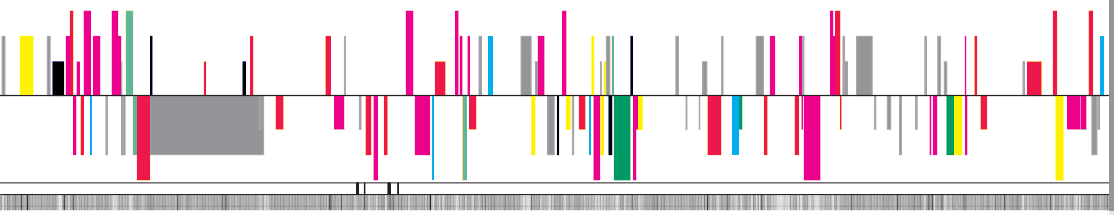


5

DNA REPLICATION, REPAIR, AND RECOMBINATION

The ability of cells to maintain a high degree of order in a chaotic universe depends upon the accurate duplication of vast quantities of genetic information carried in chemical form as DNA. This process, called *DNA replication*, must occur before a cell can produce two genetically identical daughter cells. Maintaining order also requires the continued surveillance and repair of this genetic information because DNA inside cells is repeatedly damaged by chemicals and radiation from the environment, as well as by thermal accidents and reactive molecules. In this chapter we describe the protein machines that replicate and repair the cell's DNA. These machines catalyze some of the most rapid and accurate processes that take place within cells, and their mechanisms clearly demonstrate the elegance and efficiency of cellular chemistry.

While the short-term survival of a cell can depend on preventing changes in its DNA, the long-term survival of a species requires that DNA sequences be changeable over many generations. Despite the great efforts that cells make to protect their DNA, occasional changes in DNA sequences do occur. Over time, these changes provide the genetic variation upon which selection pressures act during the evolution of organisms.

We begin this chapter with a brief discussion of the changes that occur in DNA as it is passed down from generation to generation. Next, we discuss the cellular mechanisms—DNA replication and DNA repair—that are responsible for keeping these changes to a minimum. Finally, we consider some of the most intriguing ways in which DNA sequences are altered by cells, with a focus on DNA recombination and the movement of special DNA sequences in our chromosomes called transposable elements.

THE MAINTENANCE OF DNA SEQUENCES

Although the long-term survival of a species is enhanced by occasional genetic changes, the survival of the individual demands genetic stability. Only rarely do the cell's DNA-maintenance processes fail, resulting in permanent change in the DNA. Such a change is called a **mutation**, and it can destroy an organism if it

occurs in a vital position in the DNA sequence. Before examining the mechanisms responsible for genetic stability, we briefly discuss the accuracy with which DNA sequences are maintained from one generation to the next.

Mutation Rates Are Extremely Low

The **mutation rate**, the rate at which observable changes occur in DNA sequences, can be determined directly from experiments carried out with a bacterium such as *Escherichia coli*—a resident of our intestinal tract and a commonly used laboratory organism. Under laboratory conditions, *E. coli* divides about once every 30 minutes, and a very large population—several billion—can be obtained from a single cell in less than a day. In such a population, it is possible to detect the small fraction of bacteria that have suffered a damaging mutation in a particular gene, if that gene is not required for the survival of the bacterium. For example, the mutation rate of a gene specifically required for cells to utilize the sugar lactose as an energy source can be determined (using indicator dyes to identify the mutant cells), if the cells are grown in the presence of a different sugar, such as glucose. The fraction of damaged genes is an underestimate of the actual mutation rate because many mutations are *silent* (for example, those that change a codon but not the amino acid it specifies, or those that change an amino acid without affecting the activity of the protein coded for by the gene). After correcting for these silent mutations, a single gene that encodes an average-sized protein (~10^3 coding nucleotide pairs) is estimated to suffer a mutation (not necessarily one that would inactivate the protein) once in about 10^6 bacterial cell generations. Stated in a different way, bacteria display a mutation rate of 1 nucleotide change per 10^9 nucleotides per cell generation.

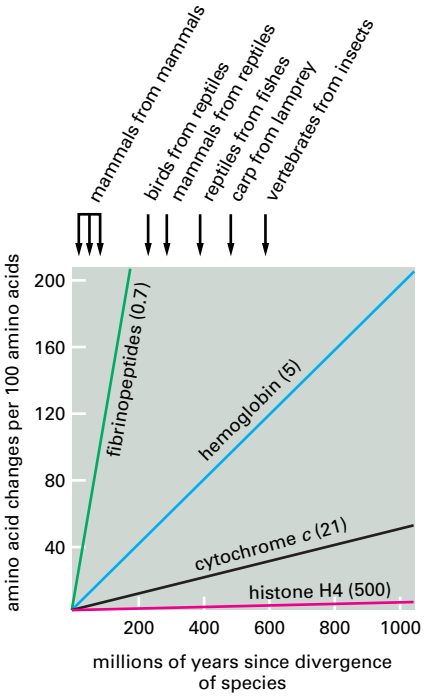
The germ-line mutation rate in mammals is more difficult to measure directly, but estimates can be obtained indirectly. One way is to compare the amino acid sequences of the same protein in several species. The fraction of the amino acids that are different between any two species can then be compared with the estimated number of years since that pair of species diverged from a common ancestor, as determined from the fossil record. Using this method, one can calculate the number of years that elapse, on average, before an inherited change in the amino acid sequence of a protein becomes fixed in an organism. Because each such change usually reflects the alteration of a single nucleotide in the DNA sequence of the gene encoding that protein, this value can be used to estimate the average number of years required to produce a single, stable mutation in the gene.

These calculations will nearly always substantially underestimate the actual mutation rate, because many mutations will spoil the function of the protein and vanish from the population because of natural selection—that is, by the preferential death of the organisms that contain them. But there is one family of protein fragments whose sequence does not seem to matter, allowing the genes that encode them to accumulate mutations without being selected against. These are the *fibrinopeptides*, 20 amino-acid fragments that are discarded from the protein *fibrinogen* when it is activated to form *fibrin* during blood clotting. Since the function of fibrinopeptides apparently does not depend on their amino acid sequence, they can tolerate almost any amino acid change. Sequence comparisons of the fibrinopeptides indicate that a typical protein 400 amino acids long would be randomly altered by an amino acid change in the germ line roughly once every 200,000 years.

Another way to estimate mutation rates is to use DNA sequencing to compare corresponding nucleotide sequences from different species in regions of the genome that do not carry critical information. Such comparisons produce estimates of the mutation rate that are in good agreement with those obtained from the fibrinopeptide studies.

E. coli and humans differ greatly in their modes of reproduction and in their generation times. Yet, when the mutation rates of each are normalized to a single round of DNA replication, they are found to be similar: roughly 1 nucleotide change per 10^9 nucleotides each time that DNA is replicated.

Figure 5–1 Different proteins evolve at very different rates. A comparison of the rates of amino acid change found in hemoglobin, histone H4, cytochrome *c*, and the fibrinopeptides. The first three proteins have changed much more slowly during evolution than the fibrinopeptides, the number in parentheses indicating how many million years it has taken, on average, for one *acceptable* amino acid change to appear for every 100 amino acids that the protein contains. In determining rates of change per year, it is important to realize that two species that diverged from a common ancestor 100 million years ago are separated from each other by 200 million years of evolutionary time.



Many Mutations in Proteins Are Deleterious and Are Eliminated by Natural Selection

When the number of amino acid differences in a particular protein is plotted for several pairs of species against the time that has elapsed since the pair of species diverged from a common ancestor, the result is a reasonably straight line: the longer the period since divergence, the larger the number of differences. For convenience, the slope of this line can be expressed in terms of a "unit evolutionary time" for that protein, which is the average time required for 1 amino acid change to appear in a sequence of 100 amino acid residues. When various proteins are compared, each shows a different but characteristic rate of evolution (Figure 5–1).

Since most DNA nucleotides are thought to be subject to roughly the same rate of random mutation, the different rates observed for different proteins must reflect differences in the probability that an amino acid change will be harmful for each protein. For example, from the data in Figure 5–1, we can estimate that about six of every seven random amino acid changes are harmful in cytochrome *c,* and that virtually all amino acid changes are harmful in histone H4. The individual animals that carried such harmful mutations were presumably eliminated from the population by natural selection.

Low Mutation Rates Are Necessary for Life as We Know It

Since so many mutations are deleterious, no species can afford to allow them to accumulate at a high rate in its germ cells. Although the observed mutation frequency is low, it is nevertheless thought to limit the number of essential proteins that any organism can encode to perhaps 60,000. By an extension of the same argument, a mutation frequency tenfold higher would limit an organism to about 6000 essential genes. In this case, evolution would probably have stopped at an organism less complex than a fruit fly.

Whereas *germ cells* must be protected against high rates of mutation to maintain the species, the *somatic cells* of multicellular organisms must be protected from genetic change to safeguard each individual. Nucleotide changes in somatic cells can give rise to variant cells, some of which, through natural selection, proliferate rapidly at the expense of the rest of the organism. In an extreme case, the result is an uncontrolled cell proliferation known as cancer, a disease that causes about 30% of the deaths each year in Europe and North America. These deaths are due largely to an accumulation of changes in the DNA sequences of somatic cells (discussed in Chapter 23). A significant increase in the mutation frequency would presumably cause a disastrous increase in the incidence of cancer by accelerating the rate at which somatic cell variants arise. Thus, both for the perpetuation of a species with a large number of genes (germ cell stability) and for the prevention of cancer resulting from mutations in somatic cells (somatic cell stability), multicellular organisms like ourselves depend on the remarkably high fidelity with which their DNA sequences are maintained.

As we see in subsequent sections, successful DNA maintenance depends both on the accuracy with which DNA sequences are duplicated and distributed to daughter cells, and on a set of enzymes that repair most of the changes in the DNA caused by radiation, chemicals, or other accidents.

Summary

In all cells, DNA sequences are maintained and replicated with high fidelity. The mutation rate, approximately 1 nucleotide change per 10^9 nucleotides each time the DNA is replicated, is roughly the same for organisms as different as bacteria and humans. Because of this remarkable accuracy, the sequence of the human genome (approximately 3×10^9 nucleotide pairs) is changed by only about 3 nucleotides each time a cell divides. This allows most humans to pass accurate genetic instructions from one generation to the next, and also to avoid the changes in somatic cells that lead to cancer.

DNA REPLICATION MECHANISMS

All organisms must duplicate their DNA with extraordinary accuracy before each cell division. In this section, we explore how an elaborate "replication machine" achieves this accuracy, while duplicating DNA at rates as high as 1000 nucleotides per second.

Base-Pairing Underlies DNA Replication and DNA Repair

As discussed briefly in Chapter 1, *DNA templating* is the process in which the nucleotide sequence of a DNA strand (or selected portions of a DNA strand) is copied by complementary base-pairing (A with T, and G with C) into a complementary DNA sequence (Figure 5–2). This process entails the recognition of each nucleotide in the DNA *template* strand by a free (unpolymerized) complementary nucleotide, and it requires that the two strands of the DNA helix be separated. This separation allows the hydrogen-bond donor and acceptor groups on each DNA base to become exposed for base-pairing with the appropriate incoming free nucleotide, aligning it for its enzyme-catalyzed polymerization into a new DNA chain.

The first nucleotide polymerizing enzyme, **DNA polymerase**, was discovered in 1957. The free nucleotides that serve as substrates for this enzyme were found to be deoxyribonucleoside triphosphates, and their polymerization into DNA required a single-stranded DNA template. The stepwise mechanism of this reaction is illustrated in Figures 5–3 and 5–4.

The DNA Replication Fork Is Asymmetrical

During DNA replication inside a cell, each of the two old DNA strands serves as a template for the formation of an entire new strand. Because each of the two daughters of a dividing cell inherits a new DNA double helix containing one old and one new strand (Figure 5–5), the DNA double helix is said to be replicated "semiconservatively" by DNA polymerase. How is this feat accomplished?

Analyses carried out in the early 1960s on whole replicating chromosomes revealed a localized region of replication that moves progressively along the parental DNA double helix. Because of its Y-shaped structure, this active region

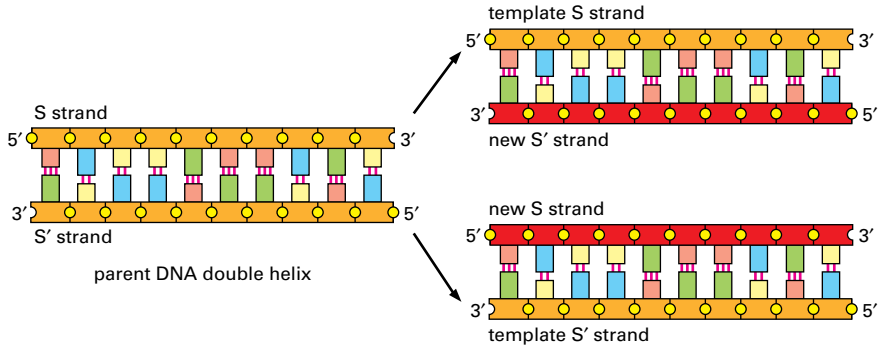


Figure 5–2 The DNA double helix acts as a template for its own duplication. Because the nucleotide A will successfully pair only with T, and G only with C, each strand of DNA can serve as a template to specify the sequence of nucleotides in its complementary strand by DNA base-pairing. In this way, a double-helical DNA molecule can be copied precisely.

Figure 5–3 The chemistry of DNA synthesis. The addition of a deoxyribonucleotide to the 3′ end of a polynucleotide chain (the *primer strand)* is the fundamental reaction by which DNA is synthesized. As shown, base-pairing between an incoming deoxyribonucleoside triphosphate and an existing strand of DNA (the *template strand)* guides the formation of the new strand of DNA and causes it to have a complementary nucleotide sequence.

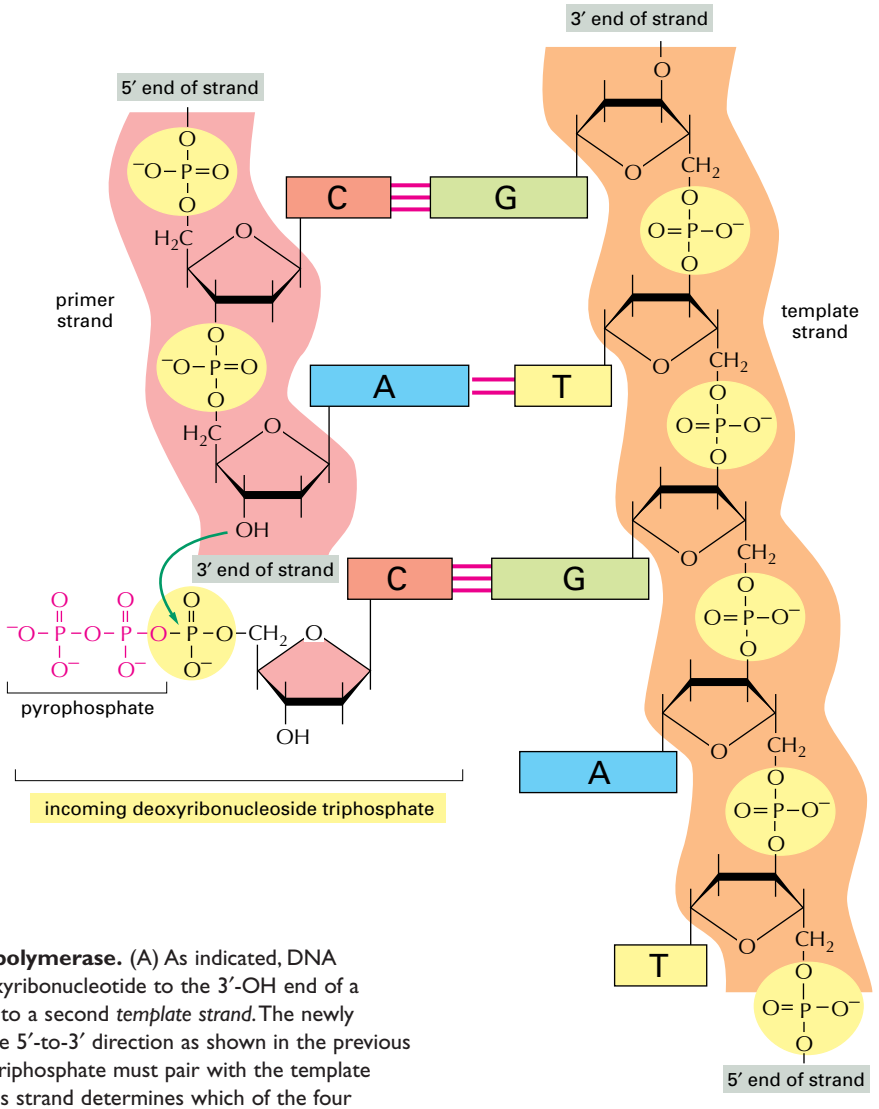
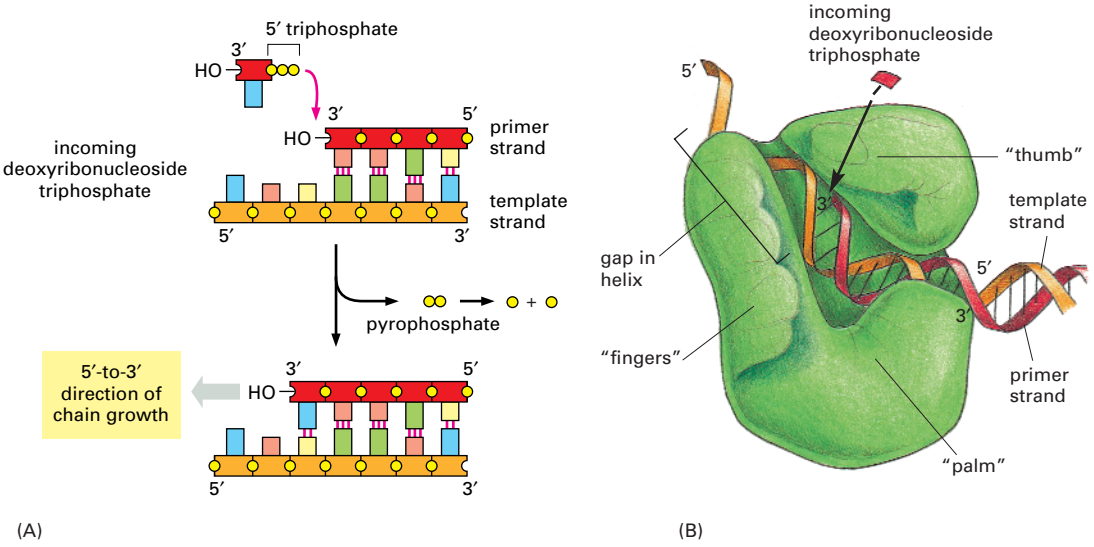


Figure 5–4 DNA synthesis catalyzed by DNA polymerase. (A) As indicated, DNA polymerase catalyzes the stepwise addition of a deoxyribonucleotide to the 3′-OH end of a polynucleotide chain, the *primer strand*, that is paired to a second *template strand*. The newly synthesized DNA strand therefore polymerizes in the 5′-to-3′ direction as shown in the previous figure. Because each incoming deoxyribonucleoside triphosphate must pair with the template strand to be recognized by the DNA polymerase, this strand determines which of the four possible deoxyribonucleotides (A, C, G, or T) will be added. The reaction is driven by a large, favorable free-energy change, caused by the release of pyrophosphate and its subsequent hydrolysis to two molecules of inorganic phosphate. (B) The structure of an *E. coli* DNA polymerase molecule, as determined by x-ray crystallography. Roughly speaking, it resembles a right hand in which the palm, fingers, and thumb grasp the DNA. This drawing illustrates a DNA polymerase that functions during DNA repair, but the enzymes that replicate DNA have similar features. (B, adapted from L.S. Beese, V. Derbyshire, and T.A. Steitz, *Science* 260:352–355, 1993.)



is called a **replication fork** (Figure 5–6). At a replication fork, the DNA of both new daughter strands is synthesized by a multienzyme complex that contains the DNA polymerase.

