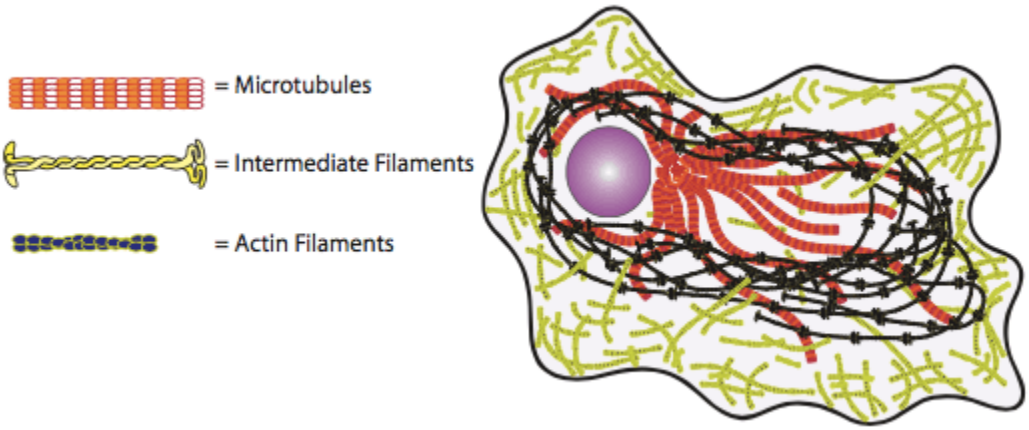


Figure 12.1.1. Cytoskeletal element distribution in a prototypical eukaryotic cell. The purple ball is the nucleus.

The three major components of the cytoskeleton are microtubules, microfilaments, and intermediate filaments. Each of these are polymers composed of repeating subunits in specific arrangements. With just a quick glance (Figure 12.1.1), it is very clear that the intermediate filaments will likely play a significantly different role from either microtubules or microfilaments. Because the IF's are made of long fibrous subunits that coil around one another to form the filament, there is clearly a great deal of contact

(which facilitates formation of hydrogen bonds, aka molecular velcro^{TM}) between subunits providing great tensile strength. It is very difficult to break these subunits apart, and thus the IF's are primarily used for long-term or permanent load-bearing purposes. Looking at the other two components of the cytoskeleton, one can see that with the globular instead of fibrous shape of the subunits, the maximum area of contact between subunits is greatly limited (think of the contact area when you push two basketballs together), making it easier to separate the subunits or break the microfilament or microtubule. The cell can use this characteristic to its advantage, by utilizing these kinds of cytoskeletal fibers in dynamic situations where formation or destruction of intermediate filaments would take far too long. We now address these three groups of cytoskeletal elements in more detail.

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12.2: Intermediate Filaments

“Intermediate filaments” is actually a generic name for a family of proteins (grouped into 6 classes based on sequence and biochemical structure) that serve similar functions in protecting and shaping the cell or its components. Interestingly, they can even be found inside the nucleus. The nuclear filamins, which constitute class V intermediate filaments, form a strong protective mesh attached to the inside face of the nuclear membrane. Neurons have neurofilaments (class IV), which help to provide structure for axons — long, thin, and delicate extensions of the cell that can potentially run meters long in large animals. Skin cells have a high concentration of keratin (class I), which not only runs through the cell, but connects almost directly to the keratin fibers of neighboring cells through a type of cellular adhesion structure called a desmosome (described in the next chapter). This allows pressure that might be able to burst a single cell to be spread out over many cells, sharing the burden, and thus protecting each member. In fact, malformations of either keratins or of the proteins forming the desmosomes can lead to conditions collectively termed *epidermolysis bullosa*, in which the skin is extraordinarily fragile, blistering and breaking down with only slight contact, compromising the patient’s first line of defense against infection.

> Most intermediate filaments fall between 50-100 kDa, including keratins (40-67 kDa), lamins (60-70 kDa), and neurofilaments (62-110 kDa). Nestin (class VI), found mostly in neurons, is an exception, at approximately 240 kDa.

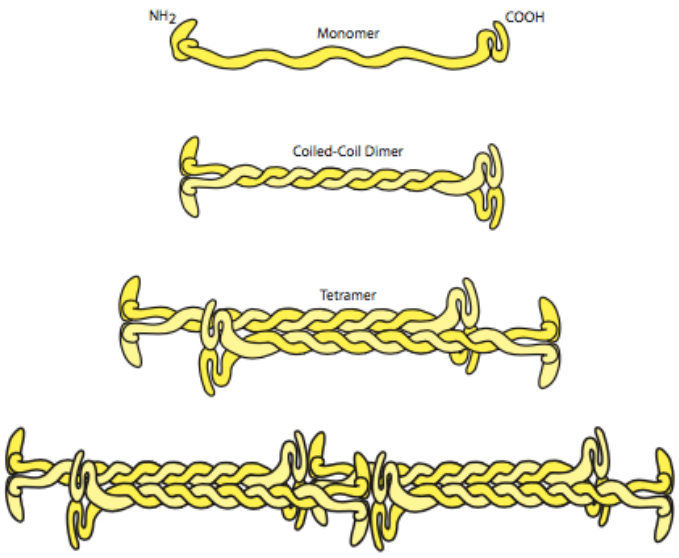


Figure 12.2.2. Intermediate filaments are composed of linear subunits that wrap around each other and interact very tightly.

Structurally, as mentioned previously, all intermediate laments start from a fibrous subunit (Figure 12.2.2). This then coils around another filamentous subunit to form a coiled-coil dimer, or protofilament. These protofilaments then interact to form tetramers, which are considered the basic unit of intermediate filament construction. Using proteins called *plectins*, the intermediate laments can be connected to one another to form sheets and meshes. Plectins can also connect the intermediate laments to other parts of the cytoskeleton, while other proteins can help to attach the IF cytoskeleton to the cell membrane (e.g. desmoplakin). The most striking characteristic of intermediate filaments is their relative longevity. Once made, they change and move very slowly. They are very stable and do not break down easily. They are not usually *completely* inert, but compared to microtubules and microfilaments, they sometimes seem to be.

> *Epidermolysis bullosa simplex* is a collection of congenital diseases caused by mutations to the keratin genes KRT5 or KRT14, or to the plectin gene PLEC1. These mutations either weaken the polymerization of keratin into filaments, or the interaction between keratin filaments. This leads to the inability of each individual cell to maintain structural integrity under pressure. Another type of EB, junctional epidermolysis bullosa (JEB), is caused by mutations to integrin receptors (b4, a6)

or laminins. This includes JEB gravis or Herlitz disease, which is the most severe, often leading to early postnatal death. JEB is also related to dystrophic epidermolysis bullosa (DEB) diseases such as Cockayne-Touraine, each of which s due to a mutation in collagen type VII. The gene products involved in JEB and DEB are discussed in more detail in the next chapter. They play a role in adhering the cells to the basement membrane, and without them, the disorganization of the cells leads to incomplete connections between the epidermal cells, and therefore impaired pressure-sharing.

Some forms of *Charcot-Marie-Tooth disease*, the most common inherited peripheral nerve disease, are also linked to mutations of intermediate filament genes. This disease, also known as peroneal muscular atrophy or hereditary motor sensory neuropathy, is a non-lethal degenerative disease primarily affecting the nerves of the distal arms and legs. There is a broad variety of CMT types and causes, the most common being malformations of Schwann cells and the myelin sheath they form. CMT type 2 is characterized by malformations of the peripheral nerve axons, and is linked to mutations of lamin A proteins and of light neurofilaments. The causal mechanism has not yet been established; however, the neurofilaments are significant elements in maintaining the integrity of long axons.

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12.3: Actin Microfilaments

Microfilaments are also known as actin filaments, filamentous actin, and f-actin, and they are the cytoskeletal opposites of the intermediate filaments. These strands are made up of small globular actin (g-actin) subunits that stack on one another with relatively small points of contact. You might envision two tennis balls, one fuzzy and the other covered in velcro hooks. Even if you push hard to mush them together, the area of contact between the balls (i.e. the area available for H-bonding between subunits) is fairly small compared to the overall surface area, or to the area of contact between IF subunits. They will hold together, but they can also fall apart with relatively little force. Contrast this with intermediate filaments, which might be represented as two ribbons of velcro hooks or loops. Considerably more work is required to take them apart. Because there are fewer H-bonds to break, the microfilaments can be deconstructed very quickly, making it suitable for highly dynamic applications.

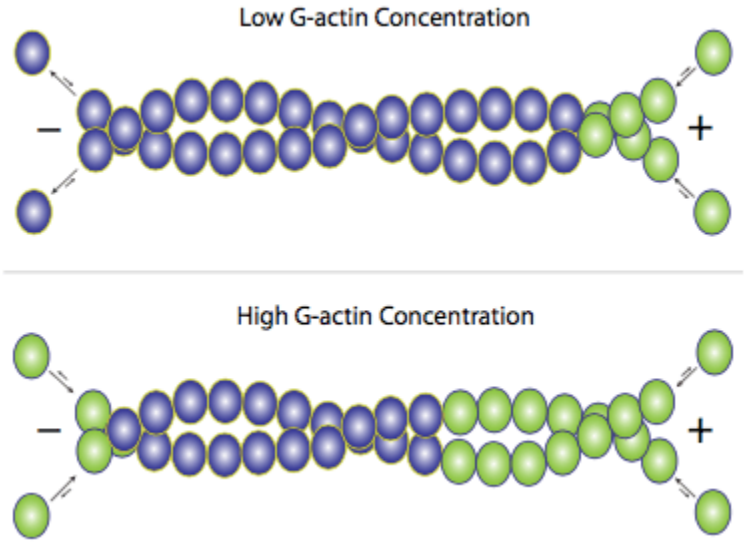


Figure 12.3.3. Actin microfilaments have a (+) and (-) end. When the free (globular) actin concentration is low, actin is primarily added to the (+) end, and lost from the (-) end. However at high levels of g-actin, new monomers can potentially add onto the filament from either end.

When the actin subunits come together to form microfilaments, they interact directionally. That is, subunits have a “top” and a “bottom”, and the top of one subunit always interacts with the bottom of another. If we go to the “bottom”-most subunit of a filament, the open end is called the minus (-) end, while the opposite end, which incidentally sees more additive action, is called the plus (+) end. Microfilaments are also said to have polarity, but again this is only in the sense of having directionality, and has nothing to do with electrical charge. Individual microfilaments can exist, but most microfilaments *in vivo* are twisted pairs. Unlike DNA; however, microfilament pairs are not antiparallel: both strands have the same directionality.

The formation of filaments from g-actin is an ATP-dependent process, although not in the conventional sense of utilizing the energy released in hydrolysis. Instead, the globular actin subunits will only bind with another g-actin subunit if it has first bound an ATP. If the g-actin has bound ADP, then it must first exchange the ADP for ATP before it can be added onto a filament. This alters the conformation of the subunit to allow for a higher-affinity interaction. A short time later, hydrolysis of the ATP to ADP (with release of P_i) weakens the affinity but does not directly cause dissolution of the subunit binding. The hydrolysis is brought about by the actin itself, which has this ATPase enzymatic activity built in.

Although f-actin primarily exists as a pair of filaments twisted around each other, addition of new actin occurs by the addition of *individual* g-actin monomers to each filament (Figure 12.3.3). Accessory proteins can be used to help or hinder either the building or breakdown of the filaments, but the primary mechanism is essentially self-regulating. When free g-actin levels are high, elongation of actin filaments is favored, and when the g-actin concentration falls, depolymerization of f-actin predominates. Under average physiological conditions, though, what is often seen in actin microfilaments is an effect called treadmilling. Since actin is mostly added onto one end but removed from the other, the net effect is that any given actin monomer in a filament is effectively moving from (+) end to (-) end even if the apparent length of the filament does not change.

In most cell types, the greatest concentration of actin-based cytoskeletal structures is found in the periphery of the cell rather

than towards the center. This fits well with the tendency of the edges of the cell to be more dynamic, constantly adjusting to sense and react to its environment. Clearly, the polymerization and depolymerization of actin filaments is much faster than for intermediate filaments. The big exception to the actin-in-periphery rule is found in muscle cells. Actin filaments, and the myosin motor proteins that work on them, are the basis for muscle cell contraction, and ll up most of the muscle cells, not just the periphery. We will discuss the role of actin in both types of cell movement later in the chapter.

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12.4: Microtubules

Microtubules are made up of two equally distributed, structurally similar, globular subunits: α and β tubulin. Like microfilaments, microtubules are also dependent on a nucleotide triphosphate for polymerization, but in this case, it is GTP.

> Microtubule stability is temperature-dependent: if cooled to 4°C, microtubules fall apart into αβ-tubulin heterodimers. Warmed back up to 37°C, the tubulin repolymerizes if there is GTP available.

Another similarity is that microtubules have a polarity in which the (-) end is far less active than the (+) end. However, unlike the twisted-pair microfilaments, the microtubules are mostly found as large 13-stranded (each strand is called a protofilament) hollow tube structures. Also, the α and β tubulin used for building the microtubules not only alternate, but they are actually added in pairs. Both the α-tubulin and β-tubulin must bind to GTP to associate, but once bound, the GTP bound to α-tubulin does not move. On the other hand, GTP bound in the β-tubulin may be hydrolyzed to GDP. GDP-bound αβ-dimers will not be added to a microtubule, so similar to the situation with ATP and g-actin, if the tubulin has GDP bound to it, it must first exchange it for a GTP before it can be polymerized. Although the affinity of tubulin for GTP is higher than the affinity for GDP, this process is usually facilitated by a GEF, or guanine nucleotide exchange factor. As the signal transduction chapter will show in more detail, this type of nucleotide exchange is a common mechanism for activation of various biochemical pathways.

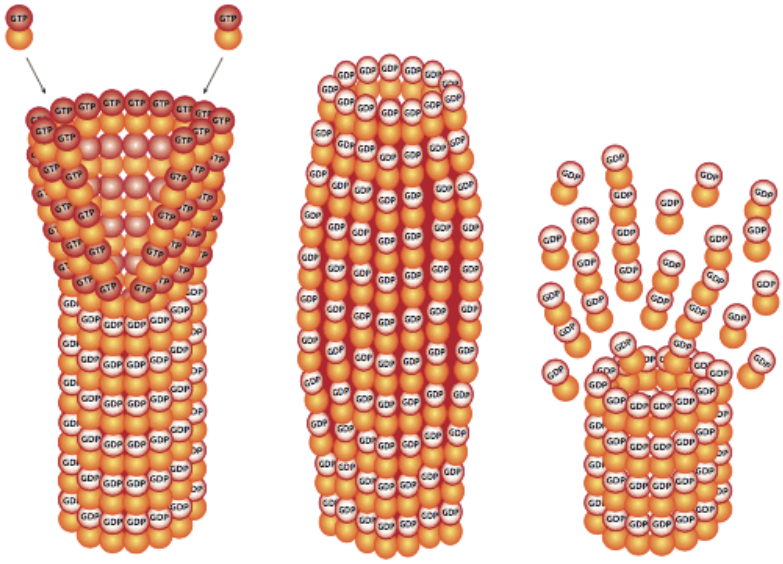


Figure 12.4.4. Microtubules exhibit dynamic instability. GTP-bound αβ-tubulin dimers are added onto the microtubule. Once the GTP is hydrolyzed, the conformational shift strains the microtubule, which will tend to break apart unless new tubulin dimers are added to stabilize the structure.

Again like actin, the tubulin itself has enzymatic activity, and over time, the GTPase activity hydrolyzes the GTP to GDP and phosphate. This changes the attachment between β-tubulin of one dimer and the α-tubulin of the dimer it is stacked on because the shape of the subunit changes. Even though it isn't directly loosening its hold on the neighboring tubulin, the shape change causes increased stress as that part of the microtubule tries to push outward. This is the basis of a property of microtubules known as *dynamic instability*. If there is nothing to stabilize the microtubule, large portions of it will fall apart. However, as long as new tubulin (which will have GTP bound) is being added at a high enough rate to keep a section of low-stress "stable"-conformation microtubule (called the GTP cap) on top of the older GDP-containing part, then it stabilizes the overall microtubule. When new tubulin addition slows down, and there is only a very small or nonexistent cap, then the microtubule undergoes a *catastrophe* in which large portions rapidly break apart. Note that this is a very different process than breakdown by depolymerization, which is the gradual loss of only a few subunits at a time from an end of the microtubule. Depolymerization also occurs, and like with actin, is determined partially by the relative concentrations of free tubulin and microtubules.

From a physical standpoint, the microtubule is fairly strong, but not very flexible. A microfilament will flex and bend when a

deforming force is applied (imagine the filament anchored at the bottom end standing straight up, and something pushing the tip to one side). The microtubule in the same situation will bend only slightly, but break apart if the deforming force is sufficient. There is, of course, a limit to the flexibility of the microfilament and eventually, it will also break. Intermediate filaments are slightly less flexible than the microfilaments, but can resist far more force that either microfilaments or microtubules.

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12.5: Microtubule Organizing Centers

Microtubules, like microfilaments, are dynamic structures, changing in length and interactions to react to intra- and extra-cellular changes. However, the general placement of microtubules within the cell is significantly different from microfilaments, although there is some overlap as well as interaction. Microfilaments do not have any kind of global organization with respect to their polarity. They start and end in many areas of the cell. On the other hand, almost all microtubules have their (-) end in a perinuclear area known as the MTOC, or microtubule organizing center and they radiate outward from that center. Since the microtubules all radiate outward from the MTOC, it is not surprising that they are concentrated more centrally in the cell than the microfilaments which, as mentioned above, are more abundant around the periphery of the cell. In some cell types (primarily animal), the MTOC contains a structure known as the *centrosome*. This consists of a centriole (two short barrel-shaped microtubule- based structures positioned perpendicular to each other) and a poorly defined concentration of pericentriolar material (PCM). The centriole is composed of nine fibrils, all connected to form a cylinder, and each also connected by radial spokes to a central axis. The electron micrograph in Figure 12.5.5 shows a cross-section of a centriole. In it, each fibril is shown to actually be a fused triplet of microtubules.

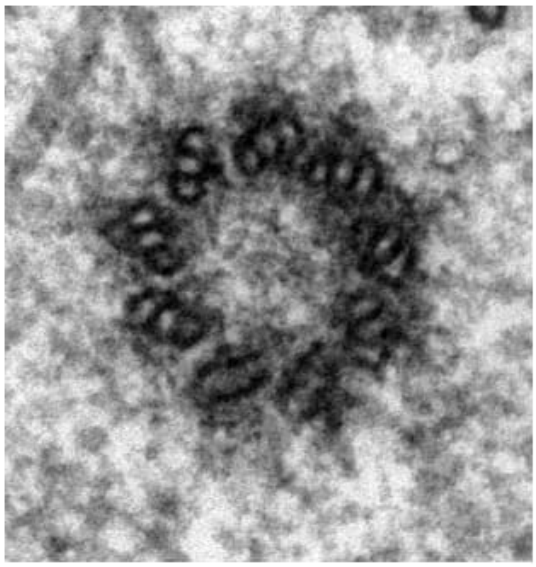


Figure 12.5.5. An electron micrograph depicting the cross-section of a centriole in an embryonic mouse brain cell. L. Howard and M. Marin-Padilla, 1985

> Inhibition of γ-tubulin function by antibody blocking, RNA interference of expression, and gene knockout confirm that without γ-tubulin function, the microtubule structures did not form. In addition, it appears to be play roles in coordination of late mitosis (anaphase onwards).

However, in each triplet, only one is a complete microtubule (designated the A tubule), while the B and C tubules do not form complete tubes (they share a wall with the A and B tubules, respectively). Interestingly, the centrioles do not appear to be connected to the cellular microtubule network. However, whether there is a defined centrosome or not, the MTOC region is the point of origin for all microtubule arrays. This is because the MTOC contains a high concentration of γ-tubulin. Why is this important? With all of the cytoskeletal elements, though it is most pronounced with microtubules, the rate of nucleation, or starting a microtubule is significantly slower than the rate of elongating an existing structure. Since it is the same biochemical interaction, the assumption is that the difficulty lies in getting the initial ring of dimers into position. The γ-tubulin facilitates this process by forming a γ-tubulin ring complex that serves as a template for the nucleation of microtubules (Figure 12.5.6).

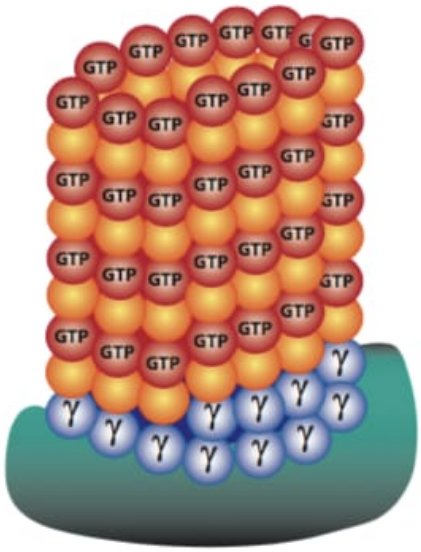


Figure 12.5.6. γ-*tubulin ring complex facilitates microtubule nucleation.*

This is true both in animal and fungal cells with a single defined MTOC, as well as in plant cells, which have multiple, dispersed sites of microtubule nucleation.

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12.6: Transport on the Cytoskeleton

While it can be useful to think of these cytoskeletal structures as analogous to an animal skeleton, perhaps a better way to remember the relative placement of the microtubules and microfilaments is by their function in transporting intracellular cargo from one part of the cell to another. By that analogy, we might consider the microtubules to be a railroad track system, while the microfilaments are more like the streets. By the same analogy, we can suggest that the microtubule network and microfilament network are connected at certain points so that when cargo reaches its general destination by microtubule (rail), then it can be taken to its specific address by microfilament. Let's extend this analogy a bit further. If the microtubules and microfilaments are the tracks and streets, then what are the trains and trucks? Ah, an astute question, Grasshopper. On the microtubules, the "trains" are one of two families of molecular motors: the kinesins and the dyneins.

We can generalize somewhat and say that the kinesins drive towards the (+) end (toward periphery of cell) while the dyneins go toward the (-) end (toward the MTOC). On actin microfilaments, the molecular motors are proteins of the myosin family. At this point, the analogies end, as the functioning of these molecular motors is very different from locomotion by train or truck. Finally, one might question the biological need for such a transport system. Again, if we analogize to human transport, then we could say that transport via simple diffusion is akin to people carrying packages randomly about the cell. That is to say, the deliveries will eventually be made, but you wouldn't want to count on this method for time-critical materials. Thus a directed, high-speed system is needed to keep cells (particularly larger, eukaryotic cells) alive.

Although this type of transport occurs in all eukaryotic cells, a particularly well-studied case is axonal transport (also called axoplasmic transport) in neurons. Here, the transport of materials from the cell body (soma) to the tips of the axons can sometimes traverse very long distances up to several meters in larger animals, and must do so in a timely manner. Axonal transport is generally classified as anterograde (from soma to axon terminal) or retrograde (from terminals back). The types of material transported in these two directions is very different: much of the anterograde transport is protein building blocks for extending the axon or synaptic vesicles containing neurotransmitters; retrograde transport is mostly endocytic vesicles and signaling molecules. Axonal transport is also categorized as fast and slow. Slow transport is primarily the movement of proteins directly bound to the motors, and they can move from from 100 mm per day (SCa, slow component a) up to 3mm/day (SCb). In comparison, fast transport is generally movement of vesicles, and can vary from 50 to 400 mm/day. The mechanism of slow transport had been debated for over a decade until 2000, when direct visualization of fluorescently labeled neurofilaments in transport showed that the actual movement of the proteins was very similar to the movement in fast axonal transport, but there were many pauses in the transport, a "stop and go" mechanism rather moving from source to destination continuously.

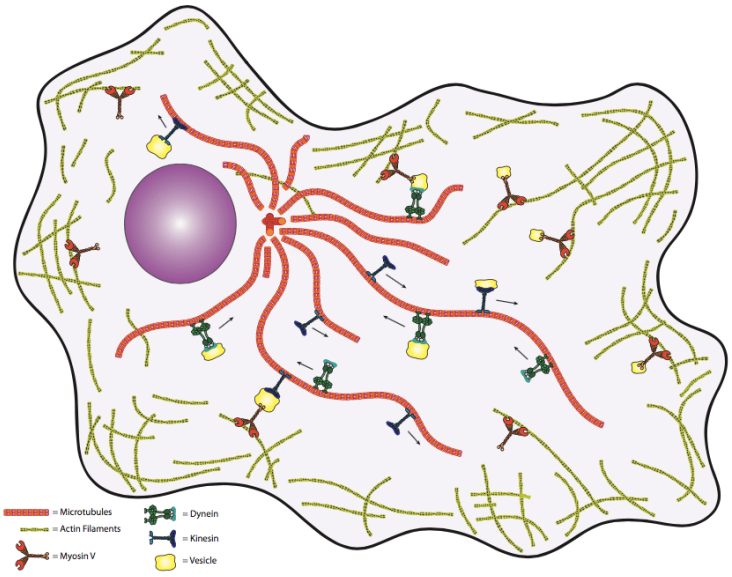


Figure 12.6.7. Transport on microtubules and microfilaments.

All of the kinesins and dyneins have a few key commonalities. There is a catalytic energy-releasing “head” connected to a hinge or neck region allowing the molecule to flex or “step”, and there is a cargo-carrying tail beyond that (Figure 12.6.8). The head of a kinesin or dynein catalyzes the hydrolysis of ATP, releasing energy to change its conformation relative to the neck and tail of the molecule, allowing it to temporarily release its grip on the microtubule, swivel its “hips” around to plant itself a “step” away, and rebind to the microtubule (Figure 12.6.9). On the actin microfilaments, the myosins, of which there are also many types (some depicted in Figure 12.6.10) are the molecular motors. Their movement is different from dyneins and kinesins, as will be described in the next section, but also uses the energy of ATP hydrolysis to provide energy for the conformational changes needed for movement. We have introduced the motors, but considering the enormous diversity in the molecules that need to be transported around a cell, it would be impossible for the motors to directly bind to all of them. In fact, the motors bind to their cargo via adapter molecules that bind the motor on one side, and a cargo molecule or vesicle on the other. Further examination of the cargo and the routing of the cargo by address markers (SNAREs) was discussed in the vesicular transport chapter.

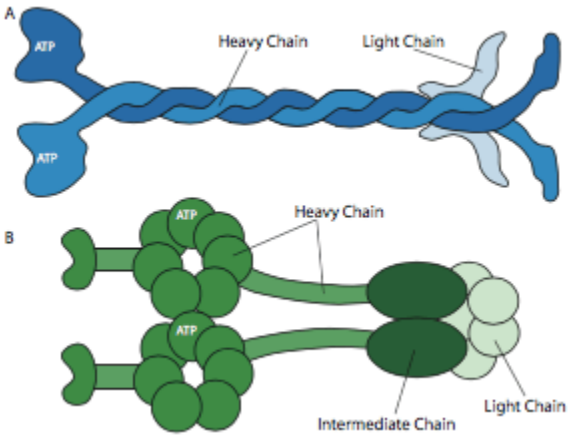


Figure 12.6.8. Kinesin (A) and Dynein (B) are motor proteins that move along microtubules. Generally, kinesins move to the (+) end while dyneins move to the (-) end. Their motor function requires ATP hydrolysis. ATP binding sites are marked in white.

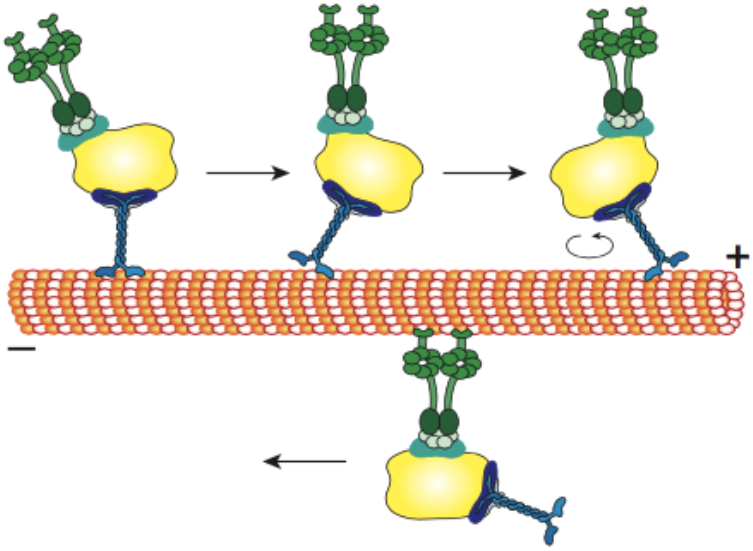


Figure 12.6.9. A cargo vesicle (yellow) can be simultaneously bound by dynein (green) and kinesin (blue) via adapter proteins. This top side also depicts the movement of the kinesin, in which binding of ATP causes one “foot” to release, and hydrolysis of ATP causes the molecule to swivel the other foot in front.

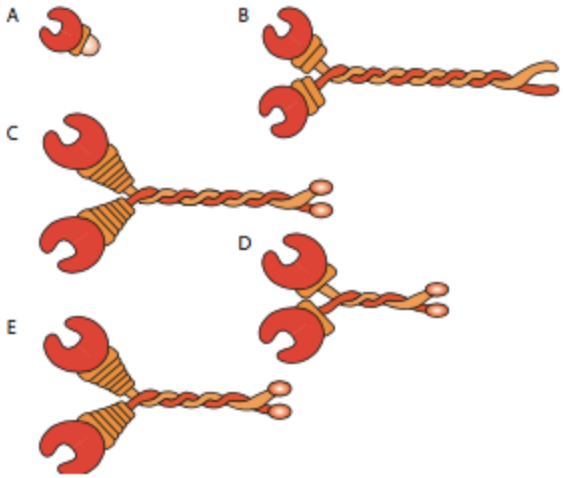


Figure 12.6.10. Selected Myosins. (A) Type I myosin, primarily for binding membranes to f-actin, including endocytic vesicles. (B) Type II myosin, binds f-actin on both ends to slide filaments against each other. (C) Type V myosin, used in vesicular transport. (D) Type VI myosin, used in endocytosis. (E) Type XI myosin, a fast myosin used in cytoplasmic streaming in plant cells.

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12.7: Actin - Myosin Structures in Muscle

The motor proteins that transport materials along the acting microfilaments are similar in some ways, such as the globular head group that binds and hydrolyzes ATP, yet different in other ways, such as the motion catalyzed by the ATP hydrolysis. Much of the f-actin and myosin in striated and cardiac muscle cells is found in a peculiar arrangement designed to provide a robust contractile response over the entire length of the cell. The sarcomere is an arrangement of alternating fibers of f-actin (also known as “thin fibers” based on their appearance in electron micrographs) and myosin II (or “thick fibers”). Although we do not normally think of the motor protein as a fiber, in this case the tails of the myosin II molecules intertwine to form a continuous fiber of myosin molecules. As the contractile cycle proceeds, the myosin molecules grip the adjacent actin fibers, and move them. In Figure 12.7.11, you can see that a sarcomere is constructed so that the stationary myosin fibers are located centrally, with two parallel sets of actin fibers interspersed between the myosin fibers, to the left and the right of the center. Note that the actin fibers do not cross the center line, and that at the center, the myosin molecules switch orientation. The physiological effect of this is that the actin filaments are all pulled inwards toward the center of the sarcomere. The sarcomere in turn, is merely one of many connected together to form a myofibril. The myofibrils extend the length of the muscle cell.

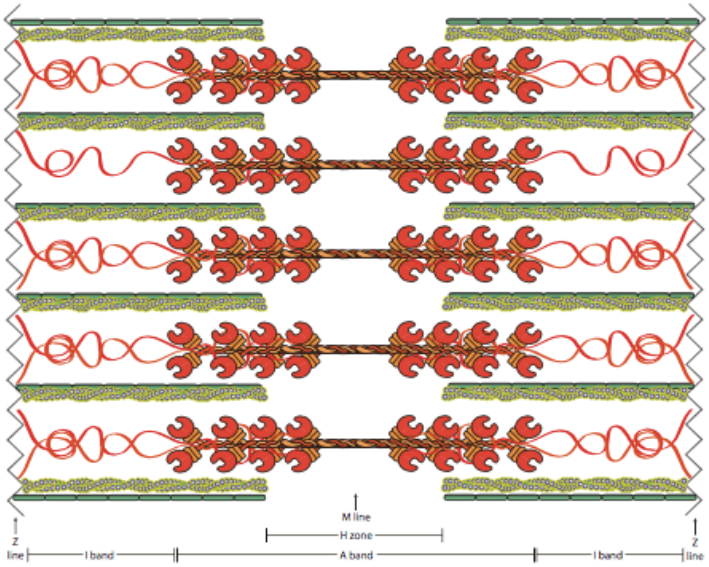


Figure 12.7.11. Sarcomere. Myosin II is depicted as in Figure 12.7.9, but here entwined with other myosins to form the thick filament. They are supported and anchored by titin (shown as long tangled orange ribbons). The myosin heads act on the actin filaments (blue), pulling them towards each other in a contractile movement.

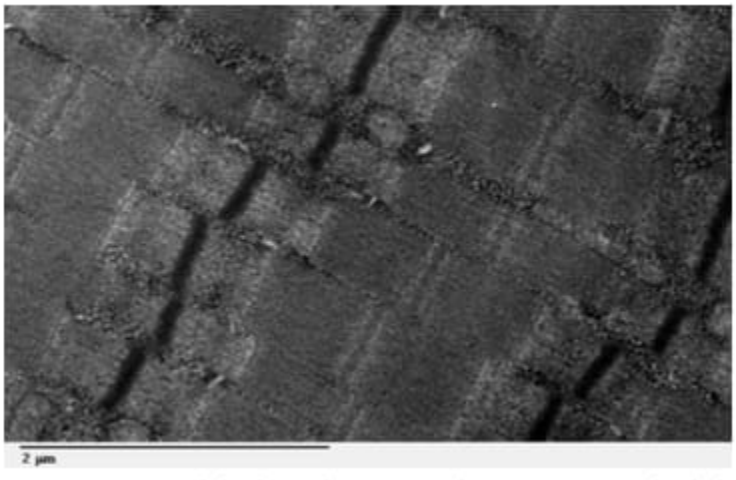


Figure 12.7.12. Human skeletal muscle is organized into sarcomeres. The dark Z lines are a clear reference point in comparing this to diagram in Figure11. This electron micrograph placed in the public domain by L. Howard.

When the myosin head is in its resting state, it is tightly attached to the actin filament. In fact, *rigor mortis* occurs in dead animals because there is no more ATP being made, and thus the sarcomeres are locked into place. Rigor begins approximately 2-3 hours after death in humans, after reserves of ATP are depleted. When the body relaxes again in about 3 days, it is due to the decomposition and breakdown of the actin and myosin proteins. However, while they are still living animals, ATP is generally available, and it can bind to the myosin head, causing it to lose affinity for the f-actin, and let go (Figure 12.7.12). At this point, no significant movement has occurred. Once the ATP is hydrolyzed though, the myosin head can reattach to the f-actin a little further down the filament than it had originally. The energy released is stored in the neck region. The ADP and P_i are still attached to the myosin head as well. The next step is for the P_i to drop off the myosin, leading to the power stroke. The neck of the myosin swivels around, leading to a translocation of the head by approximately 10 nm for myosin II. The distance of translocation varies depending on the type of myosin, but it is not yet clear whether the length of the neck is proportional to the displacement of the head. Finally, the ADP drops off the myosin head, increasing the affinity of the head for the f-actin.

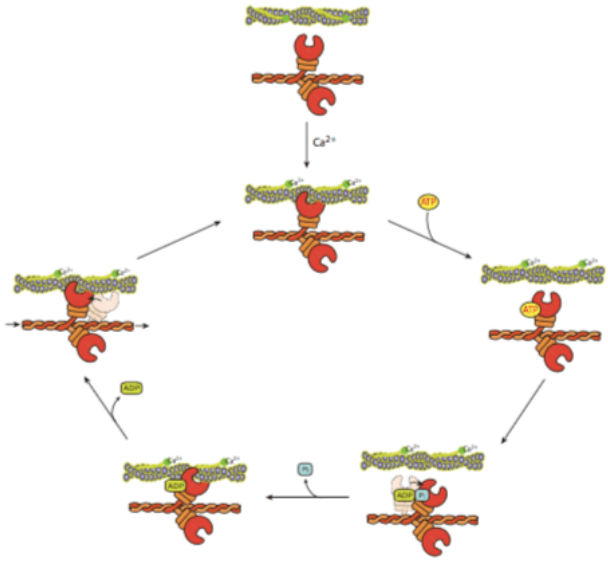


Figure 12.7.12. The myosin power stroke. Myosin can only attach to f-actin if there Ca^{2+} available to bind troponin (green) and move tropomyosin (yellow) out of the binding groove. When ATP binds to the myosin head, it releases the f-actin. Hydrolysis of the ATP leads to cocking of the myosin head (moving it relative to the f-actin). As Pi leaves the myosin head, it reattaches to the f-actin, but slightly displaced from its original binding site. ADP is then released and the myosin undergoes a power stroke in which it springs back to its original position, moving the f-actin along with it.

The sarcomere structure described in the first paragraph was incomplete in order to place the major players clearly in their roles. There are other proteins in the sarcomere with important structural and regulatory functions. One of the key regulatory components is tropomyosin. This is a fibrous protein that lies in the groove of an actin microfilament and blocks access to the myosin binding site. Tropomyosin attaches to the microfilament in conjunction with a multi-subunit troponin complex. When Ca^{2+} is available, it can bind to troponin-C, leading to a conformational change that shifts the position of tropomyosin to reveal the myosin binding site. This is the primary point of control for muscle contraction: recall that intracellular Ca^{2+} levels are kept extremely low because its primary function is in intracellular signaling. One way that the Ca^{2+} levels are kept that low is to pump it into a reservoir, such as the endoplasmic reticulum.

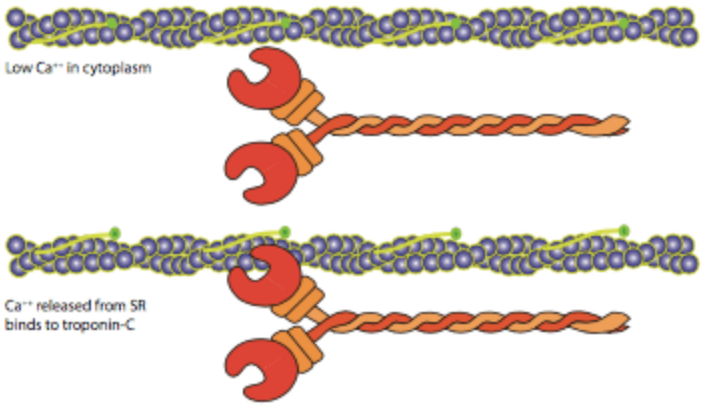


Figure 12.7.13. In low Ca^{2+} conditions, tropomyosin (yellow line) is held in the myosin-binding groove of f-actin (blue) by a tripartite troponin complex (light green). Once Ca^{2+} levels increase, it can bind to troponin-C, causing a conformational shift that moves the tropomyosin out of the way so that myosin (orange) can bind the actin microfilaments.

In muscle cells, there is a specialization of the ER called the sarcoplasmic reticulum (SR) that is rich in Ca^{2+} pumps and Ca^{2+}. When a signal is sent from a controlling nerve cell to the muscle cell, it causes a depolarization of the muscle cell membrane. This consequently depolarizes a set of membranes called the transverse tubules (T-tubules) that lie directly on parts of the sarcoplasmic reticulum. There are proteins on the t-tubule surface that directly interact with a set of Ca^{2+} channel proteins, holding the channel closed normally. When the t-tubule is depolarized, the proteins change shape, which changes the interaction with the Ca^{2+} channels on the SR, and allows them to open. Ca^{2+} rushes out of the SR where it is available to troponin-c. Troponin-C bound to Ca^{2+} shifts the tropomyosin away from the actin filament, and the myosin head can bind to it. ATP can bind the myosin head to start the power stroke cycle, and voila, we have controlled muscle cell contraction.

> The SR is a specialization of part of the endoplasmic reticulum, and contains a high concentration of Ca^{2+} ions because the SR membrane is embedded with Ca^{2+} pumps (ATPases) to keep the cytoplasmic concentration low and sequester the Ca^{2+} ions inside the SR. This is regulated by phosphorylation and [Ca^{2+}] via a regulatory protein such as phospholamban (in cardiac muscle). Phospholamban is an integral membrane protein of the SR that normally associates with and inhibits the Ca^{2+} pump. However when it is phosphorylated, or as cytoplasmic Ca^{2+} levels rise, the phospholamban releases from the Ca^{2+} pump and allows it to function.

In addition to the “moving parts”, there are also more static, structural, proteins in the sarcomere (Figure 12.7.11). *Titin* is a gigantic protein (the largest known, at nearly 3 MDa), and can be thought of as something of a bungee cord tether to the myosin fiber. Its essential purpose is to prevent the forces generated by the myosin from pulling the fiber apart. Titin wraps around the myosin fiber and attaches at multiple points, with the most medial just near the edge of the H zone. At the Z-line, titin attaches to a telethonin complex, which attach to the Z-disk proteins (antiparallel α-actinin). Titin also interacts with obscurin in the I-band region, where it may link myofibrils to the SR, and in the M-band region it can interact with the Ca^{2+}-binding protein calmodulin-1 and TRIM63, thought to acts as a link between titin and the microtubule cytoskeleton. There are multiple isoforms of titin from alternative splicing, with most of the variation coming in the I-band region.

> Disturbances to the proper formation of the titin-based support structure can be a cause of dilated cardiomyopathy, and from that, congestive heart failure. Some 20-30% of cases of dilated cardiomyopathy are familial, and mutations have been mapped to the N-terminal region of titin, where the protein interacts with telethonin. Defects in titin are also being investigated with respect to chronic obstructive pulmonary disease, and some types of muscular dystrophy.

Of course in an actual muscle (Figure 12.7.14), what happens is that nerves grow into the muscle and make synaptic connections with them. At these synaptic connections, the nerve cell releases neurotransmitters such as acetylcholine (ACh), which bind to receptors (AChR) on the muscle cell. This then opens ion channels in the muscle cell membrane, triggering a voltage change

across that membrane, which also happens to affect the nearby membrane of the transverse tubules subsequently opening Ca^{2+} channels in the SR. The contraction of sarcomeres can then proceed as already described above.

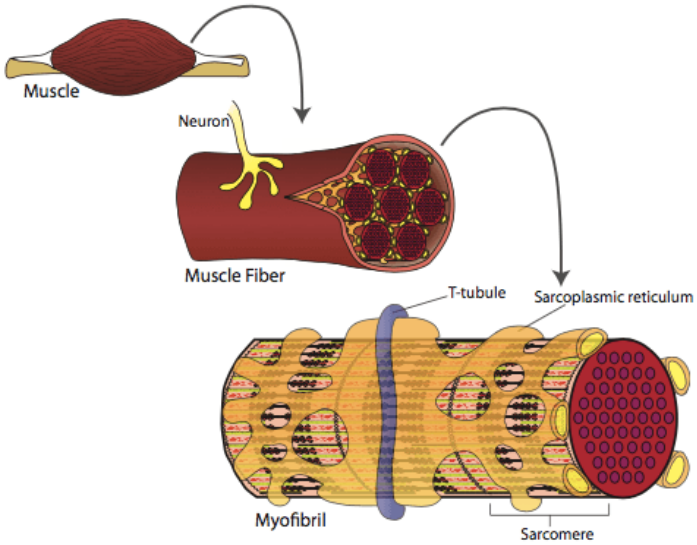


Figure 12.7.14. The sarcomere in context. The sarcomere of Figures 11 and 12 is one tiny contractile unit within an array that forms a myofibril. The myofibril is one of many within a muscle cell, surrounded by the sarcoplasmic reticulum, a specialized extension of the ER that sequesters Ca^{2+} until T-tubule excitation causes its release.

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12.8: Cytoskeletal Dynamics

In the early development of animals, there is a huge amount of cellular rearrangement and migration as the roughly spherical blob of cells called the blastula starts to differentiate and form cells and tissues with specialized functions. These cells need to move from their point of birth to their eventual positions in the fully developed animal. Some cells, like neurons, have an additional type of cell motility - they extend long processes (axons) out from the cell body to their target of innervation. In both neurite extension and whole cell motility, the cell needs to move first its attachment points and then the bulk of the cell from one point to another. This is done gradually, and uses the cytoskeleton to make the process more efficient. The major elements in cell motility are changing the point of forward adhesion, clearing of internal space by myosin-powered rearrangement of actin microfilaments and the subsequent filling of that space with microtubules.

For force to be transmitted, the membrane must be attached to the cytoskeleton. In fact, signaling from receptors in the membrane can sometimes directly induce rearrangements or movements of the cytoskeleton via adapter proteins that connect actin (or other cytoskeletal elements) to transmembrane proteins such as integrin receptors. One of the earliest experimental systems for studies of cytoskeleton-membrane interaction was the erythrocyte (red blood cell).

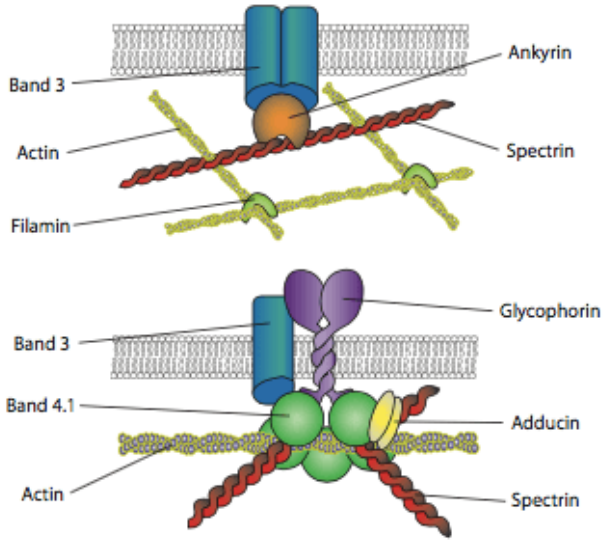


Figure 12.8.15. Membrane to microfilament linkage complexes in erythrocytes involve spectrin.

The illustrations above (Figure 12.8.15) show some of the interactions of an extensive actin microfilament network with transmembrane proteins. Ankyrin and spectrin are important linkage proteins between the transmembrane proteins and the microfilaments. This idea of building a protein complex around the cytoplasmic side of a transmembrane protein is ubiquitous, and scaffolding (linking) proteins are used not only in connecting the extracellular substrate (via transmembrane protein) to the cytoskeleton, but also to physically connect signaling molecules and thus increase the speed and efficiency of signal transduction.

Accessory proteins to actinfilaments and microtubules were briefly mentioned earlier. Among other functions, they can control polymerization and depolymerization, form bundles, arrange networks, and bridge between the different cytoskeletal networks. For actin, the primary polymerization control proteins are profilin, which promotes polymerization and thymosin β_4, which sequesters g-actin. The minus end capping proteins Arp 2/3 complex and tropomodulin, and the plus end capping proteins CapZ, severin, and gelsolin can stabilize the ends of f-actin. Finally, cofilin can increase depolymerization from the (-) end.

Profilin has two activities that promote polymerization. First, it is a nucleotide exchange factor that removes ATP bound to g-actin, and replaces it with ADP. This sounds counterintuitive, but keep reading through to the next paragraph. Second, when bound to a g-actin, it increases the rate of addition to actin microfilaments. It does so by binding to the end opposite the ATP-binding site, leaving that site and that side open to binding both ATP and the (+) end of a microfilament. Profilin can be found both in the cytoplasm at large, and associated with phospholipids (PIP_2) and membrane proteins, to control such processes as leading edge remodeling of f-actin cytoskeletal structures.

Thymosin β_4 regulates microfilament assembly by controlling the available pool of g-actin. We already stated that greater concentrations of g-actin can increase polymerization rates. However, because of the highly dynamic nature of the actin cytoskeleton, the time constraints of degrading and producing new actin would prevent the fast-response control necessary. Therefore, the optimal mechanism is to maintain a large pool of g-actin monomers, but regulate its availability by tying it up with a sequestering protein - thymosin β_4. Thymosin β_4 has a 50x higher affinity for g-actin-ATP than for g-actin-ADP, so here is where profilin comes back into the picture. Profilin exchanges the ATP of a Tβ_4-g-actin-ATP complex for an ADP. The result is that the Tβ_4 releases the g-actin-ADP, allowing it to enter the general pool for building up filaments.

Increased depolymerization and slowing or cessation of polymerization can gradually break down f-actin structures, but what if there is a need for rapid breakdown? Two of the capping proteins previously mentioned, gelsolin and severin, have an alternate mode of action that can sever actin microfilaments at any point by binding alongside an actin filament and altering the conformation of the subunit to which it is bound. The conformational change forces the actin-actin interaction to break, and the gelsolin or severin then remains in place as a (+) end capping protein.

> Gelsolin is inhibited by the phospholipid PIP_2. Phospholipase C, which breaks down PIP_2 can also increase cytosolic Ca^{2+}, which is an activator of gelsolin. Thus it is possible to rapidly upregulate gelsolin activity by PLC signaling.

On the microtubule side of things, due to dynamic instability, one might think that a severing enzyme is not needed, but in fact, spastin and katanin are microtubule-severing proteins found in a variety of cell types, particularly neurons. There is also a Tβ_4-like protein for tubulin: Op18, or stathmin, which binds to tubulin dimers (not monomers), acting to sequester them and lower the working concentration. It is regulated by phosphorylation (which turns off its tubulin binding).

> Mutations in spastin are linked to 40% of those spastic paraplegias distinguished by degeneration of very long axons. The severing ability of spastin appears to be required for remodeling of the cytoskeleton in response to neuronal damage.

Microtubule-associated proteins MAP1, MAP2, and tau (t) each work to promote assembly of microtubules, as well as other functions. MAP1 is the most generally distributed of the three, with tau being found mostly in neurons, and MAP2 even more restricted to neuronal dendrites. These and some other MAPs also act to stabilize microtubules against catastrophe by binding alongside the microtubule and reinforcing the tubulin-tubulin interactions.

> Tau has a complicated biomedical history. Its normal function is clear - assembling, stabilizing, and linking microtubules. However, it is also found in hyperphosphorylated neurofibrillary tangles that are associated with Alzheimer's disease. A cause for Alzheimer's is not yet known, so it is still unclear whether the tau protein tangles are play a major role in any of the symptoms.

Finally, with respect to microfilament and microtubule accessory proteins, there are the linkers. Some of the aforementioned MAPs can crosslink microtubules either into parallel or mesh arrays, as can some kinesins and dyneins, although they are conventionally considered to be motor proteins. On the microfilament side, there are many known proteins that crosslink f-actin, many of which are in the calponin homology domain superfamily, including fimbrin, α-actinin, β-spectrin, dystrophin, and filamin. Although they all can bind to actin, the shape of the protein dictates different types of interaction: for example, fimbrin primarily bundles f-actin in parallel to form bundles, while filamin brings actin filaments together perpendicularly to form mesh networks.

> FG Syndrome is a genetically linked disease characterized by mental retardation, enlarged head, congenital hypotonia, imperforate anus, and partial agenesis of the corpus callosum. It has been linked to mutations in several X chromosome genes, including filamin A (FLNA, FLN1, located Xq28).
>
> Mutations in dystrophin, which is a major muscle protein of the CD-domain superfamily, can result in Duchenne Muscular Dystrophy or the related but less severe Becker Muscular Dystrophy. The most distinctive feature is a progressive proximal muscular degeneration and pseudohypertrophy of the calf muscles. Onset of DMD is usually recognized before age 3 and

is lethal by age 20. However, symptoms of BMD may not present until the 20s, with good probability of long-term survival. Although it is primarily a muscle-wasting disease, dystrophin is present in other cell types, including neurons, which may explain a link to mild mental retardation in some DMD patients. Like FLNA, the dystrophin gene is also located on the X chromosome (Xp21.2).

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12.9: Cell Motility

There are a number of ways in which a cell can move from one point in space to another. In a liquid medium, that method may be some sort of swimming, utilizing ciliary or flagellar movement to propel the cell. On solid surfaces, those mechanisms clearly will not work efficiently, and the cell undergoes a crawling process. In this section, we begin with a discussion of ciliary/flagellar movement, and then consider the more complicated requirements of cellular crawling.

Cilia and flagella, which differ primarily in length rather than construction, are microtubule-based organelles that move with a back-and-forth motion. This translates to "rowing" by the relatively short cilia, but in the longer flagella, the flexibility of the structure causes the back-and-forth motion to be propagated as a wave, so the flagellar movement is more undulating or whiplike (consider what happens as you waggle a garden hose quickly from side to side compared to a short piece of the same hose). The core of either structure is called the axoneme, which is composed of 9 microtubule doublets connected to each other by *ciliary dynein* motor proteins, and surrounding a central core of two separate microtubules.

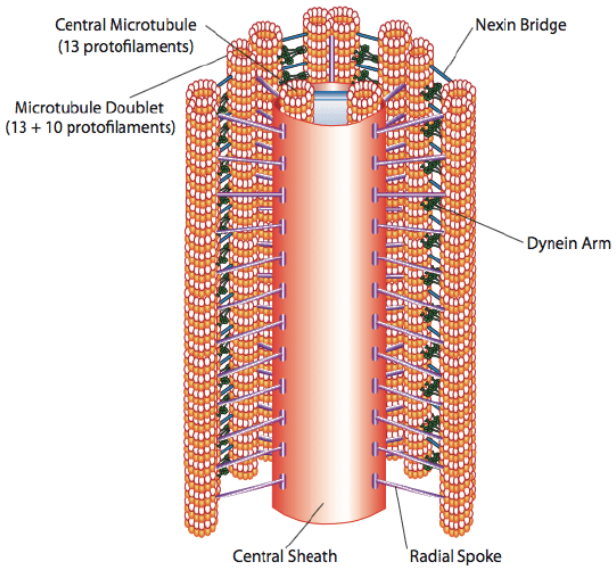


Figure 12.9.16. Partial (cutaway) diagram of an axoneme, the central bundle of cilia and flagella.

This is known as the "9+2" formation, although the nine doublets are not the same as the two central microtubules. The A tubule is a full 13-protofilaments, but the B tubule fused to it contains only 10 protofilaments. Each of the central microtubules is a full 13 protofilaments. The 9+2 axoneme extends the length of the cilium or flagellum from the tip until it reaches the base, and connects to the cell body through a *basal body*, which is composed of 9 microtubule triplets arrange in a short barrel, much like the centrioles from which they are derived.

> This section refers only to eukaryotes. Some prokaryotes also have motile appendages called flagella, but they are completely different in both structure and mechanism. The flagella themselves are long helical polymers of the protein flagellin, and the base of the flagellin fibers is connected to a rotational motor protein, not a translational motor. This motor (Figure 12.9.18)

utilizes ion (H^+ or Na^+ depending on species) down an electrochemical gradient to provide the energy to rotate as many as 100000 revolutions per minute. It is thought that the rotation is driven by conformational changes in the stator ring, nestled in the cell membrane.

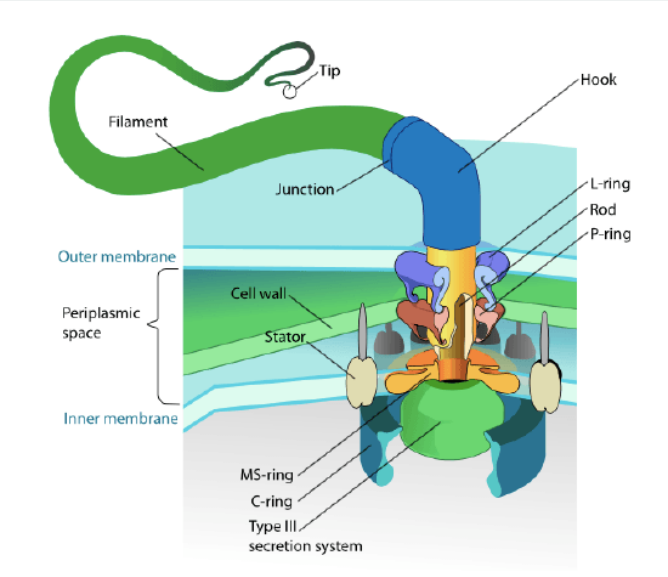


Figure 12.9.18. The bacteria flagellum is completely different from eukaryotic flagella. It is moved by a rotary motor driven by proton or Na^+ ion ow down the electrochemical gradient. Illustration released to public domain by M.R. Villareal.

The ciliary dyneins provide the motor capability, but there are two other linkage proteins in the axoneme as well. There are *nexins* that join the A-tubule of one doublet to the B-tubule of its adjacent doublet, thus connecting the outer ring. And, there are *radial spokes* that extend from the A tubule of each doublet to the central pair of microtubules at the core of the axoneme. Neither of these has any motor activity. However, they are crucial to the movement of cilia and flagella because they help to transform a sliding motion into a bending motion. When ciliary dynein (very similar to cytoplasmic dyneins but has three heads instead of two) is engaged, it binds an A microtubule on one side, a B microtubule from the adjacent doublet, and moves one relative to the other. A line of these dyneins moving in concert would thus slide one doublet relative to the other, if (and it's a big "if") the two doublets had complete freedom of movement. However, since the doublets are interconnected by the nexin proteins, what happens as one doublet attempts to slide is that it bends the connected structure instead (Figure 12.9.17). This bend accounts for the rowing motion of the cilia, which are relatively short, as well as the whipping motion of the long flagella, which propagate the bending motion down the axoneme.

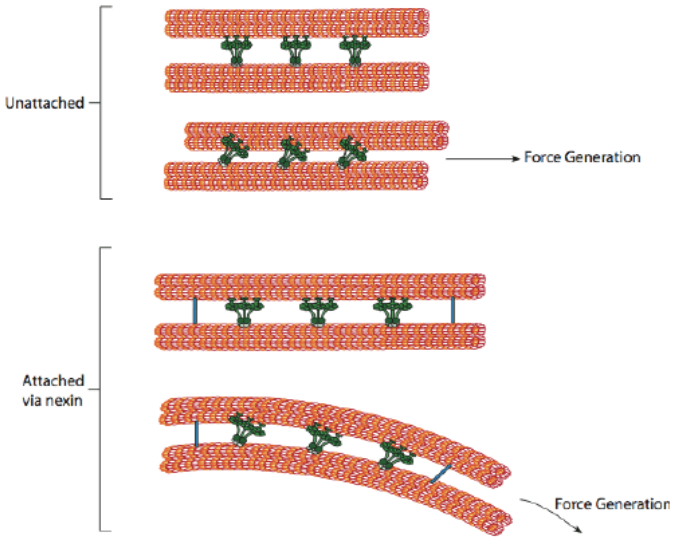


Figure 12.9.17. The nexin bridges connecting adjacent microtubule doublets transform the sliding motion generated by the ciliary dynein into a bending motion.

Although we think of ciliary and flagellar movement as methods for the propulsion of a cell, such as the flagellar swimming of sperm towards an egg, there are also a number of important places in which the cell is stationary, and the cilia are used to move fluid past the cell. In fact, there are cells with cilia in most major organs of the body. Several ciliary dyskinesias have been reported, of which the most prominent, primary ciliary dyskinesia (PCD), which includes Kartagener syndrome (KS), is due to mutation of the DNAI1 gene, which encodes a subunit (intermediate chain 1) of axonemal (ciliary) dynein. PCD is characterized by respiratory distress due to recurrent infection, and the diagnosis of KS is made if there is also *situs inversus*, a condition in which the normal left-right asymmetry of the body (e.g. stomach on left, liver on right) is reversed. The first symptom is due to inactivity of the numerous cilia of epithelial cells in the lungs. Their normal function is to keep mucus in the respiratory track constantly in motion. Normally the mucus helps to keep the lungs moist to facilitate function, but if the mucus becomes stationary, it becomes a breeding ground for bacteria, as well as becoming an irritant and obstacle to proper gas exchange.

Situs inversus is an interesting malformation because it arises in embryonic development, and affects only 50% of PCD patients because the impaired ciliary function causes randomization of left-right asymmetry, not reversal. In very simple terms, during early embryonic development, left-right asymmetry is due in part to the movement of molecular signals in a leftward ow through the embryonic node. This flow is caused by the coordinated beating of cilia, so when they do not work, the flow is disrupted and randomization occurs.

Other symptoms of PCD patients also point out the work of cilia and flagella in the body. Male infertility is common due to immotile sperm. Female infertility, though less common, can also occur, due to dysfunction of the cilia of the oviduct and fallopian tube that normally move the egg along from ovary to uterus. Interestingly there is also a low association of hydrocephalus internus (overfilling of the ventricles of the brain with cerebrospinal fluid, causing their enlargement which compresses the brain tissue around them) with PCD. This is likely due to dysfunction of cilia in the ependymal cells lining the ventricles, and which help circulate the CSF, but are apparently not completely necessary. Since CSF bulk flow is thought to be driven primarily by the systole/ diastole change in blood pressure in the brain, some hypothesize that the cilia may be involved primarily in ow through some of the tighter channels in the brain.

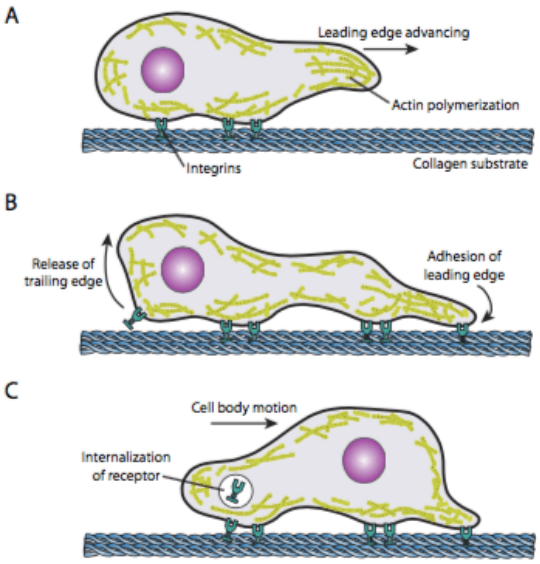


Figure 12.9.19. Cells crawl by (a) extending the leading edge primarily through remodeling of the actin cytoskeleton, (b) forming new adhesive contacts at that leading edge while releasing adhesions to the rear, and (c) bulk internal movement forward to "catch up" with the leading edge.

Cell crawling (Figure 12.9.19) requires the coordinated rearrangement of the leading edge microfilament network, extending (by both polymerization and sliding filaments) and then forming adhesions at the new forward-most point. This can take the form of filopodia or lamellipodia,and often both simultaneously. *Filopodia* are long and very thin projections with core bundles of parallel microfilaments and high concentrations of cell surface receptors. Their purpose is primarily to sense the environment. *Lamellipodia* often extend between two lopodia and is more of a broad ruffle than a finger. Internally the actin forms more into meshes than bundles, and the broader edge allows for more adhesions to be made to the substrate. The microfilament network then rearranges again, this time opening a space in the cytoplasm that acts as a channel for the movement of the microtubules towards the front of the cell. This puts the transport network in place to help move intracellular bulk material forward. As this occurs, the old adhesions on the tail end of the cell are released. This release can happen through two primary mechanisms: endocytosis of the receptor or deactivation of the receptor by signaling/conformational change. Of course, this oversimplification belies the complexities in coordinating and controlling all of these actions to accomplish directed movement of a cell.

> One model of microfilament force generation, the Elastic Brownian Ratchet Model (Mogilner and Oster, 1996), proposes that due to Brownian motion of the cell membrane resulting from continuous minute thermal fluctuation, the actin filaments that push out towards the edges of the membrane are flexed to varying degrees. If the flex is large enough, a new actin monomer can fit in between the membrane and the tip of the filament, and when the now longer filament flexes back, it can exert a greater push on the membrane. Obviously a single filament does not generate much force, but the coordinated extension of many filaments can push the membrane forward.

Once a cell receives a signal to move, the initial cytoskeletal response is to polymerize actin, building more microfilaments to incorporate into the leading edge. Depending on the signal (attractive or repulsive), the polymerization may occur on the same or opposite side of the cell from the point of signal-receptor activation. Significantly, the polymerization of new f-actin alone can generate sufficient force to move the membrane forward, even without involvement of myosin motors! Models of force generation are being debated, but generally start with the incorporation of new g-actin into a filament at its tip; that is, at the filament-membrane interface. Even if that might technically be enough, in a live cell, myosins are involved, and help to push and arrange filaments directionally in order to set up the new leading edge. In addition, some filaments and networks must be quickly severed, and new connections made, both between filaments and between filaments and other proteins such as adhesion molecules or microtubules.

How is the polymerization and actin rearrangement controlled? The receptors that signal cell locomotion may initiate somewhat different pathways, but many share some commonalities in activating one or more members of the Ras-family of small GTPases. These signaling molecules, such as Rac, Rho, and cdc42 can be activated by receptor tyrosine kinases (see RTK-Ras activation pathways, Chap. 14). Each of these has a slightly different role in cell motility: cdc42 activation leads to filopodia formation,

Rac activates a pathway that includes Arp2/3 and cofilin to lamellipodia formation, and Rho activates myosin II to control focal adhesion and stress fiber formation. A different type of receptor cascade, the G-protein signaling cascade (also Chapter 14), can lead to activation of PLC and subsequent cleavage of PIP_2 and increase in cytosolic Ca^{2+}. These changes, as noted earlier, can also activate myosin II, as well as the remodeling enzymes gelsolin, cofilin, and profilin. This breaks down existing actin structures to make the cell more fluid, while also contributing more g-actin to form the new leading edge cytoskeleton.

In vitro experiments show that as the membrane pushes forward, new adhesive contacts are made through adhesion molecules or receptors that bind the substrate (often cell culture slides or dishes are coated with collagen, filaminin, or other extracellular matrix proteins). The contacts then recruit cytoskeletal elements for greater stability to form a focal adhesion (Figure 12.9.20). However, the formation of focal adhesions appears to be an artifact of cell culture, and it is unclear if the types of adhesions that form in vivo recruit the same types of cytoskeletal components.

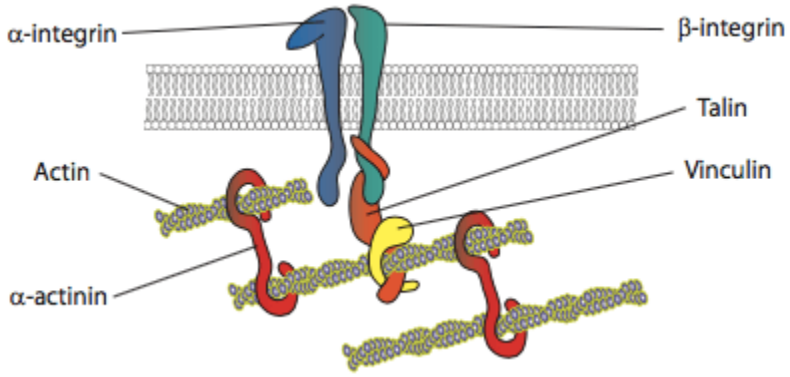


Figure 12.9.20. Focal adhesions form when integrins bind to an artificial ECM surface in cell culture dishes.

The third step to cell locomotion is the bulk movement of the cellular contents forward. The mechanisms for this phase are unclear, but there is some evidence that using linkages between the actin cytoskeleton at the leading edge and forward parts of the microtubule cytoskeleton, the microtubules are rearranged to form an efficient transport path for bulk movement. Another aspect to this may be a “corralling” effect by the actin networks, which directionally open up space towards the leading edge. The microtubules then enter that space more easily than working through a tight actin mesh, forcing flow in the proper direction.

> Much of the work on microtubule-actin interactions in cell motility has been done through research on the neuronal growth cone, which is sometimes referred to as a cell on a leash, because it acts almost independently like a crawling cell, searching for the proper pathway to lead its axon from the cell body to its proper synaptic connection (A.W. Schaefer et al, Dev. Cell 15: 146-62, 2008).

Finally, the cell must undo its old adhesions on the trailing edge. This can happen in a number of different ways. *In vitro*, crawling cells have been observed to rip themselves off of the substrate, leaving behind tiny bits of membrane and associated adhesion proteins in the process. The force generated is presumed to come from actin-myosin stress fibers leading from the more forward focal adhesions. However, there are less destructive mechanisms available to the cells. In some cases, the adhesivity of the cellular receptor for the extracellular substrate can be regulated internally, perhaps by phosphorylation or dephosphorylation of a receptor. Another possibility is endocytosis of the receptor, taking it off the cell surface. It could simply recycle up to the leading edge where it is needed (i.e. transcytosis), or if it is no longer needed or damaged, it may be broken down in a lysosome.

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13: EXTRACELLULAR MATRIX AND CELL ADHESION

Interactions between a cell and its environment or with other cells are governed by cell-surface proteins. This chapter examines a subset of those interactions: direct cell contact with either other cells or extracellular matrix (ECM). Extracellular matrix is a general term for the extremely large proteins and polysaccharides that are secreted by some cells in a multicellular organism, and which acts as connective material to hold cells in a defined space. Cell density can vary greatly between different tissues of an animal, from tightly-packed muscle cells with many direct cell-to-cell contacts to liver tissue, in which some of the cells are only loosely organized, suspended in a web of extracellular matrix.

13.1: Introduction to Extracellular Matrix and Cell Adhesion

13.2: Collagen

13.3: Proteoglycans

13.4: Fibronectins

13.5: Laminins

13.6: Integrins

13.7: Hemidesmosomes

13.8: Dystrophin Glycoprotein Complex

13.9: Desmosomes

13.10: Cadherins

13.11: Tight Junctions

13.12: Ig Superfamily CAMs

13.13: Selectins

13.14: Gap Junctions

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13.1: Introduction to Extracellular Matrix and Cell Adhesion

Interactions between a cell and its environment or with other cells are governed by cell-surface proteins. This chapter examines a subset of those interactions: direct cell contact with either other cells or extracellular matrix (ECM). Extracellular matrix is a general term for the extremely large proteins and polysaccharides that are secreted by some cells in a multicellular organism, and which acts as connective material to hold cells in a defined space. Cell density can vary greatly between different tissues of an animal, from tightly-packed muscle cells with many direct cell-to-cell contacts to liver tissue, in which some of the cells are only loosely organized, suspended in a web of extracellular matrix.

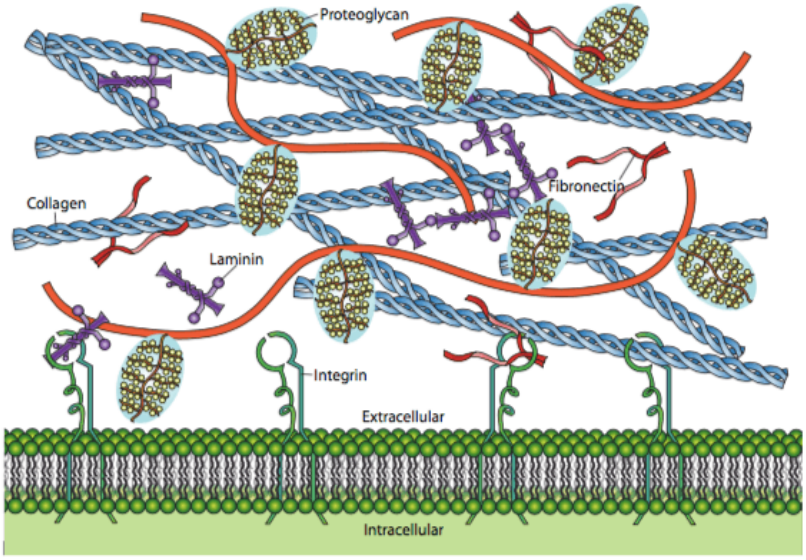


Figure 13.1.1. Extracellular matrix (ECM). Typical components include collagen, proteoglycans (with hydration shell depicted around sugars), bronectin, and laminin. The cellular receptors for a number of these ECM components are integrins, although the exact integrin αβ *pair may differ.*

ECM is a generic term encompassing mixtures of polysaccharides and proteins, including collagens, bronectins, laminins, and proteoglycans, all secreted by the cell. The proportions of these components can vary greatly depending on tissue type. Two, quite different, examples of ECM are the basement membrane underlying the epidermis of the skin, a thin, almost two-dimensional layer that helps to organize the skin cells into a nearly-impenetrable barrier to most simple biological insults, and the massive three-dimensional matrix surrounding each chondrocyte in cartilaginous tissue. The ability of the cartilage in your knee to withstand the repeated shock of your footsteps is due to the ECM proteins in which the cells are embedded, not to the cells that are actually rather few in number and sparsely distributed. Although both types of ECM share some components in common, they are clearly distinguishable not just in function or appearance, but in the proportions and identity of the constituent molecules.

> The “basal lamina” and “basement membrane” are frequently confused by students and professionals alike. The basement membrane was discovered first as a very thin layer of connective proteins just beneath an epithelial cell layer. The basal lamina was not discovered until later because it is not visible by light microscopy (normally only ~50 nm thick). Technically, the basal lamina, which consists of multiple layers itself, is a layer of ECM proteins secreted by the epithelial layer. The basal lamina and a thick reticular lamina (ECM secreted by other cell types) together form what is considered the basement membrane.
>
> The basal lamina around glomerular blood vessels in the kidneys is twice as thick (up to 100 nm) as usual, accomplishing part of the kidneys’ physiological role in blood filtration.

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13.2: Collagen

The largest and most prominent of the extracellular matrix proteins, constituting a quarter of the dry mass of the human body, are the members of the collagen family. Collagens are polymers that can be categorized into fibrillar (e.g. collagens I, II, III) and non fibrillar (e.g. collagen IV) types. The fibrillar collagens are made up of triple helical monomers of either identical (homotrimer) or different (heterotrimer) subunits. These monomers are then associated in an offset parallel interaction with other collagen monomers, leading to the formation of long fibers. Electron microscopic examination of these long fibers shows a banding pattern, which is indicative of the slight gap between monomers along the same parallel.

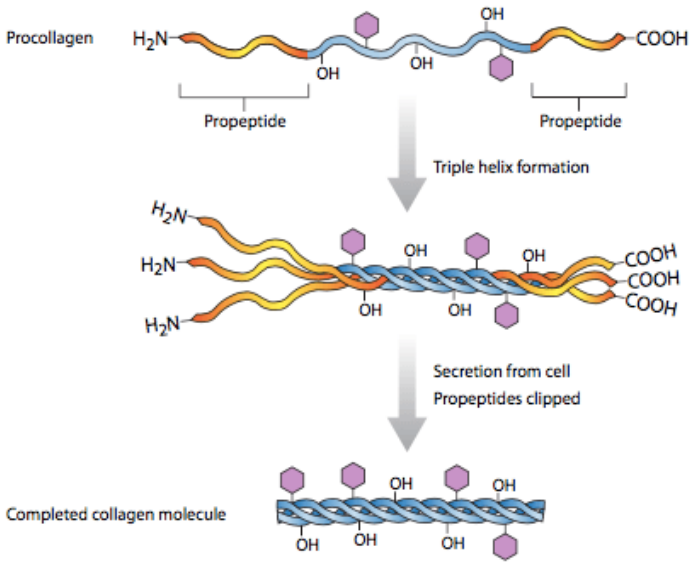


Figure 13.2.2. Collagen is a triple-helical protein consisting of three fibrillar subunits. Some of the amino acids are hydroxylated, and the protein is also glycosylated (represented by purple hexagons).