Initially, the simplest mechanism of DNA replication seemed to be the continuous growth of both new strands, nucleotide by nucleotide, at the replication fork as it moves from one end of a DNA molecule to the other. But because of the antiparallel orientation of the two DNA strands in the DNA double helix (see Figure 5–2), this mechanism would require one daughter strand to polymerize in the 5′-to-3′ direction and the other in the 3′-to-5′ direction. Such a replication fork would require two different DNA polymerase enzymes. One would polymerize in the 5′-to-3′ direction, where each incoming deoxyribonucleoside triphosphate carried the triphosphate activation needed for its own addition. The other would move in the 3′-to-5′ direction and work by so-called "head growth," in which the end of the growing DNA chain carried the triphosphate activation required for the addition of each subsequent nucleotide (Figure 5–7). Although head-growth polymerization occurs elsewhere in biochemistry (see pp. 89–90), it does not occur in DNA synthesis; no 3′-to-5′ DNA polymerase has ever been found.

How, then, is overall 3′-to-5′ DNA chain growth achieved? The answer was first suggested by the results of experiments in the late 1960s. Researchers added highly radioactive ^{3}H-thymidine to dividing bacteria for a few seconds, so that only the most recently replicated DNA—that just behind the replication fork—became radiolabeled. This experiment revealed the transient existence of pieces of DNA that were 1000–2000 nucleotides long, now commonly known as *Okazaki fragments,* at the growing replication fork. (Similar replication intermediates were later found in eucaryotes, where they are only 100–200 nucleotides long.) The Okazaki fragments were shown to be polymerized only in the 5′-to-3′chain direction and to be joined together after their synthesis to create long DNA chains.

A replication fork therefore has an asymmetric structure (Figure 5–8). The DNA daughter strand that is synthesized continuously is known as the **leading strand**. Its synthesis slightly precedes the synthesis of the daughter strand that is synthesized discontinuously, known as the **lagging strand**. For the lagging strand, the direction of nucleotide polymerization is opposite to the overall direction of DNA chain growth. Lagging-strand DNA synthesis is delayed because it must wait for the leading strand to expose the template strand on which each Okazaki fragment is synthesized. The synthesis of the lagging strand

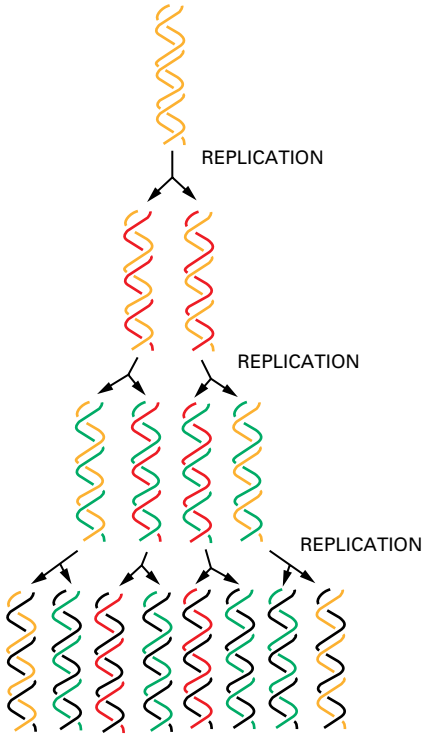


Figure 5–5 The semiconservative nature of DNA replication. In a round of replication, each of the two strands of DNA is used as a template for the formation of a complementary DNA strand. The original strands therefore remain intact through many cell generations.

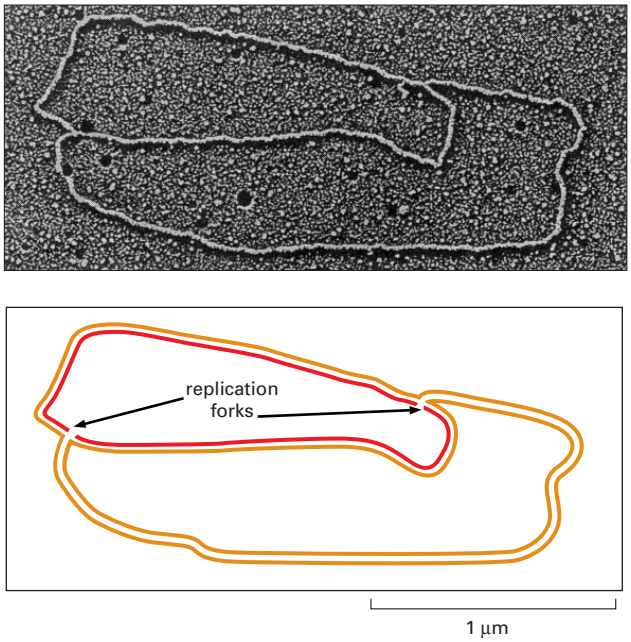


Figure 5–6 Two replication forks moving in opposite directions on a circular chromosome. An active zone of DNA replication moves progressively along a replicating DNA molecule, creating a Y-shaped DNA structure known as a replication fork: the two arms of each Y are the two daughter DNA molecules, and the stem of the Y is the parental DNA helix. In this diagram, parental strands are *orange;* newly synthesized strands are *red.* (Micrograph courtesy of Jerome Vinograd.)

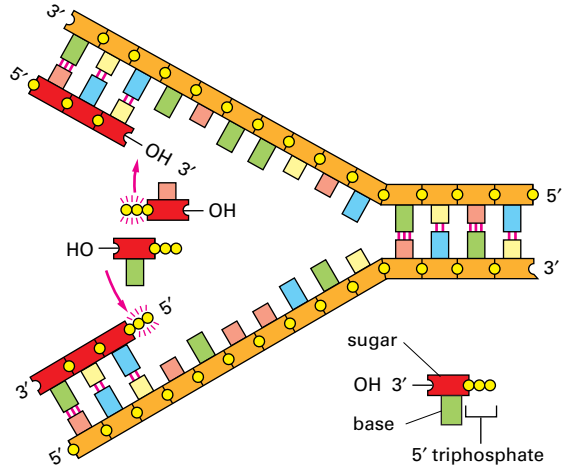


Figure 5–7 An incorrect model for DNA replication. Although it might seem to be the simplest possible model for DNA replication, the mechanism illustrated here is not the one that cells use. In this scheme, both daughter DNA strands would grow continuously, using the energy of hydrolysis of the two terminal phosphates *(yellow circles* highlighted by *red rays)* to add the next nucleotide on each strand. This would require chain growth in both the 5′-to-3′ direction *(top)* and the 3′-to-5′ direction *(bottom)*. No enzyme that catalyzes 3′-to-5′ nucleotide polymerization has ever been found.

by a discontinuous "backstitching" mechanism means that only the 5′-to-3′ type of DNA polymerase is needed for DNA replication.

The High Fidelity of DNA Replication Requires Several Proofreading Mechanisms

As discussed at the beginning of this chapter, the fidelity of copying DNA during replication is such that only about 1 mistake is made for every 10^9 nucleotides copied. This fidelity is much higher than one would expect, on the basis of the accuracy of complementary base-pairing. The standard complementary base pairs (see Figure 4–4) are not the only ones possible. For example, with small changes in helix geometry, two hydrogen bonds can form between G and T in DNA. In addition, rare tautomeric forms of the four DNA bases occur transiently in ratios of 1 part to 10^4 or 10^5. These forms mispair without a change in helix geometry: the rare tautomeric form of C pairs with A instead of G, for example.

If the DNA polymerase did nothing special when a mispairing occurred between an incoming deoxyribonucleoside triphosphate and the DNA template, the wrong nucleotide would often be incorporated into the new DNA chain, producing frequent mutations. The high fidelity of DNA replication, however, depends not only on complementary base-pairing but also on several "proofreading" mechanisms that act sequentially to correct any initial mispairing that might have occurred.

The first proofreading step is carried out by the DNA polymerase, and it occurs just before a new nucleotide is added to the growing chain. Our knowledge of this mechanism comes from studies of several different DNA polymerases, including one produced by a bacterial virus, T7, that replicates inside *E. coli*. The correct nucleotide has a higher affinity for the moving polymerase than does the incorrect nucleotide, because only the correct nucleotide can correctly base-pair with the template. Moreover, after nucleotide binding, but before the nucleotide is covalently added to the growing chain, the enzyme must undergo a conformational change. An incorrectly bound nucleotide is more likely to dissociate during this step than the correct one. This step therefore allows the polymerase to "double-check" the exact base-pair geometry before it catalyzes the addition of the nucleotide.

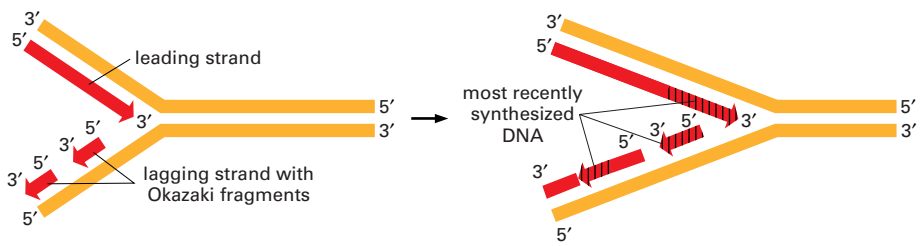


Figure 5–8 The structure of a DNA replication fork. Because both daughter DNA strands are polymerized in the 5′-to-3′ direction, the DNA synthesized on the lagging strand must be made initially as a series of short DNA molecules, called *Okazaki fragments*.

The next error-correcting reaction, known as *exonucleolytic proofreading*, takes place immediately after those rare instances in which an incorrect nucleotide is covalently added to the growing chain. DNA polymerase enzymes cannot begin a new polynucleotide chain by linking two nucleoside triphosphates together. Instead, they absolutely require a base-paired 3′-OH end of a *primer strand* on which to add further nucleotides (see Figure 5–4). Those DNA molecules with a mismatched (improperly base-paired) nucleotide at the 3′-OH end of the primer strand are not effective as templates because the polymerase cannot extend such a strand. DNA polymerase molecules deal with such a mismatched primer strand by means of a separate catalytic site (either in a separate subunit or in a separate domain of the polymerase molecule, depending on the polymerase). This *3′-to-5′ proofreading exonuclease* clips off any unpaired residues at the primer terminus, continuing until enough nucleotides have been removed to regenerate a base-paired 3′-OH terminus that can prime DNA synthesis. In this way, DNA polymerase functions as a "self-correcting" enzyme that removes its own polymerization errors as it moves along the DNA (Figures 5–9 and 5–10).

The requirement for a perfectly base-paired primer terminus is essential to the self-correcting properties of the DNA polymerase. It is apparently not possible for such an enzyme to start synthesis in the complete absence of a primer without losing any of its discrimination between base-paired and unpaired growing 3′-OH termini. By contrast, the RNA polymerase enzymes involved in gene transcription do not need efficient exonucleolytic proofreading: errors in making RNA are not passed on to the next generation, and the occasional defective RNA molecule that is produced has no long-term significance. RNA polymerases are thus able to start new polynucleotide chains without a primer.

An error frequency of about 1 in 10^4 is found both in RNA synthesis and in the separate process of translating mRNA sequences into protein sequences. This level of mistakes is 100,000 times greater than that in DNA replication, where a series of proofreading processes makes the process remarkably accurate (Table 5–1).

Only DNA Replication in the 5′-to-3′ Direction Allows Efficient Error Correction

The need for accuracy probably explains why DNA replication occurs only in the 5′-to-3′ direction. If there were a DNA polymerase that added deoxyribonucleoside triphosphates in the 3′-to-5′ direction, the growing 5′-chain end, rather than the incoming mononucleotide, would carry the activating triphosphate. In this

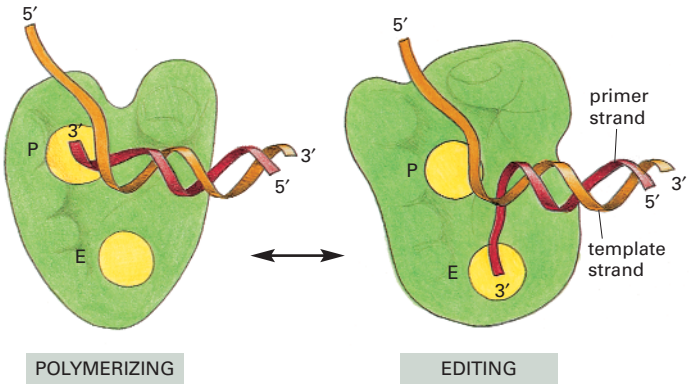


Figure 5–10 Editing by DNA polymerase. Outline of the structures of DNA polymerase complexed with the DNA template in the polymerizing mode *(left)* and the editing mode *(right)*. The catalytic site for the exonucleolytic (E) and the polymerization (P) reactions are indicated. To determine these structures by x-ray crystallography, researchers "froze" the polymerases in these two states, by using either a mutant polymerase defective in the exonucleolytic domain *(right)*, or by withholding the Mg^{2+} required for polymerization *(left)*.

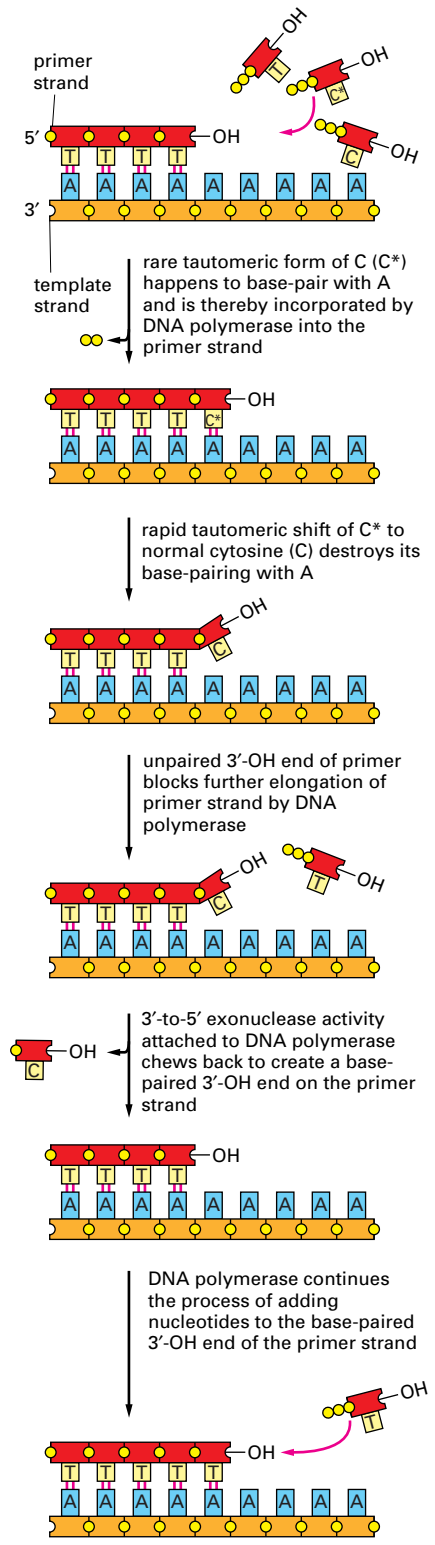


Figure 5–9 Exonucleolytic proofreading by DNA polymerase during DNA replication. In this example, the mismatch is due to the incorporation of a rare, transient tautomeric form of C, indicated by an asterisk. But the same proofreading mechanism applies to any misincorporation at the growing 3′-OH end.

TABLE 5–1 The Three Steps That Give RiseTo High-fidelity DNA Synthesis

REPLICATION STEP	ERRORS PER NUCLEOTIDE POLYMERIZED
5′→3′ polymerization	1×10^5
3′→5′ exonucleolytic proofreading	1×10^2
Strand-directed mismatch repair	1×10^2
Total	1×10^9

The third step, strand-directed mismatch repair, is described later in this chapter.

case, the mistakes in polymerization could not be simply hydrolyzed away, because the bare 5′-chain end thus created would immediately terminate DNA synthesis (Figure 5–11). It is therefore much easier to correct a mismatched base that has just been added to the 3′ end than one that has just been added to the 5′ end of a DNA chain. Although the mechanism for DNA replication (see Figure 5–8) seems at first sight much more complex than the incorrect mechanism depicted earlier in Figure 5–7, it is much more accurate because all DNA synthesis occurs in the 5′-to-3′ direction.

Despite these safeguards against DNA replication errors, DNA polymerases occasionally make mistakes. However, as we shall see later, cells have yet another chance to correct these errors by a process called *strand-directed mismatch repair*. Before discussing this mechanism, however, we describe the other types of proteins that function at the replication fork.