Like all secreted proteins, collagen I is processed in the ER (Figure 13.2.2), but not completely assembled there: the three pro-α-chains are assembled into a procollagen triple helix, which is secreted. Extracellularly, they must then be cleaved at both termini to form the active collagen protein, which is completely fibrillar. Other collagen types do not have the same cleavage, and may have globular domains at the ends of the fibrils. Collagens are also interesting for their unusual amino acid makeup. They contain a high proportion of hydroxylated amino acids, mostly prolines and lysines (Figure 13.2.3). This hydroxylation is necessary for the extensive hydrogen bonding that occurs between subunits and between monomers. The fibrils are associated with high tensile strength. An example of this would be the long collagen fibers that run parallel to the long axis of tendons and ligaments. These high-stress-bearing structures (connecting bone to muscle, and bone to bone, respectively) require the resilience that collagen fibers can provide.

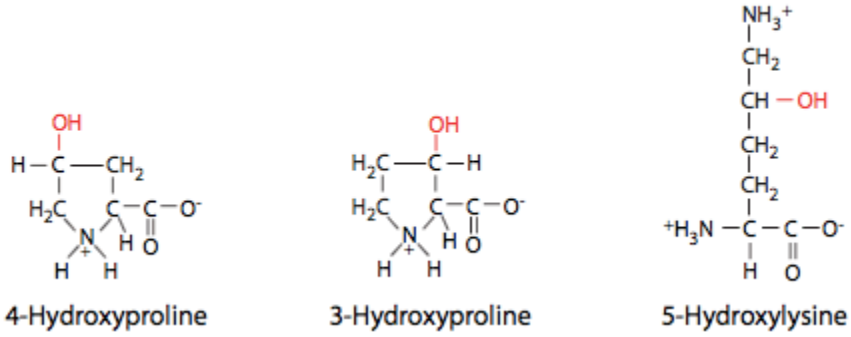


Figure 13.2.3. Collagens have a high proportion of hydroxylated prolines and lysines.

Conversely, conditions that adversely affect collagen formation can lead to serious disease conditions. In fact, a form of epidermolysis bullosa (the heritable skin blistering disease introduced in the previous chapter) is caused by mutation in collagen VII which is primarily produced by epidermal keratinocytes and secreted into the dermal-epidermal basement membrane layer. A variety of chondrodysplasias as well as bone malformations such as osteogenesis imperfecta (which can be perinatally lethal) have been linked to mutations in various collagen genes. Finally, several symptoms of scurvy are due to malformation of collagen in the ECM: weak blood vessel walls, bleeding gums and loose teeth, and fragile bones. Scurvy is a disease of ascorbic acid (vitamin C) deficiency, and the effect on ECM is due to the need for ascorbic acid as a cofactor for enzymes that hydroxylate the prolines and lysines of collagen.

Collagen is a major component of the basement membrane and basal lamina. The basal lamina is strong and flexible, able to serve as structural support for the epithelial sheets attached to it, as well as providing a semi-permeable matrix/filter that allows the passage of water and smaller molecules, but excludes larger macromolecules. The two major protein components to the basal lamina are collagen IV and laminin. Collagen IV has both long fibrillar, alpha-helical domains as well as globular domains that can interact in different orientations to form the meshwork that sets up the basement membrane. The laminin network is connected to the collagen network through entactin (nidogen) linker proteins.

An interesting application of collagen fibrils is in the cornea, the protective clear covering of the eye. The cornea is the primary protection against eye injury, and must be tough. The central layer (stroma, or substantia propria) is composed of approximately 200 layers of tightly packed, regularly spaced parallel collagen fibrils, with adjacent layers arranged so that the collagen fibrils lie perpendicularly from one layer to the next. This kind of laminar structure is used in a variety of man-made construction materials (including the ubiquitous building material, plywood) and provides great strength in a relatively small mass. Somewhat amazingly, and quite unlike plywood, the cornea is transparent. That property is thought to come from the regularity of the collagen lattice, which allows for cancellation of scattered light from one fibril by destructive interference from the scattered light of another fibril. Somewhat counterintuitively, it actually gets cloudy (due to refraction) when it absorbs fluid from the aqueous humor, and has active mechanisms to pump any such fluid back out of the cornea. This is why the cornea thickens and becomes translucent after death — the pump mechanism no longer has energy to run, and the aqueous humor diffuses into the cornea.

> The reasoning behind the use of glucosamine and chondroitin sulfate supplements by people with joint problems is that they are two of the sugars found in proteoglycans of cartilaginous tissue such as the meniscus of the knee, and in other joints. Chondroitin sulfate in particular is the major sugar in articular cartilage proteoglycans. Both are thought to stimulate GAG synthesis, and limited documentation of protease inhibitory and collagen synthesis effects have been noted. Data from rabbit models (but potential conflict of interest, Lippiello et al, 2000) suggests a therapeutic benefit from such supplements. However, human studies have so far shown no significant improvement in patients already suffering from moderate to severe arthritis and other joint-related ailments (Clegg et al, 2006). A secondary survey analysis suggested that there was some promise with regard to effects on mild to moderate cases, but the data was not significant.

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13.3: Proteoglycans

The protein component of proteoglycans are not as large as fibrillar collagens in general, but they often ll a massive volume because of heavy glycosylation. The sugars, many of which are sulfated or carboxylated, are hygroscopic to begin with, but being negatively charged, attract positive ions, which in turn brings in more water. Sugars attached to the core proteins are usually repeating disaccharide units such as chondroitin (d-Glucuronic acid and GalNAc), chondroitin sulfate, heparin (d-Glucuronic acid and GlcNAc by α/β1-4 bond), heparan sulfate, keratan sulfate (Galactose and GlcNAc), or hyaluronan (also called hyaluronic acid, composed of d-Glucuronic acid linked by β1-3 bond to GlcNAc).

> Heparin, a hypersulfated form of heparan sulfate, is also used medically as an anticlotting drug. It does so not by preventing clots directly, but by activating antithrombin III, which inhibits clotting.

As with all glycoproteins, assembly of the GAGs occurs in the Golgi, but beyond that, mechanisms for control of the extent and length of the disaccharide polymer addition is unknown. Unlike collagens and most other ECM components, proteoglycans can either be secreted or membrane bound. In fact, of the membrane bound proteoglycans, some are actually transmembrane proteins (these are designated syndecans), while other are bound to the cell surface via glycosylphosphatidylinositol (GPI) anchor (glypicans). In addition to these three basic varieties of core proteins, proteoglycans exhibit extraordinary diversity in glycosylation, ranging from the addition of only a few sugars, to well over a hundred. Interestingly, the core protein for chondroitin sulfate proteoglycans in basal lamina of muscle can be a collagen (Type XV)!

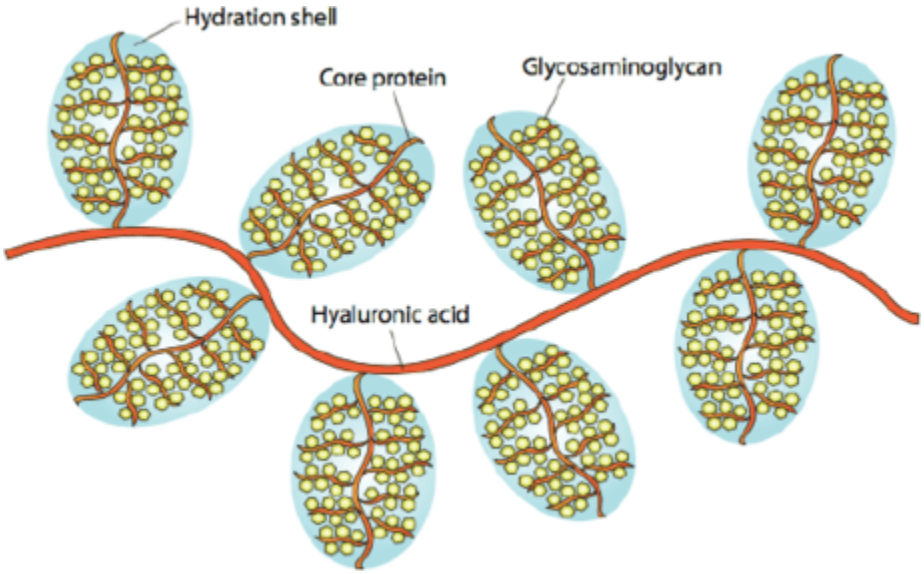


Figure 13.3.4. Proteoglycans are composed of multiple glycosaminoglycans attached to a core protein. These core proteins are sometimes attached to a hyaluronic acid molecule. Negatively charged sugars, like chondroitin sulfate or heparan sulfate, are depicted in yellow. They attract positive ions and water, forming a hydration shell around the proteoglycan. This Figure depicts aggrecan, a cartilaginous aggregate of proteoglycans assembled on a hyaluronic acid core.

One of the paradoxes of proteoglycans is that they can function either as a substrate for cells to attach to, or due to the hydration shell, they can be very effective barriers to other cells as well. This is useful during development when there is a great deal of cell migration, and there needs to be ways to segregate cells both by attracting them and repelling them. Unfortunately, this can have deleterious consequences in some situations. For example, when the brain or spinal cord is injured, a glial scar is formed, and that scar contains a chondroitin sulfate proteoglycan. Unfortunately, this proteoglycan is an inhibitor of neural growth, which contributes to the prevention of neural regeneration, and for the unlucky patient, likely paralysis or worse depending on location and severity of the lesion.

On the other hand, there is also evidence (Rolls et al, *PLoS Med.* **5**: e171, 2008) that the CSPG may be needed to activate microglia and macrophages to promote healing.

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13.4: Fibronectins

Fibronectin and laminin are significantly smaller than either collagens or proteoglycans, and play different roles in the extracellular matrix. Fibronectin is formed by the joining of two similar polypeptide subunits via a pair of disulfide bonds near the C-terminal of each (Figure 13.4.5). Each subunit is arranged as a linear sequence of 30 functional domains (varies slightly by species). Within each subunit, each domain acts as a semi-independent unit with respect to secondary and even tertiary structure. Structurally, there are three major types of domains (Figure 13.4.6) that can be distinguished not only by sequence, but by the binding sites they form. The Figure above shows binding sites for other fibronectins, fibrin, collagen, heparin, and syndecan. Fibronectin is therefore an excellent linkage protein between these different molecules to stabilize and strengthen the ECM.

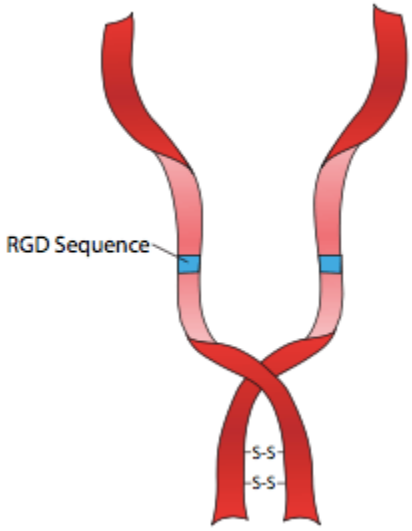


Figure 13.4.5. Fibronectin. The C-terminus of each subunit is at the bottom of the Figure. The RGD sequence is a binding site for integrin receptors.

One of the interesting aspects of fibronectin fibril formation is that the self-association site is generally hidden, but is revealed when a cell binds to the integrin-binding site. Thus cell-binding seems to nucleate the formation of fibrils, perhaps helping to form strong anchors in certain situations in which the cell is not migrating, but establishing itself permanently.

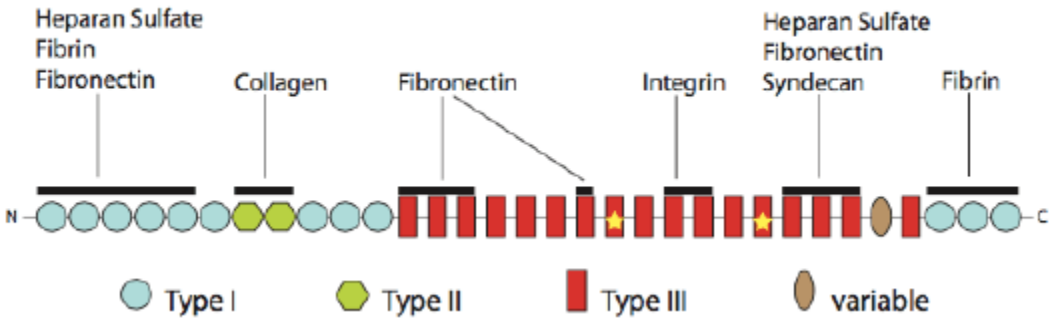


Figure 13.4.6. Each fibronectin subunit is composed of about 30 modular domains, of which there are three major structural types. Binding sites for other molecules are labeled. The two Type III domains that are marked with a star are alternatively spliced domains not found in fibronectin that circulates in the bloodstream, where it helps to promote clotting.

Importantly, in addition to linking a variety of extracellular matrix proteins together, fibronectin also has a site that binds to integrin receptors on cells. Whereas collagen for the most part acts as a passive substrate that cells are willing to attach to or crawl on, fibronectin can *actively* induce cell migration by activation of the integrins. Fibronectin expression along very specific pathways are crucial for the migration of neural crest and other cell types in development. *In vitro* experiments with fibroblasts and other cell types show a marked preference for areas coated with fibronectin over areas coated with collagen. This is also true

in vivo: an upregulation of fibronectin in response to injury promotes migration of fibroblasts and cells associated with wound healing into the lesioned area.

The integrin binding site is characterized by the presence of an arginine-glycine-aspartic acid (RGD) sequence. If this site is abolished or mutated, the mutant bronectin does not bind to cells. Similarly, if cells are treated with high concentrations of short peptides that contain the RGD sequence, those peptides bind to the integrins, and the cells ignore fibronectin. Finally, in addition to serving as a linker between other ECM proteins, or even to cells, fibronectin can form fibrils through interaction with other fibronectins.

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13.5: Laminins

Although there are many other less abundant proteins in the extracellular matrix, laminin is the final ECM molecule to be discussed in this chapter. Laminins are a family of secreted glycoproteins that are found in many ECM formations, and like fibronectin, bind to cells via integrin receptors. The laminin protein is composed of three subunits (α, β, γ) arranged in a cruciform shape. There are multiple isoforms of each subunit yielding the variety (15) of laminin proteins catalogued to date. Although laminin contains an RGD sequence like fibronectin, its role in cell adhesion is not universal. Although some cell types have been demonstrated to bind to the RGD site, others clearly bind to other domains of laminin, primarily located on the opposite end of the protein.

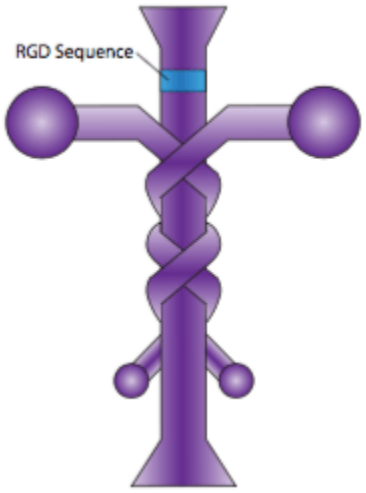


Figure 13.5.7. The cruciform structure of laminin is composed of three subunits. The arms bind collagens and sulfolipids, while the foot contains LG domains that bind integrins and carbohydrates.

> At present, there are 5 α-chain genes, 4 β-chains, and 3 γ-chains known. The α2/β1/γ1 combination is known as laminin-2 or merosin, and is found primarily in the basal lamina of striated muscle. Mutations that affect the function of this laminin cause a form of congenital muscular dystrophy.

Laminin plays a crucial role in neural development, where it acts as a guiding path along which certain axons extend to find their eventual synaptic targets. One prominent example is the retinotectal pathway that leads retinal ganglion cell axons from the eye to the brain. Another example of the role of laminin in development is the guidance of primordial germ cells (PGC). These are cells that eventually become the gametes, but need to migrate from the yolk sac, which is outside the embryo proper, to the site of gonad formation. Laminin is found along this pathway. Interestingly, as the PGCs reached a stretch of laminin very close to the final destination, the adhesion to laminin increased, and this adhesion was found to involve not integrin receptors, but an interaction with a cell surface heparan-sulfate proteoglycan. This and other evidence suggests that migration on laminin may be mediated by integrin receptors, whose adhesivity can be regulated intracellularly, while more static interactions with laminin may be mediated by other types of binding proteins. Finally, as already discussed, laminin is an important component of basal lamina, able to form fibrils and networks itself, as well as with collagen IV.

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13.6: Integrins

The integrins have thus far been introduced as receptors for fibronectin and laminin, but it is a large family with a wide variety of substrates. For example, the focal adhesion (Figure 13.6.8) shows an an integrin receptor bound to collagen. As already discussed in the previous chapter, focal adhesions are usually transient, and seen as points of contact as fibroblasts or other migratory cells crawl on a culture dish or slide coated with ECM proteins.

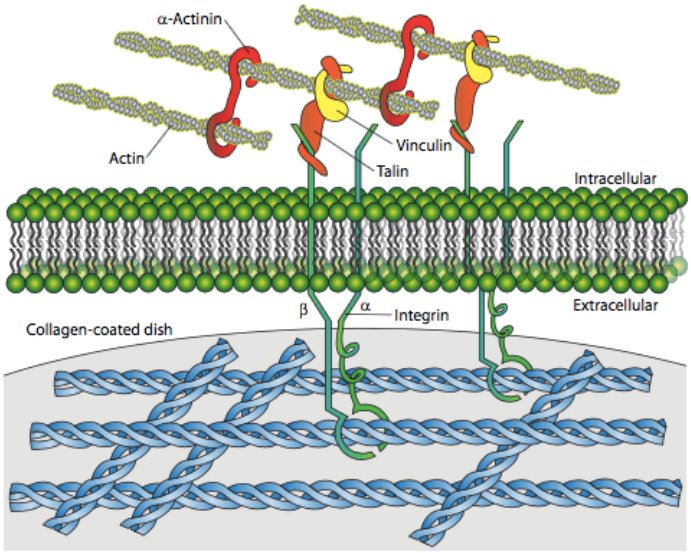


Figure 13.6.8. A focal adhesion is a dynamic point of contact formed by a cell growing on a collagen-coated dish.

In addition to collagen, fibronectins and laminins are also potential binding partners for integrins. As table 1 shows, the diversity of subunits and combinations means that integrins are involved in a wide array of cellular processes, and can bind cell surface proteins as well as ECM.

Table 1: Integrin receptors, their ligands and distribution.

Subunits	Ligand	Distribution
α1β1	mostly collagens, also laminin	widespread
α2β1	mostly collagens, also laminin	widespread
α4β1	fibronectin, VCAM-1	hematopoietic cells
α5β1	fibronectin	fibroblasts
α6β1	laminin	widespread
α6β4	laminin	epithelial cells
αLβ2	ICAM-1, ICAM-2	T-lymphocytes
αIIbβ3	fibronectin, fibrinogen	platelets

With this variety, it is not surprising that not all integrins bind RGD sequences, although most do. For example, α2β1 integrins prefer YYGDLR or FYFDLR sequences, and αIIbβ3 binds both the RGD and a KQAGDV sequence strongly. Integrin activation has been shown to initiate signaling pathways, beginning focal adhesion kinase (FAK) or a few other central kinases, which control activities from cytoskeletal rearrangement to cell survival.

Both α and β subunits are transmembrane proteins that pass through the membrane just once. Evolutionarily, they are found only in metazoan species, but they are also found in *all* metazoan species. All integrins but one, α6β4, connect to the actin microfilament cytoskeleton through the b subunit cytoplasmic domain. The α6β4 integrin links to the intermediate filament

cytoskeleton, in part because the β4 cytoplasmic domain is very large and extends further into the cytoplasm. On the extracellular side, there is a metal ion coordination site usually occupied by Mg^{2+}, that is necessary for ligand binding. There are also several other divalent ion binding sites, The receptor can be found in either an inactive (somewhat bent over towards the membrane) or an active state (straightened up). In the inactive state, the a subunit binds the β subunit closely preventing interaction with the cytoskeleton. However, once a subunit cytoplasmic domain, it displaces the a subunit, causing a slight separation of the two subunits and leading to activation of the receptor. In fact, integrins demonstrate what is known as “inside-out” signaling, in which a cellular signal (for example, from the signaling cascade of a growth factor) leads to alterations to the cytoplasmic domain and which shifts the conformation of the extracellular domain to an active straightened-up state in which it can more readily bind to ligands. This is why integrins are so well suited to focal adhesions and other “in motion” adhesions that must adhere and release quickly. Though recycling of receptors also happens, turning them on or off by inside-out signaling is an effective mechanism for fast movement.

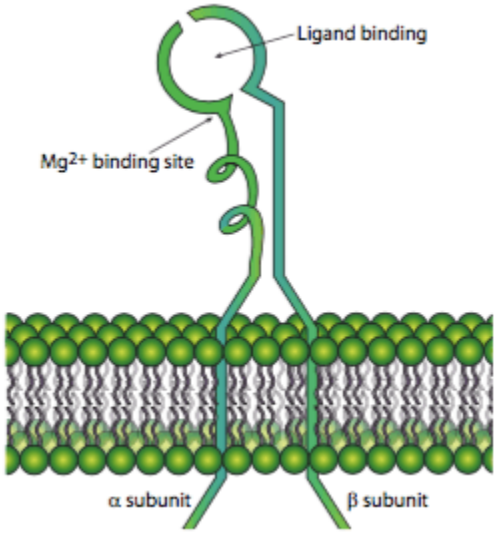


Figure 13.6.9. Integrin receptors are composed of two polypeptides that each pass through the membrane once.

As one might expect from an actin-linked structure, focal adhesions and their *in vivo* equivalents are transient, dynamic points of contact between the cell and the substrate it is crawling over. However, there are many situations in which a cell is not only stationary, it needs to be firmly attached to its substrate in order to gird itself for what- ever stressors might come to test its resolve. In these cases, the actin cytoskeleton is too ephemeral for the task.

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13.7: Hemidesmosomes

Hemidesmosomes, particularly those attaching epithelial cells to their basement membrane, are the tightest adhesive interactions in an animal body. This close contact, and the reinforced structure of these contacts, is crucial for the protective resilience of epithelial layers. Remember the α6β4 integrin? That would be the one that links with intermediate filaments instead of f-actin. Intermediate filaments, as we've already noted, are not dynamic, but about as stable as a cellular component can be. They are also very strong and are used to buttress cellular integrity. So, it is no surprise to see intermediate filaments and the α6β4 integrin playing roles in hemidesmosomes.

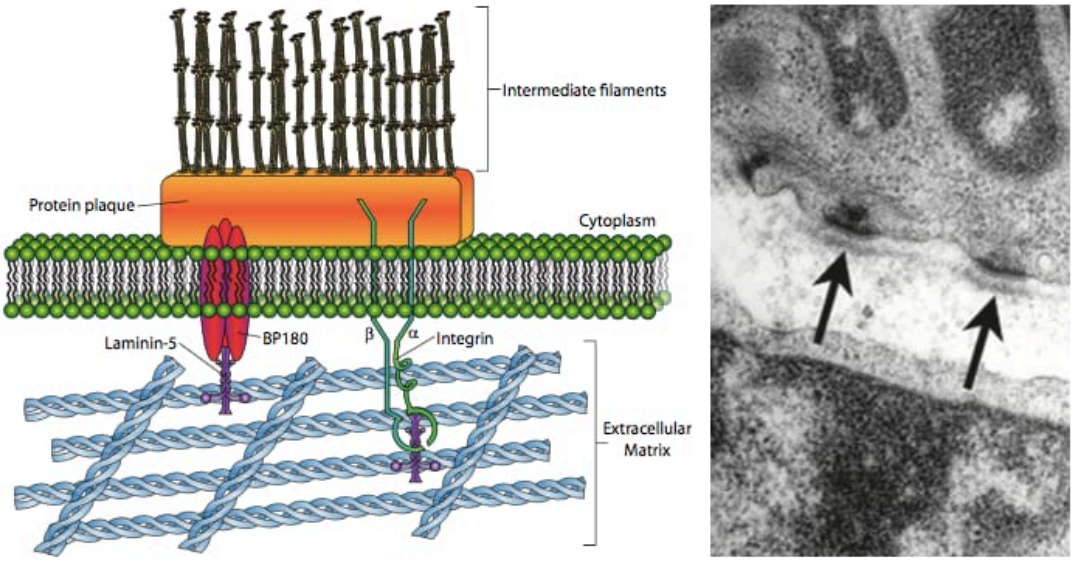


Figure 13.7.10. Hemidesmosomes. (Left) Diagram depicting involvement of intermediate filaments and a dense protein plaque reinforcing the membrane at the point of contact. (Right) This shows up as the electron dense areas pointed out by the arrows in this electron micrograph of epithelial cells in mouse trachea. Micrograph released with creative commons attribution license by Nguyen et al, Respiratory Research 7:28 (2006).

The distinguishing characteristic of hemidesmosomes though, is the electron-dense plaque. It can be thought of as reinforcement so that when the epithelium is stretched, the cell does not just pop loose leaving behind part of its membrane. The plaque contains several proteins, but the primary component are plectins, the linker proteins that help to bundle intermediate filaments, and connect them to each other as well as other cytoskeletal elements. Another major element of the plaques is BP230, which connects the plaque to keratin. On the extracellular side, in addition to the integrin already mentioned, there is also a transmembrane glycoprotein called BP180, which also binds to laminin elements of the basement membrane.

> BP230 and BP180 are named for bullous pemphigoid, the subepidermal bullous disorder characterized by chronic blistering of the skin. It is an autoimmune disorder and in which the aberrant antibody response is to these two hemidesmosomal proteins.

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13.8: Dystrophin Glycoprotein Complex

Another type of cell-ECM connection is the dystrophin glycoprotein complex (DGC) of skeletal muscle cells. Similar complexes are found in smooth muscle and in some non-muscle tissues. Muscle cells, of course are subject to frequent mechanical stress, and connectivity to the ECM is important in supporting the cell integrity. The DGC uses the large transmembrane glycoprotein, dystroglycan, as its primary binding partner to basal lamina laminin. A sarcoglycan complex and sarcospan are other major transmembrane components of the DGC, but their roles do not appear to include direct interaction with basal lamina.

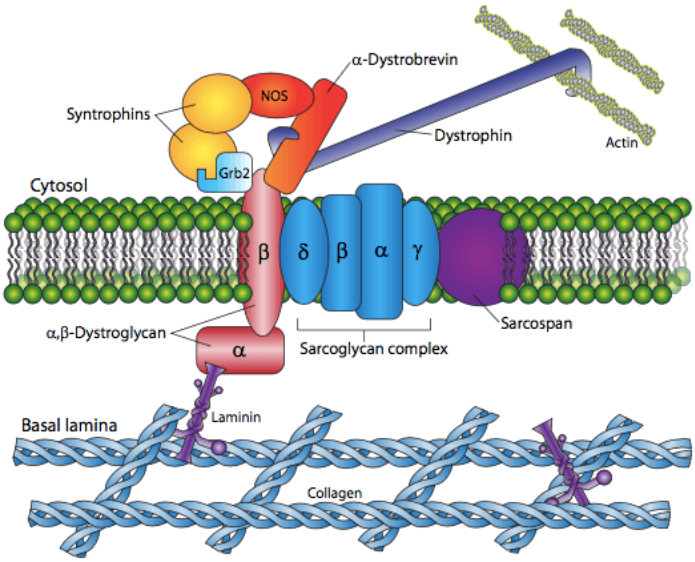


Figure 13.8.11. Dystrophin Glycoprotein Complex.

The sarcoglycan complex (consisting of 4 sarcoglycans) is postulated to act as structural reinforcement for the membrane at these contact points. The role of sarcospan, a 4-pass transmembrane protein, has not been demonstrated within the DGC, but homologous proteins in other cells are found in adhesive complexes with integrin receptors, suggesting the same function here. Although the DGCs are long-lived adhesive contact that have more in common with hemidesmosomes than focal contacts, the cytoskeletal component attached to the DGC is actin (via dystrophin), not intermediate laments. However, it is important to note that the actin cytoskeleton of muscle cells has very different functions from its counterpart in a fibroblast. Mutations to the sarcoglycans, dystroglycan, and dystrophin have all been shown to cause muscular dystrophies.

> Mutations leading to loss of sarcospan have not been linked to any muscular dystrophies. However, sarcospan is not found in muscular dystrophy patients who have mutations in the sarcoglycans. It thus appears that the tetrameric sarcoglycan complex is required for normal membrane localization of sarcospan.

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13.9: Desmosomes

Cells will form adhesive interactions with other cells as well as with ECM. Most of these interactions utilize a different set of proteins, although integrins have been found to interact with some cell adhesion proteins. An example of a cell-cell interaction with many similarities to a cell-ECM interaction, but using different adhesion molecules, is the desmosome. Like its basal-lamina-attached counterpart, the hemidesmosome, the desmosome is found in epithelial sheets, and its purpose is to link cells together so that pressure is spread across many cells rather than concentrated on one or a few. Desmosomes are necessary for the structural integrity of epithelial layers, and are the most common cell-cell junction in such tissues. The primary structural characteristic of the hemidesmosome, the dense plaque reinforcing the intracellular side of the adhesion, is also found in desmosomes, although it is composed of different proteins. In desmosomes, the plaque is composed primarily of plakoglobins and desmoplakins. The plakoglobins connect the adhesion molecules to the desmoplakins, and the desmoplakins link to intermediate laments such as keratin.

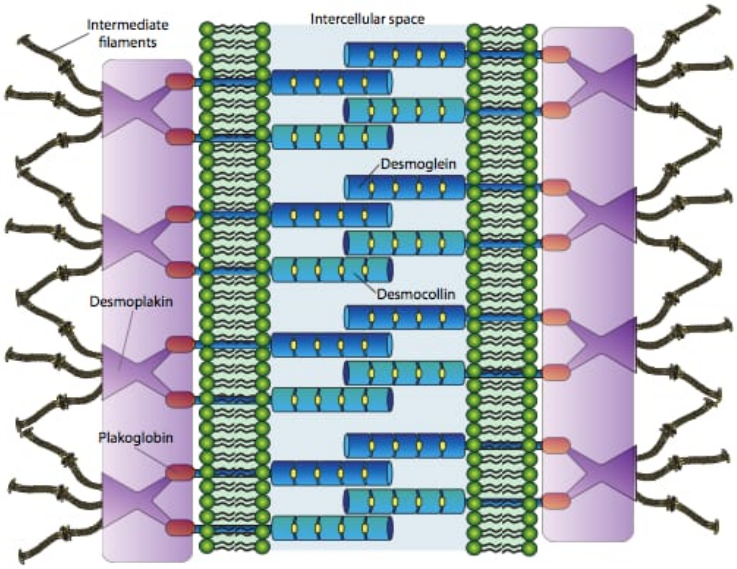


Figure 13.9.12. Desmosomes connect the intermediate laments of adjacent cells across adhesion molecules strengthened by a protein plaque. Desmoglein and desmocollin, the adhesion molecules are members of the cadherin family, and the reinforcing plaque contains plakoglobin, which connects the adhesion molecules to the IF linker, desmoplakin.

Another similarity to hemidesmosomes, and one predicted by the involvement of keratin, is the permanence of desmosomes. On the other hand, a key difference between the two types of adhesions is the adhesion proteins involved. The major proteins of the desmosome are desmoglein and desmocollin, both of which are members of the cadherin superfamily of Ca^{2+}-dependent adhesion molecules.

Mutations in desmoplakin (on chromosome 6p24) are linked to Carvajal syndrome (also known as dilated cardiomyopathy with woolly hair and keratoderma). Patients are born with woolly hair, and palmoplantar keratoderma appears within the first year. Dilation of the left ventricle and attending weakness in contractility may lead to death from heart failure in teenage years.

Pemphigus vulgaris is another rare disease involving dysfunction of desmosomes. It is an autoimmune disease targeted to the patient's own desmoglein proteins. The reduced epithelial adhesion leads to blistering of skin and mucous membranes.

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13.10: Cadherins

The cadherin superfamily is comprised of the desmogleins (of which 4 have been identified in humans) and desmocollins (3 in humans), the cadherins (>20), and the protocadherins (~20) as well as other related proteins. They share structural similarity and a dependence on Ca^{2+} for adhesive activity, and they can be found in most tissues, and for that matter, most metazoan species. Cadherins are single-transmembrane modular proteins. On the outside of the cell, the cadherin has five domains of similar but not identical structure. It was originally thought that Ca^{2+} was used between cadherins to mediate adhesion, but it is now clear that Ca^{2+} is bound in between each extracellular domain, apparently coordinating them into a more rigid structure. Cadherins can also act in *cis*, i.e. cadherins from the same cell can form dimers. This property allows a patch of cadherin adhesions such as a desmosome to "zipper" together into very strong clusters.

The cytoplasmic domain of cadherins characteristically binds to a family of proteins called the catenins, and this binding can be regulated by phosphorylation of the cadherin. The most common catenins are a and β, usually with the β-catenin acting as intermediary between cadherin and α-catenin, and the α-catenin linking them to the actin microfilaments. This kind of arrangement is found in both cells that are motile, crawling over other cells that are expressing cadherin, as well as stationary cells. Although this is not the arrangement in desmosomes, the desmosomal plaque protein plakoglobin is a member of the catenin family.

The primary binding site for cadherins appears to be the N-terminal domain (most distal extracellular), although there is evidence that as many as three domains can be involved. Cadherins mostly bind homophilically (E-cadherin binds E-cadherin on another cell, but not P-cadherin), although some cadherins can bind heterophilically (e.g. N-cadherin can bind to either N-cadherin or E-cadherin). Incidentally, these three, E- cadherin (epithelial), N-cadherin (neural), and P-cadherin (placental) are the best-studied cadherins. Both E- and P-cadherins are important in early embryonic development, while N-cadherin has been studied in the context of axon guidance in the developing nervous system. E-cadherin is also a target of scrutiny because it is also important in the metastasis of cancer. In order for a cancer cell to break from the initial tumor, it must downregulate its adhesion to neighboring cells before migrating elsewhere. This is known as the epithelial-mesenchymal transition and is accompanied by decreased E-cadherin expression.

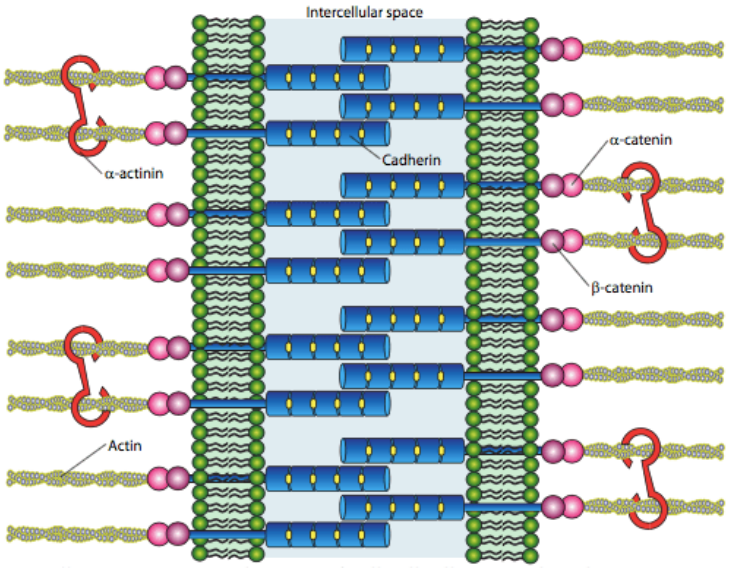


Figure 13.10.13. Adherens Junction. This type of cell-cell adhesion is based on interaction of cad- herins, which are connected intracellularly to the actin cytoskeleton through the linker proteins a- and b- catenin. Also depicted here is the actin bundling protein α-actinin.

With the patch of cadherin interactions, the adherens junction (Figure 13.10.13) looks very similar to the desmosome (Figure 13.10.12). Adherens junctions serve some of the same purposes as desmosomes: providing connectivity to neighboring cells, and reinforcing and shaping the cells. However, adherens junctions are mostly localized near the apical surface of epithelial cells, and instead of intermediate filaments, they are connected to actin microfilaments that form a circumferential belt that produce tension and shaping forces in conjunction with myosins that associate with it.

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13.11: Tight Junctions

Sometimes, holding cells together, even with great strength, is not enough. In epithelia especially, a layer of cells may need to not only hold together but form a complete seal to separate whatever is in contact with the apical side from whatever is in contact with the basal side. That would be a job for The Tight Junction! Well, more accurately, for many tight junctions in an array near the apical surface. Perhaps the best example of the utility of tight junctions is in the digestive tract. The tight junctions that form between cells of the epithelial lining of the gut separate the food and its digestion products from the body at large, forcing macromolecule nutrients to be transported through the epithelial cell by endocytosis/transcytosis to the bloodstream where they can be most efficiently distributed. The tight junctions also form in blood vessels to prevent leakage of blood, and in a variety of organs where liquids must be contained.

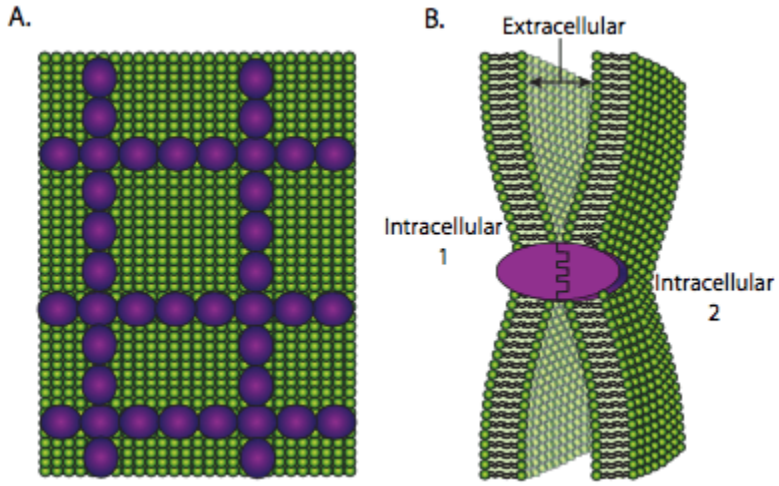


Figure 13.11.14. Tight Junctions. (A) Tight junctions are usually present in arrays that seal off one side of an epithelial layer from the other multiple times. (B) Each tight junction is formed by very small transmembrane proteins, claudins and occludins, so that the membranes of opposing cells can come into extremely close contact.

An individual tight junction is formed by the interaction of claudins and occludins. They are each 4-pass transmembrane proteins with both N- and C-termini on the cytoplasmic side; the extracellular side has a very low pro le, consisting of one (claudin) or two (occludin) small loops. Because of their small size, when they interact, the membranes are brought together very closely. In order to actually form a seal between cells though, tight junctions must be lined up in close order all the way around the cell, and in fact, usually there are multiple lines, which one could think of as “backup” in case one line develops a leak. Claudin molecules have relatively small cytoplasmic domains and it is not clear whether there are significant interactions with other proteins. However, occludin has a large C-terminal cytoplasmic domain that contains a PDZ-binding domain. PDZ is a protein interaction motif of approximately 80-90 amino acids found in a number of signaling proteins, most often in use to hold signaling complexes near the membrane by interacting with a transmembrane protein, as would be the case here with occludin. These PDZ-containing proteins both have signaling functions and can act as adapters to the cytoskeleton, primarily the actin laments. Finally, although an exact mechanism is unclear, elevated levels of Ca^{2+}, either extracellularly or perimembranously, is associated with tight junction assembly.

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13.12: Ig Superfamily CAMs

In addition to occludin and claudins, junction adhesion molecules (JAMs) have recently been found in tight junctions. These molecules are members of a gigantic superfamily of cell adhesion molecules known as the Ig (immunoglobulin domain) superfamily because all of these proteins contain an immunoglobulin loop domain that plays an important part in the adhesion mechanism. The purpose of immunoglobulins (antibodies) is to recognize and adhere to other molecules, so it makes sense that such a structural motif would also be used for other kinds of adhesion. Ig loops fall into two primary categories, again based on the loops of an immunoglobulin molecule. These are the variable (V) loop and the constant (C1) loop. Some IgSF molecules contain a C2 loop, which has amino acid homology to the V loop, but structural/size similarity to the C1 loop. The size of IgSF molecules ranges greatly, and the number of Ig loop domains and other domains (e.g. fibronectin Type III domains) can also vary significantly.

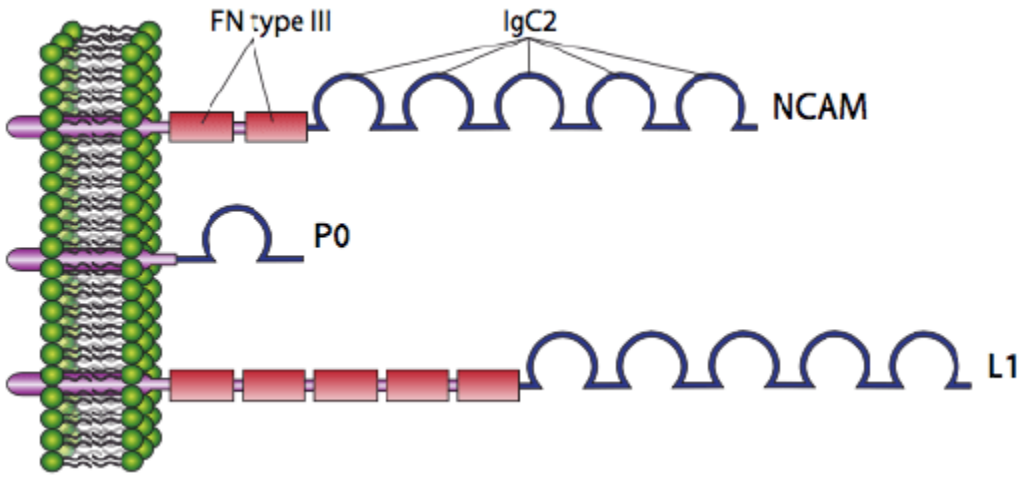


Figure 13.12.15. Ig superfamily cell adhesion molecules NCAM, P0, and L1 (top to bottom) are built with fibronectin Type II domains and Ig C2-like domains.

IgSF molecules are involved in a number of cellular processes requiring adhesion. The most obvious, of course, is the immune response in which an immunoglobulin, either secreted or on a cell, binds to a molecule foreign to the body. However, there are many other interactions outside of the immune system that involve IgSF molecules. One well-studied area is in neural development, where IgSF members L1 (L1CAM), NCAM and numerous others are expressed in specific patterns to control the routing of axons as they make their way from the neuronal cell body to their eventual connections. This path can often be very long, crossing many different cell types, and taking several turns, so a robust guidance system is crucial to make a normal functioning nervous system. Specific combinations of cell adhesion molecules (also called axon guidance molecules in this case) direct these processes even through the extraordinary density of potential (but incorrect) nerve cell targets in the brain. IgSF molecules have been found to bind both homophilically and heterophilically, and for that matter, not just to other IgSF molecules, but to adhesion molecules of other structural families such as integrins.

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13.13: Selectins

The last major cell adhesion molecule family to discuss is the selectins. Selectins bind heterophilically to oligosaccharide moieties on glycoproteins. In fact the name of the family is based on lectin, a generic term for proteins that bind sugars. The selectins, like cadherins and IgSF molecules are modular glycoproteins that pass through the membrane once. From C-terminus to N-terminus, the selectins are composed of a relatively short cytoplasmic domain, a single transmembrane domain, a series of CR (consensus repeat) or structural domains (from 2 in L-selectin to 9 in P-selectin), an EGF (epidermal growth factor) -like domain, and a lectin-like domain. The lectin-like domain binds a fairly specific oligosaccharide composed of sialic acid, galactose, GlcNAc, and fucose, of which the sialic acid and fucose are most important for recognition. Selectin-mediated adhesion is a Ca^{2+} dependent process.

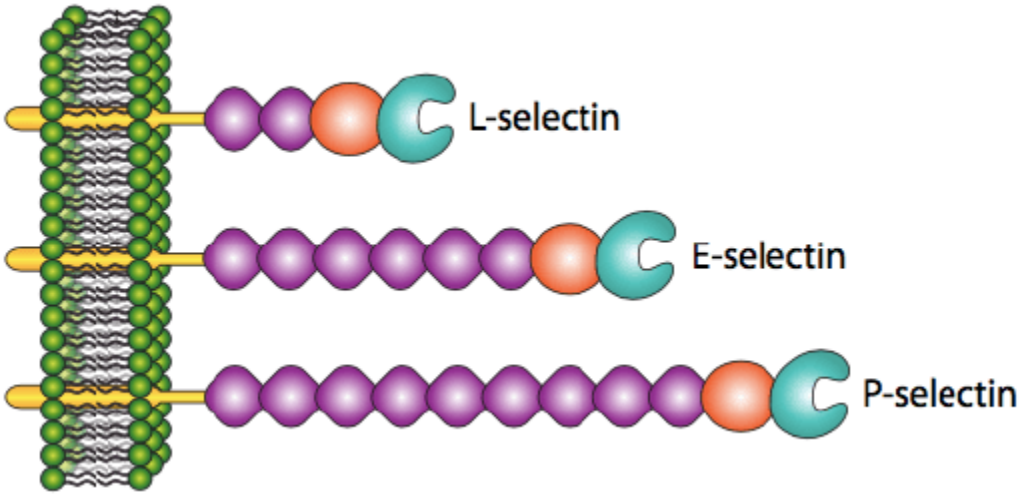


Figure 13.13.16. Selectins (L-, E-, and P-) are composed of between 2 and 9 short consensus repeat domains (purple), an EGF-like domain (orange), and the lectin-like binding domain (teal).

L-selectin, which is found on leukocytes, was the first discovered, by virtue of an interesting phenomenon called lymphocyte homing, in which lymphocytes were removed from various peripheral lymph nodes, labeled, and then injected back into the animal.

These lymphocytes would migrate back to the tissues from which they were derived without regard for the site of re-injection. The other two known selectins are E-selectin, which is expressed primarily on endothelial cells, and P-selectin, which is expressed on platelets and endothelial cells. The best-characterized ligand for selectins is PSGL-1, creatively named P-selectin glycoprotein ligand -1. Both E- and P-selectin are an important part of the inflammatory response, mediating the invasion of neutrophils from the bloodstream into the area of injury (Figure 13.13.17).

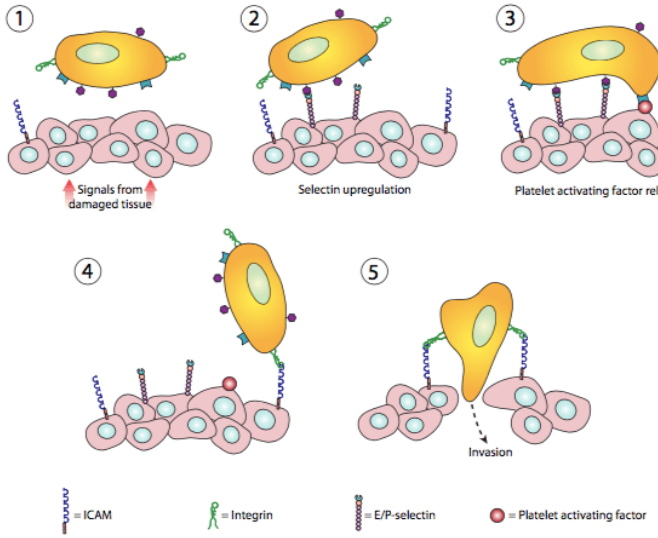


Figure 13.13.17. Neutrophil activation and invasion in the inflammatory response. First, endothelial cells in the blood vessel walls are activated by nearby damaged cells. These endothelial cells then begin to express E- and P- selectins, which bind onto neutrophils that are tumbling by in the bloodstream. This interaction slows the neutrophils down so that they are just rolling more slowly in partial contact with the endothelial cells. The endothelials release platelet activating factor, which activates α1β2 and αmβ2 integrins on neutrophils. Those integrins can bind onto the ICAM (and IgSF molecule) on the endothelial cell surface, stopping their movement. Finally the neutrophil binds to ICAMs on two adjacent cells, then remodels its cytoskeleton as it squeezes between the two to exit the bloodstream and move to the lesion site.

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13.14: Gap Junctions

Unlike the other types of cell-cell adhesion, the gap junction (sometimes called a nexus) connects not only the outside of two cells, it connects their cytoplasm as well. Each cell has a connexon (aka hemichannel) made of six connexin proteins. The connexins may be all of the same type, or combinations of different ones, of which there are 20 known in humans and mice. The connexon interacts with a connexon on an adjacent cell to connect the cytoplasm of both cells in a gap junction.

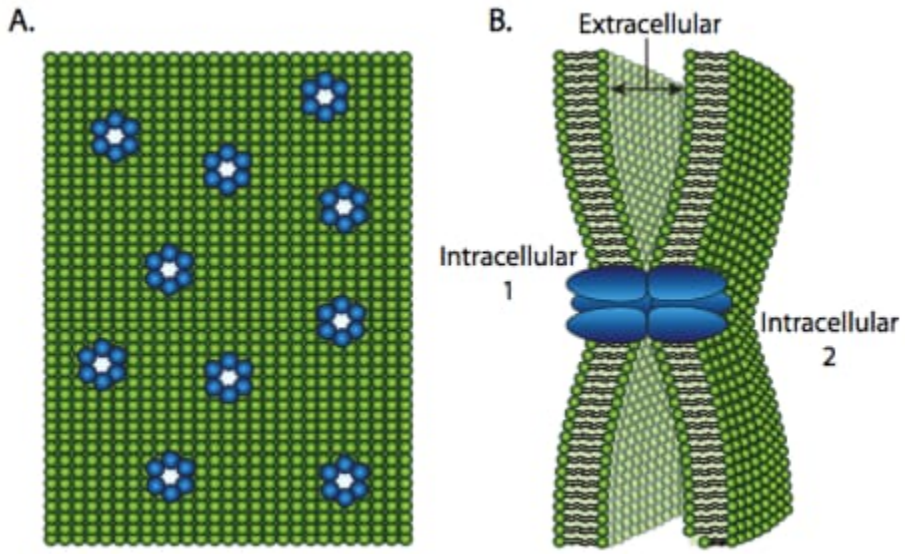


Figure 13.14.18. Gap Junctions

The gap junction pore size varies depending on the type of connexins, but generally, the molecules under 1 kDa are able to pass through while larger ones can not. Therefore, cells connected by gap junctions are electrically connected (ions can freely pass), they can share cellular energy (ATP), and second messenger signaling molecules like Ca^{2+} or IP_3, but not most proteins or nucleic acids. The pores are not always open, but are controlled by phosphorylation of several serines in the intracellular domains of each connexin.

Although they have now been found in most metazoan tissue types, they are particularly important in heart muscle. Here, the gap junctions insure efficient propagation of contractile signals so that the cardiac muscle can contract in synchrony. It is also important in cardiac development: gene knockout of connexin43, the primary heart connexin, leads to delayed looping of the ascending limb of the embryonic heart tube, which means malformations especially in the right ventricle, tricuspid valve, and subpulmonary outflow tract.

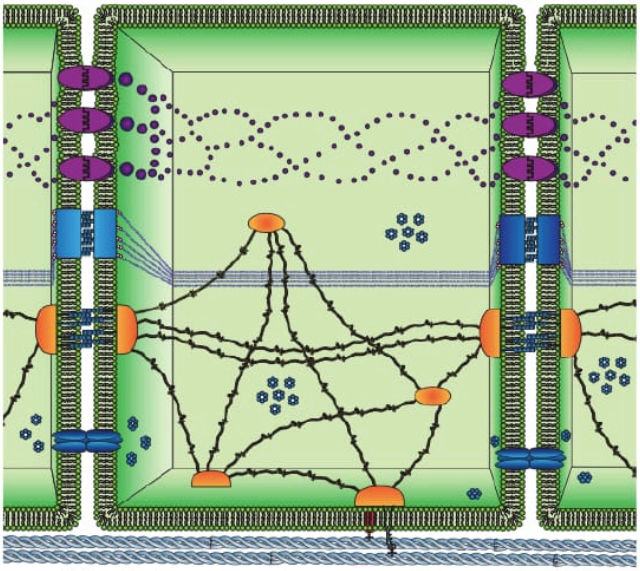


Figure 13.14.19. An overview of cellular adhesions. From top to bottom, there are tight junctions (purple), adherens junctions with f-actin (blue), desmosomes (orange) connected to intermediate filaments, and gap junctions (blue). There are also hemidesmosomes (orange) on the basal surface attached to the basement membrane

When most people, including most biologists, think about neuronal connections and synapses, they think of chemical synapses in which one cell signals to another by release of neurotransmitters. However, it is now well established that in the CNS, electrical synapses through gap junctions are a significant part of the repertoire of neural communication. The retina is an excellent example with numerous gap junctions between neurons. In fact, light-activated neurotransmitters can activate protein kinase pathways that phosphorylate connexins, thus altering conductance through the gap junctions. A striking example is the gap junction-based electrical coupling of cone photoreceptor neurons. They are coupled near the base of the cells, so that excitation of one drives excitation of several others. This is important in generating a clear visual signal because the primary reaction, phototransduction, is a dirty process. Due to the simple presence of random photons bouncing about, the signal to noise ratio of light-induced excitation is very low. However, because electrical coupling sums up the signal of near neighbors but not the background noise, the signal output from these neurons has an improvement in signal to noise ratio of ~77%! This topic is reviewed in Bloomfield and Volgyi, *Nature Reviews (Neuroscience)*, **10**:495-506, 2009.

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14: SIGNAL TRANSDUCTION

Metazoan organisms are not just conglomerations of cells that happen to stick together. The cells each have specific functions that must be coordinated with one another in order to assure the survival of the organism and thus the shared survival of the component cells. If coordination is required, then a method of communication between cells is also required. In fact, it is even more complicated than that because the communications between the cells only scratches the surface and the intracellular communication that goes on to coordinate multiple cellular activities in response to an external signal is usually far more complex than the initial transmission of that signal.

14.1: Introduction to Signal Transduction

14.2: Receptors and Ligands

14.3: 7-TM receptors (G-protein-coupled)

14.4: Receptor Tyrosine Kinases

14.5: Calcium Ion Signaling

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14.1: Introduction to Signal Transduction

There are three primary modes of intercellular communication. These are

1. direct contact between signaling molecules bound to the membranes of two adjacent cells,
2. short-range soluble signals that diffuse over short distances, and
3. long-range soluble signals that are secreted into the circulation to be carried anywhere in the body.

An example of juxtacrine signaling is exemplified by the activity of some cell adhesion or ECM proteins, such as laminin, that do not just allow a cell to move over them, but act as signals to promote increased motility. This likely happens by activation of integrin receptors on the moving cell, which then initiate and coordinate changes through the rest of the cell to accomplish the change in activity. Another example is the Delta-Notch pathway used in embryonic patterning.

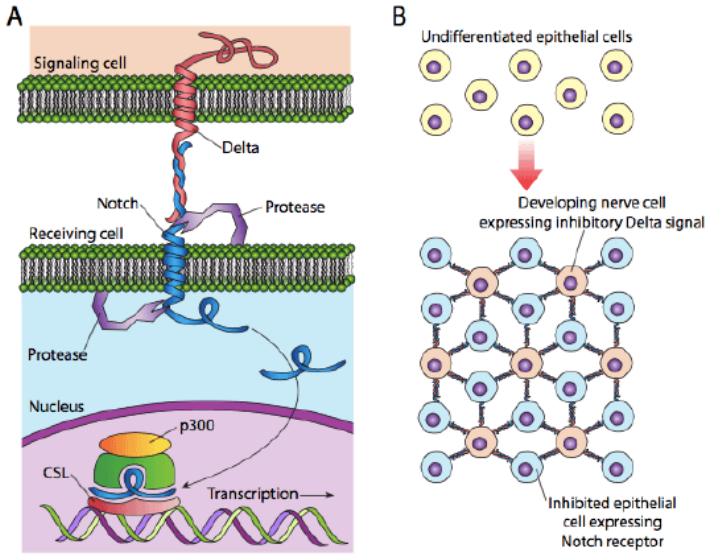


Figure 14.1.1. Delta-Notch signaling.

Delta, a transmembrane protein on the signaling cell, binds to Notch, a receptor on the receiving cell. Notch alters its conformation, allowing its cytoplasmic domain to be cut off by g-secretase. The cytoplasmic domain then translocates into the nucleus, where it acts as an activating transcription factor by binding with CSL. In the example sketched in Figure 14.1.1B, stochastic upregulation of delta in one cell activates notch in the surrounding cells, which then activates a specific differentiation pathway for them. Thus the central cell may be a sensory neuron, like a hair cell, while those immediately surrounding it are support cells like glia. This type of signaling imposes a spacing pattern on the expression of neurons (or other cell).

> The Delta-Notch pathway is well characterized and somewhat more complicated than portrayed in the paragraph above. The cleavage of Notch involves two proteases and two sites. Once the Notch cytoplasmic domain binds to CSL, it displaces a number of co-repressors bound to the CSL, and also recruits MAM (Mastermind-1) as a coactivator. MAM recruits histone acetylases to allow further increase transcription of targeted genes, but also recruits kinases that initiate the process of targeting the Notch cytoplasmic domain for ubiquitin-mediated destruction. The expression of Notch-controlled genes is thus self-regulated and shuts off soon after Delta is no longer available. Reviewed in R.A. Kovall, *Curr. Opin. Struct. Biol.* **17**: 117-27, 2007.

Diffusion limited signals from near neighbors is called *paracrine* signaling, and some- times the signals can act on receptors right on the cell that secreted the signal, which would be *autocrine* signaling. Paracrine signals are only active if they can bind to a cell above a critical concentration to activate a signaling pathway. Therefore, as the signals diffuse away from the source, there is a cutoff, beyond which the concentration of signal is insufficient to activate a receiving cell. Growth factors are often paracrine signals. Although they do often encourage growth, they are also often survival factors. In that context, Nerve Growth Factor (NGF)

is secreted by target cells that then reward the neurons that make the right connections by providing NGF for their survival. Those neurons that head off in the wrong direction, are unable to obtain NGF, and they do not survive, promoting efficiency and a better signal:noise ratio within the nervous system.

Endocrine signaling is essentially whole-body signaling. A signal produced by a hormone-producing gland is secreted into the bloodstream, where it becomes accessible to nearly any cell in the body. Of course, not every cell will respond to the hormone: like every other case of intercellular signaling, response is wholly dependent on receptors, so only the cells that have receptors to recognize the signal will react. For example, estrogen is released into the circulation, but in females, only some organs show significant impact when estrogen levels are significantly altered. Most tissues are unaffected. Endocrine signals may circulate in other extracellular fluids such as lymph.

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14.2: Receptors and Ligands

A protein that happens to bind something is not necessarily a receptor. A receptor is defined as a protein that binds to an extracellular ligand, and then undergoes a conformational or biochemical shift in such a way that it initiates a chain of intracellular events by which the cell reacts to the extracellular signal. What are these ligands and their receptors?

Intercellular signals span a very wide range of molecule types. Some are simple gases, like NO, while others are amino acids or derivatives, including glutamate, dopamine, or epinephrine. Lipids such as steroids (e.g. estrogen, cortisol) or eicosanoids (e.g. prostaglandins, leukotrienes) can be intercellular messengers. Finally many signals are peptides or even complex proteins (recall our juxtacrine signaling example). Although most are recognized by cell surface receptors, this is not always the case since, for example, steroids are lipid-soluble and can diffuse through the plasma membrane.

Receptors are a far less varied group of molecules, since they are all proteins, though it must be said that they represent many different protein structures and functions. In general, receptors are very speci c for their ligands, but the specificity is not mutual: ligands can be rather promiscuous and bind with multiple receptors. This is part of the coordination aspect of signaling, though as a single ligand can initiate different effects in different cells depending on what receptor is expressed. The remainder of this chapter will delve into some of the intracellular signaling cascades that are characteristic of particular receptor types.

Because receptors, even at high density, represent only a minute fraction of the surface area of the cell, and therefore an even tinier fraction of the volume of the cell, the activation of a receptor must be amplified in order for it to initiate cellular activities (e.g. locomotion, growth, cell cycle progression). Thus one of the rst things a receptor does upon activation is to initiate a signaling cascade. This aptly named sequence of events begins with the receptor activating an enzyme. The enzyme may be the cytoplasmic domain of the receptor itself, or it may be an independent protein but closely linked to the receptor. The enzyme does what enzymes do: it rapidly converts substrate molecules into product molecules. In this case, sometimes the product is an activator for another enzyme, and sometimes, the substrate is an inactive enzyme and the product is an activated enzyme. Either way, because of the high activity rates, the single activation of the receptor has increased first to tens or hundreds of enzyme activations, and each of those activates hundreds, and so on, so that the effect of the receptor can be rapidly distributed throughout the cell.

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14.3: 7-TM receptors (G-protein-coupled)

The 7-transmembrane receptors, or G-protein-coupled receptors are, unsurprisingly, a family of proteins that pass through the cell membrane 7 times. The amino terminal is extracellular and the carboxyl terminal is intracellular. Figure 14.3.2 shows the transmembrane regions spread out for clarity, but the transmembrane domains actually form together in more of a cylindrical shape.

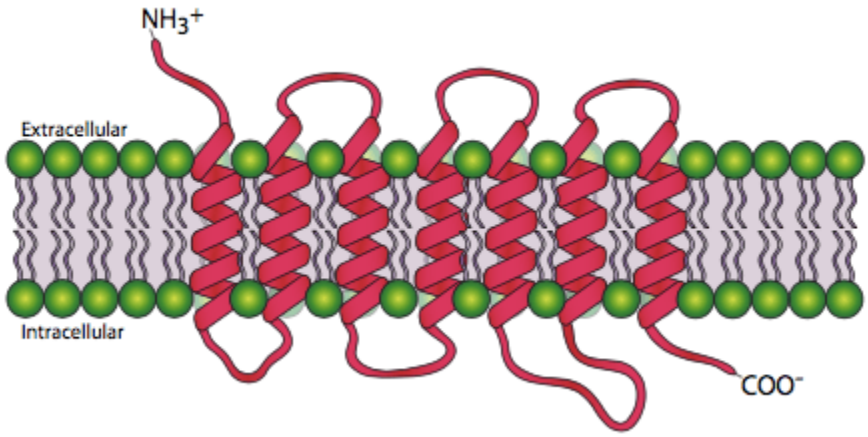


Figure 14.3.5. Activation of the 7-TM receptor rhodopsin by light.

7-TM proteins are used as receptors for neurotransmitters such as epinephrine (β-adrenergic receptor), acetylcholine (muscarinic receptor), and serotonin, as well as hormones like glucagon or thyroid-stimulating hormone, and even non-molecular ligands such as light! Rhodopsins are a class of 7-TM receptors that are activated when they absorb a photon (Figure 14.3.5). Activating this family of receptors, whether by photon or by more conventional ligand binding, induces a conformational change in the cytoplasmic domain that alters the interaction between the receptor and a protein complex known as a heterotrimeric G protein.

The heterotrimeric G protein consists of an a, b, and g subunit, of which the a subunit can bind either GTP or GDP, and can hydrolyze GTP to GDP. When the 7-TM receptor is inactive, the G protein complex is usually nearby associated with the membrane by myristoylation or palmitoylation of the a subunit and farnesylation or geranylgeranylation of the g subunit. Once the 7-TM receptor is activated, it associates with the heterotrimeric G-protein, which causes the Ga to let go of the GDP and bind to a GTP. This then dissociates the Ga from the other two subunits. It can then associate with and activate an enzyme to expand the signaling cascade. One of the two classical path- ways starts with Ga activation of adenylate (adenylyl) cyclase. Adenylate cyclase (AC) converts ATP to cAMP. Since ATP is plentiful and AC is a relatively fast enzyme, the rst ampli cation of the signal comes with generation of the “second messenger” molecule cAMP. Each cAMP molecule can then activate other enzymes, the primary one being protein kinase A. PKA can then phosphorylate a variety of substrates to alter cellular activity by gene expression, molecular motors, or metabolic changes.

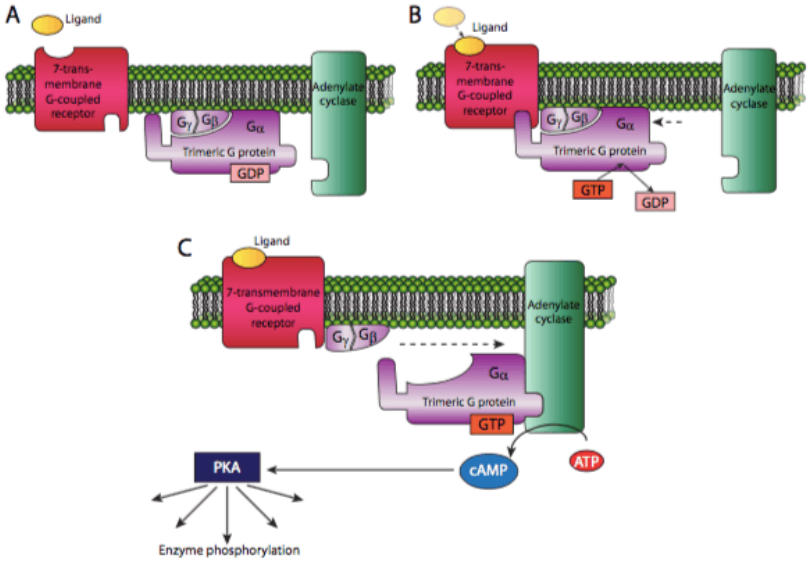


Figure 14.3.3. The heterotrimeric G-protein can act as a timed activator of adenylate cyclase.

The other classical pathway for 7-TM receptors is the activation of phosphlipase Cb, also by Ga. PLCb actually produces two second messenger molecules: it hydrolyzes phosphatidylinositol into diacylgylcerol (DAG) and inositol triphosphate (IP_3). IP_3 primarily induces the release of Ca^{2+} from the endoplasmic reticulum. The DAG can activate protein kinase C. PKC is also activated by Ca^{2+} and both Ca^{2+} and DAG can activate PKC synergistically. Protein kinase C is an important central kinase that has been shown to phosphorylate and control the activity of numerous other enzymes from cytoskeletal elements to transcription factors.

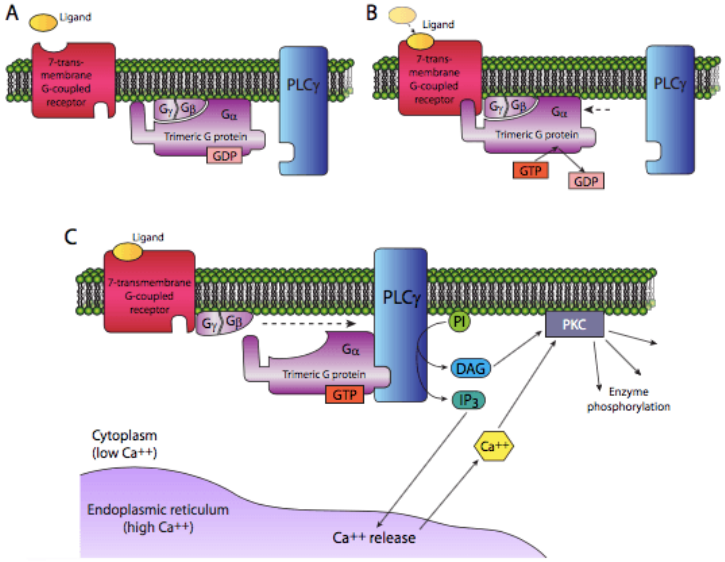


Figure 14.3.4. G-protein activated Phospholipase C. (A) The G-protein is inactive with GDP bound. (B) Upon ligand binding, G-protein binds receptor, exchanges GDP for GTP, and Ga dissociates. (C) Ga-GTP activates PLCg, which produces DAG and IP_3. The latter induces Ca^{2+} release into the cytoplasm and together with DAG, activates PKC.

An interesting variation from the classic 7-TM pathways starts with the rhodopsin receptors in rod cells. These receptors bind photons for activation, and engage a heterotrimeric G protein. The Ga-GTP then binds to the g subunit of phosphodiesterase (PDE), activating it and catalyzing conversion of cGMP to GMP. As cGMP decreases, ion channels close, polarizing the membrane and changing the signal from the rod cell to the brain (via connecting neurons).

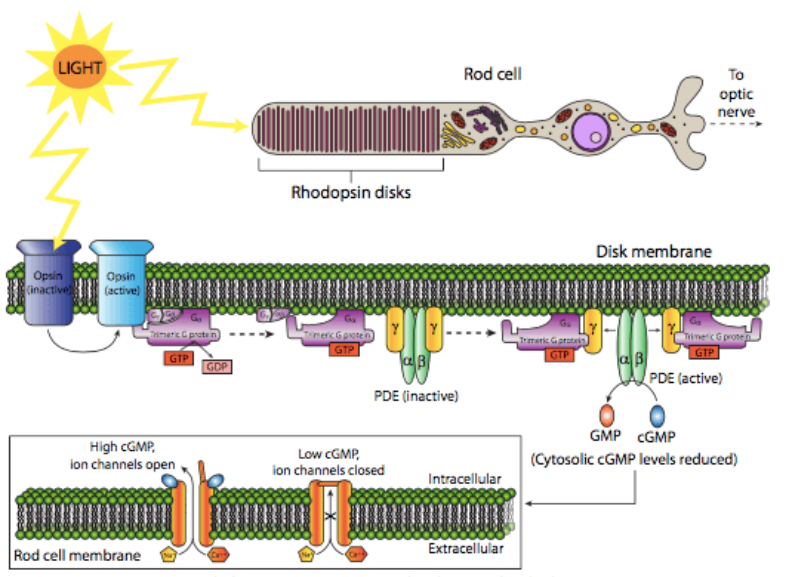


Figure 14.3.5. Activation of the 7-TM receptor rhodopsin by light.

Second messengers must have two properties. They must be small enough to diffuse effectively, and the cell must be able to generate them quickly. Ca^{2+} and cAMP fall into this category. Furthermore they can both be removed from circulation fairly quickly: the former by Ca^{2+} pumps in the ER and Golgi, and the latter by phosphodiesterase activity. When the G-protein-pathway was discovered, the use of lipid second messengers was surprising. Membrane phospholipids were largely ignored at the time as simple static components of membranes. It is now clear that some of the phospholipids are biochemically active, with several enzymes that modify them, including phospholipases, phospholipid kinases, and phospholipid phosphatases. Some of these enzymes have a variety of functions because their substrate of product may be an important messenger molecule. For example, PI3K (phosphatidylinositol-3-kinase) is a central signaling kinase because its product, PIP3 (phosphatidylinositol (3,4,5)-triphosphate) is an activator for Akt/PKB and other enzymes that can activate several signaling pathways.

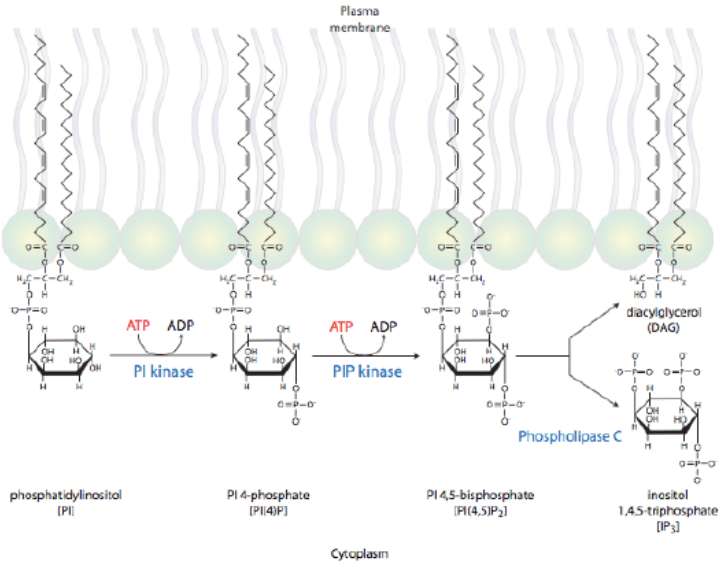


Figure 14.3.6. Modifications of Phosphatidylinositol generate various biologically active species.

The activation must eventually end, and it does so when Ga hydrolyzes the GTP bound to it. In this way, the Ga acts as a kind of timer for the signaling cascade. This is im- portant because for signaling to be effective, it must be tightly controlled. Very early in this course, it was pointed out that Ca^{2+} is kept at a very low concentration in the cytoplasm of the cell because we want Ca^{2+}-sensitive mechanisms to be able to react quickly to an influx of calcium, but we equally want to be able to quickly turn off the signal as needed, and that is obviously much easier to do by sequestering a small amount of Ca^{2+} than a lot of it. Similarly, if sustained activity of a recipient cell is called for, it is accomplished by continuous activation of the receptor and not by a long-lasting effect from a single activation. This ensures that if a cellular effect must be abruptly and quickly cut off, it can be accomplished without a significant lag period between cessation of hormone secretion and cessation of intracellular signaling.

The receptor is a part of another shutoff mechanism as well: to prevent overstimulation, the receptors are desensitized for a short time after they have activated. G-protein-coupled receptor kinase (GRK) phos- phorylates the 7-TM receptor. The phosphorylation creates recognition sites for arrestins. The arrestins have a variety of functions, the simplest of which is to act as a competitive inhibitorofG-proteinbindingbythe receptor. This is a relatively short- lived desensitization.

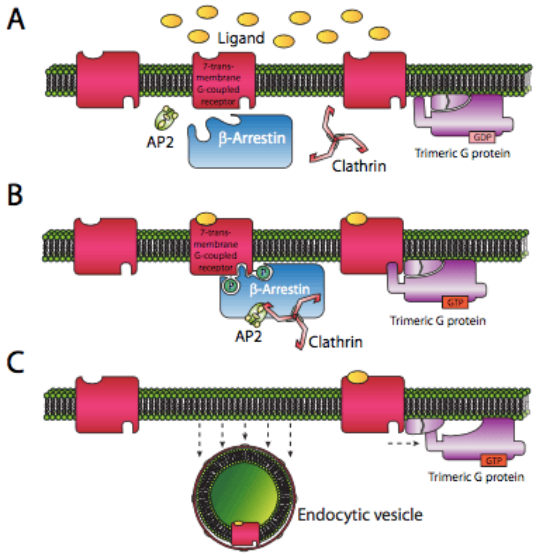


Figure 14.3.7. (A) After GRK phosphorylates a 7-TM receptor, arrestin can bind. In some cases, arresting can also bind AP1 andclathrin,(B)nucleatingtheformationofaclathrin-coated vesicle and endocytosis of the receptor (C).