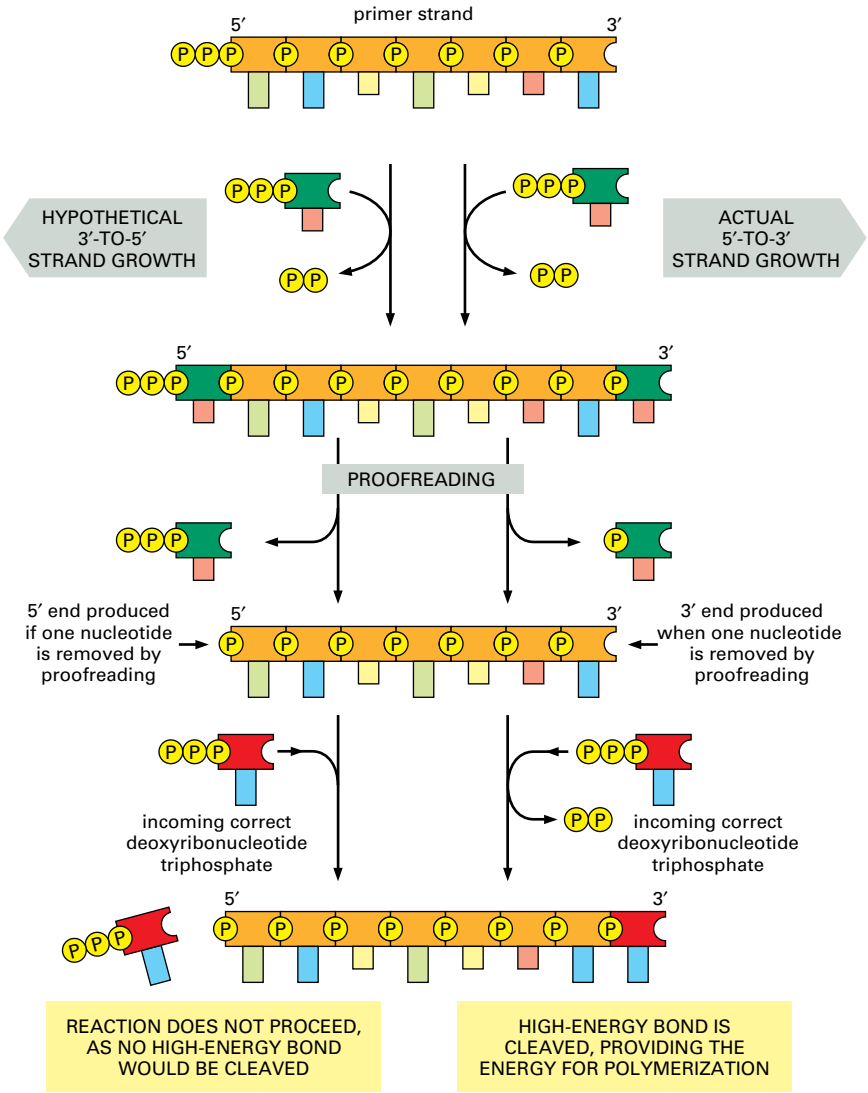


Figure 5–11 An explanation for the 5′-to-3′ direction of DNA chain growth. Growth in the 5′-to-3′ direction, shown on the right, allows the chain to continue to be elongated when a mistake in polymerization has been removed by exonucleolytic proofreading (see Figure 5–9). In contrast, exonucleolytic proofreading in the hypothetical 3′-to-5′ polymerization scheme, shown on the left, would block further chain elongation. For convenience, only the primer strand of the DNA double helix is shown.

A Special Nucleotide-Polymerizing Enzyme Synthesizes Short RNA Primer Molecules on the Lagging Strand

For the leading strand, a special primer is needed only at the start of replication: once a replication fork is established, the DNA polymerase is continuously presented with a base-paired chain end on which to add new nucleotides. On the lagging side of the fork, however, every time the DNA polymerase completes a short DNA Okazaki fragment (which takes a few seconds), it must start synthesizing a completely new fragment at a site further along the template strand (see Figure 5–8). A special mechanism is used to produce the base-paired primer strand required by this DNA polymerase molecule. The mechanism involves an enzyme called **DNA primase**, which uses ribonucleoside triphosphates to synthesize short **RNA primers** on the lagging strand (Figure 5–12). In eucaryotes, these primers are about 10 nucleotides long and are made at intervals of 100–200 nucleotides on the lagging strand.

The chemical structure of RNA was introduced in Chapter 1 and described in detail in Chapter 6. Here, we note only that RNA is very similar in structure to DNA. A strand of RNA can form base pairs with a strand of DNA, generating a DNA/RNA hybrid double helix if the two nucleotide sequences are complementary. The synthesis of RNA primers is thus guided by the same templating principle used for DNA synthesis (see Figures 1–5 and 5–2).

Because an RNA primer contains a properly base-paired nucleotide with a 3′-OH group at one end, it can be elongated by the DNA polymerase at this end to begin an Okazaki fragment. The synthesis of each Okazaki fragment ends when this DNA polymerase runs into the RNA primer attached to the 5′ end of the previous fragment. To produce a continuous DNA chain from the many DNA fragments made on the lagging strand, a special DNA repair system acts quickly to erase the old RNA primer and replace it with DNA. An enzyme called **DNA ligase** then joins the 3′ end of the new DNA fragment to the 5′ end of the previous one to complete the process (Figures 5–13 and 5–14).

Why might an erasable RNA primer be preferred to a DNA primer that would not need to be erased? The argument that a self-correcting polymerase cannot start chains *de novo* also implies its converse: an enzyme that starts chains anew cannot be efficient at self-correction. Thus, any enzyme that primes the synthesis of Okazaki fragments will of necessity make a relatively inaccurate copy (at least 1 error in 10^5). Even if the copies retained in the final product constituted as little as 5% of the total genome (for example, 10 nucleotides per 200-nucleotide DNA fragment), the resulting increase in the overall mutation rate would be enormous. It therefore seems likely that the evolution of RNA rather than DNA for priming brought a powerful advantage to the cell: the ribonucleotides in the primer automatically mark these sequences as "suspect copy" to be efficiently removed and replaced.

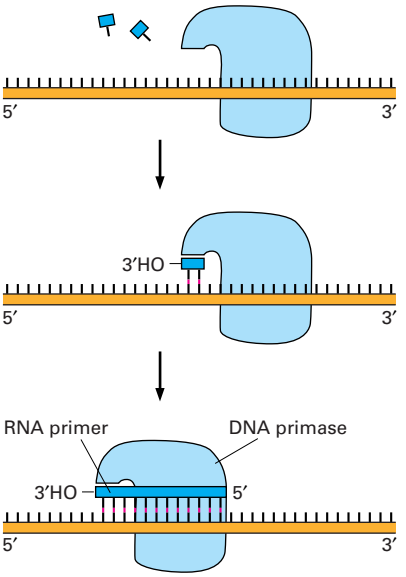


Figure 5–12 RNA primer synthesis. A schematic view of the reaction catalyzed by *DNA primase*, the enzyme that synthesizes the short RNA primers made on the lagging strand using DNA as a template. Unlike DNA polymerase, this enzyme can start a new polynucleotide chain by joining two nucleoside triphosphates together. The primase synthesizes a short polynucleotide in the 5′-to-3′ direction and then stops, making the 3′ end of this primer available for the DNA polymerase.

Special Proteins Help to Open Up the DNA Double Helix in Front of the Replication Fork

For DNA synthesis to proceed, the DNA double helix must be opened up ahead of the replication fork so that the incoming deoxyribonucleoside triphosphates can form base pairs with the template strand. However, the DNA double helix is very stable under normal conditions; the base pairs are locked in place so strongly that temperatures approaching that of boiling water are required to separate the two strands in a test tube. For this reason, DNA polymerases and

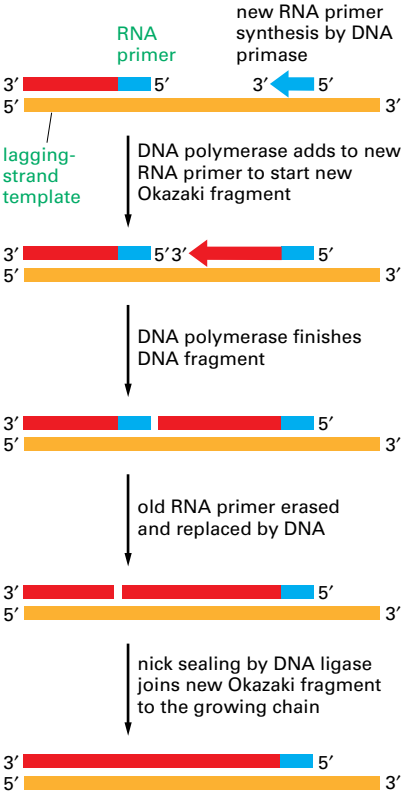


Figure 5–13 The synthesis of one of the many DNA fragments on the lagging strand. In eucaryotes, RNA primers are made at intervals spaced by about 200 nucleotides on the lagging strand, and each RNA primer is approximately 10 nucleotides long. This primer is erased by a special DNA repair enzyme (an RNAse H) that recognizes an RNA strand in an RNA/DNA helix and fragments it; this leaves gaps that are filled in by DNA polymerase and DNA ligase.

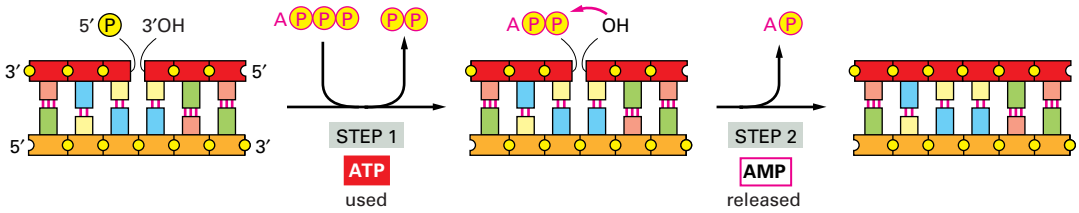


Figure 5–14 The reaction catalyzed by DNA ligase. This enzyme seals a broken phosphodiester bond. As shown, DNA ligase uses a molecule of ATP to activate the 5′ end at the nick (step 1) before forming the new bond (step 2). In this way, the energetically unfavorable nick-sealing reaction is driven by being coupled to the energetically favorable process of ATP hydrolysis.

DNA primases can copy a DNA double helix only when the template strand has already been exposed by separating it from its complementary strand. Additional replication proteins are needed to help in opening the double helix and thus provide the appropriate single-stranded DNA template for the DNA polymerase to copy. Two types of protein contribute to this process—DNA helicases and single-strand DNA-binding proteins.

DNA helicases were first isolated as proteins that hydrolyze ATP when they are bound to single strands of DNA. As described in Chapter 3, the hydrolysis of ATP can change the shape of a protein molecule in a cyclical manner that allows the protein to perform mechanical work. DNA helicases use this principle to propel themselves rapidly along a DNA single strand. When they encounter a region of double helix, they continue to move along their strand, thereby prying apart the helix at rates of up to 1000 nucleotide pairs per second (Figures 5–15 and 5–16).

The unwinding of the template DNA helix at a replication fork could in principle be catalyzed by two DNA helicases acting in concert—one running along the leading strand template and one along the lagging strand template. Since the two strands have opposite polarities, these helicases would need to move in opposite directions along a DNA single strand and therefore would be different enzymes. Both types of DNA helicase exist. In the best understood replication systems, a helicase on the lagging-strand template appears to have the predominant role, for reasons that will become clear shortly.

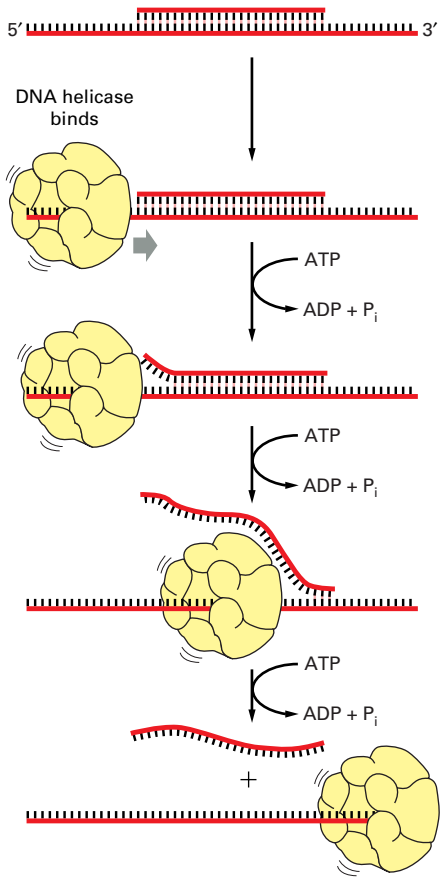


Figure 5–15 An assay used to test for DNA helicase enzymes. A short DNA fragment is annealed to a long DNA single strand to form a region of DNA double helix. The double helix is melted as the helicase runs along the DNA single strand, releasing the short DNA fragment in a reaction that requires the presence of both the helicase protein and ATP. The rapid step-wise movement of the helicase is powered by its ATP hydrolysis (see Figure 3–76).

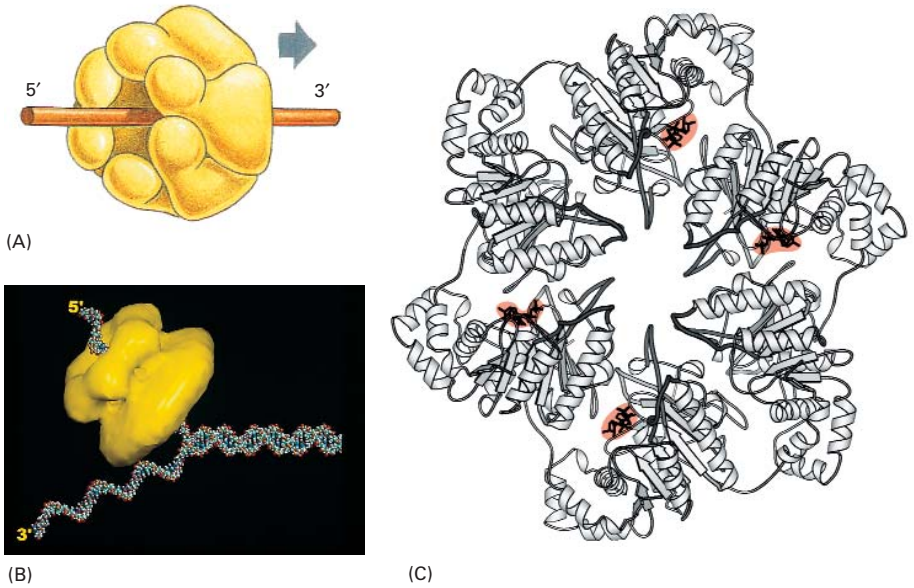


Figure 5–16 The structure of a DNA helicase. (A) A schematic diagram of the protein as a hexameric ring. (B) Schematic diagram showing a DNA replication fork and helicase to scale. (C) Detailed structure of the bacteriophage T7 replicative helicase, as determined by x-ray diffraction. Six identical subunits bind and hydrolyze ATP in an ordered fashion to propel this molecule along a DNA single strand that passes through the central hole. *Red* indicates bound ATP molecules in the structure. (B, courtesy of Edward H. Egelman; C, from M.R. Singleton et al., *Cell* 101:589–600, 2000. © Elsevier.)

Single-strand DNA-binding (SSB) proteins, also called *helix-destabilizing proteins,* bind tightly and cooperatively to exposed single-stranded DNA strands without covering the bases, which therefore remain available for templating. These proteins are unable to open a long DNA helix directly, but they aid helicases by stabilizing the unwound, single-stranded conformation. In addition, their cooperative binding coats and straightens out the regions of single-stranded DNA on the lagging-strand template, thereby preventing the formation of the short hairpin helices that readily form in single-strand DNA (Figures 5–17 and 5–18). These hairpin helices can impede the DNA synthesis catalyzed by DNA polymerase.

A Moving DNA Polymerase Molecule Stays Connected to the DNA by a Sliding Ring

On their own, most DNA polymerase molecules will synthesize only a short string of nucleotides before falling off the DNA template. The tendency to dissociate quickly from a DNA molecule allows a DNA polymerase molecule that has just finished synthesizing one Okazaki fragment on the lagging strand to be recycled quickly, so as to begin the synthesis of the next Okazaki fragment on the same strand. This rapid dissociation, however, would make it difficult for the polymerase to synthesize the long DNA strands produced at a replication fork were it not for an accessory protein that functions as a regulated clamp. This

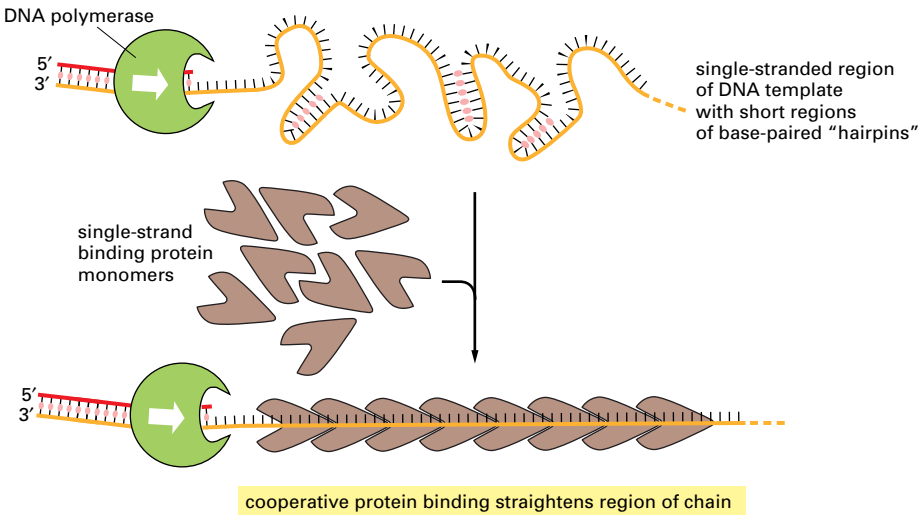


Figure 5–17 The effect of single-strand DNA-binding proteins (SSB proteins) on the structure of single-stranded DNA. Because each protein molecule prefers to bind next to a previously bound molecule, long rows of this protein form on a DNA single strand. This *cooperative binding* straightens out the DNA template and facilitates the DNA polymerization process. The "hairpin helices" shown in the bare, single-stranded DNA result from a chance matching of short regions of complementary nucleotide sequence; they are similar to the short helices that typically form in RNA molecules (see Figure 1–6).

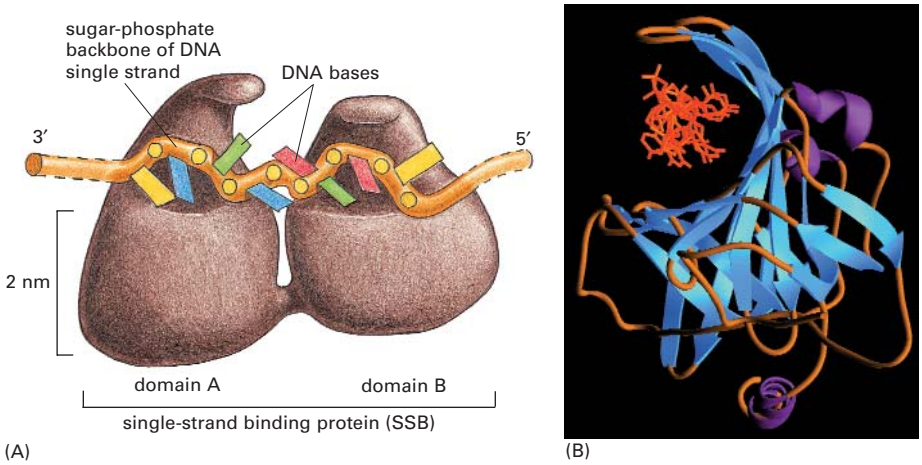


Figure 5–18 The structure of the single-strand binding protein from humans bound to DNA. (A) A front view of the two DNA binding domains of RPA protein, which cover a total of eight nucleotides. Note that the DNA bases remain exposed in this protein–DNA complex. (B) A diagram showing the three-dimensional structure, with the DNA strand *(red)* viewed end-on. (B, from A. Bochkarev et al., *Nature* 385:176–181, 1997. © Macmillan Magazines Ltd.)

clamp keeps the polymerase firmly on the DNA when it is moving, but releases it as soon as the polymerase runs into a double-stranded region of DNA ahead.