For longer desensitization, arrestin binds to AP2 and clathrin, nucleating formation of a clathrin-coated endocytic vesicle. This removes the receptor from the cell surface, desensitizing the cell for a much longer period of time than simple competition between arrestin and G-protein.

The arrestins have another potential function. They can act as scaffolding proteins that bring a completely different signaling complex to the 7-TM receptor. Figure 14.3.8 shows an example in which the 7-TM receptor is used to activate a Jun transcription factor.

The b-arrestin brings AJK-1, MKKY, and JNK-1 to activate JNK-1, which can then phosphorylate Jun. This allows translocation of phospho-Jun into the nucleus and subsequent dimerization, converting it into an active transcription factor.

What are some of the cellular actions that can be evoked by 7-TM receptor activation? Ca^{2+} dynamics will be addressed in a separate section. IP_3 has been shown to evoke contraction of smooth and skeletal muscle, actin polymerization and cell shape changes, calcium release from intracellular stores, opening of potassium channels, and membrane depolarization. cAMP has been implicated in control of glycogen breakdown and gluconeogenesis, triacyglycerol metabolism, secretion of estrogens by ovarian cells, secretion of glucocorticoids, and increased permeability of kidney cells to water.

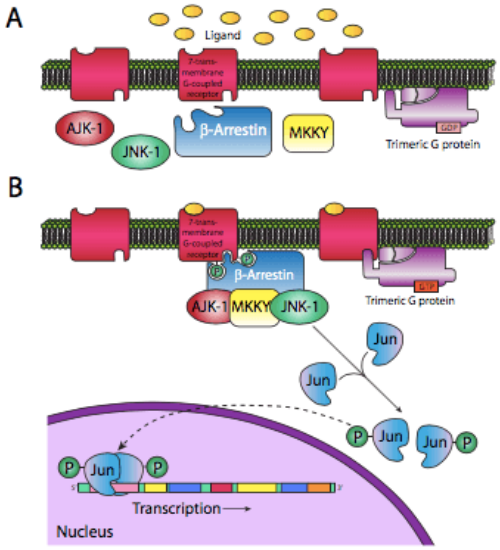


Figure 14.3.8. Interestingly, arrestins can also act to initiate a completely different type of signaling cascade from a 7-TM receptor. Here, the Jun transcription factor is activated.

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14.4: Receptor Tyrosine Kinases

In contrast to the 7-TM receptors, the receptor tyrosine kinases (RTK) pass through the membrane only once, and have a built-in enzyme domain - a protein tyrosine kinase. RTKs must dimerize to be functional receptors, although individual RTKs can bind to their ligands. The ligands also dimerize, and when a dimerized receptor is activated, the kinase domains cross-phosphorylate the cytoplasmic domain on the other receptor unit.

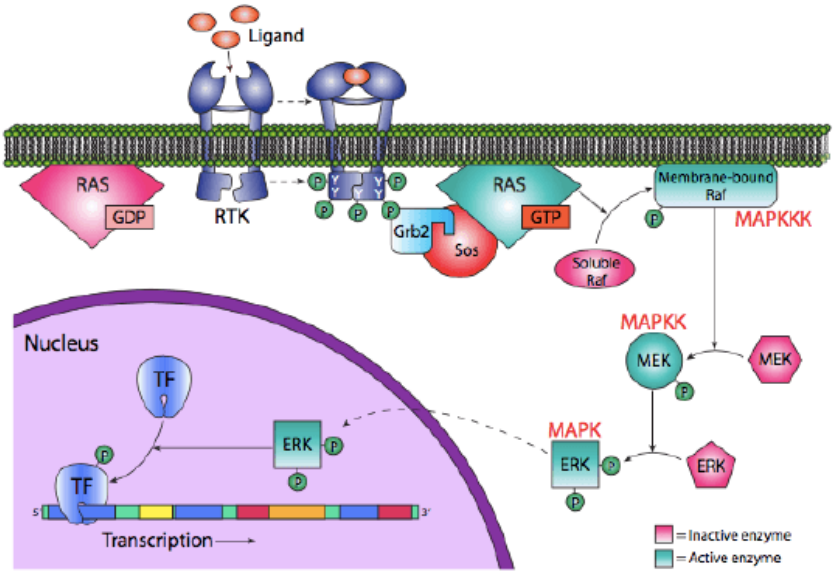


Figure 14.4.9. Receptor Tyrosine Kinases can activate the MAP pathway.

This phosphorylation is necessary to form recognition sites for scaffolding or effector proteins. Figure 14.4.9 shows an example of an adapter protein, Grb2, which binds to a phosphorylated SH2/SH3 type domain on the receptor as well as to Sos (a guanine nucelotide exchange factor), which binds to and activates the GTPase Ras by exchanging a GDP for a GTP. This is the start of a very common RTK intracellular signaling pathway, the MAP kinase pathway. Following activation of Ras, it can activate Raf by phosphorylation and translocating it from the cytoplasm to the inner surface of the plasma membrane. Raf is a Ser/Thr kinase (also known by the unwieldy but fun to say, MAP kinase kinase kinase) that phosphorylates MEK (aka MAP kinase kinase). MEK is interesting because it is a dual-specificity kinase, phosphorylating both Ser/Thr sites as well as Tyr sites. The targets we are particularly interested here though, are MAP kinases (mitogen activated protein kinase), also known as ERKs (extracellular signal regulated kinases).

Each kinase along the canonical MAP kinase pathway has other potential substrates besides the next one in the MAPK sequence, so the variety of cellular responses that can be initiated by this pathway is very broad. There are at least 20 classes of RTK by structural similarity, including the fibroblast growth factor receptor (FGFR) class, epidermal growth factor receptor (EGFR) class, neurotrophin receptor (Trk) class, and insulin receptor class. Some growth factors not only induce growth, but survival, and sometimes proliferation. In fact, mutations to growth factors can be oncogenic (cancer-causing).

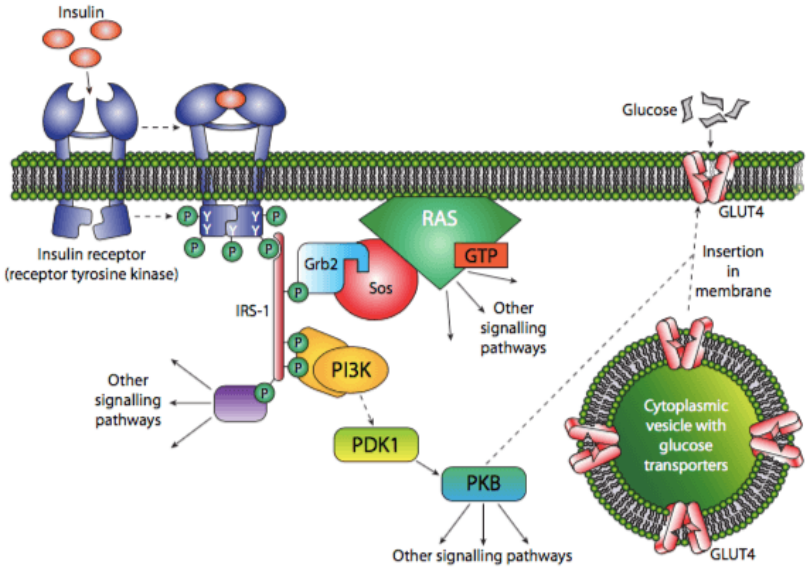


Figure 14.4.10. Insulin receptor signaling pathways.

One of the aspects of cell signaling that make studying it both fun and frustrating is the immensity of possibilities. The insulin receptor example above (Figure 14.4.10) demonstrates this. When the receptor is activated, the IRS-1 scaffolding protein binds to it, and brings with it binding sites to recruit a number of different signaling molecules such as Grb2-Sos-Ras to head down the MAPK pathway, but also PI3K, which can lead to activation of PDK1 and Protein Kinase B, important in regulation of glucose transport. PKB (also known as Akt), is also an important mediator of cell survival (by inhibiting BAD), cell proliferation, and angiogenesis.

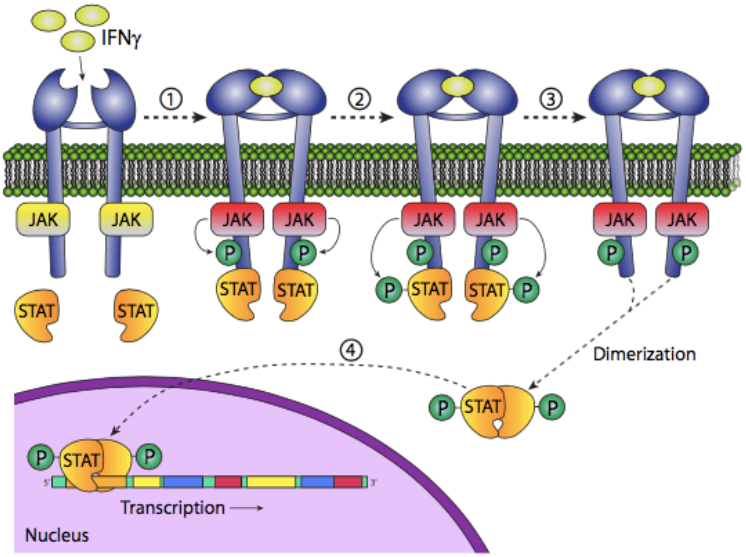


Figure 14.4.11. The JAK-STAT pathway.

Activation of cytokine receptors can initiate the JAK-STAT pathway. Cytokines are generally immunomodulatory signals, some of which act as hormones and others in a paracrine fashion. Interferon-γ is an example (Figure 14.4.11) of a cytokine, and the inactive RTK receptor binds to JAK (Janus kinase) in the inactive state. Upon ligand binding to the dimerized receptor, the JAK units are activated and the phosphorylate the receptor. This receptor phosphorylation leads to binding of STATs (the creatively named “signal transducers and activators of transcription”), which are then phosphorylated by the still-active JAK. Upon phosphorylation, the STAT-P proteins dissociate from the receptor and dimerize in the cytoplasm, where they are bound by importins and translocated into the nucleus where they act as transcription factors.

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14.5: Calcium Ion Signaling

Signaling by increasing cytosolic calcium is an important and ubiquitous intracellular coordination mechanism. We already saw that release of Ca^{2+} in muscle cells is required to allow contraction of each sarcomere, and the positioning of the sarcoplasmic reticulum makes possible rapid changes in concentration nearly simultaneous across the entire cell. Another extremely important physiological mechanism that relies on calcium is fertilization. Immediately upon penetration of the egg by the sperm, a wave of intracellular Ca^{2+} increase spreads across the egg starting from the point of fertilization. This activates CaMKII (a kinase) and calcineurin (a phosphatase). Both are needed to overcome meiotic arrest and may also be necessary in initial embryonic development by control of chromatin decondensation, nuclear-envelope formation, and the movement and fusion of the two nuclei.

The aforementioned CaMKII is Ca^{2+}/calmodulin-dependent kinase II, and illustrates a fairly common theme, which is the use of Ca^{2+}-binding proteins as intermediate Ca^{2+} sensing activators. Calmodulin is a ubiquitous calcium-binding protein in eukaryotes and its importance is highlighted by the extraordinarily high homology across species. In normal cytosolic Ca^{2+} levels, the relatively low affinity of the 4 Ca^{2+} binding sites on calmodulin are unfilled. But when Ca^{2+} concentrations rise, they occupy the sites, causing a conformational change in calmodulin and allowing it to interact with other proteins. In addition to calmodulin, troponin-C, and PKC, a few other important calcium-sensitive proteins are calsequestrin, a Ca^{2+} buffer protein, gelsolin, the f-actin severing enzyme, the protease calpain, and calretinin, an activator of guanylyl cyclase (which makes the second messenger cGMP).

Guanylate (guanylyl) cyclase is also an important player in signal transduction by nitric oxide (NO). Nitric oxide is a gas produced by the action of nitric oxide synthase (NOS) on the substrate amino acid arginine. It is used as a super-soluble signal that passes through cells easily. However, it requires relatively high concentrations for physiological effect, so it is strictly a paracrine factor working on near neighbors. Perhaps the best studied example of NO signaling is vasodilation, in which the NOS-expressing endothelial cells of a blood vessel release NO to the smooth muscle cells surrounding them. The NO binds to and stimulates guanylate cyclase. The resulting increase in cGMP concentration leads to relaxation through multiple targets of protein kinase G.

> Sildena l (Viagra) and its chemical siblings take advantage of this pathway by inhibiting cGMP-specific phosphodiesterases (PDE5) which normally break down cGMP to limit the response to NO. However, it should be noted that though PDE5 expression is limited, it is expressed not only in the genitalia but in the retina as well.

Finally, no discussion of signal transduction would be complete without at least a fleeting mention of the extraordinary crosstalk (Figure 14.5.12) that can occur between the different pathways mentioned.

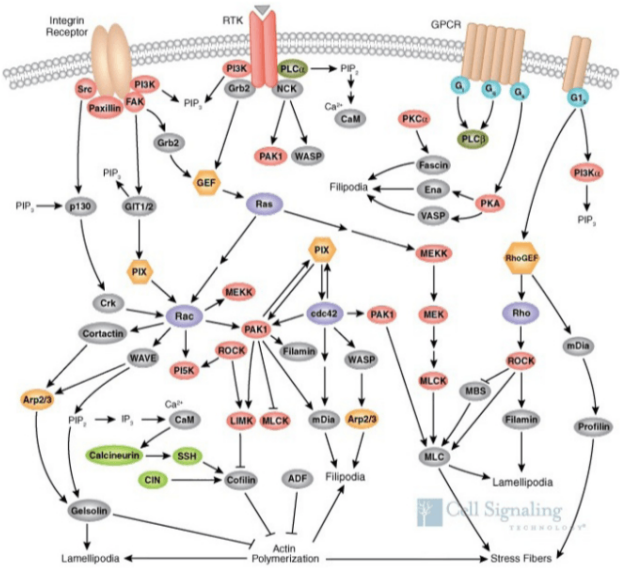


Figure 14.5.12. Signal transduction in actin dynamics. This Figure comes from Cell Signaling Technology, Inc.

The Figure represents only one small part of the signaling that happens inside a moving cell. Not only are some parts of the cell forming filopodia to help determine where to go, other parts or ruffling up the lamellipodia, and still others inducing motor proteins to rearrange the cytoskeleton in the proper way to facilitate bulk transport internally even as the leading edge of the cell is thrusting forward to make contacts externally. All of this must be coordinated by crosstalk between signaling systems as depicted, not to mention signaling related to metabolism, or gene expression, or even cell cycle, all of which are happening simultaneously inside the cell.

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15: CELL CYCLE

Cells, whether prokaryotic or eukaryotic, eventually reproduce or die.

15.1: The Prokaryotic Cell Cycle

15.2: The Eukaryotic Cell Cycle

15.3: Controlling the Cell Cycle

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15.1: The Prokaryotic Cell Cycle

Cells, whether prokaryotic or eukaryotic, eventually reproduce or die. For prokaryotes, the mechanism of reproduction is relatively simple, since there are no internal organelles. The process consists of three distinct but short phases: first, a growth phase in which the mass of the cell is increased, then the chromosomal replication phase, and finally the chromosomes are separated and the cells are physically split into two independent new cells. In bacteria, these are referred to as the B, C, and D periods, respectively. Initiation of the reproductive process appears to be primarily a function of cell size. The length of the overall cell cycle is determined by the B period, as the C and D periods have relatively fixed time constraints. The length of B is determined, in part, by environmental conditions and the gain in cell mass. Generation times for bacteria can vary from under half an hour to several days, although most bacterial cultures in laboratory settings and nutrient-rich media have generation times under a day.

DNA replication has already been covered in detail in Chapter 7. In bacteria, the process is initiated at the origin of replication by DnaA. However, in archaea, synchronous initiation of replication at multiple sites on the chromosome as well as recognition proteins homologous to eukaryotic ORC proteins suggests that there are similarities between archaebacterial and eukaryotic DNA replication to be explored.

Once the DNA is replicated and moved to opposite sides of the cell, the midcell septum forms to split the cell. At least 9 gene products are involved in this process including FtsZ, the prokaryotic tubulin homologue that forms a circumferential ring, FtsI, a peptidoglycan synthetase involved in septum formation, FtsL, whose function is unclear but is involved in ingrowth of the cell wall at the septum, and ZipA, which anchors the FtsZ ring. The ring contracts, pulling the membrane in with it. Eventually the membrane is pinched in enough to fuse and generate two completely separate cytoplasmic compartments. Other septation enzymes make cell wall components that ll in as the septum forms simultaneously with membrane/FtsZ contraction, and the cells separate.

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15.2: The Eukaryotic Cell Cycle

Most eukaryotic cells undergo a reproductive cycle to generate either another copy of themselves or to generate gametes (sex cells), and in doing so require a complex mechanism to govern the safe and accurate replication of their much larger (than prokaryote) genomes. Immediately following mitosis, the newly created cells are in the G_1 phase. This is largely a growth phase, during which there is a lot of biosynthesis of proteins, lipids, and carbohydrates. However, there is no synthesis of new DNA at this time. G_1 is the longest of the cell cycle phases in many cell types, and most of the physiological activity of a cell happens during G_1. Following G_1, the next phase of the cell cycle is the S phase, during which synthesis of new DNA occurs. In other words, the genome is being replicated during this phase; thus at the end of S phase, the cell has twice the normal amount of DNA. After S phase, the cell proceeds into G_2, which provides an opportunity for the cell to perform a self-assessment and make final preparations (such as more cell growth, repairs of DNA) as necessary before it finally heads into mitosis. Mitosis, or M phase, is primarily

1. the breakdown of the nucleus,
2. re-distribution of the DNA to opposite sides of the cell, and
3. formation of two new nuclei around that DNA, and cytokinesis, the final splitting of the cell itself.

As the cell progresses through the various phases of mitosis, and for that matter, the phases of the cell cycle overall, it does so in a specific and controlled manner, with checkpoints that “ask” if the cell is ready for the next step: is it big enough, is the DNA healthy, etc. so that the cell has the best chance of generating healthy daughter cells. For example, if the cell cycle runs too rapidly through each phase, then there is not enough time for the cell to build up its mass in preparation for reproduction, and that leads to abnormally small daughter cells, and potentially even daughter cells that are too small to survive. If a cell undergoes mitosis with damaged or mutated DNA, then that may increase the likelihood of a pathological mutation surviving and harming the organism by turning into a cancerous tumor.

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15.3: Controlling the Cell Cycle

There are three major checkpoints for cell cycle control (Figure 15.3.1). The first regulates the transition from G_1 to S phase. Recall that G_1 can be a very long phase, even (in the case of G_0) as long as the lifespan of the cell. However, once the cell reaches S phase, it is committed to going through S, G_2, and M phases to reproduce. This is because once S phase has begun, there is more than the normal diploid complement of DNA inside the cell. Over time this would confuse the cell (e.g., by overexpression of duplicated genes) as it tried to use the DNA to direct RNA and protein synthesis, and it could become sick and die. The second major checkpoint regulates entry into mitosis. Once mitosis begins, most of the metabolic activity of the cell is shut down, and the cell concentrates its resources on dividing the nuclear and cellular material equally to support the life of both resulting daughter cells. If the cell needs more time to make final repairs on the DNA or even to bulk up a little, this checkpoint can hold the cell in G_2 a little longer for those things to happen. Finally, the third major checkpoint occurs during mitosis, and regulates the transition from metaphase into anaphase. Since the sister chromatids are being split apart and moved to opposite poles to form the new nuclei, it is important that all of them are perfectly lined up at metaphase and the proteins holding them together have dropped off. If they do not split evenly, the daughter cells will have abnormal numbers of chromosomes (aneuploidy) usually leading to deleterious consequences.

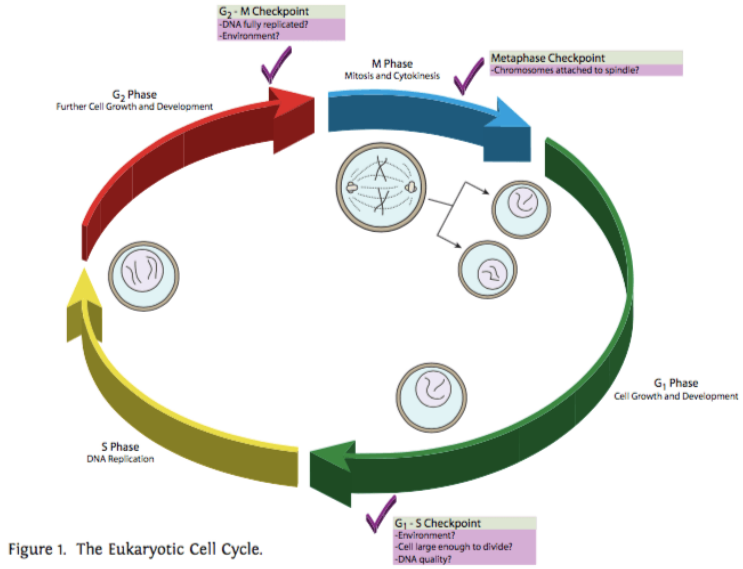


Figure 1. The Eukaryotic Cell Cycle.

Figure 15.3.2). Interestingly, the intracellular level of cdks is fairly constant. The level of cyclins, on the other hand, uctuates dramatically depending on the state of the cell with respect to the cell cycle.

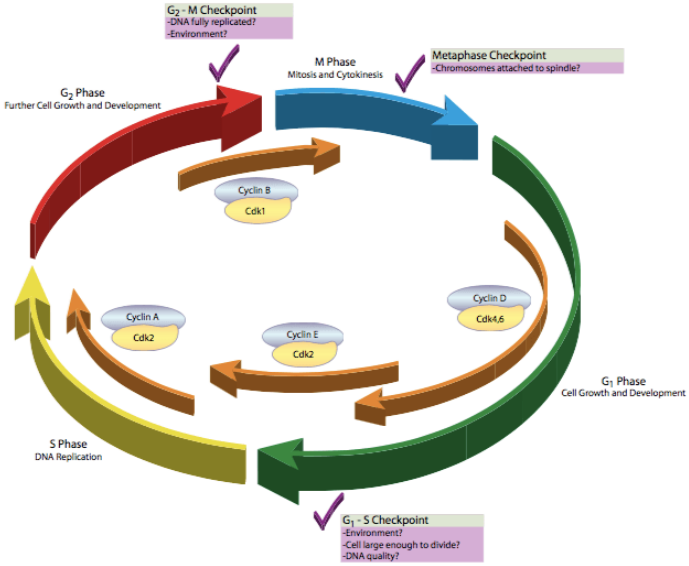


Figure 15.3.2. Cyclins are involved in control of the cell cycle.

The methodology of some of the early experiments is perfectly suited to explaining how this works. The seminal paper in this field was a 1971 paper in J. Exp. Zool. by Masui and Markert. In it, they examined frog (Xenopus laevis) eggs that were arrested at G_2. The oocytes arrest for about 8 months naturally in order to build up the mass needed to start a new organism once it has been fertilized. The basic question being asked is what is causing the eggs to come out of G_2 and into M phase? It was already known that the hormone progesterone can trigger this transition, but what are the intracellular players in the change in cell state? Masui and Markert decided to test whether there was a cytoplasmic molecule that was responsible. They took a small amount of cytoplasm from an M-phase egg and injected it into a G_2-arrested egg. This triggered the maturation of the G2-arrested egg and pushed it into M phase, even without progesterone. The activity was called maturation promoting factor (MPF), and was hypothesized to be a soluble, cytosolic protein.

In later experiments, other investigators attempted to find the specific protein trigger, and from there, presumably, the rest of the mechanism. Fractionating the M-phase oocyte cytoplasm by column chromatography, a protein, named cyclin B, was found to rise and fall in concentration in direct synchronization with MPF activity. Furthermore, addition of cyclin B alone was sufficient to rescue MPF activity from M-phase cytoplasmic extract that had been depleted by RNase treatment (preventing synthesis of any new proteins, including cyclin B, and abolishing MPF activity). This clearly places cyclin B in the forefront of the maturation mechanism, but there was one major issue: cyclin B had no enzymatic activity. How was it effecting the changes needed for progress from G_2 to M phase?

This problem was answered by experiments on a very different organism, the fission yeast, *Schizosaccharomyces pombe*. Because they have a very short cycle time, a relatively small genome, and they can be given random mutations *en masse* by irradiation or chemical treatment, yeast are excellent model organisms for many types of biological study. After random mutation of a population of yeast, they can be screened for mutations of particular types, such as cell division cycle (cdc). When the mutations are sequenced and identified, they are often named by the type of mutation and order of discovery. Cdc2, it turns out, showed two interesting phenotypes when mutated in opposite directions. Mutations that knocked out function of cdc2 caused the formation of extremely large yeast that do not undergo cell division, while mutations that made cdc2 overactive caused the formation of rapidly dividing very small cells. The interpretation was that when cdc2 is missing or inactive, the cells cannot progress to mitosis, so they stay in G2 accumulating bulk material in preparation for a cell split that never comes. Conversely, when cdc2 is overactive, it drives the cell quickly into mitosis, even if it has not been in G_2 long enough to synthesize enough mass to form two normal-sized cells. This ties cdc2 nicely to cell cycle regulation, and it even has an enzymatic activity: it is a kinase. This made it a perfect candidate as a first-order coordinator of cellular events because phosphorylation is fast, phosphorylation usually activates some other enzyme, and kinases usually act on an array of targets, not just one. So we now have a cyclin (identified as cdc13 in *S. pombe*) and a cyclin-dependent kinase that work together to promote cell cycle progression into M phase.

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15.4: Activation and inactivation of the cyclin-cdk complex

As more mutant yeast were being screened for changes to their cell cycle, two other genes were found in which mutations gave rise to similar phenotypes. Nonfunctional cdc25 or overactive wee1 mutants generated the overly large cells with a single nucleus, and conversely, overactive cdc25 or inactive wee1 generated many severely undersized cells. Both cdc25 and wee1 gene products interact with cdk, and in fact, they are positive and negative regulators of cdk, respectively. Acting together with one more enzyme, CAK (cdk-activating kinase), they activate the cdk (Figure 15.4.3).

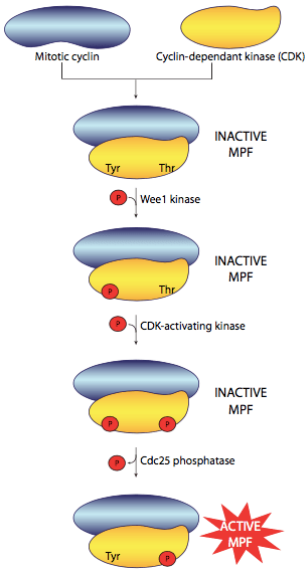


Figure 15.4.3. Activation of mitotic cyclin/cdk complex

Using the mitotic cyclin/cdk complex as an example, the cyclin (cdc13) and cdk (cdc2) come together to form an inactive complex. The cdk is then phosphorylated by wee1, a kinase. The phosphate it puts on tyrosine-15 is needed for the rest of the activation sequence, but it is inhibitory: it actually *prevents* final activation. But once Tyr-15 is phosphorylated, CAK can phosphorylate a neighboring threonine (Thr-161), which is required for activation. Finally, cdc25, a protein phosphatase, removes the phosphate on Tyr-15, allowing activation of the cdk by the phosphorylated Thr-161, and the MPF is finally on its way. There is self-amplification of the activation as well, because one of the targets of MPF is cdc25, so there is a positive feedback loop in which the activity of cdc25 is upregulated by phosphorylation.

As you will see in a later section of this chapter, MPF performs many functions, some of which prevent progress of mitosis past anaphase. Therefore, there must be a way to turn off MPF (and for that matter, any cyclin/cdk complex) quickly and completely when the cell reaches the appropriate stage of the cell cycle. This is borne out by time-course studies of MPF activity, which show a precipitous drop in activity in anaphase. This coincides with a depletion of the cyclin B (cdc13 in *S. pombe*) due to a combination of turning off transcription of the gene, and specific proteolytic degradation. The degradation pathway is now well understood, and is an interesting example of a sort of feedback regulation.

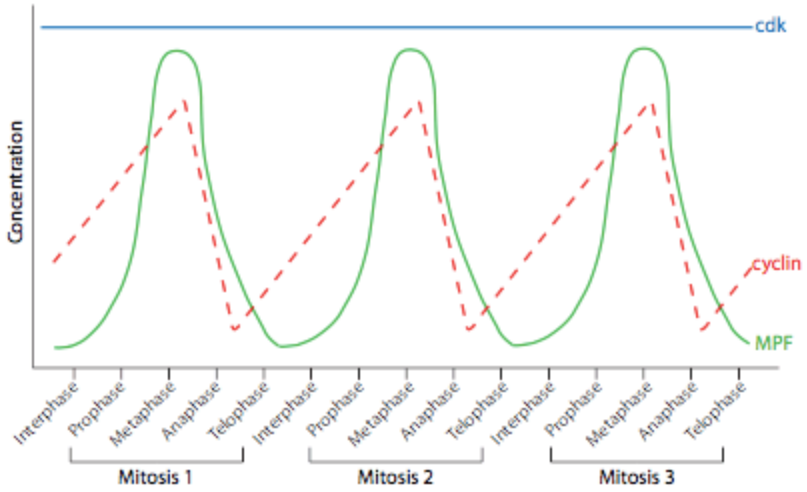


Figure 15.4.4. MPF activity and cyclin B protein expression rise as the cell enters mitosis but drop just before anaphase. However, cdk levels remain steady throughout the cell cycle.

Essentially, MPF ensures its own destruction: one of its phosphorylation targets is cdc20. Upon phosphorylation, cdc20 is activated and then activates anaphase promoting complex (APC). APC is a ubiquitin ligase (type E3) that polyubiquitinates the cyclin of the MPF complex, making it a target for proteolytic degradation by a proteosome. Note that only the cyclin is destroyed, while the kinase is left alone. Without the cyclin, the kinase is inactive and must wait for cyclin levels to rise again before it can be re- activated by a fresh round of phosphorylation and dephosphorylation.

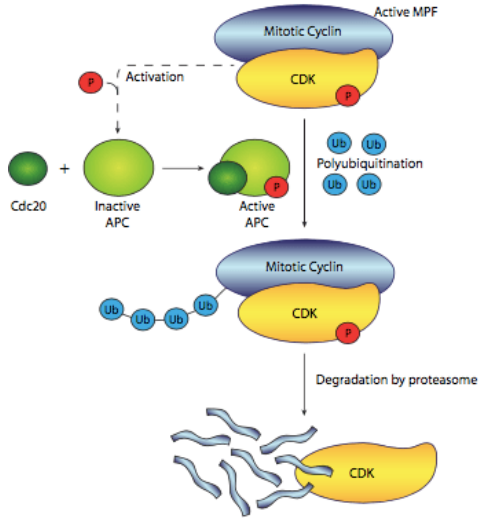


Figure 15.4.5. MPF and other cyclin/cdk complexes are inactivated by destroying the cyclin.

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15.5: Pre-mitotic Phases

G_1/G_0 Phase

The G_1 phase is the state a cell is in immediately following cytokinesis. At that point, the cells will be somewhat undersized, and need to take up materials and energy sources, and convert them to cellular components in order to support the eventual cell division. During this time, the cell goes about doing its “normal” business - an endocrine cell makes and secretes hormones, an intestinal epithelial cell absorbs nutrients from the gut and passes them on to the bloodstream, a neuron conducts signals, etc. Most types of cells spend the majority of their cycle in G_1, although there are exceptions, such as the frog oocytes mentioned earlier. The length of G_1 is generally constant for a given cell type under normal conditions, but can vary greatly between different cell types. Post-mitotic cells, which have left the cell cycle and will no longer divide, are in G_1 until they die, barring reactivation of the cell cycle by stress conditions. This continuous G_1-like state is referred to as G_0.

For those cells preparing to move from G_1 into S, cyclins D and E, and cdk 2, 4, and 6 predominate, with activation of cyclin D complexes preceding activation of cyclin E complexes. Two major questions are asked by the cell: is the DNA undamaged and complete, and is the extracellular environment favorable for cell division? The cellular sensors for these conditions then link to cyclin complexes effect restriction points on cell cycle progression. The extracellular environment questions can be a tricky one, because this can include more than just assessment of nutrient availability or predatory threats; it can also be a requirement for an external trigger such as a mitogenic hormone or paracrine signal. In fact, nearly all normal animal cells require an extracellular signal to progress through the G1/S checkpoint. The cyclin E/cdk2 combination is the principal regulator of entry into S phase and DNA replication.

> The active cyclin E/cdk2 complex phosphorylates the tumor suppressor protein Rb (retinoblastoma), which causes E2F to translocate to the nucleus and turn on genes needed for entry into S phase.

S phase

The mechanisms of DNA replication were discussed in Chapter 7. It is important to note that once a cell has entered S phase, it has essentially committed to going through cell division. Cells do not cope well with extra copies of chromosomes, and a cell that went through S phase without going through mitosis would likely have major malfunctions in gene regulation. For similar reasons, the cell must only undergo DNA replication once per cell division. The cyclinA/ cdk2 complex plays a key role in initiation of repli- cation by activating the pre-replicative complex. It also phosphorylates cdc6, causing it to dissociate from the ORC, and consequently the rest of the pre-RC. This prevents immediate re-use of this origin of replication, and since the phosphorylation of cdc6 allows it to be recognized by a ubiquitin ligase complex, it is tagged for proteolysis In addition to DNA replication, S phase is also the cell cycle stage in which centrosomes are duplicated in animal cells. The cyclin E/cdk2 combination licenses the duplication of centrosome, phosphorylating nucleophosmin, which then dissociates from the cen- trosome. This helps to trigger the centrosome duplication. Nucleophosmin does not reassociate with centrosomes until telophase, when it is no longer phosphorylated. Plk4 (Polo-family kinase 4) activity is necessary for centriole duplication, and appears to initiate the centriole assembly mechanism.

> The ubiquitin ligase complex, SCF, is made up of three major proteins and several minor species. Skp1 (S-phase kinase-associated protein 1) can be an RNA polymerase elongation factor, but in this complex links the other two proteins together. Cul1 (Cullin 1) is an E3 type ubiquitin ligase. Finally, an F-box family protein like Rbx1 (Ring-box 1), that heterodimerizes with cullin-1 and may also recruit E2 ubiquitinating enzyme.. In addition to cdc6, it also recognizes and ubiquitinates CKIs (cyclin complex kinase inhibitors) such as p27, which is involved in processes such as DNA repair and error-checking.

G_2 Phase

The G_2 phase begins when DNA replication has completed. Having said that, before the cell is allowed out of G_2 and on to M phase, it must pass a DNA fidelity checkpoint, ensuring that not only has replication been fully completed, but that there are no major errors. G_2 is a relatively short phase (compared to G_1) in most cell types, and it is spent building up energy and material stores for cell division and checking the DNA. If everything is ok, and the cyclin B/cdk1 complex has been activated, the cell proceeds to M phase.

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15.6: Mitosis

Mitosis consists of prophase, metaphase, anaphase, and telophase, with distinct cellular activities characterizing each phase. This completes the duplication of the nucleus, and is followed by cytokinesis, in which the cell divides to produce two daughter cells.

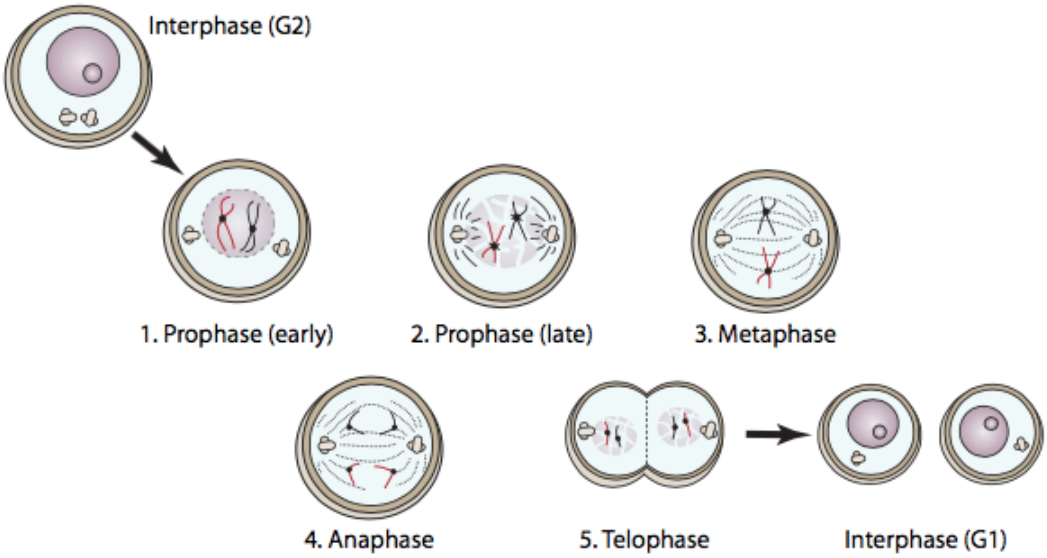


Figure 15.6.6. Mitosis. During mitosis, the nuclear envelope breaks apart to allow the spindle access to the chromosomes. Once they have been moved to opposite ends, the nuclear membrane reforms around each set. Finally, cytokinesis divides the cell into two new daughter cells.

Prophase is the preparation of each component for this complex cellular dance. The DNA condenses (it is wrapped around itself tightly to make it a smaller and stronger package) so that it is less susceptible to breakage during movement across the cell. In doing so, most of the DNA becomes transcriptionally inactive. The Golgi bodies and the endoplasmic reticulum begin to break apart into membranous vesicles that can be more easily and evenly distributed across the cell so that both daughter cells receive about the same. The centrosomes (in animal cells) move from their original position near the nucleus toward opposite sides of the cell, to establish the poles of the mitotic spindle.

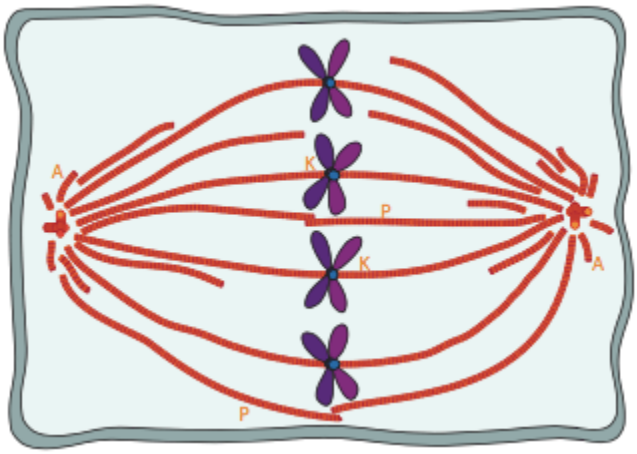


Figure 15.6.7. The mitotic spindle. The spindle is made of microtubules that originate from the centrosomes, which have migrated to opposite sides of the cell. There are three types of spindle microtubules: the kinetochore microtubules (K), polar microtubules (P), and astral microtubules (A).

MPF phosphorylates microtubule motor proteins and microtubule associated proteins (MAPs) to alter the normal microtubule dynamics and allow the massive reorganization into a mitotic spindle to occur. For example, one target of MPF is PRC1, a bundling

protein that is inactivated by phosphorylation, thus allowing individual microtubules to move to new locations more easily than a large bundle could. Other effects are inactivation of stabilizing MAPs, which leads to greater lability of microtubules due to increased incidences of catastrophe. The motor protein targets of MPF are in the kinesin family and the phosphorylation is necessary for some of them to bind to the mitotic spindle.

Prometaphase is sometimes considered a separate phase but is also referred to as late prophase, and is primarily defined by the breakup of the nuclear envelope. This process is induced by MPF phosphorylation of the nuclear lamins. Adorned with negative charges from the phosphates, the lamins refuse to associate with one another any longer, leading to the breakdown of the nuclear lamina. As the lamins dissociate, the nuclear envelope remains bound to them, and fragments. This nuclear fragmentation must happen so that the mitotic spindle can reach inside and attach to the chromosomes.

Some of the microtubules of the mitotic spindle attach to the chromosomes via the kinetochore proteins, which link the spindle microtubules to the centromere region of each chromosome. These are known as kinetochore microtubules (Figure 15.6.8). There are two other types of microtubules in the mitotic spindle (Figure 15.6.7): the polar microtubules that reach across the cell and interact with one another to help maintain the separation of the centrosomes and defining the overall length of the spindle, and the aster microtubules that are generally short, radiating out from, and stabilizing the centrosome. Remember that the DNA replicated earlier in S phase, and thus sister chromatids are still partially attached. Visually, the centromere region appears narrower or more compressed than the rest of the chromosome, and generally lies near the middle. The centromere contains repeated sequences that are involved in kinetochore binding and assembly.

> In primates, the repeating motif is known as alpha satellite DNA, which is made of multiple instances of tandem repeats of a core ~170bp sequence over a centromeric DNA span over a megabase in length. Similar repeats are found in various other vertebrates as well. In other eukaryotes, the size and sequence may vary; for example, much shorter repeats of ~5bp are found in centromeric DNA measuring 200-600kb in *Drosophila* chromosomes, and S. *pombe* has centromeric DNA well under 10kb.

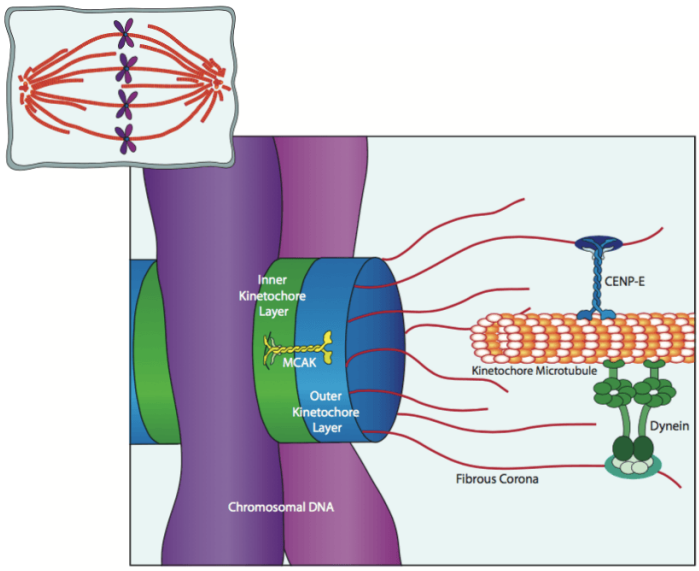


Figure 15.6.8. The kinetochore assembles on the centromere of the chromosome. Spindle microtubules attach to the fibrous corona of the kinetochore through kinesins and dyneins.

The kinetochores attaching to the centromere DNA are trilaminar protein structures consisting of an inner layer, an outer layer, and a fibrous corona. The kinetochore microtubules of the mitotic spindle are primarily attached to the fibrous corona. As depicted in the Figure, it is attached through CENP-E, a kinesin, and dynein motor proteins that bind along the barrel of the microtubule. In fact, sometimes the first contact between a chromosome (via the kinetochore) and a spindle microtubule is somewhere in the middle of the microtubule, and a combination of microtubule dynamics and motor protein activity move the chromosome to the distal end of the microtubule. This is facilitated by MCAK (mitotic centromere-associated kinesin), which is associated with the kinetochore core proteins and plays a role in depolymerizing microtubules near the (+) end.

The transition from the interphase microtubule cytoskeleton to a mitotic spindle require a number of molecular motors to move the centrosomes, align the microtubules, and expand the spindle. These are depicted in Figure 15.6.9. Initially, as the duplicated centrosomes move away from each other along with some of the cytoskeletal microtubules, the microtubules will interact at various angles. Because the polar microtubules that help to expand or maintain the spindle width must ineract in parallel, cytoplasmic dyneins bind to the eventual polar microtubules and by moving one along the other, bring them into parallel (9a). Once in that position, BimC and other kinesins take over as the primary motors along polar microtubules. They create an outward pushing force by holding onto a microtubule facing one direction, and driving along a parallel MT facing the opposite direction towards the (+) end (9b). Finally, cytosolic dyneins attached to cortical cytoskeleton pull on the astral microtubules, which pulls the spindle ends further from center (9c).

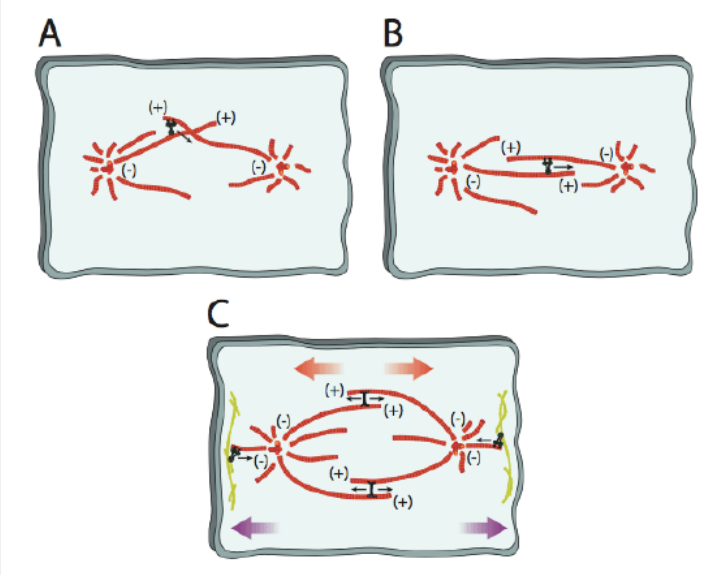


Figure 15.6.9. Molecular motors set up the mitotic spindle.

In fact, there appear to be two mechanisms at work: the bub1/bub2 system works in the tension sensing pathway, while another metaphase protein, mad2 appears to be important in suspending mitosis upon disconnection of the kinetochore with the spindle microtubule.

As the nuclear envelope is breaking apart, the mitotic spindle microtubules are undergoing increased dynamic instability, cycling between periods of growth spurts (polymerization) and rapid shortening (catastrophic disassembly), searching for chromosomes to connect to. Once the kinetochore microtubules connect to the chromosomes, the microtubule dynamics shift. The microtubule will primarily undergo shortening if it is beyond the center of the spindle and primarily lengthening if it is short of center. Since eventually each set of sister chromatids is connected to microtubules on both kinetochores, each chromatid is connected to one shortening and one lengthening microtubule. As the chromosomes approach the center of the mitotic spindle, the rate of microtubule shortening/lengthening slows. The sister chromatids are pushed and pulled by the spindle microtubules until they are all lined up along the midline of the mitotic spindle, which in most (but not all) cases is also the midline of the cell. Once they are all lined up, the cell is considered to have reached metaphase. Unlike the other phases, metaphase is a relatively static phase - it is a checkpoint for lining up the chromosomes.

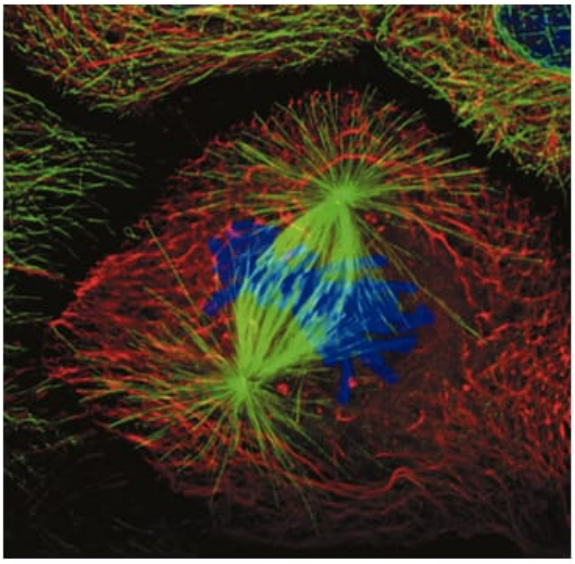


Figure 15.6.10. A cell at metaphase. Microtubules are stained green, f-actin is stained red, and chromosomes, with centromeres lined up along the midline, are stained blue. Note the surrounding cells, which are not in mitosis, with their MT and MF cytoskeletons more overlapped. This photo released to public domain by the US government.

The chromosomes must be properly aligned to ensure that both daughter cells receive the proper complement of chromosomes. How does the cell know when the chromosomes have reached the center of the spindle? An elegantly simple experiment demonstrated that the general mechanism is a tension check - if the two microtubules connecting to the pair of sister chromatids from each side are of the same length, they should be exerting equal tension on the chromosomes. If the microtubule-kinetochore connection is severed at metaphase, the cell will be prevented from progressing (Nicklas, R.B., et al, *J. Cell Biol.* **130**: 929-39, 1995). However, if an equivalent tension is applied by tugging on the chromosome with a glass microneedle, progression of mitosis is restored!

In addition to the tension check, there is another condition that must be met for continuation of mitosis: the MPF must be inactivated. As outlined earlier, MPF in part leads to its own inactivation by activating the anaphase-promoting complex (APC), which polyubiquitinates the cyclin, leading to its destruction and thus MPF-cdk inactivation. APC also tags securin for destruction. Securin is a protein that binds and inhibits the proteolytic enzyme, separase, the activation of which is needed to allow the sister chromatids to separate, which in turn, is necessary for anaphase to proceed.

Barring pathological situations, if and only if the chromosomes all line up at the metaphase plate will the cell proceed to the next stage of mitosis: anaphase. The sister chromatids separate and are pulled toward opposite poles of the mitotic spindle. Somewhat perversely, even as the chromosomes move towards the spindle poles, the poles themselves move outward slightly. Separation of the sister chromatids requires the dissociation of the molecular “glue” holding them together: the cohesin proteins. The cohesins bind to both molecules of DNA and hold them together shortly after replication back in S phase. As anaphase approaches, the enzyme separase is activated, which then cuts the cohesin molecules. Once all of the cohesin molecules are cut, the sister chromatids can finally be separated. The removal of the cohesins proceeds roughly inwards from the distal points of the chromosomes to the centromere, which is generally the last region of attachment.

> A cohesin is a multimer of four subunits, Scc1, Scc3, Smc1, and Smc3 in yeast. An additional protein has also been observed in Xenopus. The SCC1 protein is cleaved by separin in yeast, but in metazoans, SCC1 may be removed from chromosomes by another method as well. It is phosphorylated, which decreases its affinity for DNA, and may expose a site for separase-catalyzed hydrolysis.
>
> Separase also promotes anaphase by activating Cdc14, a phosphatase needed to dephosphorylate the cdk substrates that had been phosphorylated by the cyclin-cdk complexes of early mitosis. In addition, Cdc14 is also required for cytokinesis in the yeast *S. cerevisiae* and nematode *C. elegans*.

Anaphase can actually be divided into two stages, sometimes referred to as early and late or A and B. At first, the kinetochore microtubules are shortening from both ends, and kinesin-family motors pull the microtubules back toward the spindle poles.

As late anaphase starts, polar microtubules elongate, and an additional chromatid-separating force is applied by kinesin-family motor proteins [kinesin-5] that push the polar microtubules against one another to increase the separation between the poles. Dynein-family motors help to direct movement of the poles as well, through their attachment to the aster microtubules and the cortical (peripheral) cytoskeleton.

When both sets of chromosomes arrive at their respective poles, telophase begins. Technically, it was slowly building up since anaphase: when MPF was inactivated by APC, its ability to phosphorylate nuclear lamins was ended. Protein phosphatases in the cell remove the phosphate groups, allowing the lamins to once again interact with one another, and by telophase they are reconstituting the nuclear lamina and the nuclear envelope. Since the lamins and other nuclear membrane proteins also interact with DNA, the nuclear membrane fragments dispersed back in late prophase now coalesce around each set of DNA to form the new nuclear envelopes. The other fragmented membranous organelles (ER, Golgi) also start to re-form. By the end of telophase, the product is a single large cell with two complete nuclei on opposite sides. The next and last step, cytokinesis, splits the cell into two separate and independent daughter cells. In animal cells, cytokinesis is similar to the tightening of a drawstring in the middle of the cell, pulling the “waist” in until all edges meet, and two separate cells result. This contractile ring is composed of actin (structural) and myosin (motive) subunits. These proteins, using ATP for energy, ratchet themselves closer and closer together similar to the actin-myosin “power stroke” described for muscle cell sarcomeres, also primarily made from actin and myosin. This mechanism is universal for animal cells, but the placement of the ring is not always in the center of the cell. The ring often coincides with the center of the cell, but is in fact positioned by the metaphase plate (i.e. the center of mitotic spindle). The most obvious example of a metaphase plate that does not coincide with the center of the cell is found in the formation of egg cells. Because the purpose of an egg cell is to provide all of the material necessary to make a viable new organism upon fertilization (the sperm contributes negligible biomass beyond the genetic material), it divides asymmetrically, with the mitotic spindle located far to one side of the cell (Figure 15.6.11).

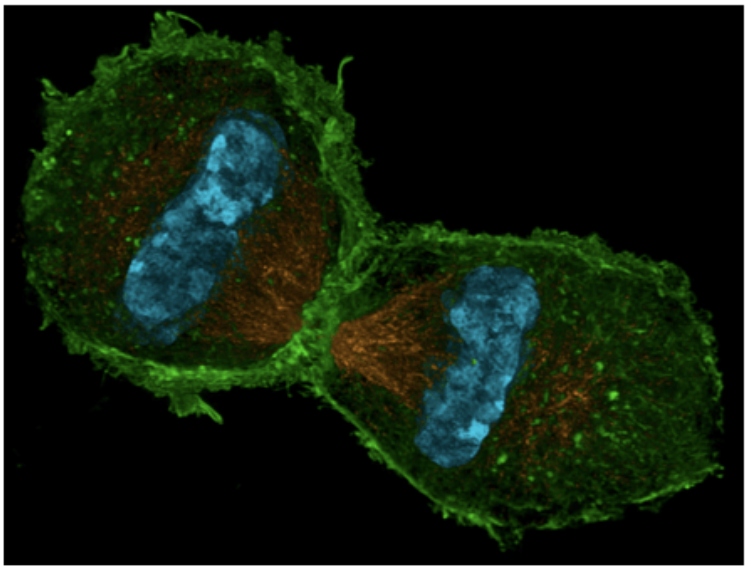


Figure 15.6.11. Telophase/Cytokinesis. The contractile ring and other actin structures are stained green, the microtubules are orange, and the chromosomes are blue. Photo released to public domain by US government.

When cytokinesis occurs, one daughter cell, the presumed oocyte, is very large, while the other cell, called a polar body, has minimal cytoplasmic material surrounding the nucleus. The contractile ring works in animal cells because the cell membrane is flexible. In plant cells, the cell membrane is firmly attached to a rigid cell wall, and thus cannot be pulled in. So, the plant cell ingeniously builds a wall down the middle of the cell using specialized vesicles that originate from part of the Golgi, and which contain the materials necessary to form a cell wall. The vesicles travel along the *phragmoplast*, a structure built from the mitotic spindle microtubules, and as the vesicles line up along the middle of the cell, they begin to fuse to form bigger vesicles and then a large disk-like vesicle, the cell plate. Eventually they reach the cell membrane itself, and fusing with that leads to formation of a new cell wall, and two complete and independent cells.

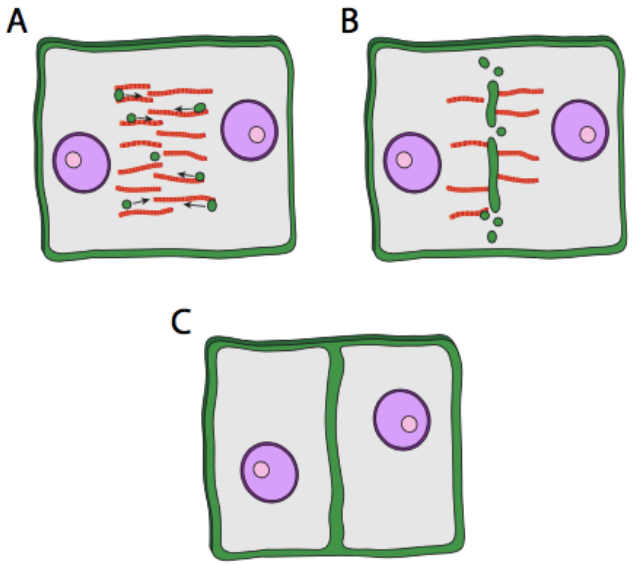


Figure 15.6.12. Cytokinesis in plant cells. Golgi-derived vesicles filled with cell wall material travel along the phragmoplast and fuse in the center to form a new cell wall.

The contents of the vesicles traveling along the phragmoplast are not well described. Callose, a glucose polysaccharide with β1-3 linkages is known to be present in the developing cell plate, but has not been found in the Golgi or vesicles. Interestingly, once the cell plate has fused completely with the existing cell walls, callose gradually disappears. It is thought that the same enzyme system that synthesized callose may switch to synthesizing cellulose as the cell plate matures.

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15.7: Cell Death

A cell may die either intentionally (usually referred to as apoptosis or programmed cell death, though also once known also as “cellular suicide”), or unintentionally (necrosis). The microscopic observation of these two processes shows strikingly different mechanisms at work. In apoptosis, the cell begins to shrink and lose shape as the cytoskeleton is degraded, then the organelles appear to pack together, except for the nucleus. Inside the nucleus, the chromatin condenses and attaches to the nuclear envelope, which then loses its integrity and starts to break apart. The cell membrane begins to show irregularities, descriptively known as blebs, and eventually, the cell breaks apart into vesicles that are neatly cleaned up by phagocytes drawn to the site by apoptotic signals emitted by the dying cell. Necrosis, on the other hand, is quite literally a mess. The cell appears to swell and the plasma membrane begins to lose its integrity. It is soon catastrophically leaking cytoplasm, and leaves behind cell debris that can accumulate and trigger necrotic death of adjacent cells.

Figure 15.7.13. (A) A cell underdying by necrosis is disorganized, generally bursts and leaks its contents. (B) A cell undergoing apoptosis first subdivides itself, digesting itself in an orderly fashion and compartmentalizing everything for scavenging by phagocytes.

Apoptosis is ultimately put into action by a cascade of caspases, a family of proteolytic enzymes. This family of enzymes is generally produced as proenzymes that are activated by other members of the caspase family. Thus a cascade effect occurs, after the initial trigger activating one set of caspases, they can then cleave a variety of proteins including procaspases that are thereby activated and can hydrolyze even more proteins, including yet another type of procaspase, and so on. Of course, other enzymes are also activated and participate by widening the response, activating other groups of proteases and apoptotic enzymes. Triggering the apoptotic cascade is usually one of two general pathways: an internal trigger, arising from damage to the mitochondria, and an external trigger, started by binding an extracellular signal molecule to activate a “death receptor”. Although there are many variations on both triggers, they follow similar paths to the examples we will use here.

If you recall the section on electron transport in oxidative phosphorylation, then you may also recall the soluble electron carrier, cytochrome c. This protein is exclusively found in the mitochondrial matrix under normal circumstances, so its presence in the cytoplasm can be taken to indicate mitochondria in distress. Given the importance of mitochondria in providing the energy for most aerobic cells to carry out their normal life, such distress is an early indicator that the cell will die soon. The diagram below shows a sample pathway that can cause cytochrome c leakage from the mitochondria, but mitochondria can also just “get old”, and if the cell is “programmed” (by transcription factors) not to replace failing components, then as the mitochondrial membranes lose integrity and allow cytochrome c out, it is a clear signal to initiate termination protocols, to use the parlance of science fiction novels.

Figure 15.7.14. Apoptotic signaling cascades may be initiated by leakage of cytochrome c into cytoplasm.

The cytochrome c is bound by APAF-1 (apoptotic protease activating factor 1) which oligomerizes to form an apoptosome made of 7 APAF-1 moecules and 7 cytochrome c molecules. The apoptosome binds and activates procaspase-9 to initiate a caspase cascade that continues with activation of procaspase-3. When the mitochondria leaks cytochrome c, it also leaks another apoptotic protein, SMAC/Diablo. This protein, among other functions, inhibits IAP (inhibitor of apoptosis)-family proteins. The IAP proteins normally inhibit caspase activation both directly and indirectly to prevent cell death, and SMAC/Diablo blocks that inhibition.

Figure 15.7.15. Apoptotic signaling cascades may be started by external activation of a “death receptor” such as FasR.

When death receptors are activated, the subsequent caspase cascade does not involve the mitochondria or APAF-1. The best studied case, FasR (Fas receptor) activates caspases 2, 8, and 10 by clipping procaspases and by releasing caspases from inhibiting complexes. These activate caspases 3, 6, and 7, which leads to the final stages of apoptosis. In both internally and externally triggered apoptosis, the final steps are the same: some of the final targets of the caspases are the nuclear lamins and ICAD (inhibitor of caspase-activated DNase). Destroying the nuclear lamins leads to fragmentation of the nuclear envelope, while removing ICAD activated the caspase-activated DNase (CAD) which then be- gins to digest the DNA.

Why does the apoptosis mechanism exist? There are two major (and many other) reasons for apoptosis. The first is developmental. In the development of an organism, the most effective strategy is often to have overgrowth of cells that are then pruned back to the proper formations. Examples of this are the apoptotic death of tissue between initially connected fingers and toes (we humans start with webbed fingers and toes embryonically), and death of unconnected or improperly connected neurons. The latter case also illustrates a fundamental principle in mammalian cell biology, and most other vertebrates as well: cells require

signals (trophic factors) to stay alive. In this example, the neurons that do not make proper connections to a target cell do not receive the necessary trophic factor (secreted by the target). This leads to apoptotic death of the unconnected neuron. In fact, if apoptosis is blocked due to mutation to a gene in the pathway, there is severe overgrowth of the brain and spinal cord, causing serious mal- function and craniofacial deformities. Thus in development, apoptosis is necessary to control the growth of different parts of a metazoan organism.

The other major function for apoptosis is to kill dangerous cells. In some cases, these may be cells infected by a pathogen. In others, the cells have accumulated mutations that do have affected the DNA error-correction system or cell-cycle checkpoints. When the former occurs, each generation has an increased likelihood of even more mutations. It is important to activate apoptosis in such cells before they have a chance to acquire errors that removes all cell cycle checkpoints, allowing unchecked cell proliferation. This could lead to tumor formation and potentially cancer (see next chapter). When such cells need to be killed for the benefit of the organism, it may happen by the triggering of an internal sensor such as mitochondrial damage, or by external means, such as an immune system cell recognizing an infected cell.

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15.8: Meiosis

In metazoa, there are two situations in which a cell gives rise to daughter cells. The first, and by far most common, is mitosis. The second is meiosis. Meiosis is the process by which gametes (sex cells) are generated. Animals and plants are generated by sexual reproduction (if this is news to you, please consider majoring in something other than biology). These organisms start life through the fusion of two cells: a sperm and an egg. Both contribute genetic material to the new organism. In order to maintain the proper number of chromosomes in each generation, the gametes each contribute one set of chromosomes, so that the fertilized egg and all other cells in the organism have two sets of chromosomes — one from each parent. The purpose of meiosis, and its primary difference with mitosis, is not generating daughter cells that are exact replicates, but generating daughter cells that only have half the amount of genetic material as the original cell.

Let us take a look at this situation selfishly: meiosis in human beings. Almost every cell in your body has a nucleus containing 46 chromosomes, a set of 23 from your father, and a set of 23 from your mother. The only exceptions are the gametes: the spermatocytes in men and the oocytes in women. The somatic cells are said to be 2n or diploid, that is having 2 sets of chromosomes, and the gametes are 1n or haploid, having only one set of chromosomes. Sometimes, meiosis can be a little confusing to students because it occurs in the same part of the cell cycle as mitosis, which is to say after G_2. Because of this, the cell entering meiosis actually has 4 sets of chromosomes, since the DNA has already undergone replication in S phase.

> Mature red blood cells contain no nucleus, and some muscle cells, while multinucleated because they form from the fusion of several myoblasts, nevertheless have 46 chromosomes in each of the nuclei. *Polyploidy*, while uncommon in humans, is a normal state for many organisms. The frog, *Xenopus laevis*, a common research animal, is tetraploid.

Meiosis consists of two consecutive meiotic divisions each of which has phases similar to mitosis: prophase, metaphase, anaphase, telophase, and each of which finishes with complete cytokinesis. Note that immediately following meiotic telophase I, the cell divides, and both daughter cells are immediately in prophase II. There is no intervening G_1, S, or G_2 phase.

Prophase I of meiosis begins very similarly to prophase of mitosis: MPF (mitotic-cdk) activation, chromosome condensation, spindle formation and nuclear envelope breakdown. However, compared to mitosis, meiotic prophase I lasts for a very long time and can be subdivided into five stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During leptotene, the two sets (maternal and paternal) of sister chromatids for each chromosome condense, align and form a structure known as a bivalent. To clarify, this bivalent consists of four copies of a given chromosome: two copies each of the maternal chromosome and of the paternal chromosome. Because the maternal and paternal versions of a given chromosome are kept in extremely close proximity for an extended period of time, there is a greater chance of a *recombination*, or crossing over and exchange of homologous pieces of each chromosome.

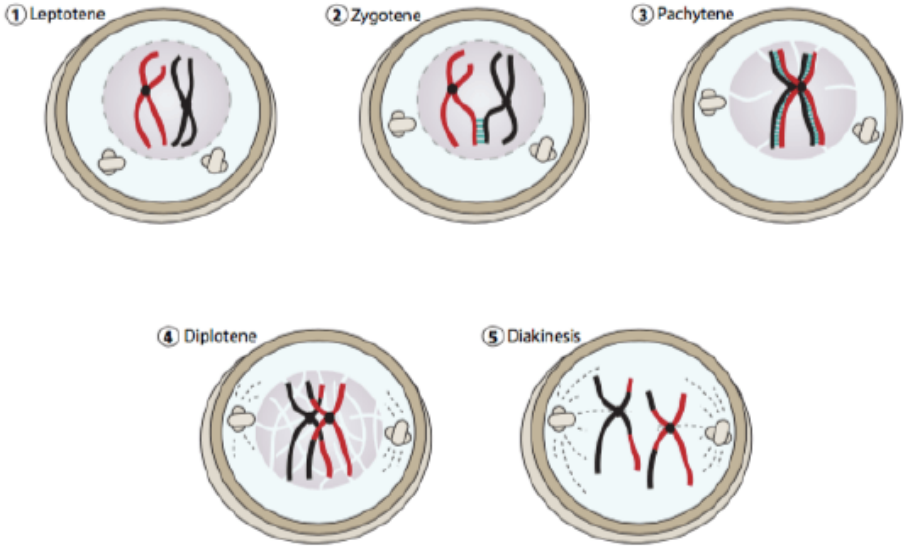


Figure 15.8.16. The five stages of Meiotic Prophase I.

Recombination occurs when a piece of the paternal chromosome is swapped for the homologous piece of DNA on the matching maternal chromosome (or vice versa). Note that sister chromatids (i.e. exact copies) do not recombine - only homologous non-sister chromatids can recombine. Obviously, this kind of a DNA swap must be done carefully and with equivalence, so that the resultant DNA on each side contains all the genetic information it is supposed to, and no more information than it is supposed to. In order to ensure this precision in recombination, the non-sister homologous chromatids are held together in a synaptonemal complex (SC). This ladder-like complex begins to form in the zygotene stage of prophase I and completes in pachytene. The complete SC consists of proteinaceous lateral elements (aka axial elements) that run along the length of the chromatids and a short central element composed of fibrous proteins forming the rungs of the ladder perpendicular to the two lateral elements. The central element is formed of transverse lament dimers that interact with one another in offset fashion, as well as with the lateral elements. These lament proteins (e.g. SCP1 (mouse), Zip1p (yeast)) have central coiled-coil regions that function as protein interaction domains. Although SCP3 and therefore complete lateral element formation are unnecessary for a functional synaptonemal complex, condensin and cohesin do appear to be necessary for proper transverse lament attachment of the lateral elements.

Recombination may occur with or without the formation of double-strand breaks, and in fact, can occur without the formation of the synaptonemal complex, although the SC probably enhances the efficiency of recombination. In *S. pombe*, meiosis occurs without the formation of a synaptonemal complex, but there are small discontinuous structures somewhat similar to parts of the SC. In the fruit fly, *Drosophila melanogaster*, females undergo meiosis using a synaptonemal complex, but males do not undergo meiotic recombination, and their chromosomes do not form synaptonemal complexes. In most cases, recombination is preceded by the formation of recombination nodules, which are protein complexes that form at potential points for recombination. The best studied mechanism for meiotic recombination involves a double-stranded break of one of the chromosomes initiated by the meiosis-specific endonuclease, Spo11. The 5' ends (one in each direction) of this cut are degraded slightly to form 3' single-stranded overhangs. This leads to the formation of Holliday junctions with a strand from one chromosome acting as a template for a missing portion of the homologous cut chromosome. This may be resolved one of two ways, with or without a crossover, as illustrated (Figure 15.8.17).

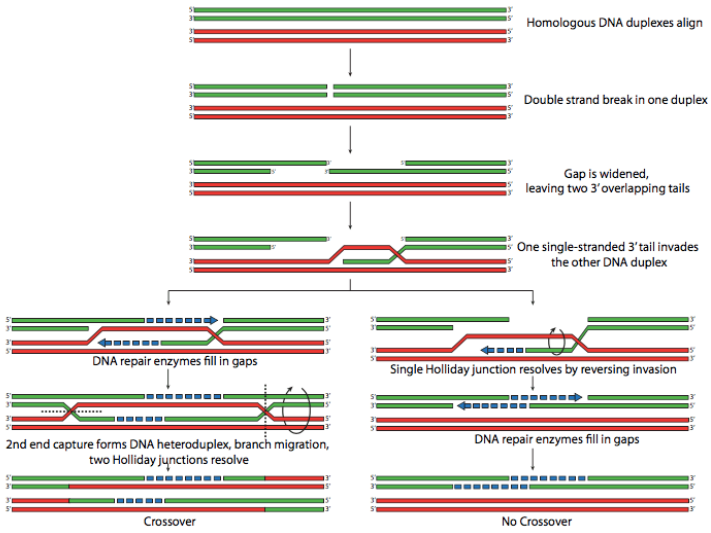


Figure 15.8.17. Recombination of homologous chromosomes.

The recombination is initiated in pachytene and completes in diplotene, at which time the synaptonemal complex breaks down. As the chromatids begin to separate, chias- mata become apparent at some of the recombination sites. As prophase completes, the chiasmata resolve from the center of the chromosomes to the ends.

As the cell goes from meiotic prophase I to meiotic metaphase I, another difference between mitosis and meiosis is revealed: the chromosomes line up at the metaphase plate as tetrads rather than as pairs. Because of this, when they pull apart in anaphase, sets of sister chromatids segregate to opposite poles. Of course, due to recombination, the sister chromatids are unlikely to still be identical.

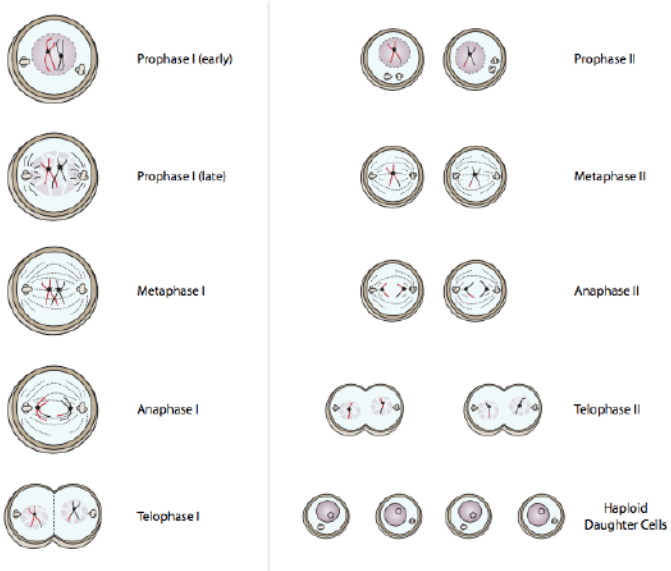


Figure 15.8.18. Meiosis generates 4 haploid daughter cells from one diploid precursor. To do so, it undergoes a two rounds of meiotic nuclear and cell division.

After a conventional anaphase and telophase, the cell splits, and immediately the daughter cells begin the second meiotic division (Figure 15.8.18, right side). In some cell types, chromosomes do not decondense in meiotic telophase I, but if they have, they re-condense in meiotic prophase II. Prophase II proceeds similarly to *mitotic* prophase, in that there is no formation of synaptonemal complexes or recombination. At metaphase II, the sister chromatids line up along the metaphase plate just as in mitosis, although now there are only 2n chromosomes in the cell, while in mitosis there would have been 4n (because the DNA has replicated). Again, nishing the rest of the division almost exactly like mitosis, the sister chromatids pull apart in anaphase II, the nucleus reforms in telophase II, and the final cytokinesis generates a total of four cells from the original one that entered into

meiosis, each containing 1n chromosomes.

Egg cells, as genetic and bulk material donors, need to be large but sperm cells, as genetic donors only, do not. The diagram below depicts the generation of the egg cells. Only one oocyte is generated from a meiotic event; the other three daughter cells are termed polar bodies, and contain so little cytoplasmic material that they are only viable for a short time. The asymmetric distribution of cytoplasm in the first meiotic division for oocytes is due to the position of the meiotic spindle in the periphery of the cell rather than centered. Since the center of the spindle determines the position of the contractile ring for cytokinesis, this leads to unevenly sized daughter cells.

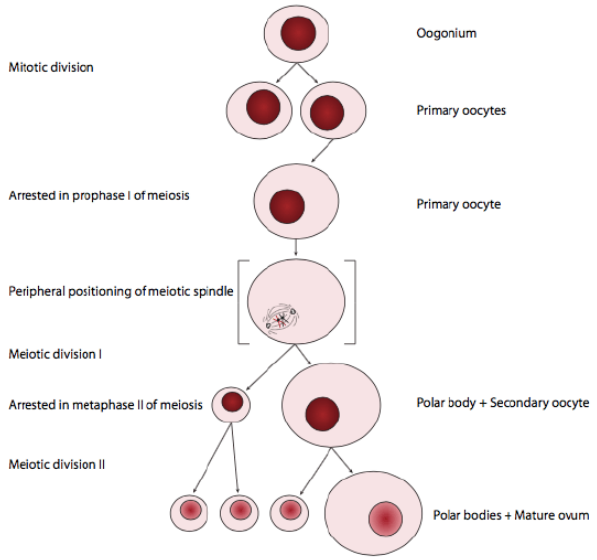


Figure 15.8.19. Oogenesis.