How can a clamp prevent the polymerase from dissociating without at the same time impeding the polymerase's rapid movement along the DNA molecule? The three-dimensional structure of the clamp protein, determined by x-ray diffraction, reveals that it forms a large ring around the DNA helix. One side of the ring binds to the back of the DNA polymerase, and the whole ring slides freely along the DNA as the polymerase moves. The assembly of the clamp around DNA requires ATP hydrolysis by a special protein complex, the clamp loader, which hydrolyzes ATP as it loads the clamp on to a primer-template junction (Figure 5–19).

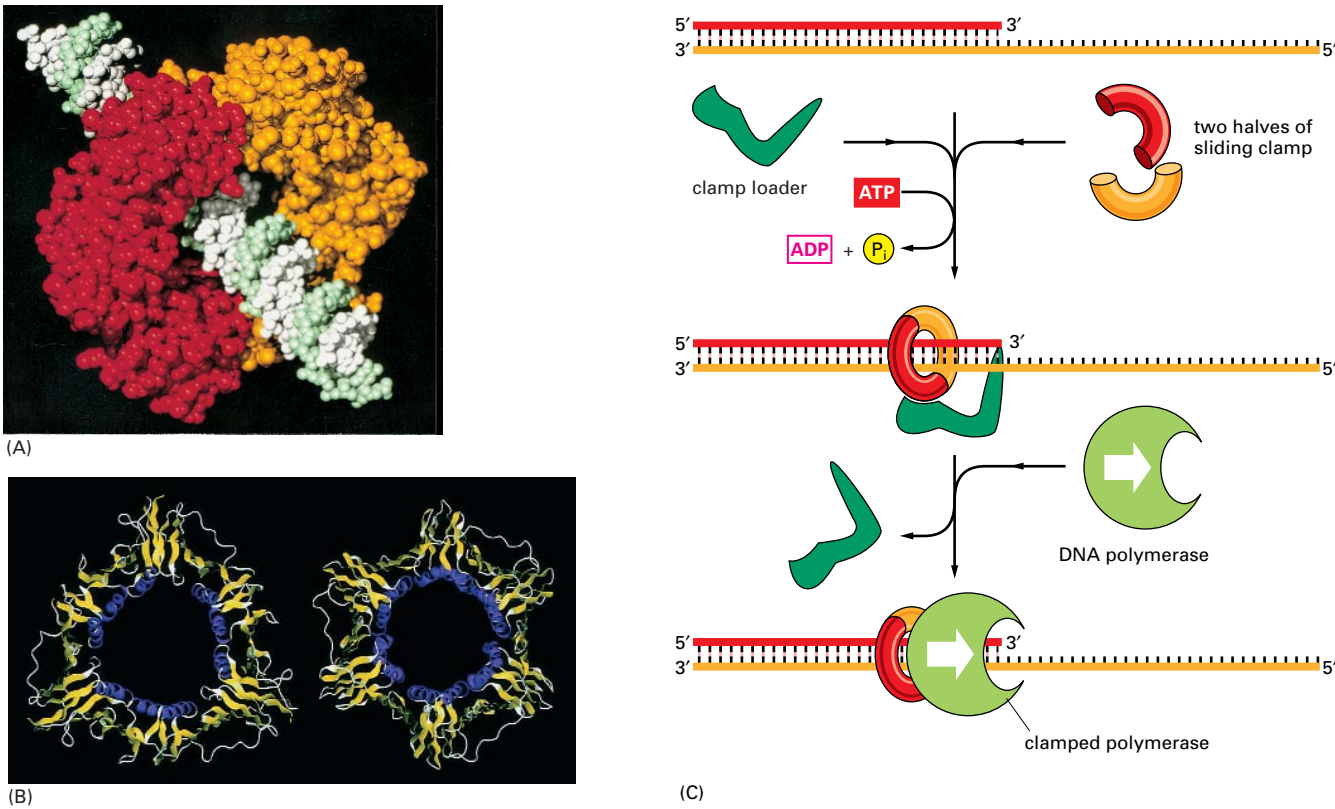


Figure 5–19 The regulated sliding clamp that holds DNA polymerase on the DNA. (A) The structure of the clamp protein from *E. coli*, as determined by x-ray crystallography, with a DNA helix added to indicate how the protein fits around DNA. (B) A similar protein is present in eucaryotes, as illustrated by this comparison of the *E. coli* sliding clamp *(left)* with the PCNA protein from humans *(right)*. (C) Schematic illustration showing how the clamp is assembled to hold a moving DNA polymerase molecule on the DNA. In the simplified reaction shown here, the clamp loader dissociates into solution once the clamp has been assembled. At a true replication fork, the clamp loader remains close to the lagging-strand polymerase, ready to assemble a new clamp at the start of each new Okazaki fragment (see Figure 5–22). (A and B, from X.-P. Kong et al., *Cell* 69:425–437, 1992. © Elsevier.)

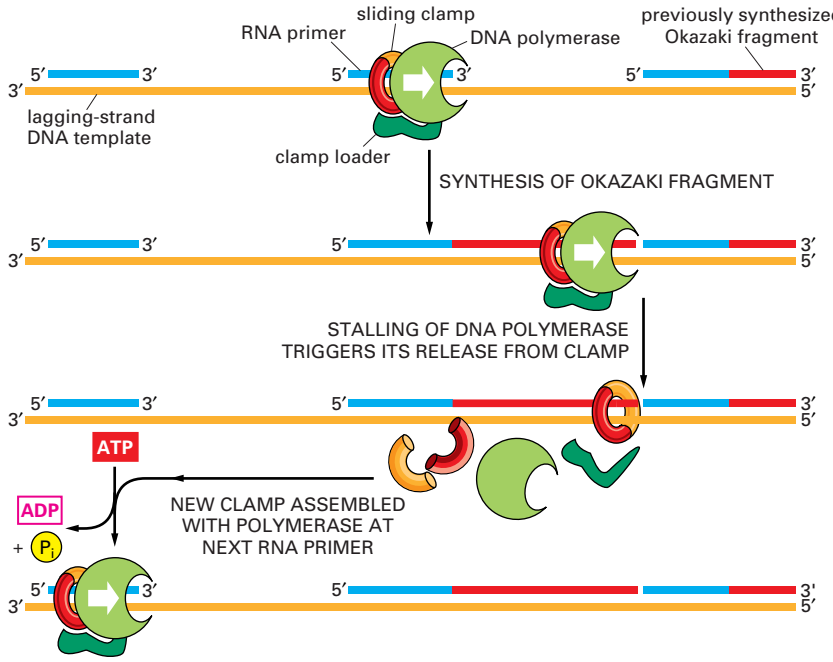


Figure 5–20 A cycle of loading and unloading of DNA polymerase and the clamp protein on the lagging strand. The association of the clamp loader with the lagging-strand polymerase shown here is for illustrative purposes only; in reality, the clamp loader is carried along with the replication fork in a complex that includes both the leading-strand and lagging-strand DNA polymerases (see Figure 5–22).

On the leading-strand template, the moving DNA polymerase is tightly bound to the clamp, and the two remain associated for a very long time. However, on the lagging-strand template, each time the polymerase reaches the 5′ end of the preceding Okazaki fragment, the polymerase is released; this polymerase molecule then associates with a new clamp that is assembled on the RNA primer of the next Okazaki fragment (Figure 5–20).

The Proteins at a Replication Fork Cooperate to Form a Replication Machine

Although we have discussed DNA replication as though it were performed by a mixture of proteins all acting independently, in reality, most of the proteins are held together in a large multienzyme complex that moves rapidly along the DNA. This complex can be likened to a tiny sewing machine composed of protein parts and powered by nucleoside triphosphate hydrolyses. Although the replication complex has been most intensively studied in *E. coli* and several of its viruses, a very similar complex also operates in eucaryotes, as we see below.

The functions of the subunits of the replication machine are summarized in Figure 5–21. Two DNA polymerase molecules work at the fork, one on the leading strand and one on the lagging strand. The DNA helix is opened by a DNA polymerase molecule clamped on the leading strand, acting in concert with one or more DNA helicase molecules running along the strands in front of it. Helix opening is aided by cooperatively bound molecules of single-strand DNA-binding protein. Whereas the DNA polymerase molecule on the leading strand can operate in a continuous fashion, the DNA polymerase molecule on the lagging strand must restart at short intervals, using a short RNA primer made by a DNA primase molecule.

The efficiency of replication is greatly increased by the close association of all these protein components. In procaryotes, the primase molecule is linked directly to a DNA helicase to form a unit on the lagging strand called a **primosome**. Powered by the DNA helicase, the primosome moves with the fork, synthesizing RNA primers as it goes. Similarly, the DNA polymerase molecule that synthesizes DNA on the lagging strand moves in concert with the rest of the proteins, synthesizing a succession of new Okazaki fragments. To accommodate this arrangement, the lagging strand seems to be folded back in the manner shown in Figure 5–22. This arrangement also facilitates the loading of the

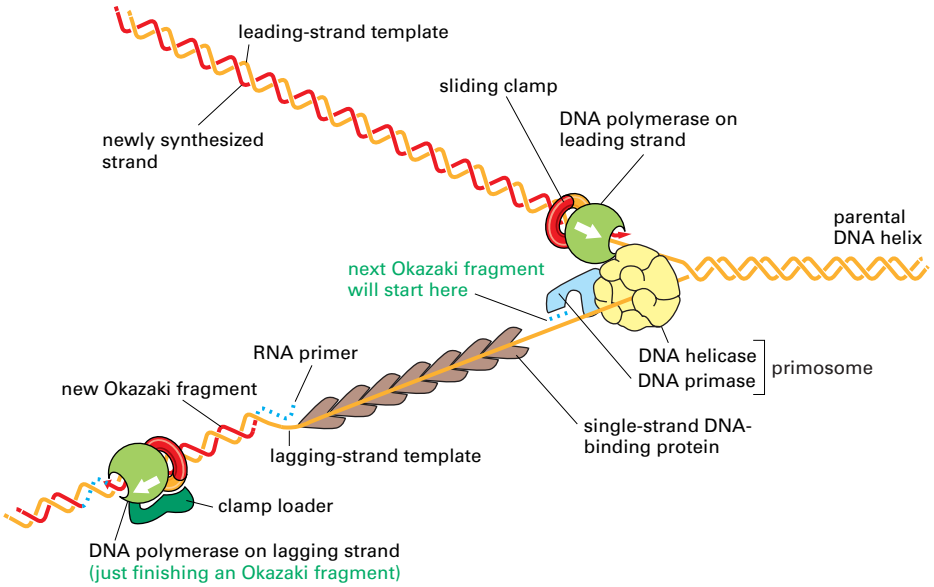


Figure 5–21 The proteins at a bacterial DNA replication fork. The major types of proteins that act at a DNA replication fork are illustrated, showing their approximate positions on the DNA.

polymerase clamp each time that an Okazaki fragment is synthesized: the clamp loader and the lagging-strand DNA polymerase molecule are kept in place as a part of the protein machine even when they detach from the DNA. The replication proteins are thus linked together into a single large unit (total molecular weight $>10^6$ daltons) that moves rapidly along the DNA, enabling DNA to be synthesized on both sides of the replication fork in a coordinated and efficient manner.

On the lagging strand, the DNA replication machine leaves behind a series of unsealed Okazaki fragments, which still contain the RNA that primed their synthesis at their 5′ ends. This RNA is removed and the resulting gap is filled in by DNA repair enzymes that operate behind the replication fork (see Figure 5–13).

A Strand-directed Mismatch Repair System Removes Replication Errors That Escape from the Replication Machine

As stated previously, bacteria such as *E. coli* are capable of dividing once every 30 minutes, making it relatively easy to screen large populations to find a rare mutant cell that is altered in a specific process. One interesting class of mutants contains alterations in so-called *mutator genes,* which greatly increase the rate of spontaneous mutation when they are inactivated. Not surprisingly, one such mutant makes a defective form of the 3′-to-5′ proofreading exonuclease that is a part of the DNA polymerase enzyme (see Figures 5–9 and 5–10). When this activity is defective, the DNA polymerase no longer proofreads effectively, and many replication errors that would otherwise have been removed accumulate in the DNA.

The study of other *E. coli* mutants exhibiting abnormally high mutation rates has uncovered another proofreading system that removes replication errors made by the polymerase that have been missed by the proofreading exonuclease. This **strand-directed mismatch repair** system detects the potential for distortion in the DNA helix that results from the misfit between non-complementary base pairs. But if the proofreading system simply recognized a mismatch in newly replicated DNA and randomly corrected one of the two mismatched nucleotides, it would mistakingly "correct" the original template strand to match the error exactly half the time, thereby failing to lower the overall error rate. To be effective, such a proofreading system must be able to distinguish and remove the mismatched nucleotide only on the newly synthesized strand, where the replication error occurred.

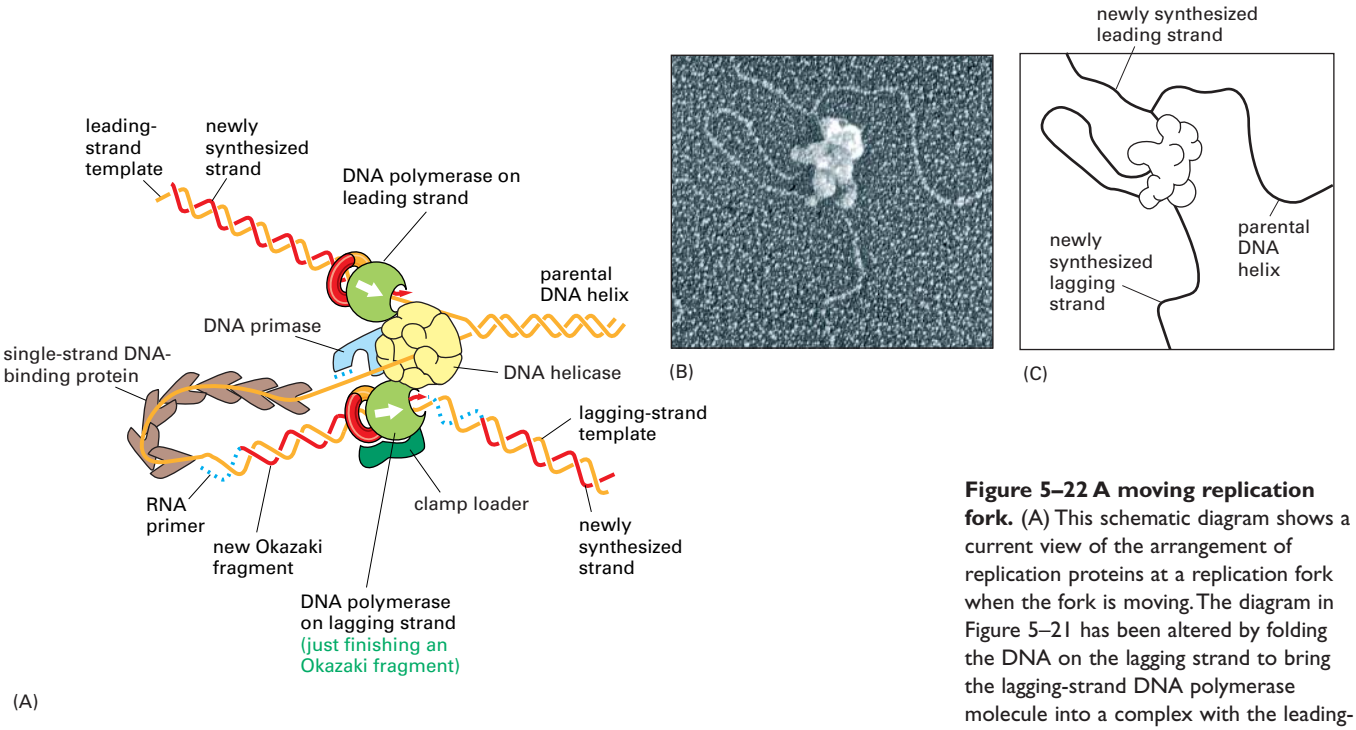


Figure 5–22 A moving replication fork. (A) This schematic diagram shows a current view of the arrangement of replication proteins at a replication fork when the fork is moving. The diagram in Figure 5–21 has been altered by folding the DNA on the lagging strand to bring the lagging-strand DNA polymerase molecule into a complex with the leading-strand DNA polymerase molecule. This folding process also brings the 3′ end of each completed Okazaki fragment close to the start site for the next Okazaki fragment (compare with Figure 5–21). Because the lagging-strand DNA polymerase molecule remains bound to the rest of the replication proteins, it can be reused to synthesize successive Okazaki fragments. In this diagram, it is about to let go of its completed DNA fragment and move to the RNA primer that will be synthesized nearby, as required to start the next DNA fragment. Note that one daughter DNA helix extends toward the bottom right and the other toward the top left in this diagram. Additional proteins help to hold the different protein components of the fork together, enabling them to function as a well-coordinated protein machine. The actual protein complex is more compact than indicated, and the clamp loader is held in place by interactions not shown here. (B) An electron micrograph showing the replication machine from the bacteriophage T4 as it moves along a template synthesizing DNA behind it. (C) An interpretation of the micrograph is given in the sketch: note especially the DNA loop on the lagging strand. Apparently, the replication proteins became partly detached from the very front of the replication fork during the preparation of this sample for electron microscopy. (B, courtesy of Jack Griffith.)

The strand-distinction mechanism used by the mismatch proofreading system in *E. coli* depends on the methylation of selected A residues in the DNA. Methyl groups are added to all A residues in the sequence GATC, but not until some time after the A has been incorporated into a newly synthesized DNA chain. As a result, the only GATC sequences that have not yet been methylated are in the new strands just behind a replication fork. The recognition of these unmethylated GATCs allows the new DNA strands to be transiently distinguished from old ones, as required if their mismatches are to be selectively removed. The three-step process involves recognition of a mismatch, excision of the segment of DNA containing the mismatch from the newly synthesized strand, and resynthesis of the excised segment using the old strand as a template—thereby removing the mismatch. This strand-directed mismatch repair system reduces the number of errors made during DNA replication by an additional factor of 10^2 (see Table 5–1, p. 243).

A similar mismatch proofreading system functions in human cells. The importance of this system is indicated by the fact that individuals who inherit one defective copy of a mismatch repair gene (along with a functional gene on the other copy of the chromosome) have a marked predisposition for certain types of cancers. In a type of colon cancer called *hereditary nonpolyposis colon cancer (HNPCC),* spontaneous mutation of the remaining functional gene produces a clone of somatic cells that, because they are deficient in mismatch proofreading, accumulate mutations unusually rapidly. Most cancers arise from cells that have accumulated multiple mutations (discussed in Chapter 23), and cells deficient in mismatch proofreading therefore have a greatly enhanced chance of becoming cancerous. Fortunately, most of us inherit two good copies of each gene that encodes a mismatch proofreading protein; this protects us, because it is highly unlikely that both copies would mutate in the same cell.

In eucaryotes, the mechanism for distinguishing the newly synthesized strand from the parental template strand at the site of a mismatch does not depend on DNA methylation. Indeed, some eucaryotes—including yeasts and *Drosophila*—do not methylate any of their DNA. Newly synthesized DNA strands are known to be preferentially *nicked*, and biochemical experiments reveal that such *nicks* (also called *single-strand breaks)* provide the signal that directs the mismatch proofreading system to the appropriate strand in a eucaryotic cell (Figure 5–23).

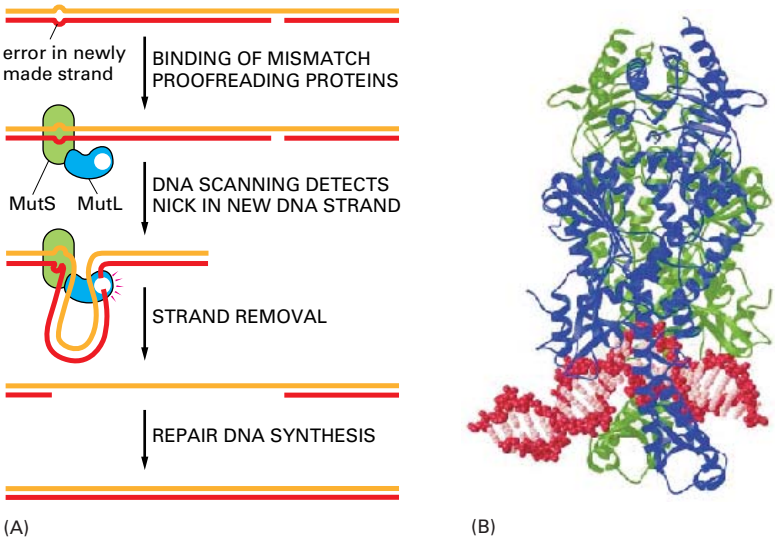


Figure 5–23 A model for strand-directed mismatch repair in eucaryotes. (A) The two proteins shown are present in both bacteria and eucaryotic cells: MutS binds specifically to a mismatched base pair, while MutL scans the nearby DNA for a nick. Once a nick is found, MutL triggers the degradation of the nicked strand all the way back through the mismatch. Because nicks are largely confined to newly replicated strands in eucaryotes, replication errors are selectively removed. In bacteria, the mechanism is the same, except that an additional protein in the complex (MutH) nicks unmethylated (and therefore newly replicated) GATC sequences, thereby beginning the process illustrated here. (B) The structure of the MutS protein bound to a DNA mismatch. This protein is a dimer, which grips the DNA double helix as shown, kinking the DNA at the mismatched base pair. It seems that the MutS protein scans the DNA for mismatches by testing for sites that can be readily kinked, which are those without a normal complementary base pair. (B, from G. Obmolova et al., *Nature* 407:703–710, 2000. © Macmillan Magazines Ltd.)

DNA Topoisomerases Prevent DNA Tangling During Replication

As a replication fork moves along double-stranded DNA, it creates what has been called the "winding problem." Every 10 base pairs replicated at the fork corresponds to one complete turn about the axis of the parental double helix. Therefore, for a replication fork to move, the entire chromosome ahead of the fork would normally have to rotate rapidly (Figure 5–24). This would require large amounts of energy for long chromosomes, and an alternative strategy is used instead: a swivel is formed in the DNA helix by proteins known as **DNA topoisomerases**.

A DNA topoisomerase can be viewed as a reversible nuclease that adds itself covalently to a DNA backbone phosphate, thereby breaking a phosphodiester bond in a DNA strand. This reaction is reversible, and the phosphodiester bond re-forms as the protein leaves.

One type of topoisomerase, called *topoisomerase I,* produces a transient single-strand break (or nick); this break in the phosphodiester backbone allows the two sections of DNA helix on either side of the nick to rotate freely relative to each other, using the phosphodiester bond in the strand opposite the nick as a swivel point (Figure 5–25). Any tension in the DNA helix will drive this rotation in the direction that relieves the tension. As a result, DNA replication can occur with the rotation of only a short length of helix—the part just ahead of the fork. The analogous winding problem that arises during DNA transcription (discussed in

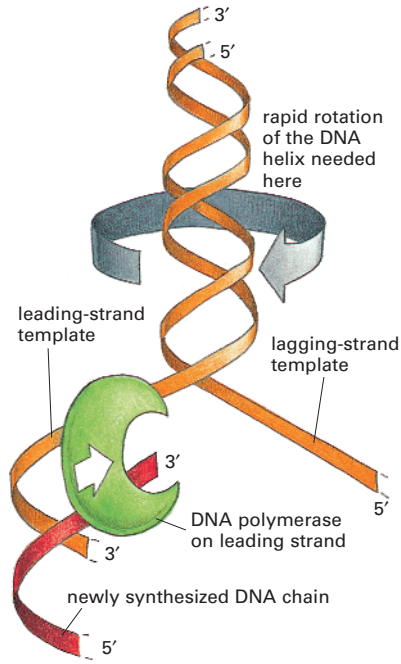


Figure 5–24 The "winding problem" that arises during DNA replication. For a bacterial replication fork moving at 500 nucleotides per second, the parental DNA helix ahead of the fork must rotate at 50 revolutions per second.

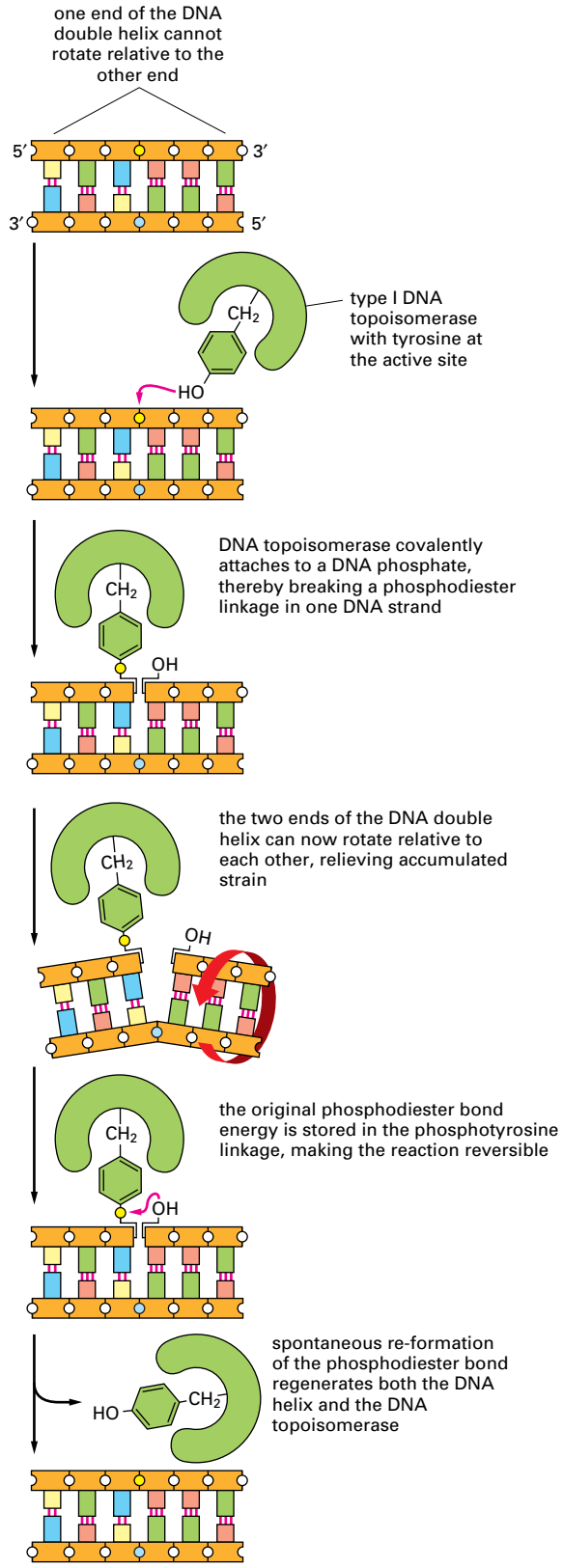


Figure 5–25 The reversible nicking reaction catalyzed by a eucaryotic DNA topoisomerase I enzyme. As indicated, these enzymes transiently form a single covalent bond with DNA; this allows free rotation of the DNA around the covalent backbone bonds linked to the *blue* phosphate.

Chapter 6) is solved in a similar way. Because the covalent linkage that joins the DNA topoisomerase protein to a DNA phosphate retains the energy of the cleaved phosphodiester bond, resealing is rapid and does not require additional energy input. In this respect, the rejoining mechanism is different from that catalyzed by the enzyme DNA ligase, discussed previously (see Figure 5–14).

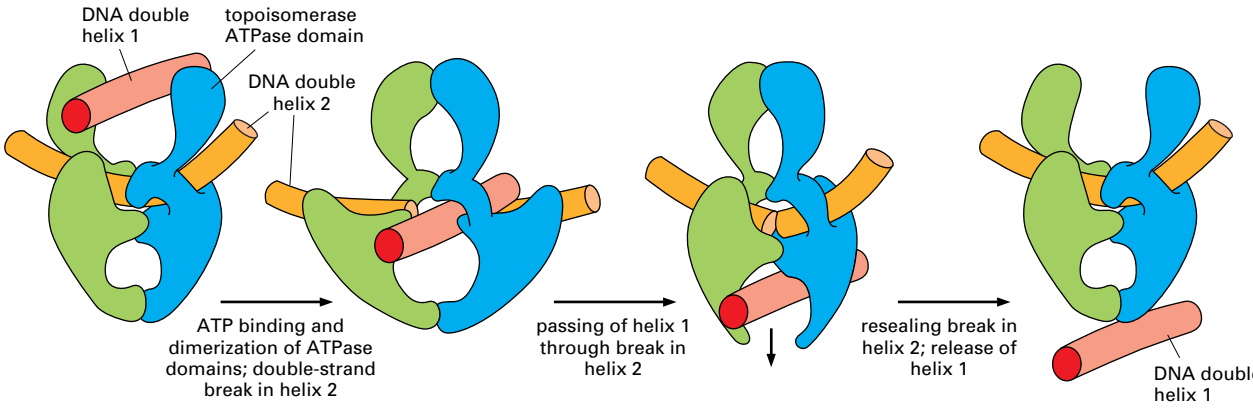


Figure 5–26 A model for topoisomerase II action. As indicated, ATP binding to the two ATPase domains causes them to dimerize and drives the reactions shown. Because a single cycle of this reaction can occur in the presence of a non-hydrolyzable ATP analog, ATP hydrolysis is thought to be needed only to reset the enzyme for each new reaction cycle. This model is based on structural and mechanistic studies of the enzyme. (Modified from J.M. Berger, *Curr. Opin. Struct. Biol.* 8:26–32, 1998.)

A second type of DNA topoisomerase, *topoisomerase II*, forms a covalent linkage to both strands of the DNA helix at the same time, making a transient *double-strand break* in the helix. These enzymes are activated by sites on chromosomes where two double helices cross over each other. Once a topoisomerase II molecule binds to such a crossing site, the protein uses ATP hydrolysis to perform the following set of reactions efficiently: (1) it breaks one double helix reversibly to create a DNA "gate;" (2) it causes the second, nearby double helix to pass through this break; and (3) it then reseals the break and dissociates from the DNA (Figure 5–26). In this way, type II DNA topoisomerases can efficiently separate two interlocked DNA circles (Figure 5–27).

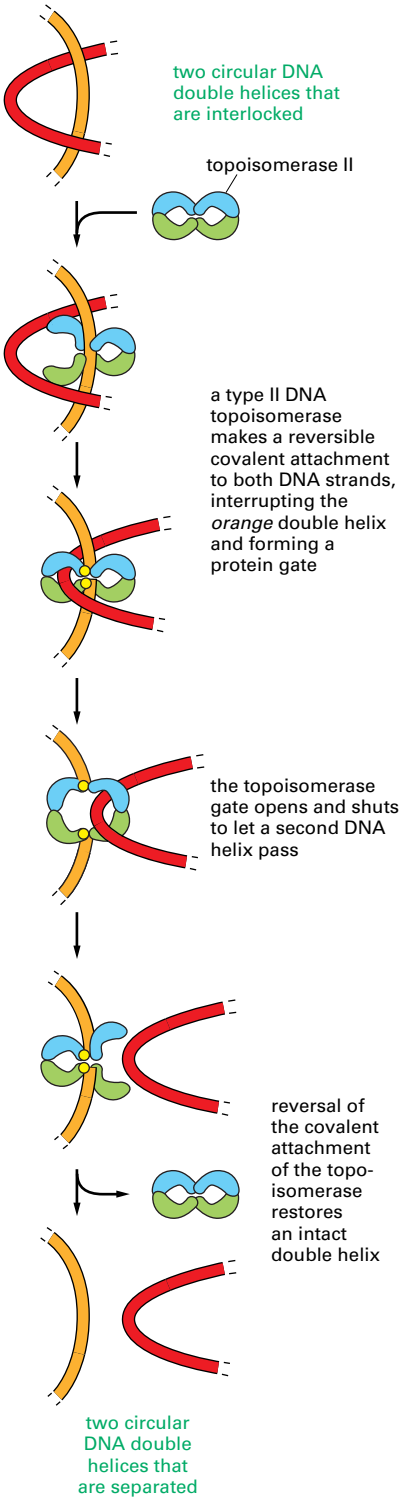
The same reaction also prevents the severe DNA tangling problems that would otherwise arise during DNA replication. This role is nicely illustrated by mutant yeast cells that produce, in place of the normal topoisomerase II, a version that is inactive at 37˚C. When the mutant cells are warmed to this temperature, their daughter chromosomes remain intertwined after DNA replication and are unable to separate. The enormous usefulness of topoisomerase II for untangling chromosomes can readily be appreciated by anyone who has struggled to remove a tangle from a fishing line without the aid of scissors.

DNA Replication Is Similar in Eucaryotes and Bacteria

Much of what we know about DNA replication was first derived from studies of purified bacterial and bacteriophage multienzyme systems capable of DNA replication *in vitro*. The development of these systems in the 1970s was greatly facilitated by the prior isolation of mutants in a variety of replication genes; these mutants were exploited to identify and purify the corresponding replication proteins. The first mammalian replication system that accurately replicated DNA *in vitro* was described in the mid-1980s, and mutations in genes encoding nearly all of the replication components have now been isolated and analyzed in the yeast *Saccharomyces cerevisiae*. As a result, a great deal is known about the detailed enzymology of DNA replication in eucaryotes, and it is clear that the fundamental features of DNA replication—including replication fork geometry and the use of a multiprotein replication machine—have been conserved during the long evolutionary process that separates bacteria and eucaryotes.

There are more protein components in eucaryotic replication machines than there are in the bacterial analogs, even though the basic functions are the same. Thus, for example, the eucaryotic single-strand binding (SSB) protein is formed from three subunits, whereas only a single subunit is found in bacteria. Similarly, the DNA primase is incorporated into a multisubunit enzyme called

Figure 5–27 The DNA-helix-passing reaction catalyzed by DNA topoisomerase II. Identical reactions are used to untangle DNA inside the cell. Unlike type I topoisomerases, type II enzymes use ATP hydrolysis and some of the bacterial versions can introduce superhelical tension into DNA. Type II topoisomerases are largely confined to proliferating cells in eucaryotes; partly for that reason, they have been popular targets for anticancer drugs.



DNA polymerase α. The polymerase α begins each Okazaki fragment on the lagging strand with RNA and then extends the RNA primer with a short length of DNA, before passing the 3′ end of this primer to a second enzyme, DNA polymerase δ. This second DNA polymerase then synthesizes the remainder of each Okazaki fragment with the help of a clamp protein (Figure 5–28).

As we see in the next section, the eucaryotic replication machinery has the added complication of having to replicate through nucleosomes, the repeating structural unit of chromosomes discussed in Chapter 4. Nucleosomes are spaced at intervals of about 200 nucleotide pairs along the DNA, which may explain why new Okazaki fragments are synthesized on the lagging strand at intervals of 100–200 nucleotides in eucaryotes, instead of 1000–2000 nucleotides as in bacteria. Nucleosomes may also act as barriers that slow down the movement of DNA polymerase molecules, which may be why eucaryotic replication forks move only one-tenth as fast as bacterial replication forks.

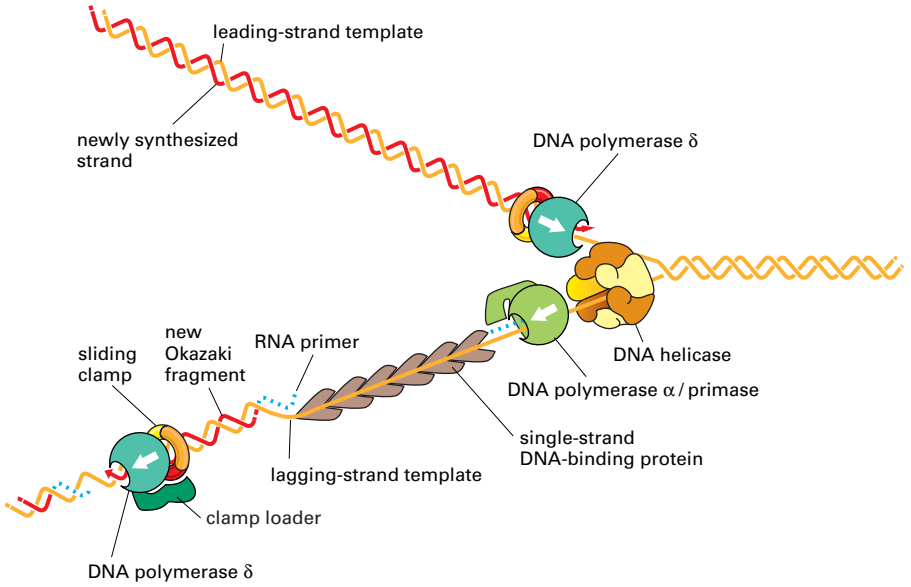


Figure 5–28 A mammalian replication fork. The fork is drawn to emphasize its similarity to the bacterial replication fork depicted in Figure 5–21. Although both forks use the same basic components, the mammalian fork differs in at least two important respects. First, it uses two different DNA polymerases on the lagging strand. Second, the mammalian DNA primase is a subunit of one of the lagging-strand DNA polymerases, DNA polymerase α, while that of bacteria is associated with a DNA helicase in the primosome. The polymerase α (with its associated primase) begins chains with RNA, extends them with DNA, and then hands the chains over to the second polymerase (δ), which elongates them. It is not known why eucaryotic DNA replication requires two different polymerases on the lagging strand. The major mammalian DNA helicase seems to be based on a ring formed from six different Mcm proteins; this ring may move along the leading strand, rather than along the lagging-strand template shown here.