The generation of the very small sperm is a different mechanism altogether. In the meiotic steps of spermatogenesis, the cell divisions are equal, with the meiotic spindle aligned with the center of the cell, and the cells have equal amounts of cytoplasm, much like an average cell that has undergone mitosis. The streamlined, minimal-cytoplasm mature sperm is a product of post-meiotic differentiation, in which it gains the flagellar tail, and ejects most of its cytoplasmic material, keeping only some mitochondria to power the flagella, and an acrosomal vesicle, that contains the enzymes and other molecules needed to reach and fuse with (i.e. fertilize) an egg.

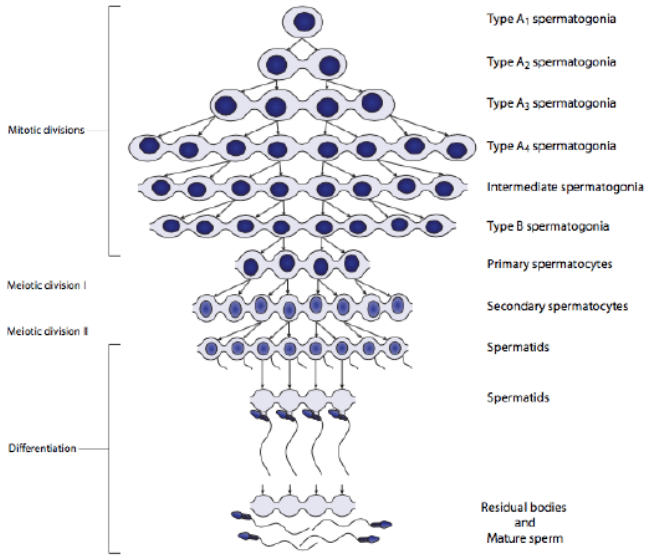


Figure 15.8.20. Spermatogenesis.

Not all organisms reproduce with the human-like egg and sperm mechanism, i.e. gametic meiosis. As just described, in a gametic

meiosis life cycle, meiosis generates haploid gametes, which then fuse/fertilize to become a diploid zygote. The zygote becomes a multicellular diploid organism, and once it reaches sexual maturity can make more haploid gametes via meiosis. The only multicellular state is diploid, and the gametes are haploid.

A common variation is sporic meiosis, used in all plants and many types of algae. In this usage, “spore” refers to eukaryotic spores, and not to bacterial endospores, which are simply dormant bacteria. Sporic meiosis does not directly produce gametes. Instead, meiosis produces haploid spores, which can develop by mitosis in haploid multicellular organisms. These organisms (termed gametophytes) can produce (still haploid) gametes by mitosis, that when fused/fertilized form a diploid zygote. This zygote can then develop into a diploid multicellular form called the sporophyte. Finally, the sporophyte is able to generate more spores by meiosis.

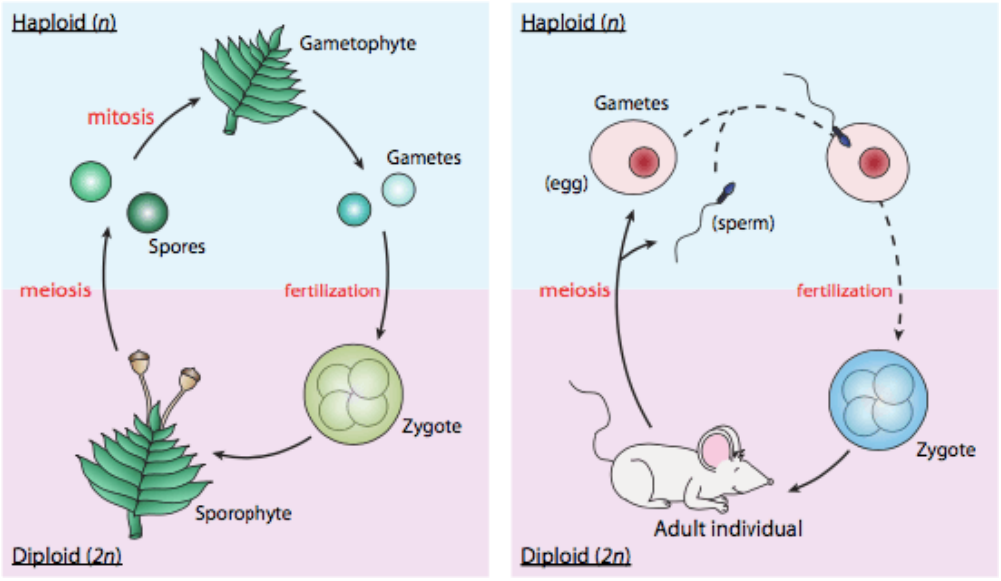


Figure 15.8.21. Gametic meiosis (left) and Sporic meiosis (right).

An example of this type of life-cycle and the role of meiosis is found in moss. What we think of as the body of the moss is actually a gametophyte, made up of haploid cells generated by mitotic division of a haploid spore. These gametophytes generate either sperm or eggs in specialized structures in their distal tips, and under the right conditions (e.g. rain) the sperm is carried to the eggs and fertilization occurs. The fertilized (diploid) egg now develops by mitotic division and differentiation into a sporophyte. In this case, the sporophyte is a specialized reproductive structure on the tip of the moss, and is also diploid. On the tip of the sporophyte is the sporangium, which is where meiosis takes place to generate haploid spores. The spores may then be dispersed (by wind or rain) and begin the cycle again by dividing and forming a new gametophyte.

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16: VIRUSES, CANCER, AND THE IMMUNE SYSTEM

At this point, you should be fairly comfortable with the basic concepts of cell biology. The purpose of this chapter is to build on that basic knowledge and put it together into more complex systems. In addition, we will introduce some more advanced variations on some of the mechanisms and structures that were discussed in earlier chapters. The three topics, viruses, cancer, and immunity, are not only relevant as current news topics, but relate to one another through multiple pathways, which is why they are lumped together.

16.1: Viruses

16.2: Viral Life Cycles

16.3: Cancer

16.4: Oncogenes

16.5: Tumor Suppressor Genes

16.6: Human Cancers

16.7: Metastasis

16.8: The Immune System

16.9: DNA Rearrangement

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16.1: Viruses

Though a virus has both genetic material and protein components, it is not a living organism. It does not contain the capability of self-replication, and is completely reliant on the cellular biochemistry of whatever host cell it has infected. The minimal definition of a virus is a nucleic acid genome inside of a protein shell, or capsid. There are variations of this, such as virions (infectious viral unit) that have a membrane coat outside of the capsid, or some that have enzymes inside of the capsid alongside the genome.

> *Viroids* are also infectious non-living particles, but they are only genetic material, RNA, and have no protein capsid. To date, they are only known to infect plant hosts, and apparently spread by direct or extremely close contact with an infected plant. These infectious pathogenic RNA molecules are single-stranded and circular, and relatively small, roughly 200-400 nucleotides.

Again, none of these viral variations are able to fully replicate without cellular machinery. The cells that viruses can infect range across most living organisms. There are viruses specific for humans, some that only infect particular animals, some that infect plants, and even viruses that use bacteria as hosts. In current media reports of viral outbreaks in recent years, HIV, avian flu, swine flu, much is made of the origin of the virus with respect to its host/target organisms. However, most viruses are very specific about the cells that they infect. The narrow host range may be not only to particular species, but particular cell types within a particular species. Viruses with a broad host range are relatively rare. However, this does not preclude the possibility of new strains of virus evolving with different host ranges from their ancestral virii.

Viruses may have either RNA or DNA genomes, that may be linear or circular, and single or double-stranded. There are fewer variations of **capsid** structure. In general, capsids fall into two categories: helical and icosahedral. Helical capsids are actually made up of globular subunits that associate into a helical cylinder, with the genome lying inside an interior groove of the helix (Figure $16.1.1C$). The icosahedral capsids are also made up of many subunits that together form an approximately 20-sided polygon made from sides that are equilateral (or nearly so) triangles. If you play Dungeons and Dragons or know someone who does, then you have probably seen dice (a "d20") of this shape. Of course with capsids, but not dice, there can be some variation in the number of sides, the shape of the triangles, and the number of subunits that make up each face.

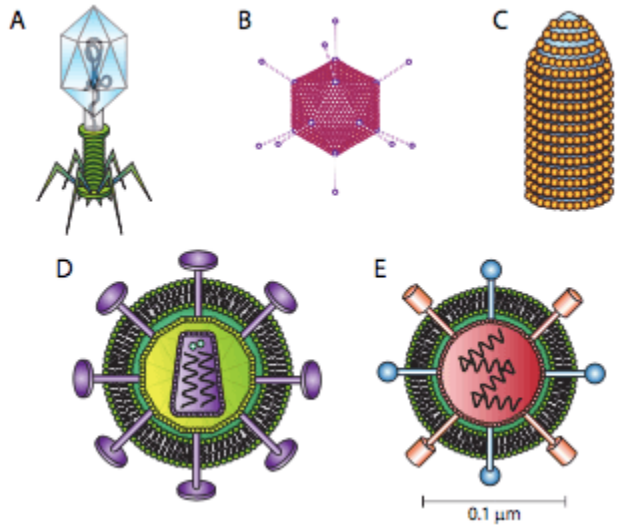


Figure $16.1.1$: Viruses. (A) a T-4 bacteriophage, (B) adenovirus, (C) tobacco mosaic virus, (D) human immunodeciency virus, (E) influenza virus.

Virus	Class	Disease
Adenovirus	Adeno	Febrile respiratory disease; pharyngocojunctival fever
Epstein-Barr	Herpes	Infectious monoculeosis
Hepatitis A	Picorna	Acute hepatitis

Virus	Class	Disease
Hepatitis B	Hepadna	Acute/chronic hepatitis; Hepatic cirrhosis; hepatocellular carcinoma
Hepatitis C	Flavi	Similar to Hepatitis B
Herpes Simplex Type 1	Herpes	Cold sores, pharyngitis, gingivostomatitis
Herpes Simplex Type 2	Herpes	Aseptic meningitis; genital herpes
HIV	Retro	AIDS
Influenza	Orthomyxo	Influenza
Measles	Paramyxo	Measles
Mumps	Paramyxo	Mumps
HPV	Papilloma	Cervical cancer
Poliovirus	Picorna	Polliomyelitis
Rabies	Rhabdo	Rabies
Rubella	Toga	German measles

External to the capsid, some viruses also have a membrane coat (viral *envelope*). As will be more clearly explained soon, this phospholipid bilayer comes from viruses that exit a host cell by exocytosis. Because it came from a host cell, the membrane can be used as a ruse by the virus to fool other potential host cells into misrecognizing the virus as a normal cell or cell debris, based on the receptors that recognize cellular proteins on the membrane. It may then be taken into the cell by receptor-mediated endocytosis in a mistaken attempt to recycle old cell debris, where it can proceed to infect the overgenerous host.

There are two classification systems for viruses, the International Committee on Taxonomy of Viruses (ICTV) has a Linnaean-like taxonomic system based on shared structural or biochemical properties (but not host specificity). Another system, also in use, is the Baltimore classification, in which viruses are classified into seven categories by the mechanism of mRNA production. That is, type I are the dsDNA viruses that make mRNA the “normal” way by direct transcription of the genome, type II are ssDNA viruses that must first make a complementary strand to become dsDNA before transcription, type VI are ssRNA viruses that use reverse transcriptase (detailed later in this section) to first convert the RNA to a DNA intermediate before transcription, and so on.

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16.2: Viral Life Cycles

Lytic “life” cycle of viruses

Viruses can interact with their hosts in two distinct ways: the *lytic pathway* and the *lysogenic pathway*. Some viruses are able to switch between the two pathways while others only use one. The distinguishing characteristic of the lytic life cycle is catastrophic death of the host cell by lysis and simultaneous release of viral particles. In Figure 16.2.2, the stages of the lytic pathway are depicted. In this case, a T4 bacteriophage (the term “phage” is used for bacterial viruses) is used as an example. In step 1, the virus attaches to the cell wall. In step 2, the virus injects its genetic material (dsDNA) into the cytoplasm of the bacteria. In step 3, the viral DNA is being replicated and the genes on the viral DNA are being transcribed and translated into viral proteins. Expression from the host genomic DNA is arrested. In step 4, viruses are assembled from the proteins and DNA. And finally, once the viral factory has used up the cell’s energy and material resources in making more viruses, it performs a final coup de grace, as cell is destroyed to free the viruses to exit and find more host cells. The T4 phage used in this example only undergoes this pathway and not the lysogenic pathway.

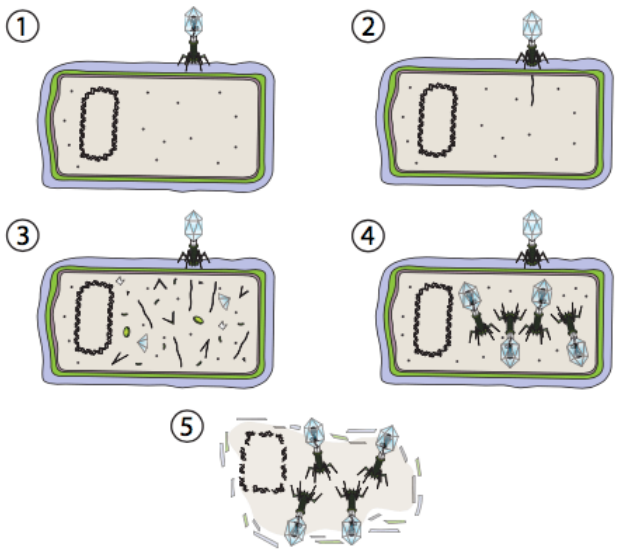


Figure 16.2.2. The lytic pathway

In eukaryotes, the mechanism is slightly more complicated by the nucleus. The DNA is transported into the nucleus, where the transcription and replication take place. Although the viral mRNA is transported out to the cytoplasm for translation as expected, the resulting capsid proteins are then imported back into the nucleus, where the virion particles are assembled. Lytic plant and animal viruses with RNA genomes can bypass the nucleus altogether, and the genome replication, protein synthesis, and particle assembly all occur in the cytoplasm.

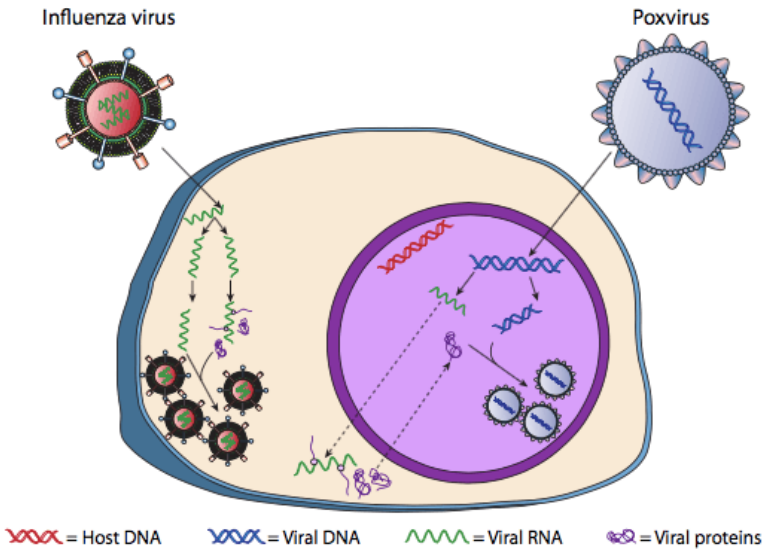


Figure 16.2.3. Lytic viruses in eukaryotes: an RNA virus (influenza) replicates completely in the cytoplasm, while the DNA virus (poxvirus) uses both cytoplasm and nucleus.

The lytic pathway can produce a huge number of viral particles between infection and lysis, as many as several tens of thousands, for example from a rabies-infected cell. Therefore, this pathway is well suited for conditions in which potential host cells are plentiful. On the other hand, this is a waste of resources if there are relatively few potential hosts. Imagine a few bacteria that have floated off from the colony: if a phage infected a bacteria in the main colony, commandeering the bacteria to create thousands of viral particles, most of those particles would infect new hosts and make many thousands more soldiers in this viral army. But if the virus infected one of the breakaway bacteria, then once it killed its host by lysis, the viral particles would have few, if any, other potential hosts, and eventually all the viral particles just break down from various environmental conditions. What would a better survival strategy for the virus in such a situation?

The Lysogenic Pathway

A better option for some bacterial viruses is called the lysogenic pathway. The bacteriophage that have this option, as well as a lytic pathway, are known as temperate phage. In this pathway, the virus goes into dormancy by integrating into the host genome, and remaining transcriptionally quiescent until environmental conditions change and reflect a likelihood of more host cells to infect (Figure 16.2.4).

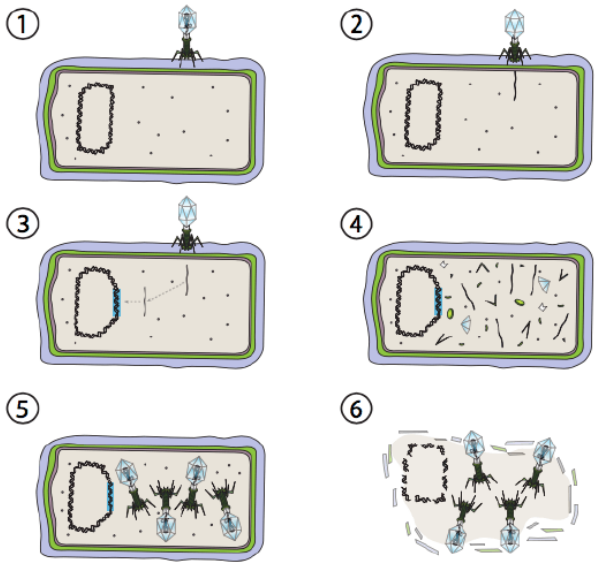


Figure 16.2.4. The lysogenic pathway

Lambda (λ) is an example of a temperate bacteriophage. The initial stages of infection and genome injection are the same as the lytic cycle, but under conditions that encourage lysogeny, the viral genome is integrated into the host genome in step 3. In λ integration into *E. coli*, this occurs by reciprocal recombination at a 15-base pair sequence known as the att λ site and is facilitated by the Int gene product. As long as the environmental conditions are not conducive to bacterial reproduction (and thus limited number of possible host cells), the viral genome remains mostly hidden and inactive. The only significant exception is a gene encoding a λ repressor that prevents the next step and keeps the virus dormant.

That next step is the excision of the λ phage DNA from the host chromosome, and subsequent replication and transcription of the viral DNA (Figure 16.2.4, Step 4). Then, like before, the final steps are assembly and accumulation of virions, and eventual breakdown of cellular structure and release of the viral particles.

In order for packaging into the tight space constraints afforded by capsids, viral genomes must be highly economical. For example, the HIV genome (Figure 16.2.6) has several genes that overlap.

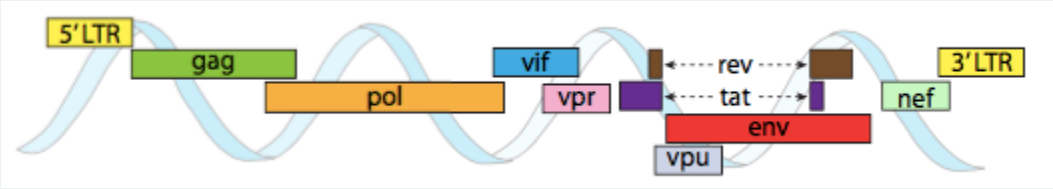


Figure 16.2.6. The HIV genome

Or, in the case of curtoviruses, ssDNA plant viruses (e.g. beet curly top virus), the genome not only has over- Rep lapping genes, it is even bi-directional (Figure 16.2.7) encoding gene products in both strands of DNA after the ssDNA has been converted to dsDNA.

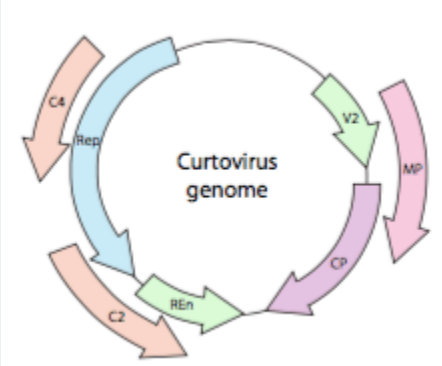


Figure 16.2.7. A curtovirus genome

Given the need for economy, what genes are found in viruses? One of the most studied viral genomes, bacteriophage λ, contains genes encoding five transcriptional control proteins (which ones are expressed depends on whether the phage is in a lysogenic or lytic mode), a binding protein that controls degradation of a transcriptional activator, 17 capsid proteins, an excisionase that controls excision and insertion of the phage genome in the host genome, an integration protein that inserts the phage genome into the host's, and 3 genes participating in lysis of the host cell.

The HIV genome depicted above is much smaller than λ, at around 9 kilobases compared to 48 kb, but again, the theme is to use cellular proteins when possible, and encode viral genes if necessary. So, *gag* encodes capsid proteins, *pol* encodes reverse transcriptase, integrase, and HIV protease (which cleaves the gag and pol gene products into their functional proteins), *vif* acts against a common host cell antiviral enzyme, *vpr* regulates nuclear import, *tat* strongly increases transcription of HIV genes, *rev* exports viral RNA from the nucleus, *vpu* is needed for budding of particles from the host, *env* encodes viral envelope glycoproteins, and *nef* promotes survival of infected cells. The LTR regions are very strong promoters to drive high expression of these genes.

Although it is not referred to as lysogeny, some animal viruses can behave similarly. The most prominent example is the Baltimore Class VI viruses - commonly known as retroviruses, one of which is HIV. The path of a retrovirus through a eukaryotic host cell is

depicted below (g. 5).

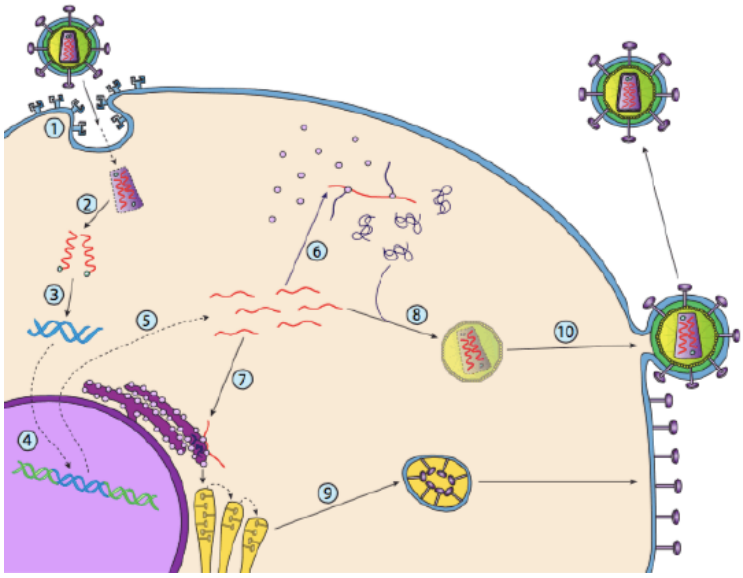


Figure 16.2.5. Infection and reproduction pathway of retrovirus in a eukaryotic host cell.

HIV has an envelope, which is studded with transmembrane proteins that are recognized by the host cell, binding the virus to the cell surface and initiating receptor-mediated endocytosis (1). After the endocytosis, the membrane envelope of the virion and the vesicular membrane fuse to release the capsid and its contents (2). After the capsid dissociates in the cytoplasm, the two strands of viral RNA are released along with a special polymerase: reverse transcriptase, which reads an RNA template and synthesizes DNA. Reverse transcriptase also uses that new DNA to synthesize a complementary DNA strand so that it eventually produces a double- stranded DNA version of the viral genome (3). This viral dsDNA is transported into the nucleus where it integrates into the host genome using another viral protein, integrase

(4). The integrated viral DNA is called a provirus. The provirus can lay dormant, but if it is activated, then it is transcribed and the resulting viral RNA is transported out of the nucleus (5). Some of the viral RNA encodes enzymes like reverse transcriptase and integrase, or capsid proteins, all of which are made in the cytoplasm (6), but some encode membrane bound glycoproteins, which are translocated into the ER (7) and eventually processed through the Golgi and incorporated into the plasma membrane (9). Once the virion has been assembled (8), it binds to the viral transmembrane proteins, nucleating an exocytic “vesicle” (10) which is the virion complete with viral envelope.

Recent structural examination of the HIV genome suggests that the structure of HIV RNA itself may play a significant role in its propagation inside of host cells. Figure 16.2.8, from Watts et al, *Nature* **460**:711-716, 2009, shows a predicted secondary structure of the genome. The authors suggest that the RNA structure actually may interact with ribosomal elongation to control the folding of the viral proteins. They also postulate the extension of this argument to include important genetic information encoded not just in the nucleotide sequence, but the secondary structure and tertiary structure of any RNA virus.

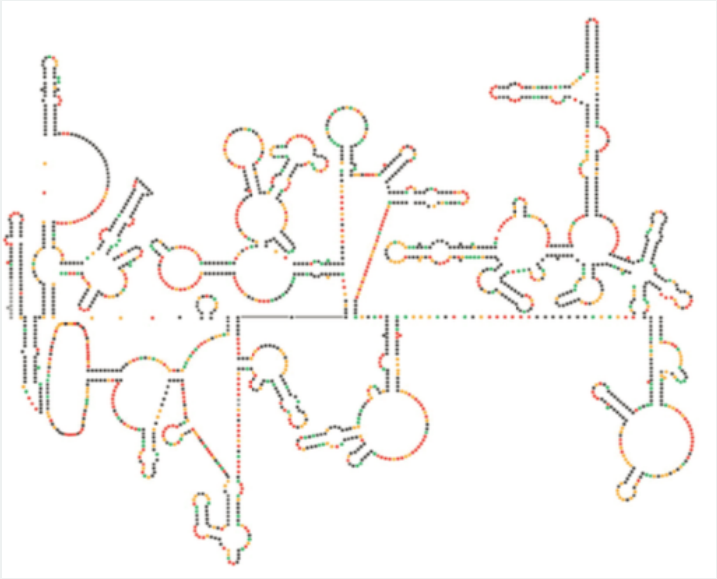


Figure 16.2.8. Secondary structure of HIV-1. (reprinted by permission from Macmillan Publishers Ltd: Nature 460:697, 2009)

In considering viruses with respect to the rest of this integrative chapter, there are two overriding ideas to keep in mind. First, viral survival is based on numbers: it needs to make huge numbers of its components to cast as wide a net for new host cells as possible. To do this, viral promoters are usually much stronger than host cell promoters, simultaneously driving more viral gene expression while preventing host gene expression (by dedicating cellular resources to virus production). Second, because of fast generation times, the rate of viral mutation and evolution is far faster than normal eukaryotic genomes. In addition, if the virus uses its own polymerase (such as reverse transcriptase or RNA replicase), the mutation rate rises even more because there is no error-checking by viral polymerases.

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16.3: Cancer

Cancer encompasses a set of genetic diseases that lead to uncontrolled cell proliferation in multicellular organisms. The discussion of cancer also happens to be useful in a cell biology course, because it ties together many of the concepts that you just spent most of the semester learning. Although it can be caused in part by an outside agent, the development of cancer is essentially a series of uncorrected mistakes by a cell's regular processes. It can strike plants as well as animals, and because of intense research and subsequent deeper understanding of the cellular events that lead to cancer, it can now be treated in humans with some degree of success, depending on the type, location, and progression of the tumor.

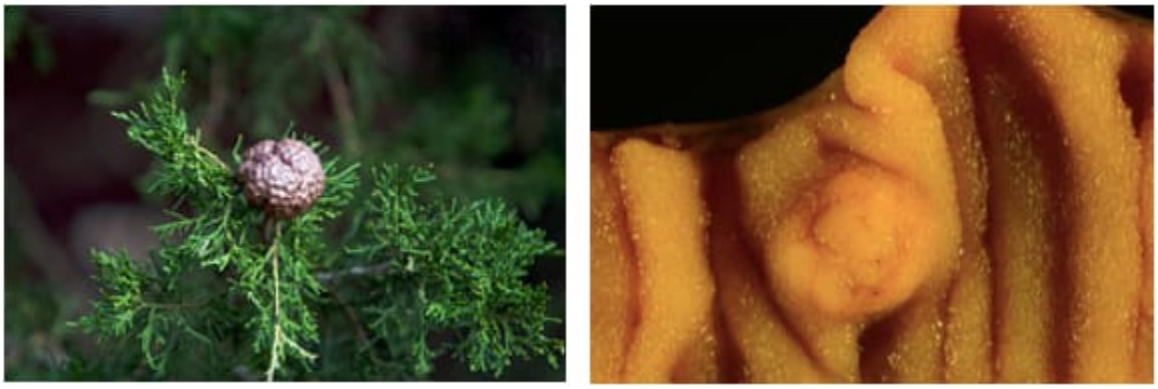


Figure 16.3.9. (left) A tumor on a cypress branch. (right) A tumor of the small intestine. Cypress tumor photo by W. Calder, cc licensed 2009. Small bowel tumor by E. Uthman, public domain 1999.

Abnormal replication of a cell generally leads to the formation of a tumor, which is simply a solid mass of abnormally growing cells, usually clonal colonies of one or a few original tumorigenic cells. However, a tumor is not necessarily cancerous. A benign tumor is one that is ensconced within an extracellular matrix sheath, does not spread beyond that sheath, and whose growth is slow or limited. In contrast, a cancerous or malignant tumor grows quickly due to uncontrolled proliferation, expands significantly beyond its original boundaries, invading new tissue, and can metastasize, spreading through the circulatory system. Once this happens, not only is it no longer possible to remove all of the cancerous cells by surgical excision of the primary tumor, it is also nearly impossible to know how many secondary tumors have formed or where they formed, since the metastatic cancer cells in the bloodstream may theoretically exit almost anywhere. However, in reality, certain tumors metastasize preferentially to particular target tissues/organs, presumably based on molecular markers on the surface of the cells or in the extracellular matrix. Metastasis is considered the greatest medical problem with respect to cancer treatment. If cancer is detected after metastasis has occurred, the chances of survival drop dramatically.

At the cellular level, cancerous cells differ from normal cells in a number of important ways. Normal cells are regulated by the cells around them, and by adulthood, most cells are inhibited from proliferation by contact with their neighboring cells. *In vitro*, this can be demonstrated by the observation that non-cancerous proliferative cells such as epithelial cells can proliferate until the culture dish bottom is completely covered (confluence), but once that happens, proliferation stops. This phenomenon is known as contact inhibition. If cancerous cells are allowed to proliferate in culture, they do not stop after the surface is covered, and instead can mound up on one another. The cell surface and internal cellular organization of cancer cells is often disorganized in comparison to normal cells. Finally, cancer cells usually appear de-differentiated in comparison to their original cell type. If the original cell type was a at cell, the cancerous cell would be more rounded and three-dimensional. This is an expected consequence of becoming a cancerous cell. Not only is proliferation deregulated, cell surface protein expression is altered to promote metastasis.

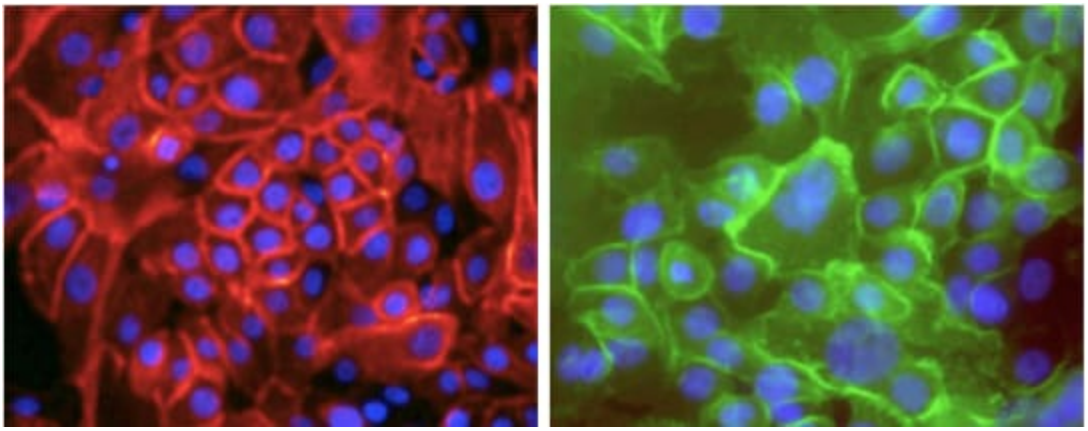


Figure 16.3.10. Normal human breast cells in culture at left. At right, similar cultured cells that have been transformed (i.e. they are now cancerous). Note the irregularity of both cell and nuclear morphology. Membranes are arbitrarily stained in different colors; chromosomes are stained blue in both panels. Photos from Ince et al, Cancer Cell 12:160-170, 2007.

Differentiation is a key part of normal metazoan development. All cells come from the fertilized egg, and even after several divisions, the cells are very similar. Eventually, though, they begin to specialize for their particular physiological functions, whether as lung cells, brain cells, or bone cells, and that process of specialization is differentiation. In cancer cells, this process is partially reversed, as the cell reverts to a less specialized, more primitive state.

Cancer is considered a *genetic* disease because it is caused by alterations to the DNA. However, it is rarely an *inherited* disease. An inherited disease would mean a disease that can be passed from one generation to the next, implying that the disease-causing DNA mutation is found in the gametes (sperm or egg) of the stricken adult. Most cancers are due to spontaneously arising mutation in the DNA of one or a few somatic cells, and not a systemic aberration. Spontaneous mutation in the germ cells are possible, but most potentially cancer-causing ones lead to non-viable offspring. So, although it is exceedingly rare for cancer to be inherited, however, it is much more common to inherit a predisposition or increased chance of developing a cancer.

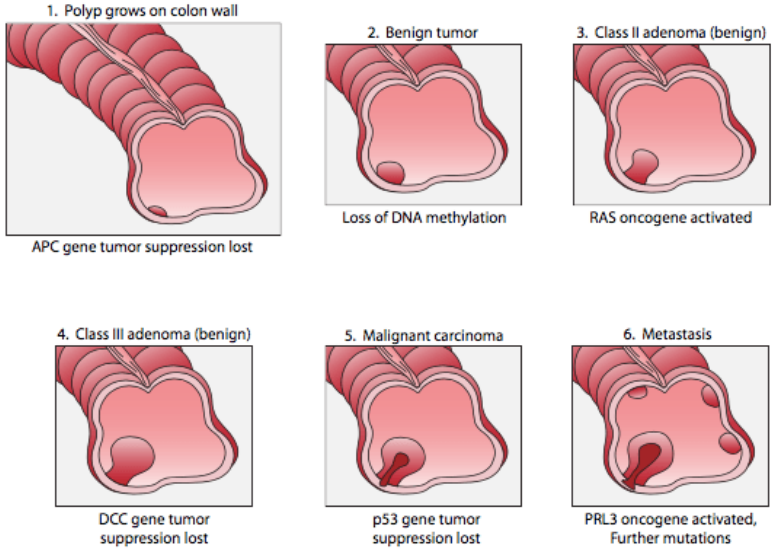


Figure 16.3.11. Development of colon cancer takes time and multiple mutations.

An individual cancer-causing mutation generally creates a problem that can be corrected by some other cellular mechanism. Therefore, development of cancer comes about through the accumulation of multiple mutations and not the acquisition of just one. The best studied example of this gradual development of cancer is colon cancer (Figure11). There is a fairly characteristic progression of mutations in the genes APC, RAS, DCC, TP53, and PRL3. Note that the progression depicted here is not inevitable: the presence of polyps does not lead invariably to colon cancer. Furthermore, intervention can be highly successful if it occurs early in the progression, so oncologists need to consider a range of risk factors in weighing the cost and benefits of medical

intervention. RAS and PRL3 are oncogenes, while APC, TP53, and DCC are tumor suppressor genes.

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16.4: Oncogenes

Oncogenes are generally dominant gain-of-function mutations of normal cellular genes called protooncogenes. These protooncogenes are themselves positive regulators of the cell cycle, but they are regulated by other factors, either extracellular signals or intracellular mechanisms. Mutations that turn them into oncogenes specifically remove all or some of this regulation. They thus become overactive, and try to push the cell cycle forward leading to increased proliferation. These mutations can also be classified into a few general mechanistic categories.

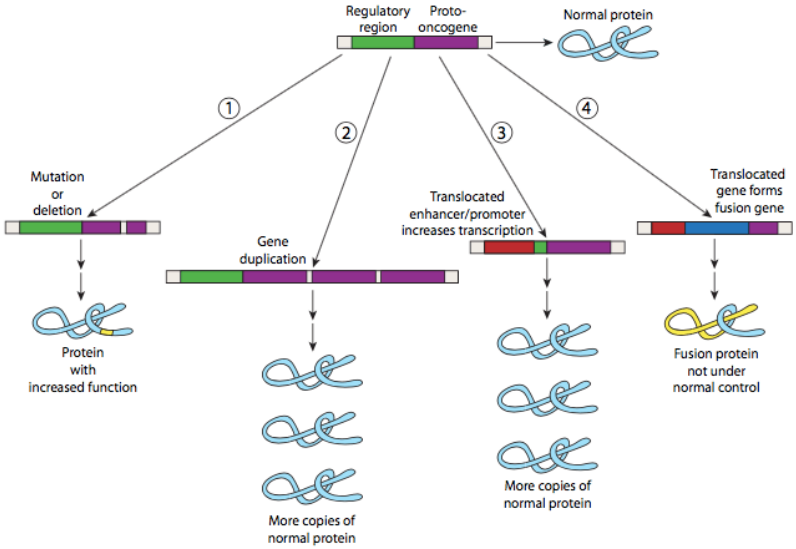


Figure 16.4.12. Conversion of protooncogenes to oncogenes. (1) Due to mutation of the coding region, the protein has higher physiological activity. (2) Gene duplication leads to multiple copies of the gene each expressed normally, making many more copies of the protein than normal. (3) Mutation of the regulatory region or translocation of a stronger enhancer or promoter to the protooncogene can lead to enhance transcription, and therefore more protein. (4) Translocation of another gene inline with part of the coding region, can put the activity of the protooncogene under the control of modifiers of the translocated gene, and thus lead to overactivity.