Summary

DNA replication takes place at a Y-shaped structure called a replication fork. A self-correcting DNA polymerase enzyme catalyzes nucleotide polymerization in a 5′-to-3′ direction, copying a DNA template strand with remarkable fidelity. Since the two strands of a DNA double helix are antiparallel, this 5′-to-3′ DNA synthesis can take place continuously on only one of the strands at a replication fork (the leading strand). On the lagging strand, short DNA fragments must be made by a "back-stitching" process. Because the self-correcting DNA polymerase cannot start a new chain, these lagging-strand DNA fragments are primed by short RNA primer molecules that are subsequently erased and replaced with DNA.

DNA replication requires the cooperation of many proteins. These include (1) DNA polymerase and DNA primase to catalyze nucleoside triphosphate polymerization; (2) DNA helicases and single-strand DNA-binding (SSB) proteins to help in opening up the DNA helix so that it can be copied; (3) DNA ligase and an enzyme that degrades RNA primers to seal together the discontinuously synthesized lagging-strand DNA fragments; and (4) DNA topoisomerases to help to relieve helical winding and DNA tangling problems. Many of these proteins associate with each other at a replication fork to form a highly efficient "replication machine," through which the activities and spatial movements of the individual components are coordinated.

THE INITIATION AND COMPLETION OF DNA REPLICATION IN CHROMOSOMES

We have seen how a set of replication proteins rapidly and accurately generates two daughter DNA double helices behind a moving replication fork. But how is this replication machinery assembled in the first place, and how are replication forks created on a double-stranded DNA molecule? In this section, we discuss how DNA replication is initiated and how cells carefully regulate this process to ensure that it takes place at the proper positions on the chromosome and also at the appropriate time in the life of the cell. We also discuss a few of the special problems that the replication machinery in eucaryotic cells must overcome. These include the need to replicate the enormously long DNA molecules found in eucaryotic chromosomes, as well as the difficulty of copying DNA molecules that are tightly complexed with histones in nucleosomes.

DNA Synthesis Begins at Replication Origins

As discussed previously, the DNA double helix is normally very stable: the two DNA strands are locked together firmly by a large number of hydrogen bonds formed between the bases on each strand. To be used as a template, the double helix must first be opened up and the two strands separated to expose unpaired bases. As we shall see, the process of DNA replication is begun by special *initiator proteins* that bind to double-stranded DNA and pry the two strands apart, breaking the hydrogen bonds between the bases.

The positions at which the DNA helix is first opened are called **replication origins** (Figure 5–29). In simple cells like those of bacteria or yeast, origins are specified by DNA sequences several hundred nucleotide pairs in length. This DNA contains short sequences that attract initiator proteins, as well as stretches of DNA that are especially easy to open. We saw in Figure 4–4 that an A-T base pair is held together by fewer hydrogen bonds than a G-C base pair. Therefore, DNA rich in A-T base pairs is relatively easy to pull apart, and regions of DNA enriched in A-T pairs are typically found at replication origins.

Although the basic process of replication fork initiation, depicted in Figure 5–29 is the same for bacteria and eucaryotes, the detailed way in which this process is performed and regulated differs between these two groups of organisms. We first consider the simpler and better-understood case in bacteria and then turn to the more complex situation found in yeasts, mammals, and other eucaryotes.

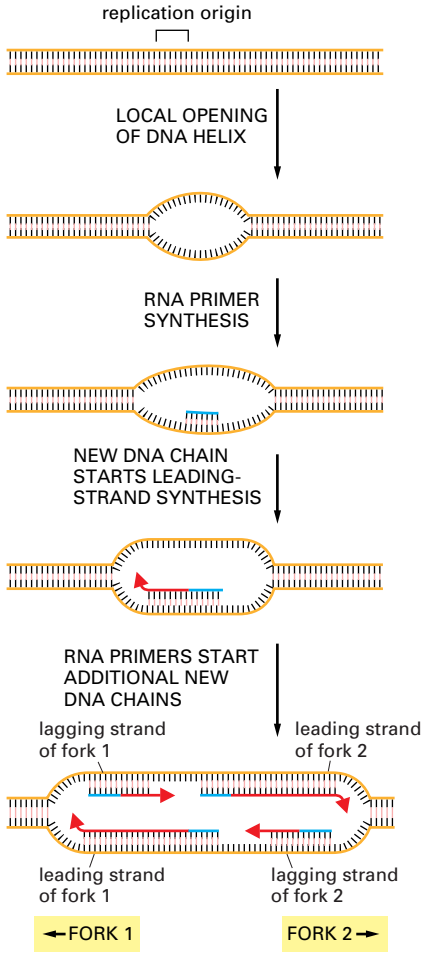
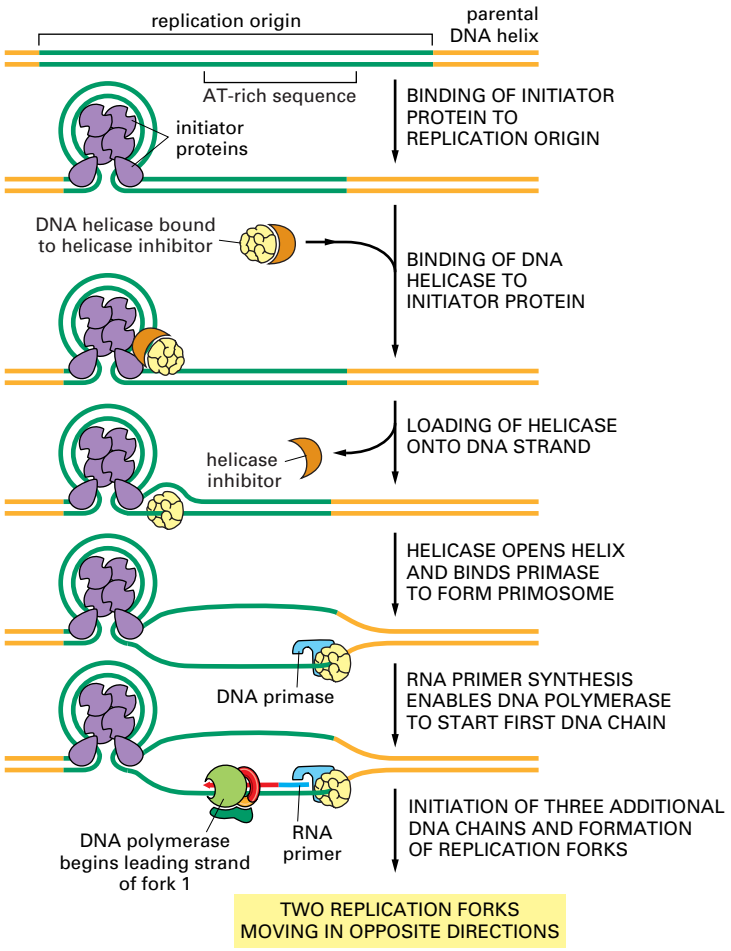


Figure 5–29 A replication bubble formed by replication fork initiation. This diagram outlines the major steps involved in the initiation of replication forks at replication origins. The structure formed at the last step, in which both strands of the parental DNA helix have been separated from each other and serve as templates for DNA synthesis, is called a *replication bubble*.

Figure 5–30 DNA replication of a bacterial genome. It takes *E. coli* about 40 minutes to duplicate its genome of 4.6×10^6 nucleotide pairs. For simplicity, no Okazaki fragments are shown on the lagging strand. What happens as the two replication forks approach each other and collide at the end of the replication cycle is not well understood, although the primosome is disassembled as part of the process.

Bacterial Chromosomes Have a Single Origin of DNA Replication

The genome of *E. coli* is contained in a single circular DNA molecule of 4.6×10^6 nucleotide pairs. DNA replication begins at a single origin of replication, and the two replication forks assembled there proceed (at approximately 500–1000 nucleotides per second) in opposite directions until they meet up roughly halfway around the chromosome (Figure 5–30). The only point at which *E. coli* can control DNA replication is initiation: once the forks have been assembled at the origin, they move at a relatively constant speed until replication is finished. Therefore, it is not surprising that the initiation of DNA replication is a highly regulated process. It begins when initiator proteins bind in multiple copies to specific sites in the replication origin, wrapping the DNA around the proteins to form a large protein–DNA complex. This complex then binds a DNA helicase and loads it onto an adjacent DNA single strand whose bases have been exposed by the assembly of the initiator protein–DNA complex. The DNA primase joins the helicase, forming the primosome, which moves away from the origin and makes an RNA primer that starts the first DNA chain (Figure 5–31). This quickly leads to the assembly of the remaining proteins to create two replication forks, with protein complexes that move away from the origin in opposite directions. These protein machines continue to synthesize DNA until all of the DNA template downstream of each fork has been replicated.



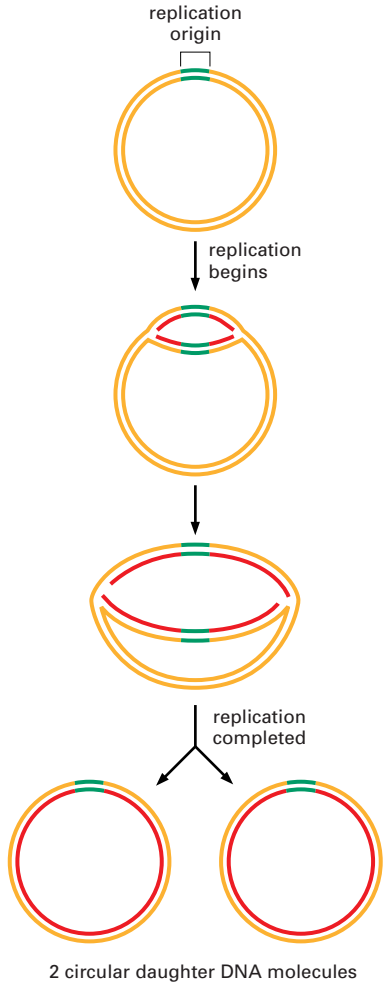


Figure 5–31 The proteins that initiate DNA replication in bacteria. The mechanism shown was established by studies *in vitro* with a mixture of highly purified proteins. For *E. coli* DNA replication, the major initiator protein is the dnaA protein; the primosome is composed of the dnaB (DNA helicase) and dnaG (DNA primase) proteins. In solution, the helicase is bound by an inhibitor protein (the dnaC protein), which is activated by the initiator proteins to load the helicase onto DNA at the replication origin and then released. This inhibitor prevents the helicase from inappropriately entering other single-stranded stretches of DNA in the bacterial genome. Subsequent steps result in the initiation of three more DNA chains (see Figure 5–29) by a pathway whose details are incompletely specified.

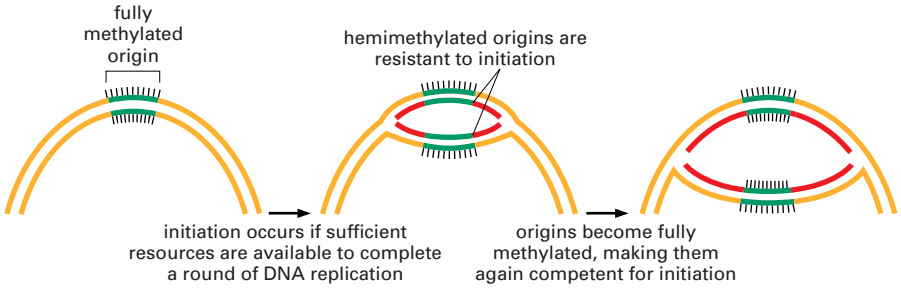


Figure 5–32 Methylation of the *E. coli* replication origin creates a refractory period for DNA initiation. DNA methylation occurs at GATC sequences, 11 of which are found in the origin of replication (spanning about 250 nucleotide pairs). About 10 minutes after replication is initiated, the hemimethylated origins become fully methylated by a DNA methylase enzyme. As discussed earlier, the lag in methylation after the replication of GATC sequences is also used by the *E. coli* mismatch proofreading system to distinguish the newly synthesized DNA strand from the parental DNA strand; in that case, the relevant GATC sequences are scattered throughout the chromosome. A single enzyme, the *dam* methylase, is responsible for methylating *E. coli* GATC sequences.

In *E. coli,* the interaction of the initiator protein with the replication origin is carefully regulated, with initiation occurring only when sufficient nutrients are available for the bacterium to complete an entire round of replication. Not only is the activity of the initiator protein controlled, but an origin of replication that has just been used experiences a "refractory period," caused by a delay in the methylation of newly synthesized A nucleotides. Further initiation of replication is blocked until these As are methylated (Figure 5–32).

Eucaryotic Chromosomes Contain Multiple Origins of Replication

We have seen how two replication forks begin at a single replication origin in bacteria and proceed in opposite directions, moving away from the origin until all of the DNA in the single circular chromosome is replicated. The bacterial genome is sufficiently small for these two replication forks to duplicate the genome in about 40 minutes. Because of the much greater size of most eucaryotic chromosomes, a different strategy is required to allow their replication in a timely manner.

A method for determining the general pattern of eucaryotic chromosome replication was developed in the early 1960s. Human cells growing in culture are labeled for a short time with ^{3}H-thymidine so that the DNA synthesized during this period becomes highly radioactive. The cells are then gently lysed, and the DNA is streaked on the surface of a glass slide coated with a photographic emulsion. Development of the emulsion reveals the pattern of labeled DNA through a technique known as *autoradiography*. The time allotted for radioactive labeling is chosen to allow each replication fork to move several micrometers along the DNA, so that the replicated DNA can be detected in the light microscope as lines of silver grains, even though the DNA molecule itself is too thin to be visible. In this way, both the rate and the direction of replication-fork movement can be determined (Figure 5–33). From the rate at which tracks of replicated DNA increase in length with increasing labeling time, the replication forks are

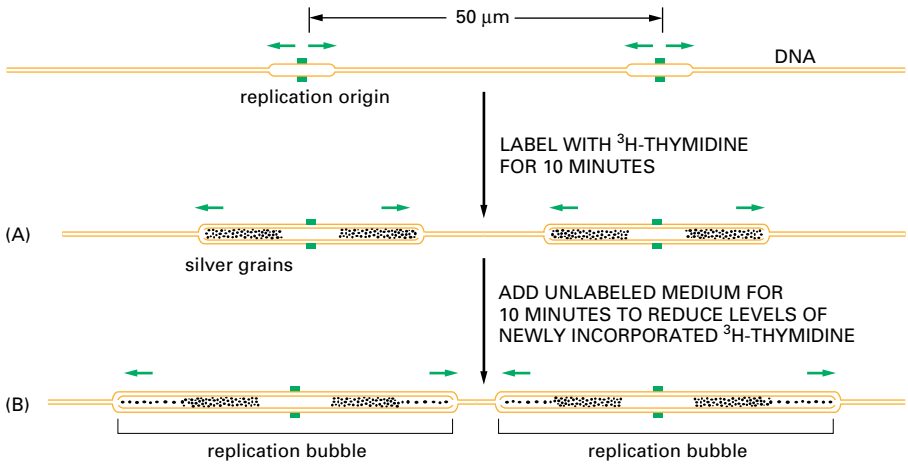


Figure 5–33 The experiments that demonstrated the pattern in which replication forks are formed and move on eucaryotic chromosomes. The new DNA made in human cells in culture was labeled briefly with a pulse of highly radioactive thymidine (^{3}H-thymidine). (A) In this experiment, the cells were lysed, and the DNA was stretched out on a glass slide that was subsequently covered with a photographic emulsion. After several months the emulsion was developed, revealing a line of silver grains over the radioactive DNA. The *brown* DNA in this figure is shown only to help with the interpretation of the autoradiograph; the unlabeled DNA is invisible in such experiments. (B) This experiment was the same except that a further incubation in unlabeled medium allowed additional DNA, with a lower level of radioactivity, to be replicated. The pairs of dark tracks in (B) were found to have silver grains tapering off in opposite directions, demonstrating bidirectional fork movement from a central replication origin where a replication bubble forms (see Figure 5–29). A replication fork is thought to stop only when it encounters a replication fork moving in the opposite direction or when it reaches the end of the chromosome; in this way, all the DNA is eventually replicated.

estimated to travel at about 50 nucleotides per second. This is approximately one-tenth of the rate at which bacterial replication forks move, possibly reflecting the increased difficulty of replicating DNA that is packaged tightly in chromatin.

An average-sized human chromosome contains a single linear DNA molecule of about 150 million nucleotide pairs. To replicate such a DNA molecule from end to end with a single replication fork moving at a rate of 50 nucleotides per second would require $0.02 \times 150 \times 10^6 = 3.0 \times 10^6$ seconds (about 800 hours). As expected, therefore, the autoradiographic experiments just described reveal that many forks are moving simultaneously on each eucaryotic chromosome. Moreover, many forks are found close together in the same DNA region, while other regions of the same chromosome have none.

Further experiments of this type have shown the following: (1) Replication origins tend to be activated in clusters, called *replication units,* of perhaps 20–80 origins. (2) New replication units seem to be activated at different times during the cell cycle until all of the DNA is replicated, a point that we return to below. (3) Within a replication unit, individual origins are spaced at intervals of 30,000–300,000 nucleotide pairs from one another. (4) As in bacteria, replication forks are formed in pairs and create a replication bubble as they move in opposite directions away from a common point of origin, stopping only when they collide head-on with a replication fork moving in the opposite direction (or when they reach a chromosome end). In this way, many replication forks can operate independently on each chromosome and yet form two complete daughter DNA helices.

In Eucaryotes DNA Replication Takes Place During Only One Part of the Cell Cycle

When growing rapidly, bacteria replicate their DNA continually, and they can begin a new round before the previous one is complete. In contrast, DNA replication in most eucaryotic cells occurs only during a specific part of the cell division cycle, called the *DNA synthesis phase* or **S phase** (Figure 5–34). In a mammalian cell, the S phase typically lasts for about 8 hours; in simpler eucaryotic cells such as yeasts, the S phase can be as short as 40 minutes. By its end, each chromosome has been replicated to produce two complete copies, which remain joined together at their centromeres until the *M phase* (M for *mitosis),* which soon follows. In Chapter 17, we describe the control system that runs the cell cycle and explain why entry into each phase of the cycle requires the cell to have successfully completed the previous phase.

In the following sections, we explore how chromosome replication is coordinated within the S phase of the cell cycle.

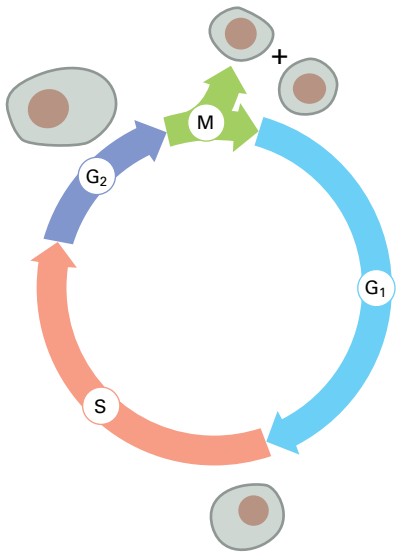


Figure 5–34 The four successive phases of a standard eucaryotic cell cycle. During the G_1, S, and G_2 phases, the cell grows continuously. During M phase growth stops, the nucleus divides, and the cell divides in two. DNA replication is confined to the part of interphase known as S phase. G_1 is the gap between M phase and S phase; G_2 is the gap between S phase and M phase.

Different Regions on the Same Chromosome Replicate at Distinct Times in S Phase

In mammalian cells, the replication of DNA in the region between one replication origin and the next should normally require only about an hour to complete, given the rate at which a replication fork moves and the largest distances measured between the replication origins in a replication unit. Yet S phase usually lasts for about 8 hours in a mammalian cell. This implies that the replication origins are not all activated simultaneously and that the DNA in each replication unit (which, as we noted above, contains a cluster of about 20–80 replication origins) is replicated during only a small part of the total S-phase interval.

Are different replication units activated at random, or are different regions of the genome replicated in a specified order? One way to answer this question is to use the thymidine analogue bromodeoxyuridine (BrdU) to label the newly synthesized DNA in synchronized cell populations, adding it for different short periods throughout S phase. Later, during M phase, those regions of the mitotic chromosomes that have incorporated BrdU into their DNA can be recognized by their altered staining properties or by means of anti-BrdU antibodies. The

results show that different regions of each chromosome are replicated in a reproducible order during S phase (Figure 5–35). Moreover, as one would expect from the clusters of replication forks seen in DNA autoradiographs (see Figure 5–33), the timing of replication is coordinated over large regions of the chromosome.

Highly Condensed Chromatin Replicates Late, While Genes in Less Condensed Chromatin Tend to Replicate Early

It seems that the order in which replication origins are activated depends, in part, on the chromatin structure in which the origins reside. We saw in Chapter 4 that heterochromatin is a particularly condensed state of chromatin, while transcriptionally active chromatin has a less condensed conformation that is apparently required to allow RNA synthesis. Heterochromatin tends to be replicated very late in S phase, suggesting that the timing of replication is related to the packing of the DNA in chromatin. This suggestion is supported by an examination of the two X chromosomes in a female mammalian cell. While these two chromosomes contain essentially the same DNA sequences, one is active for DNA transcription and the other is not (discussed in Chapter 7). Nearly all of the inactive X chromosome is condensed into heterochromatin, and its DNA replicates late in S phase. Its active homologue is less condensed and replicates throughout S phase.

These findings suggest that those regions of the genome whose chromatin is least condensed, and therefore most accessible to the replication machinery, are replicated first. Autoradiography shows that replication forks move at comparable rates throughout S phase, so that the extent of chromosome condensation seems to influence the time at which replication forks are initiated, rather than their speed once formed.

The above relationship between chromatin structure and the timing of DNA replication is also supported by studies in which the replication times of specific genes are measured. The results show that so-called "housekeeping" genes, which are those active in all cells, replicate very early in S phase in all cells tested. In contrast, genes that are active in only a few cell types generally replicate early in the cells in which the genes are active, and later in other types of cell.

The relationship between chromatin structure and the timing of replication has been tested directly in the yeast *S. cerevisiae*. In one case, an origin that functioned late in S phase, and was found in a transcriptionally silent region of a yeast chromosome, was experimentally relocated to a transcriptionally active region. After the relocation, the origin functioned early in the S phase, indicating that the time in S phase when this origin is used is determined by the origin's location in the chromosome. However, studies with additional yeast origins have revealed the existence of other origins that initiate replication late, even when present in normal chromatin. Thus, the time at which an origin is used can be determined both by its chromatin structure and by its DNA sequence.

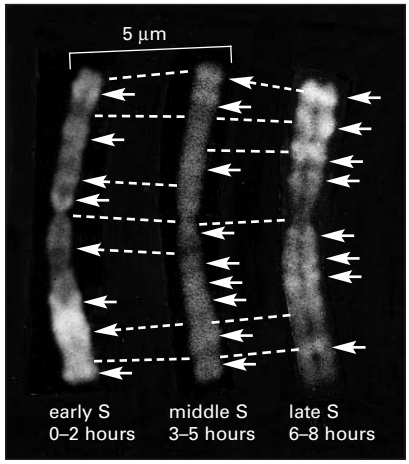


Figure 5–35 Different regions of a chromosome are replicated at different times in S phase. These light micrographs show stained mitotic chromosomes in which the replicating DNA has been differentially labeled during different defined intervals of the preceding S phase. In these experiments, cells were first grown in the presence of BrdU (a thymidine analog) and in the absence of thymidine to label the DNA uniformly. The cells were then briefly pulsed with thymidine in the absence of BrdU during early, middle, or late S phase. Because the DNA made during the thymidine pulse is a double helix with thymidine on one strand and BrdU on the other, it stains more darkly than the remaining DNA (which has BrdU on both strands) and shows up as a bright band *(arrows)* on these negatives. Broken lines connect corresponding positions on the three identical copies of the chromosome shown. (Courtesy of Elton Stubblefield.)

Well-defined DNA Sequences Serve as Replication Origins in a Simple Eucaryote, the Budding Yeast

Having seen that a eucaryotic chromosome is replicated using many origins of replication, each of which "fires" at a characteristic time in S phase of the cell cycle, we turn to the nature of these origins of replication. We saw earlier in this chapter that replication origins have been precisely defined in bacteria as specific DNA sequences that allow the DNA replication machinery to assemble on the DNA double helix, form a replication bubble, and move in opposite directions to produce replication forks. By analogy, one would expect the replication origins in eucaryotic chromosomes to be specific DNA sequences too.

The search for replication origins in the chromosomes of eucaryotic cells has been most productive in the budding yeast *S. cerevisiae.* Powerful selection methods to find them have been devised that make use of mutant yeast cells defective for an essential gene. These cells can survive in a selective medium

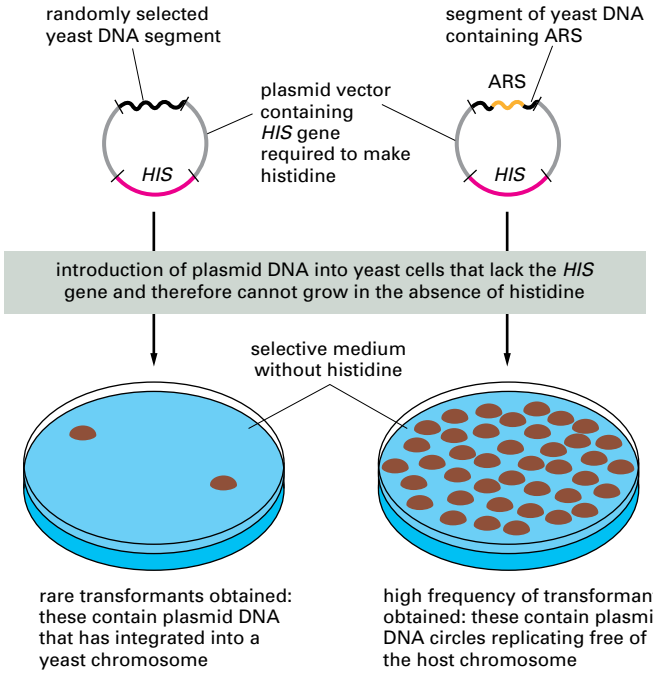


Figure 5–36 The strategy used to identify replication origins in yeast cells. Each of the yeast DNA sequences identified in this way was called an autonomously replicating sequence (ARS), since it enables a plasmid that contains it to replicate in the host cell without having to be incorporated into a host cell chromosome.

only if they are provided with DNA that carries a functional copy of the missing gene. If a circular bacterial plasmid with this gene is introduced into the mutant yeast cells directly, it will not be able to replicate because it lacks a functional origin. If random pieces of yeast DNA are inserted into this plasmid, however, only those few plasmid DNA molecules that contain a yeast replication origin can replicate. The yeast cells that carry such plasmids are able to proliferate because they have been provided with the essential gene in a form that can be replicated and passed on to progeny cells (Figure 5–36). A DNA sequence identified by its presence in a plasmid isolated from these surviving yeast cells is called an *autonomously replicating sequence (ARS)*. Most ARSs have been shown to be authentic chromosomal origins of replication, thereby validating the strategy used to obtain them.

For budding yeast, the location of every origin of replication on each chromosome can be determined (Figure 5–37). The particular chromosome shown—chromosome III from the yeast *S. cerevisiae*—is less than 1/100 the length of a typical human chromosome. Its origins are spaced an average of 30,000 nucleotides apart; this density of origins should permit a yeast chromosome to be replicated in about 8 minutes.

Genetic experiments in *S. cerevisiae* have tested the effect of deleting various sets of the replication origins on chromosome III. Removing a few origins has little effect, because replication forks that begin at neighboring origins of replication can continue into the regions that lack their own origins. However, as more replication origins are deleted from this chromosome, the chromosome is gradually lost as the cells divide, presumably because it is replicated too slowly.

A Large Multisubunit Complex Binds to Eucaryotic Origins of Replication

The minimal DNA sequence required for directing DNA replication initiation in the yeast *S. cerevisiae* has been determined by performing the experiment

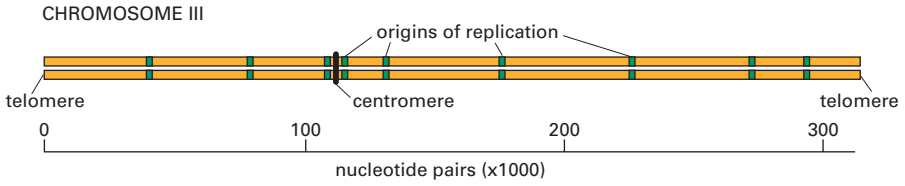


Figure 5–37 The origins of DNA replication on chromosome III of the yeast *S. cerevisiae*. This chromosome, one of the smallest eucaryotic chromosomes known, carries a total of 180 genes. As indicated, it contains nine replication origins.

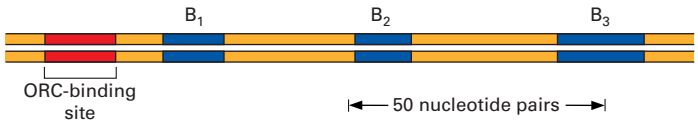


Figure 5–38 An origin of replication in yeast. Comprising about 150 nucleotide pairs, this yeast origin (identified by the procedure shown in Figure 5–36) has a binding site for ORC, a complex of proteins that binds to every origin of replication. The origin depicted also has binding sites (B1, B2, and B3) for other required proteins, which can differ between various origins. Although best characterized in yeast, a similar ORC is used to initiate DNA replication in more complex eucaryotes, including humans.

shown in Figure 5–36 with smaller and smaller DNA fragments. Each of the yeast replication origins contains a binding site for a large, multisubunit initiator protein called **ORC**, for **origin recognition complex**, and several auxiliary binding sites for proteins that help attract ORC to the origin DNA (Figure 5–38).

As we have seen, DNA replication in eucaryotes occurs only in the S phase. How is this DNA replication triggered, and how does the mechanism ensure that a replication origin is used only once during each cell cycle?

As we discuss in Chapter 17, the general answers to these two questions are now known. In brief, the ORC-origin interaction is a stable one that serves to mark a replication origin throughout the entire cell cycle. A prereplicative protein complex is assembled on each ORC during G_1 phase, containing both a hexameric DNA helicase and a helicase loading factor (the Mcm and Cdc6 proteins, respectively). S phase is triggered when a protein kinase is activated that assembles the rest of the replication machinery, allowing an Mcm helicase to start moving with each of the two replication forks that form at each origin. Simultaneously, the protein kinase that triggers S phase prevents all further assembly of the Mcm protein into prereplicative complexes, until this kinase is inactivated at the next M phase to reset the entire cycle (for details, see Figure 17–22).

The Mammalian DNA Sequences That Specify the Initiation of Replication Have Been Difficult to Identify

Compared with the situation in budding yeasts, DNA sequences that specify replication origins in other eucaryotes have been more difficult to define. In humans, for example, the DNA sequences that are required for proper origin function can extend over very large distances along the DNA.

Recently, however, it has been possible to identify specific human DNA sequences, each several thousand nucleotide pairs in length, that serve as replication origins. These origins continue to function when moved to a different chromosomal region by recombinant DNA methods, as long as they are placed in a region where the chromatin is relatively uncondensed. One of these origins is the sequence from the β-globin gene cluster. At its normal position in the genome, the function of this origin depends critically upon distant DNA sequences (Figure 5–39). As discussed in Chapter 7, this distant DNA is known to have a decondensing effect on the chromatin structure that surrounds the origin and includes the β-globin gene; the more open chromatin conformation that results is apparently required for this origin to function, as well as for the β-globin gene to be expressed.

We know now that a human ORC complex homologous to that in yeast cells is required for replication initiation and that the human Cdc6 and Mcm proteins likewise have central roles in the initiation process. It therefore seems likely that the yeast and human initiation mechanisms will turn out to be very similar. However, the binding sites for the ORC protein seem to be less specific in humans than they are in yeast, which may explain why the replication origins of humans are longer and less sharply defined than those of yeast.

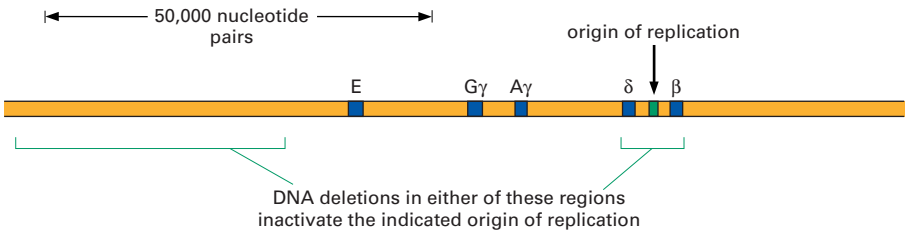


Figure 5–39 Deletions that inactivate an origin of replication in humans. These two deletions are found separately in two individuals who suffer from *thalassemia,* a disorder caused by the failure to express one or more of the genes in the β-globin gene cluster shown. In both of these deletion mutants, the DNA in this region is replicated by forks that begin at replication origins outside the β-globin gene cluster. As explained in the text, the deletion on the *left* removes DNA sequences that control the chromatin structure of the replication origin on the *right*.

New Nucleosomes Are Assembled Behind the Replication Fork

In this and the following section we consider several additional aspects of DNA replication that are specific to eucaryotes. As discussed in Chapter 4, eucaryotic chromosomes are composed of the mixture of DNA and protein known as chromatin. Chromosome duplication therefore requires not only that the DNA be replicated but also that new chromosomal proteins be assembled onto the DNA behind each replication fork. Although we are far from understanding this process in detail, we are beginning to learn how the *nucleosome,* the fundamental unit of chromatin packaging, is duplicated. A large amount of new histone protein, approximately equal in mass to the newly synthesized DNA, is required to make the new nucleosomes in each cell cycle. For this reason, most eucaryotic organisms possess multiple copies of the gene for each histone. Vertebrate cells, for example, have about 20 repeated gene sets, most sets containing the genes that encode all five histones (H1, H2A, H2B, H3, and H4).

Unlike most proteins, which are made continuously throughout interphase, histones are synthesized mainly in S phase, when the level of histone mRNA increases about fiftyfold as a result of both increased transcription and decreased mRNA degradation. By a mechanism that depends on special properties of their 3′ ends (discussed in Chapter 7), the major histone mRNAs become highly unstable and are degraded within minutes when DNA synthesis stops at the end of S phase (or when inhibitors are added to stop DNA synthesis prematurely). In contrast, the histone proteins themselves are remarkably stable and may survive for the entire life of a cell. The tight linkage between DNA synthesis and histone synthesis presumably depends on a feedback mechanism that monitors the level of free histone to ensure that the amount of histone made exactly matches the amount of new DNA synthesized.

As a replication fork advances, it must somehow pass through the parental nucleosomes. *In vitro* studies show that the replication apparatus has a poorly understood intrinsic ability to pass through parental nucleosomes without displacing them from the DNA. The chromatin-remodeling proteins discussed in Chapter 4, which destabilize the DNA–histone interface, likely facilitate this process in the cell.

Both of the newly synthesized DNA helices behind a replication fork inherit old histones (Figure 5–40). But since the amount of DNA has doubled, an equal amount of new histones is also needed to complete the packaging of DNA into chromatin. The addition of new histones to the newly synthesized DNA is aided by *chromatin assembly factors (CAFs)*, which are proteins that associate with replication forks and package the newly synthesized DNA as soon as it emerges from the replication machinery. The newly synthesized H3 and H4 histones are rapidly acetylated on their N-terminal tails (discussed in Chapter 4); after they have been incorporated into chromatin, these acetyl groups are removed enzymatically from the histones (Figure 5–41).

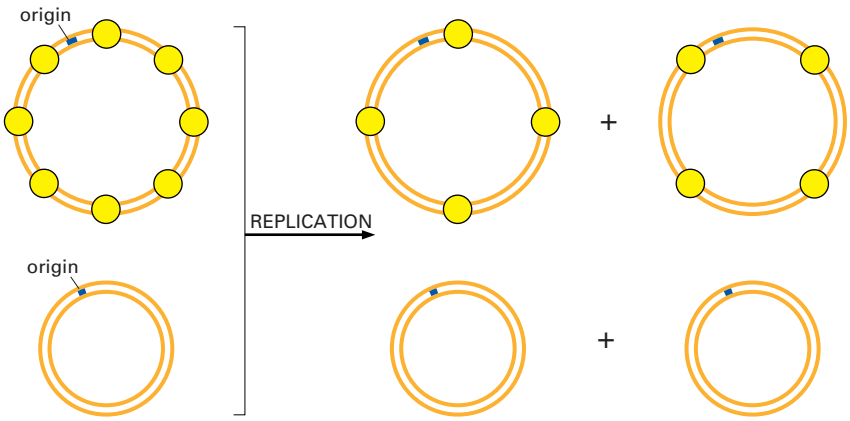


Figure 5–40 A demonstration that histones remain associated with DNA after the replication fork passes. In this experiment, performed *in vitro,* a mixture of two different-sized circular molecules of DNA (only one of which is assembled into nucleosomes) are replicated with purified proteins. After a round of DNA replication, only the daughter DNA molecules that derived from the nucleosomal parent have inherited nucleosomes. This experiment also demonstrates that both newly synthesized DNA helices inherit old histones.