These (Figure 16.4.12) are mutations to the coding region that increase physiological activity, gene duplications resulting in more copies of the gene at the DNA level and thus more at the protein level, mutations to the regulatory region of the gene or that alter regulation of gene expression, thus increasing copy number of the protein, and finally, translocations that replace part of the coding region, resulting in a chimeric protein whose activity may be under a different control scheme than normal.

Examples of two types of mutations are illustrated in Figure 16.4.13 with a mitogen receptor as the protooncogene. In the first case, the transmembrane portion of the receptor has been mutated, causing an amino acid change that alters the conformation not just of the transmembrane region, but of the cytoplasmic kinase domain, which becomes constitutively active, regardless of whether a ligand has bound outside or not. In the second case, the entire extracellular domain has been removed due to a mutation of an amino acid codon into a stop codon or translocation, and the resulting receptor is always active, also independent of ligand binding.

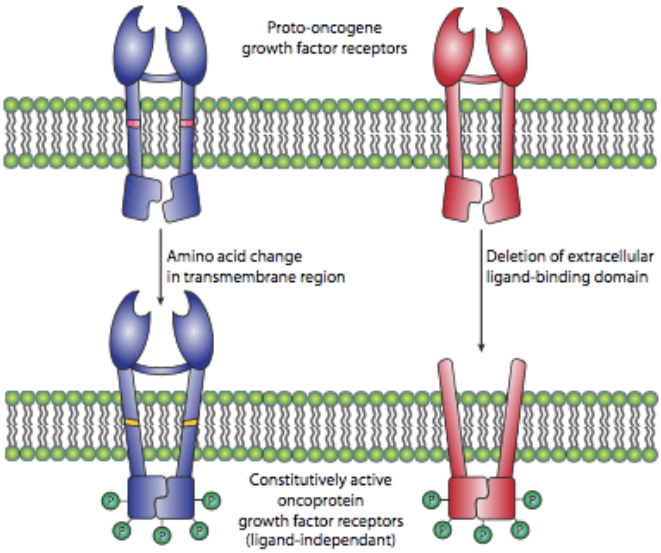


Figure 16.4.13. Conversion of a mitogen receptor protooncogene into an oncogene by point mutation leading to amino acid change in the transmembrane region (left) or by truncation of ligand-binding domain.

Some kinds of retroviral infection can accomplish the conversion of a protooncogene to an oncogene by inserting viral DNA near the promoter region of the protooncogene. Because the viral promoters tend to be very strong, they can induce overexpression of the protooncogene product. In avian species, avian leukosis virus is known to cause tumors by insertion near the c-myc oncogene, while in humans, another retrovirus, HTLV (human T-lymphotropic virus), can cause acute disease (tropical spastic paraparesis), but may also cause T-cell leukemia and lymphoma.

What functions are characteristic of protooncogenes? Mitogen receptors, as already described above, and exemplified by the receptor tyrosine kinases EGFR (epidermal growth factor receptor), VEGFR (vascular endothelial growth factor receptor), RON (recepteur d'origine nantais, a macrophage stimulating protein receptor), and ErbB2 (also HER2/neu, another human EGF receptor). Growth factors themselves may also be pro- tooncogenes, such as FGF-5, one of several oncogenes in the broblast growth factor family, or c-sis, an oncogenic form of PDGF (platelet-derived growth factor). Signal cascade proteins, often either tyrosine or serine/threonine kinases or other regulatory enzymes, are a large group of protooncogenes (e.g. Src family tyrosine kinases, BTK family tyrosine kinases, cyclin-dependent Ser/Thr kinases, Ras-family small GTPases). Finally, various transcription factors (e.g. Ets, Myc, E2F families), can effectively be mutated into oncogenes.

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16.5: Tumor Suppressor Genes

Tumor suppressor genes normally do what would be expected from their name. Whereas the oncogenes mostly drive the cell cycle forward, the tumor suppressor genes' primary functions are to temporarily stall the cell cycle so that DNA repair mechanisms can have time to work. However, if repair is unsuccessful after a few attempts, the tumor suppressor gene product may then trigger apoptosis rather than allow a damaged cell to replicate and potentially create another genetically damaged cell. Thus, the presence of an oncogene in a cell will not necessarily lead to development of cancer because a functioning tumor suppressor gene might prevent the cell from replicating. Equally, if a tumor suppressor gene is knocked out but there is no oncogene present, then the cell is unlikely to be immediately cancerous because although a cellular "emergency brake" is nonfunctional, if there is nothing to drive the cell through its cycle any faster or more frequently than usual, then the "brake" is never needed anyway.

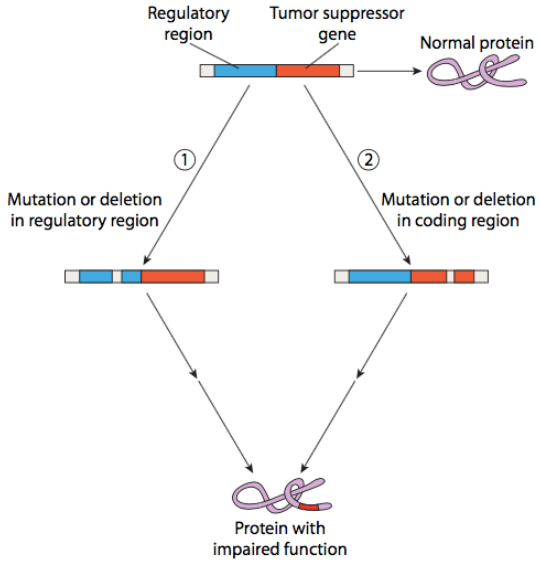


Figure 16.5.14. Tumor suppressor gene mutations can lead to cancer.

Like oncogenes, tumor suppressor genes can work (or not work, as would be the case in cancer) in several ways. Here is an example with the breast cancer-associated genes, BRCA1 and BRCA2. These gene products are involved in DNA repair (chapter 7). When BRCA1 or BRCA2 is knocked out, the cell loses its ability to use that DNA repair pathway. There are other repair pathways, and even if there weren't there may not be any serious lesions to the DNA, so the cell could behave normally for the time being. What is important from a cancer standpoint, is that each safety/repair mechanism that is lost increases the likelihood that an additional mutation may cause the cell to become cancerous.

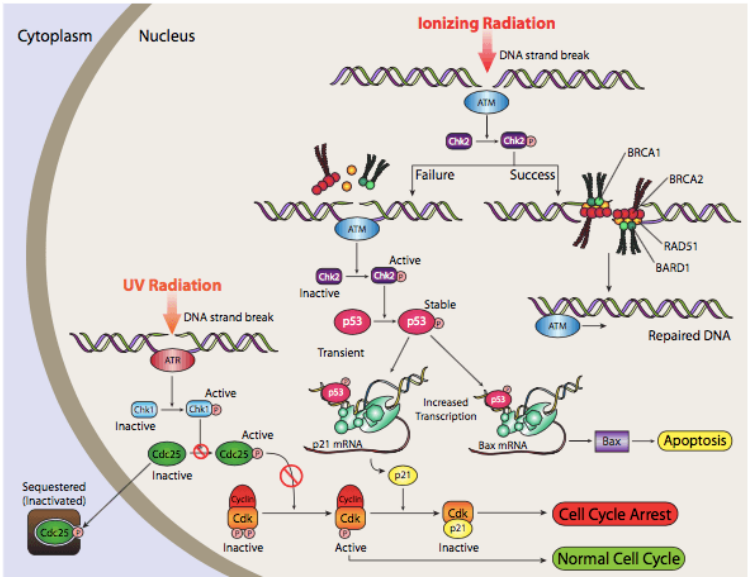


Figure 16.5.15. Cell cycle arrest due to DNA damage. ATM detects the double strand break, and activates Chk2 and BRCA1. Chk2 also activates BRCA1, which with BRCA2 forms a repair complex. However, if BRCA1 is not immediately available, the cell needs to go into a holding pattern until one becomes available. Therefore, Chk2 activates p53, which induces transcription of p21, which binds to cdk, preventing association with cyclin, and thus preventing cell cycle progress. If this continues for long, some of the p53 activates transcription of Bax, which will induce apoptosis to kill off a cell with damaged DNA. When p53 is hit with a loss of function mutation, the cell does not die, and it attempts to replicate even with damaged DNA, which may lead to more mutations in the subsequent generation, if it is successful in reproduction. Without p53, the accumulation of errors in successive generations increases. The mechanism of buying time for the cell to make repairs is not limited to the ATM-BRCA situation. The left side of the Figure shows another response to DNA damage that leads to cell cycle arrest.

It should be clear now how recessive loss-of-function mutations in a tumor suppressor gene can lead to an inherited predisposition to cancer. As diploid organisms, we have two copies of each gene in our cells, so losing one to mutation does not wipe out the protective function. Thus, if nothing happens to the other one, then the cell is fine. It is just a question of probability. Losing the function of one is a very low probability event, but the probability of losing both copies is extremely small. Thus, even though it is “only 1 step” on the way to losing the protection of this particular tumor suppressing function, it is a very large difference in probabilities. Of course, keep in mind that even complete loss of a single tumor suppressor gene is usually not enough to lead immediately to cancer, and still other mutations must occur to take advantage of the weakened cell defenses and push it towards a cancerous state.

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16.6: Human Cancers

Although some oncogenes and tumor suppressor genes have a restricted distribution that hints at likely tumor locations, many of the genes are widespread and even ubiquitous. It is presently unclear, therefore, why certain types of cancer are linked to particular mutated genes, but there are a number of strongly correlated cases.

> Cancers are classified by the tissue type in which the tumors first arise. Thus, *carcinomas*, which are the most common type (~85% of human cancers), come from epithelial cells arising from either the embryonic ectoderm (skin and nerve cells) or endoderm (gut lining). *Leukemias* (~4%) arise from white blood cells. *Lymphomas* (~5%) reflect aberrant growth of lymphocytes in spleen or lymph nodes. *Sarcomas* (~2%) arise from connective tissue of mesodermal origin, such as bone cancers.

Retinoblastoma is a cancer of the eye that usually strikes at a relatively young age. It has been linked to the RB gene, which encodes a repressor of E2F, a transcription factor that would normally turn on genes needed for S phase progression. Only 10% of individuals who inherit the RB loss-of-function mutation escape the development of the cancer. It also turns out that people with the RB mutation have a higher incidence of developing other tumors as well, although generally later in life. Perhaps the higher rate of damage to retinal cells (due to light exposure) leads to greater susceptibility.

Breast cancer is another disease that has strong links to mutations in certain genes. Loss of function mutations to the BRCA1 gene encoding a DNA repair protein lead to a five-fold higher risk of developing breast cancer in a woman's lifetime. Although mutations to other tumor suppressors (including p53, PTEN, CHEK2, ATM) most hereditary breast cancers have a link to BRCA1 or BRCA2 mutation. On the oncogene side, breast cancer tumors consistently show expression of CYCD1 (a cyclin) mutations, and depending on the type of tumor, HER2/Neu may be linked as well.

Lung cancers are among the most common - the second highest in men (prostate is higher) and women (breast is higher) alike, and make up approximately 1 in 3 cancer deaths annually. Several oncogenes of the myc family: N-myc, L-myc, and c-myc, as well as H-ras have been linked to various lung cancers. Loss of p53 and RB are also associated with the development of lung cancers, and perhaps not coincidentally, tobacco smoking is associated with p53 mutations. Interestingly, despite being so common, so far, there have been no particular oncogenes associated with prostate cancer, nor any hint of prostate-specific tumor suppressor susceptibilities.

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16.7: Metastasis

The onset of metastasis signals a drastic change in the prognosis of a cancer patient. While pre-metastatic tumors can certainly be dangerous or painful, treatment can be fairly localized, e.g. surgical excision and directed radiation therapy. Once the tumor metastasizes it must be treated systemically due to the potential for secondary tumors literally anywhere in the body. This presents a problem because the tumor cells are derived from the body's cells and are mostly indistinguishable by the body's immune system. The primary mechanism for anti-cancer drugs is to target fast proliferation, since most cancer cells proliferate much faster than most normal cells, but this still kills off some of the body's naturally fast-proliferating cells, such as the epithelial cells lining the gut. More recently, other approaches to anti-cancer drug treatments have been developed; most notably, anti-angiogenesis drugs to starve tumors by preventing them from developing or recruiting new blood vessels. As tumors grow, the ability to absorb nutrients from the environment decreases for the innermost cells of a solid tumor.

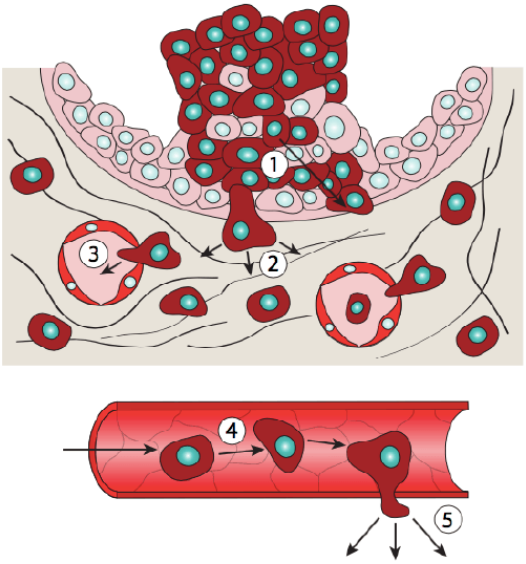


Figure 16.7.16. Metastasis.

Metastasis (Figure 16.7.16) starts with downregulation of cell-cell adhesions (1). This may include inside-out signaling to integrin receptors, or downregulation of cadherin expression, and other methods for allowing the cell to separate from the rest of the tumor. Non-metastatic tumors are surrounded by a capsule of extracellular matrix that contains the tumor in its location. To escape this capsule, the metastasizing cell must secrete proteases (usually metalloproteases) that can break down the ECM proteins (2). Once out into the looser connective mesenchymal tissue, the metastatic cell increases its locomotive activity and heads for a blood vessel. Intravasation (3) into a small, low-flow blood vessel allows the cell to be carried to nearly any destination in the body by the circulatory system (4). At some point, the metastatic cell will attach to the interior wall of a blood vessel, and exit the circulation (5). The molecules and situations that determine the point of exit are not clear yet, although there are clearly preferred sites of metastasis for some types of tumors. Presumably, there is specific recognition and adhesion occurring based on cell adhesion molecule expression on tumor cell and target tissue surfaces.

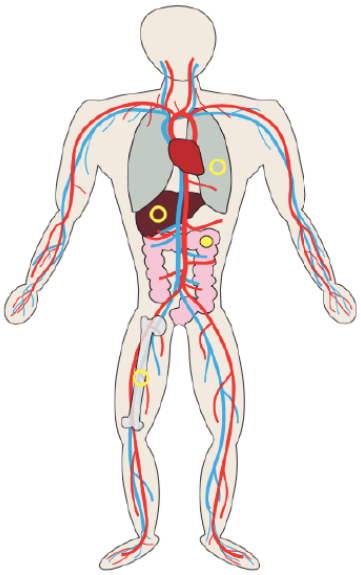


Figure 16.7.17. Common sites (open yellow circles) for the metastasis of colon cancers (filled yellow circle).

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16.8: The Immune System

Immunology is a full semester course at most universities, so this section will only touch on a few basic concepts that should be easily accessible to the student who has nearly completed the cell biology course. At its core, immunology is about adaptation. That is, since an animal has no preconception of the various potential infections it may be subject to, it must have a system in place that is flexible enough to deal with almost anything that comes along. Obviously, the systems are not perfect, but considering the wide range of pathogens, immune systems are remarkably efficient. In humans, there are two types of immune response to infection: the innate response, which is relatively nonspecific, and the adaptive, or acquired, response, which has more specificity.

The innate immune responses are common to all animals, and act on large classes of pathogens. For example, Toll-like receptors on phagocytes recognize a variety of bacterial surface molecules such as the flagellin specific to bacterial flagella, or the peptidoglycan components of bacterial cell walls. When these receptors are activated, the phagocyte goes into action, enveloping the offending bacteria or virus, and breaking it down. This depends on recognizing the external surface, so bacteria or viruses that do not have a recognizable molecule on their surface are able to escape this particular line of defense.

Defensins, which are found on a variety of surfaces (skin, cornea, gut) as well as in circulation, are small (18-45 amino acids) cysteine-rich cationic proteins that bind to a variety of pathogenic viruses, bacteria, and fungi. It is unclear how they may work against viruses, other than perhaps attacking infected host cells, but against bacteria and fungi the mode of operation is generally to bind to the cell membrane and form a pore that allows ions and other small molecules to ow out killing the pathogen. Complement, a group of proteins (~20) circulating in the blood, can act similarly against pathogenic cells.

Finally, natural killer (NK) cells, lymphocytes that target any cell that does not carry cell surface proteins that are normally found on cells from the animal, can kill not only attacking cells, but virally infected cells that have stopped producing their normal proteins (including the recognition protein) because they are busy producing viral proteins. NK cells can even be effective against some cancer cells if they have downregulated cell surface protein expression as part of their de-differentiation and de-adhesion.

The adaptive immune system, which is only found in vertebrates, is what most people think of when the human immune system is mentioned. We and other vertebrates also have an innate immune system, but all the molecules and cells that normally come to mind — antibodies, T-cells, B-cells — are part of the adaptive immune response. There are two components to the adaptive response, a humoral response, in which proteins (antibodies) floating in the blood bind to the infectious agent and prevent if from binding to cells or targeting it for the cellular response, which is mediated by T cells that can specifically recognize and kill the targeted pathogen.

Antibodies

Front and center in the adaptive immune response are antibodies. These proteins may be either secreted by or attached to the surface of B cells, the lymphocytes that differentiate either in bone marrow (adult) or liver (fetus), as opposed to those called T cells, which differentiate in the thymus gland. Incidentally, if you see sweetbreads on a menu, that would be thymus. Yum. You can take that seriously or sarcastically depending on how you think my tastes run.

Back to the antibodies. The different types of antibodies, IgA, IgD, IgE, IgG, and IgM, are all based on the IgG structure (Figure 16.8.18), which is roughly Y-shaped, and composed of two heavy chains and two light chains. These chains have disulfide bond-stabilized loops (recall the Ig-like loops in the cell adhesion molecules a few chapters back?), and the combination of the distal light chain loop and distal heavy chain loop make the antigen binding site. The antigen is defined as the molecule, or more specifically the part of a molecule that is recognized by the particular antibody. Since the antibody is meant to mediate highly specific recognition of a wide variety of invading pathogens, there must be a way to create at least as many different antibodies. This is made possible by the process of *DNA rearrangement*. This mechanism is also used to generate diversity in T-cell receptors, which are quite different structurally, but also need to be available with an extremely wide variety of specific binding sites.

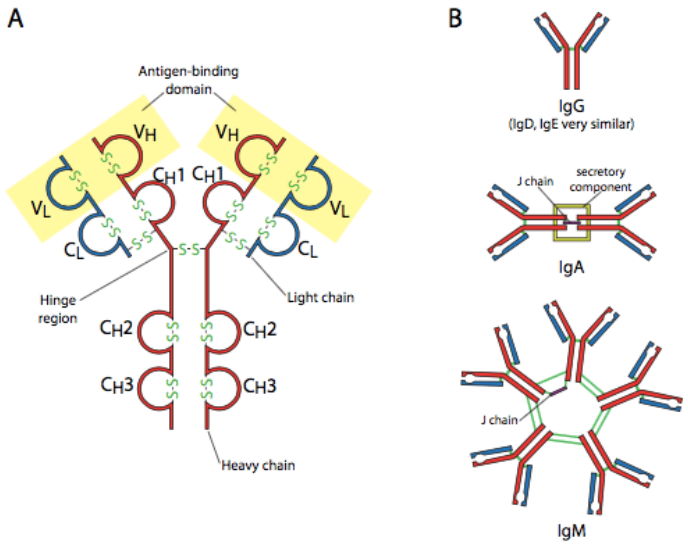


Figure 16.8.18. Antibodies. (A) an antibody is composed of two heavy chains (red) and two light chains (blue). Each has a variable region, and constant region(s). (B) IgA and IgM are built upon multiple IgG-like structures.

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16.9: DNA Rearrangement

One of the central assumptions throughout our study of the cell has been that although the RNA and proteins in any cell may differ, any cell of a given organism other than the gametes should have the same DNA. This is not the case with B cells or T cells. In these cells, part of the maturation process is to create a unique arrangement of different domains to form a specific antibody (or T-cell receptor). The germline DNA, or the DNA that is found in all other somatic cells of the organism, contains many different such segments, but only a few are put together to make the antibody/TCR. This is a stochastic process, and with this kind of rearrangement happening on both heavy chain genes and two different light chain genes (designated κ and λ), there are well over 10 trillion (1013) different combinations for generating immunoglobulins in humans, and even more combinations for T-cell receptors! How is this accomplished?

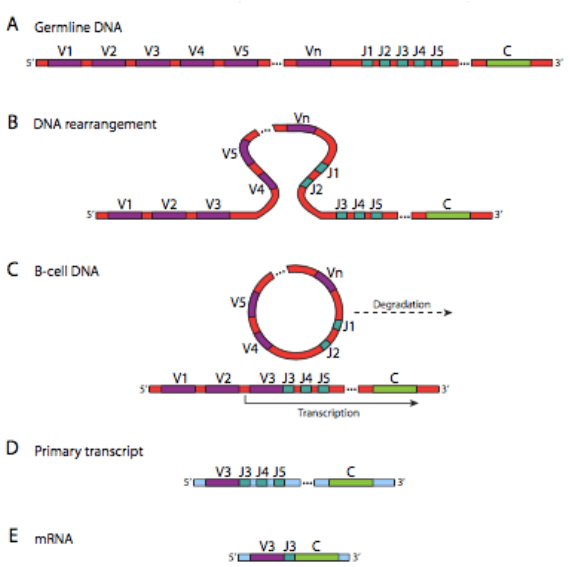


Figure 16.9.19. DNA rearrangement of a k light chain gene. (A) The germline DNA has approximately 40 V region genes, 5 J segments, and one C region gene. (B) By action of the V(D)J recombinase, a random V region is brought close to a random J region, and the intervening sequence is cut out (C). (D) The gene may still contain multiple J segments, but RNA splicing removes all but one, leaving the final mRNA (E) with one V, one J, and one C region.

Figure 16.9.19 shows the DNA rearrangements that take place in generating κ chain diversity. The λ chain locus has a slightly different arrangement, and has only 30 V genes, with 4 J segments and 4 C genes. The heavy chain has an extra domain: there are 40 V genes, which are linked to one of 25 D segments, then 6 potential J segments, and one C gene. These rearrangements, although they look something like the RNA splicing that we saw earlier in this course, are happening at the DNA level. Once it has happened, that cell and its progeny can no longer make the other combinations because those parts of the genome have been cut out and destroyed. This is distinctly different from alternative splicing of RNA, in which the genetic information is still there, and under different conditions could still generate other variations of the gene product.

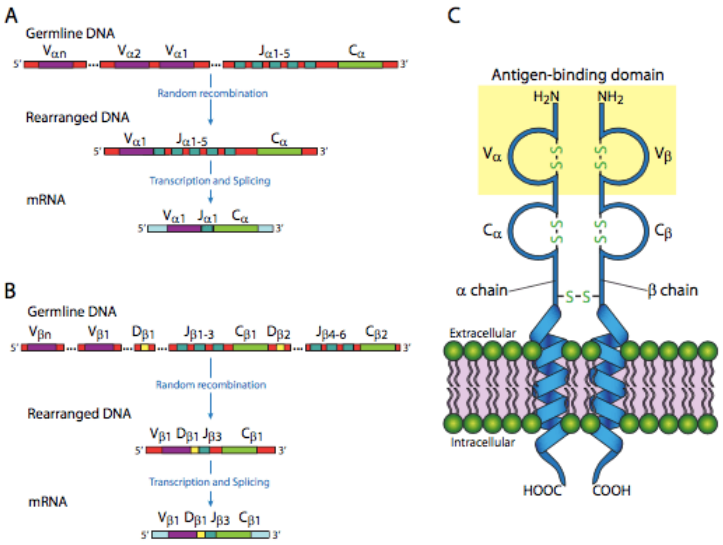


Figure 16.9.20. T-cell receptor genes also under DNA rearrangement to generate diversity like antibodies. In fact, there is actually greater diversity in TCR than in immunoglobins. (A) rearrangement of α chain and (B) β chain (C) protein structure of TCR.

The enzyme that produces this diversity is a complex called the V(D)J recombinase. The recombination occurs in two parts: first double-stranded breaks are made at re- combination signal sequence (RSS) sites, then the breaks are repaired by the general double-stranded break repair mechanism. Depending on which J segment is chosen, there may be more than one left in the gene after the rearrangement. However, only the one closest to the V segment is used, and the others are spliced out of the primary transcript (by normal RNA splicing) in the process of connecting the C segment to the V and J for the final mRNA. Although this process generates great diversity, there is another mechanism that can generate further diversity under certain circumstances.

> RAG1 and RAG2, lymphocyte-specific recombination activating genes, recognize the RSS sites and make the double-stranded cuts. Once the cuts are made, the excised portion joins its ends together to form a circular signal joint (SJ) which is then degraded. The coding portions have asymmetric cut ends that fold into hairpin formations and prevent their fusion. These hairpins are broken by Artemis, a nuclease recruited by DNA-dependent protein kinase (DNA-PK), which also brings together XRCC4, XLF, DNA ligase IV, and a DNA polymerase. The XRCC and XLF alight the DNA ends, recruits a terminal transferase that randomly adds nucleotides to the ends, then DNA polymerase λ or μ fills in the overhangs, and the ligase completes the join. Interestingly, this process adds even more variability to the immunoglobulin, since Artemis cuts the hairpin at random, and the terminal transferase also adds nucleotides at random.

Somatic hypermutation causes rearranged V segments to mutate at 10^5 times the rate of other DNA! This mechanism is carried out by Activation-Induced Cytidine Deaminase (AID), which converts cytidines to uracils, generating a G:U mismatch that is “corrected” by repair polymerases without strong error-correction. This hypermutation is initiated by the activation of a B cell by recognizing and binding to a ligand. As we will see in the next paragraphs, when that happens, the B cell initiates rapid proliferation and this is when the somatic hypermutation takes effect, so that many of the B cells will carry highly similar but subtly different antibodies than the initial B cell that recognized and was activated by the antigen. The idea is some of these subtle mutation may lead to antibodies with higher affinity for the antigen and therefore faster response the next time this particular pathogen tries to infect the organism.

The reason that this kind of DNA rearrangement is necessary is that antibody “design” is not a reactive system, but a proactive system. A common misconception is that the immune system encounters a pathogen and creates antibodies that t it. Unfortunately, there is no known mechanism by which a cell can “feel” the shape of something and create a protein that matches it. Instead, the immune system preemptively makes as many different antibodies (and TCRs) as possible, so that initially, most of the B and T cells in the body are actually genetically different. If an infection occurs, most of the B and T cells will bump into the pathogen and bounce right off, not recognizing it, but some of them will have the right antibody combination to bind to a part of the pathogenic invader. Although B cells can recognize surface antigens on their own, in most cases, a helper T cell is needed to activate the B cell (Figure 16.9.21).

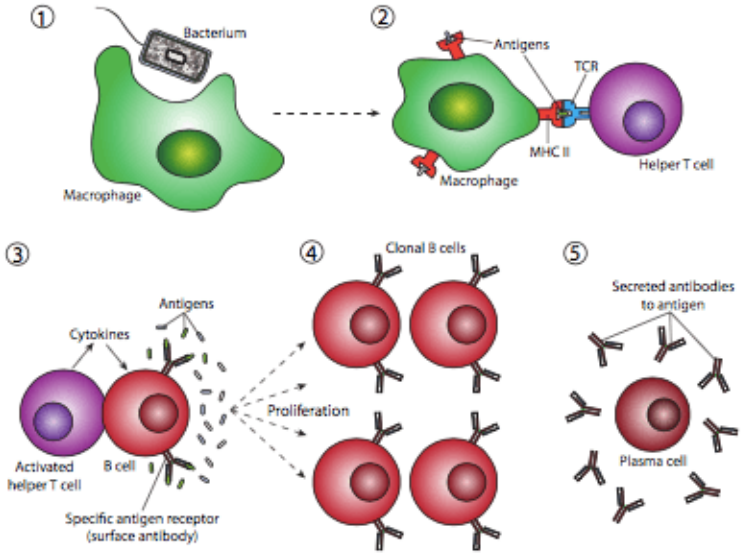


Figure 16.9.21. Activation of B cells by helper T-cells.

This process is initiated by a macrophage non-specifically ingesting a pathogen (1), breaking it apart, and presenting bits of it on its cell surface in partnership with MHC (major histocompatibility complex). A helper T-cell with a TCR that can recognize the antigen presented by the macrophage binds to it (2) and that leads to activation of the T cell. The activated helper T-cell binds to and activates a B cell (3) that has also bound to the antigen of interest, leading to massive B-cell proliferation (4), thus providing the body with many more copies of cells that have the right antibody to locate and fught the infection. This does two things: it provides lymphocyte reinforcements to specifically deal with a particular pathogen (but not other B cells, Figure 16.9.22), and once the pathogen has been eliminated, there is a larger circulating pool of these cells to respond more quickly to any subsequent infection by this particular type of pathogen.

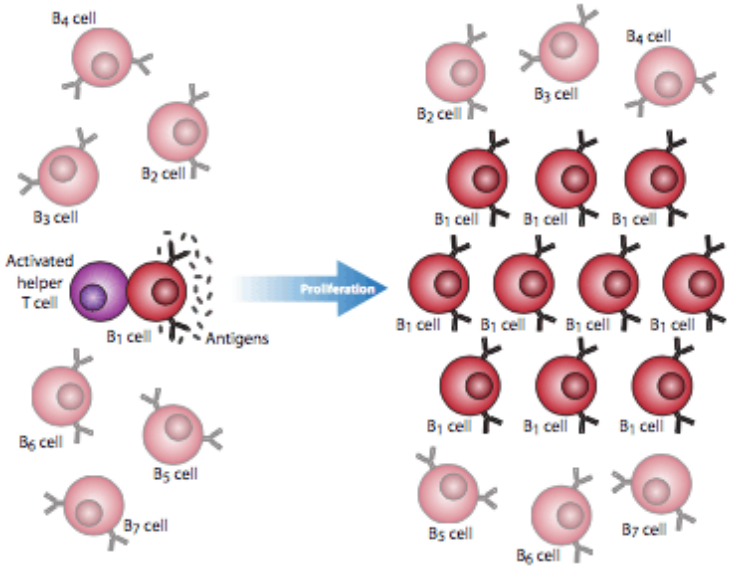


Figure 16.9.22. Amplification of only those B cells possessing antibodies that can recognize the infectious particle(s).

Finally, some of the B cells will differentiate into plasma cells (Figure 16.9.21-5), that secrete antibodies into the blood- stream to provide a humoral response. Others become memory cells, which are like B cells in that the antibody is on its cell surface and not secreted, but they can be thought of as “pre-activated” and can respond more quickly than naive B cells to re-infection.

The big question that should have been lurking in the back of your mind through all this is, how do the antibodies and T-cell receptors tell what’s foreign and what’s part of one’s own body? We’ll get to that shortly. First, recall the activation of the helper T-cell. It occurs when the T-cell receptor recognizes an antigen from an ingested pathogen being presented by the MHC class II molecule on an antigen presenting cell (e.g. a macrophage).

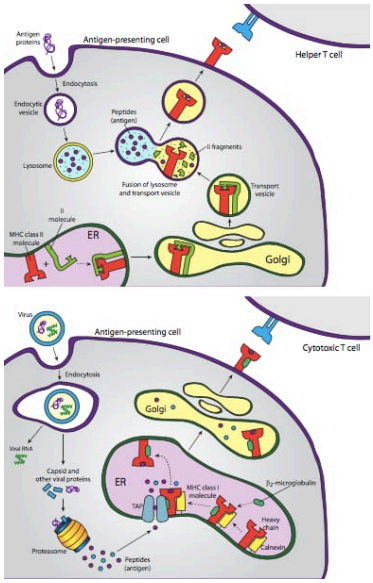


Figure 16.9.23. Antigen pre- sentation by MHC class II (top) and class I (bottom) to T cells.

Figure 16.9.23 shows the pathway from ingestion of the pathogen to the presentation of its molecular parts on an MHC molecule. A similar pathway also applies to presentation of antigens on MHC class I molecules in conjunction with cytotoxic (killer) T cells. The cytotoxic T cells work against compromised cells, whether they are infected by a virus or another pathogen (Figure 16.9.24).

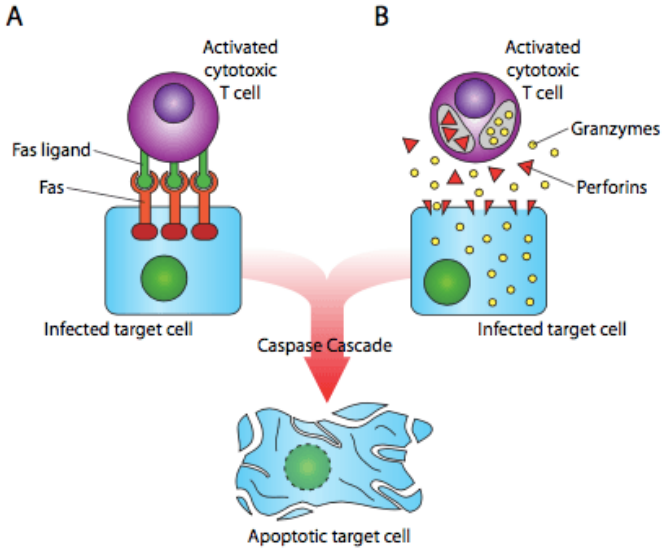


Figure 16.9.24. Cytotoxic T cells rst recog- nize an infected cell by the T cell receptor, thich then leads to (A) binding of T-cell Fas ligand to Fas on the target cell, or alternatively, (B) secretion of granzymes and perforins. Both lead to activation of a caspase cascade and subsequent apop- tosis of the infected cell.

There are two major pathways to killing the infected cell. One is the activation of a “death receptor”, Fas, which induces a signal transduction cascade to activate caspases and apoptosis. The other pathway is the release of granzymes and perforins. The perforins drill into the membrane of the target cell and become pores that allow, among other things, granzymes to enter the cell, where they activate caspases by proteolysis, and again induce apoptosis. An important part of this is that the T cell receptor recognizes the antigen in combination with the MHC molecule that is presenting it. Furthermore, there are many variations of MHC molecules due there being 6 loci each with many known alleles.

So, what does any of this have to do with self vs non-self recognition? Early in the development of the immune system, the MHC does not present bits of digested pathogens, it presents bits of the organism’s own cells that have gone through a proteasome or lysosome. At this early time, T cells behave somewhat differently, and if the T cell receptor binds strongly, the T cell commits apoptosis. This gets rid of TCR genes that strongly react to the organism’s own cells. If the T cells do not react at all, apoptosis

is also invoked, because the TCR genes that cannot recognize the MHC will not be useful in an immune response. Only those T cells that very weakly bind to the self-presenting MHC survive (Figure 16.9.25).

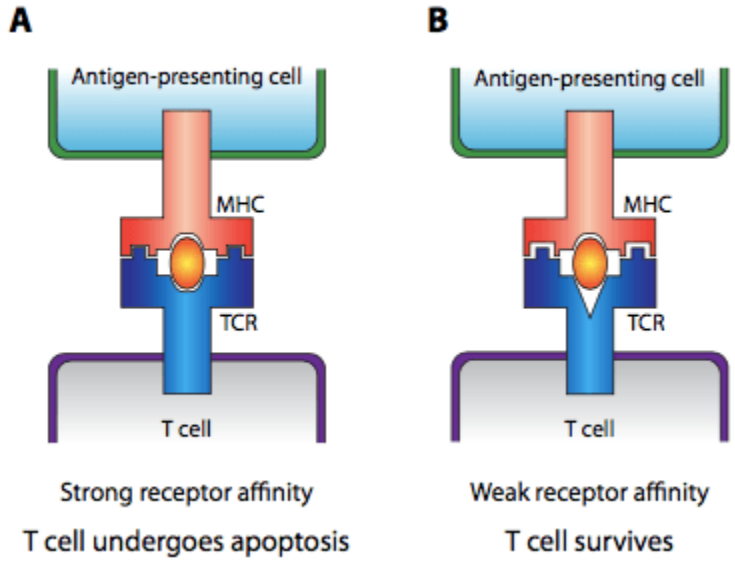


Figure 16.9.25. Self-recognition by T-cell receptor binding of MHC proteins presenting antigens derived from the organism's own cells. If there is strong recognition (A), the T cell dies to prevent cytotoxic attacks on its own cells. If it is weak and the TCR recognizes the MHC but not the antigen, the T cell survives (B).

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