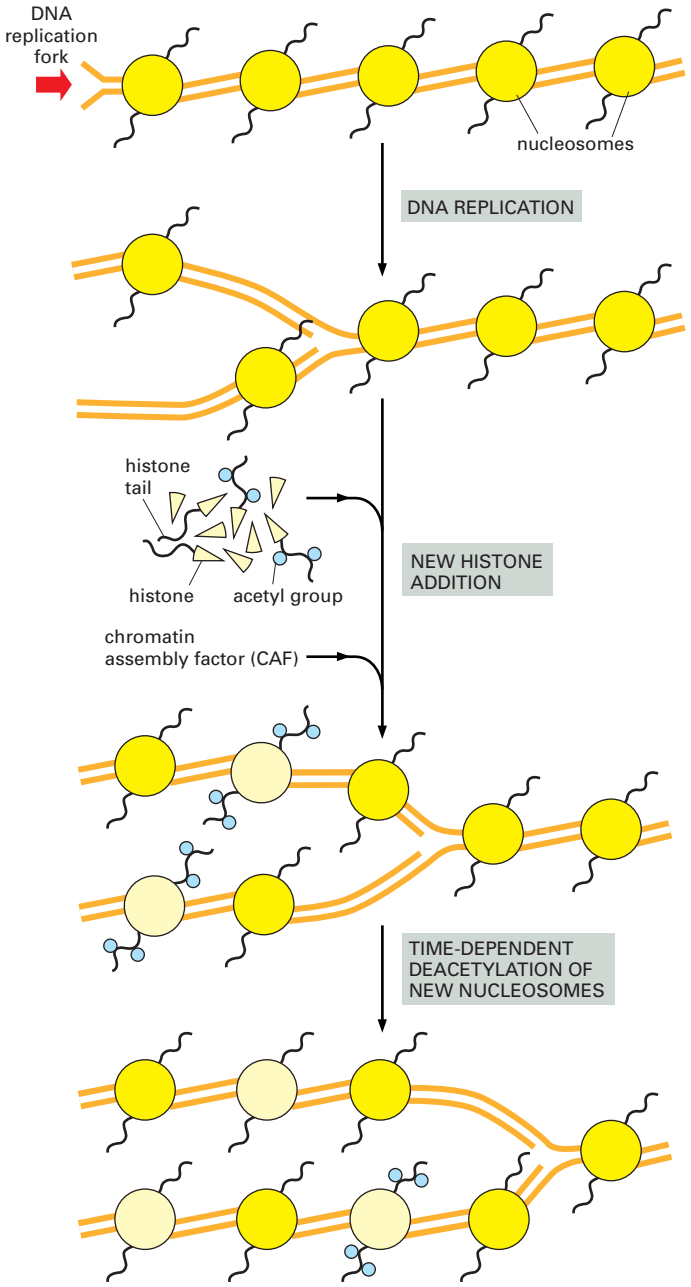


Figure 5–41 The addition of new histones to DNA behind a replication fork. The new nucleosomes are those colored *light yellow* in this diagram; as indicated, some of the histones that form them initially have specifically acetylated lysine side chains (see Figure 4–35), which are later removed.

Telomerase Replicates the Ends of Chromosomes

We saw earlier that, because DNA polymerases polymerize DNA only in the 5′-to-3′ direction, synthesis of the lagging strand at a replication fork must occur discontinuously through a backstitching mechanism that produces short DNA fragments. This mechanism encounters a special problem when the replication fork reaches an end of a linear chromosome: there is no place to produce the RNA primer needed to start the last Okazaki fragment at the very tip of a linear DNA molecule.

Bacteria solve this "end-replication" problem by having circular DNA molecules as chromosomes (see Figure 5–30). Eucaryotes solve it in an ingenious way: they have special nucleotide sequences at the ends of their chromosomes, which are incorporated into *telomeres* (discussed in Chapter 4), and attract an enzyme called **telomerase**. Telomere DNA sequences are similar in organisms as diverse as protozoa, fungi, plants, and mammals. They consist of many tandem repeats of a short sequence that contains a block of neighboring G nucleotides. In humans, this sequence is GGGTTA, extending for about 10,000 nucleotides.

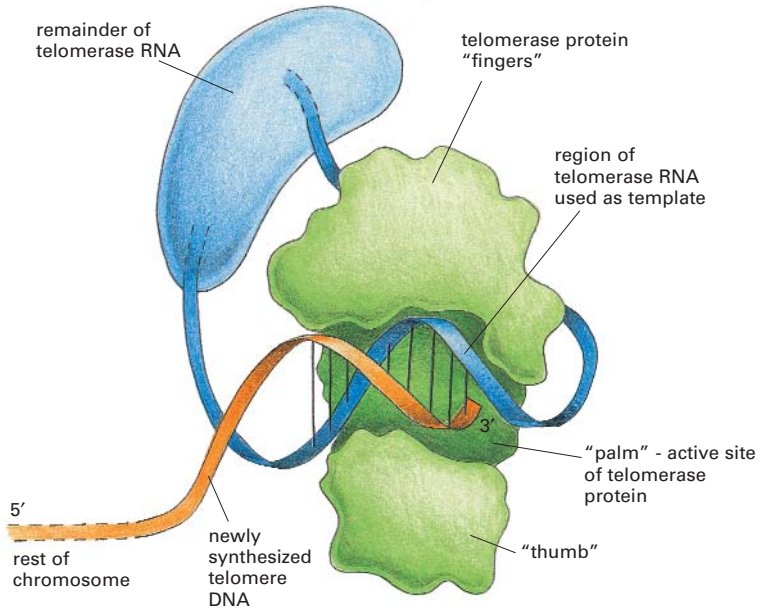


Figure 5–42 The structure of telomerase. The telomerase is a protein–RNA complex that carries an RNA template for synthesizing a repeating, G-rich telomere DNA sequence. Only the part of the telomerase protein homologous to reverse transcriptase is shown here *(green)*. A reverse transcriptase is a special form of polymerase enzyme that uses an RNA template to make a DNA strand; telomerase is unique in carrying its own RNA template with it at all times. (Modified from J. Lingner and T.R. Cech, *Curr. Opin. Genet. Dev.* 8:226–232, 1998.)

Telomerase recognizes the tip of a G-rich strand of an existing telomere DNA repeat sequence and elongates it in the 5′-to-3′ direction. The telomerase synthesizes a new copy of the repeat, using an RNA template that is a component of the enzyme itself. The telomerase enzyme otherwise resembles other *reverse transcriptases*, enzymes that synthesize DNA using an RNA template (Figure 5–42). The enzyme thus contains all the information used to maintain the characteristic telomere sequences. After several rounds of extension of the parental DNA strand by telomerase, replication of the lagging strand at the chromosome end can be completed by using these extensions as a template for synthesis of the complementary strand by a DNA polymerase molecule (Figure 5–43).

The mechanism just described ensures that the 3′ DNA end at each telomere is always slightly longer than the 5′ end with which it is paired, leaving a protruding single-stranded end (see Figure 5–43). Aided by specialized proteins, this protruding end has been shown to loop back to tuck its single-stranded terminus into the duplex DNA of the telomeric repeat sequence (Figure 5–44). Thus, the normal end of a chromosome has a unique structure, which protects it from degradative enzymes and clearly distinguishes it from the ends of the broken DNA molecules that the cell rapidly repairs (see Figure 5-53).

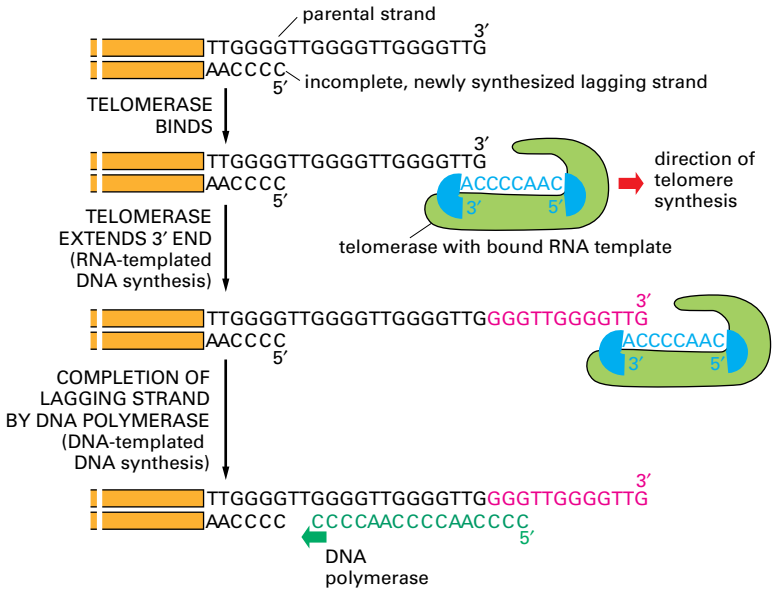


Figure 5–43 Telomere replication. Shown here are the reactions involved in synthesizing the repeating G-rich sequences that form the ends of the chromosomes (telomeres) of diverse eucaryotic organisms. The 3′ end of the parental DNA strand is extended by RNA-templated DNA synthesis; this allows the incomplete daughter DNA strand that is paired with it to be extended in its 5′ direction. This incomplete, lagging strand is presumed to be completed by DNA polymerase α, which carries a DNA primase as one of its subunits (see Figure 5–28). The telomere sequence illustrated is that of the ciliate *Tetrahymena,* in which these reactions were first discovered. The telomere repeats are GGGTTG in the ciliate *Tetrahymena,* GGGTTA in humans, and $G_{1–3}A$ in the yeast *S. cerevisiae*.

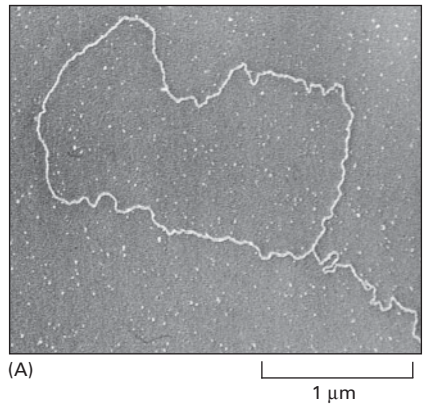


Figure 5–44 The t-loops at the end of mammalian chromosomes. (A) Electron micrograph of the DNA at the end of an interphase human chromosome. The chromosome was fixed, deproteinated, and artificially thickened before viewing. The loop seen here is approximately 15,000 nucleotide pairs in length. (B) Model for telomere structure. The insertion of the single-stranded end into the duplex repeats to form a t-loop is carried out and maintained by specialized proteins, schematized in *green*. In addition it is possible, as shown, that the chromosome end is looped once again on itself through the formation of heterochromatin adjacent to the t-loop (see Figure 4–47). (A, from J.D. Griffith et al., *Cell* 97:503–514, 1999. © Elsevier.)

Telomere Length Is Regulated by Cells and Organisms

Because the processes that grow and shrink each telomere sequence are only approximately balanced, a chromosome end contains a variable number of telomeric repeats. Not surprisingly, experiments show that cells that proliferate indefinitely (such as yeast cells) have homeostatic mechanisms that maintain the number of these repeats within a limited range (Figure 5–45).

In the somatic cells of humans, the telomere repeats have been proposed to provide each cell with a counting mechanism that helps prevent the unlimited proliferation of wayward cells in adult tissues. According to this idea, our somatic cells are born with a full complement of telomeric repeats; however, the telomerase enzyme is turned off in a tissue like the skin, so that each time a cell divides, it loses 50–100 nucleotides from each of its telomeres. After many cell generations, the descendent cells will inherit defective chromosomes (because their tips cannot be replicated completely) and consequently will withdraw permanently from the cell cycle and cease dividing—a process called *replicative cell senescence* (discussed in Chapter 17). In theory, such a mechanism could provide a safeguard against the uncontrolled cell proliferation of abnormal cells in somatic tissues, thereby helping to protect us from cancer.

The idea that telomere length acts as a "measuring stick" to count cell divisions and thereby regulate the cell's lifetime has been tested in several ways. For certain types of human cells grown in tissue culture, the experimental results support such a theory. Human fibroblasts normally proliferate for about 60 cell divisions in culture before undergoing replicative senescence. Like most other somatic cells in humans, fibroblasts fail to produce telomerase, and their

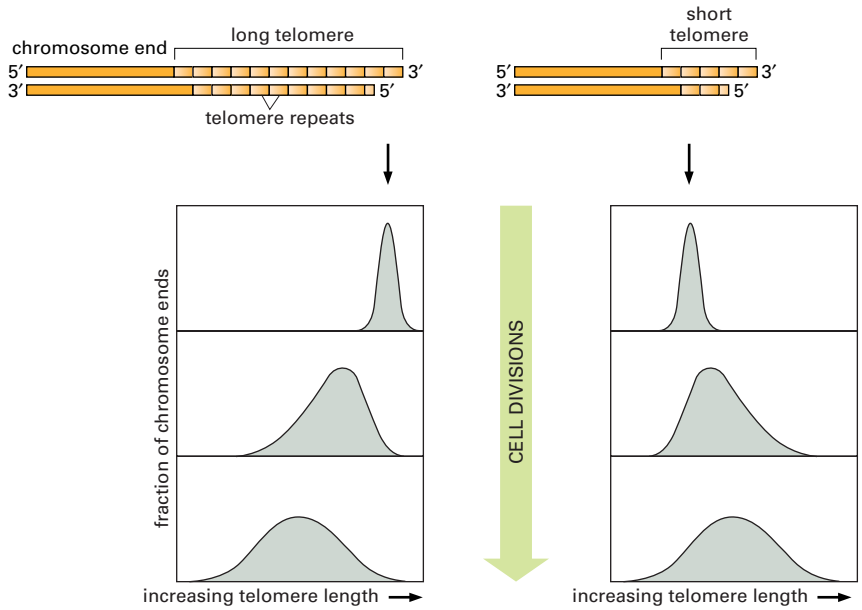


Figure 5–45 A demonstration that yeast cells control the length of their telomeres. In this experiment, the telomere at one end of a particular chromosome is artificially made either longer *(left)* or shorter *(right)* than average. After many cell divisions, the chromosome recovers, showing an average telomere length and a length distribution that is typical of the other chromosomes in the yeast cell. A similar feedback mechanism for controlling telomere length has been proposed to exist for the cells in the germ-line cells of animals.

telomeres gradually shorten each time they divide. When telomerase is provided to the fibroblasts by inserting an active telomerase gene, telomere length is maintained and many of the cells now continue to proliferate indefinitely. It therefore seems clear that telomere shortening can count cell divisions and trigger replicative senescence in human cells.

It has been proposed that this type of control on cell proliferation is important for the maintenance of tissue architecture and that it is also somehow responsible for the aging of animals like ourselves. These ideas have been tested by producing transgenic mice that lack telomerase. The telomeres in mouse chromosomes are about five times longer than human telomeres, and the mice must therefore be bred through three or more generations before their telomeres have shrunk to the normal human length. It is therefore perhaps not surprising that the mice initially develop normally. More importantly, the mice in later generations develop progressively more defects in some of their highly proliferative tissues. But these mice do not seem to age prematurely overall, and the older animals have a pronounced tendency to develop tumors. In these and other respects these mice resemble humans with the genetic disease *dyskeratosis congenita,* which has also been attributed to premature telomere shortening. Individuals afflicted with this disease show abnormalities in various epidermal structures (including skin, nails, and tear ducts) and in the production of red blood cells.

It is clear from the above observations that controlling cell proliferation by the removal of telomeres poses a risk to an organism, because not all of the cells that lack functional telomeres in a tissue will stop dividing. Others apparently become genetically unstable, but continue to divide giving rise to variant cells that can lead to cancer. Thus, one can question whether the observed absence of telomerase from most human somatic cells provides an evolutionary advantage, as suggested by those who postulate that telomere shortening tends to protect us from cancer and other proliferative diseases.

Summary

The proteins that initiate DNA replication bind to DNA sequences at a replication origin to catalyze the formation of a replication bubble with two outward-moving replication forks. The process begins when an initiator protein–DNA complex is formed that subsequently loads a DNA helicase onto the DNA template. Other proteins are then added to form the multienzyme "replication machine" that catalyzes DNA synthesis at each replication fork.

In bacteria and some simple eucaryotes, replication origins are specified by specific DNA sequences that are only several hundred nucleotide pairs long. In other eucaryotes, such as humans, the sequences needed to specify an origin of DNA replication seem to be less well defined, and the origin can span several thousand nucleotide pairs.

Bacteria typically have a single origin of replication in a circular chromosome. With fork speeds of up to 1000 nucleotides per second, they can replicate their genome in less than an hour. Eucaryotic DNA replication takes place in only one part of the cell cycle, the S phase. The replication fork in eucaryotes moves about 10 times more slowly than the bacterial replication fork, and the much longer eucaryotic chromosomes each require many replication origins to complete their replication in a typical 8-hour S phase. The different replication origins in these eucaryotic chromosomes are activated in a sequence, determined in part by the structure of the chromatin, with the most condensed regions of chromatin beginning their replication last. After the replication fork has passed, chromatin structure is re-formed by the addition of new histones to the old histones that are directly inherited as nucleosomes by each daughter DNA molecule.

Eucaryotes solve the problem of replicating the ends of their linear chromosomes by a specialized end structure, the telomere, which requires a special enzyme, telomerase. Telomerase extends the telomere DNA by using an RNA template that is an integral part of the enzyme itself, producing a highly repeated DNA sequence that typically extends for 10,000 nucleotide pairs or more at each chromosome end.

DNA REPAIR

Although genetic variation is important for evolution, the survival of the individual demands genetic stability. Maintaining genetic stability requires not only an extremely accurate mechanism for replicating DNA, but also mechanisms for repairing the many accidental lesions that occur continually in DNA. Most such spontaneous changes in DNA are temporary because they are immediately corrected by a set of processes that are collectively called **DNA repair**. Of the thousands of random changes created every day in the DNA of a human cell by heat, metabolic accidents, radiation of various sorts, and exposure to substances in the environment, only a few accumulate as mutations in the DNA sequence. We now know that fewer than one in 1000 accidental base changes in DNA results in a permanent mutation; the rest are eliminated with remarkable efficiency by DNA repair.

The importance of DNA repair is evident from the large investment that cells make in DNA repair enzymes. For example, analysis of the genomes of bacteria and yeasts has revealed that several percent of the coding capacity of these organisms is devoted solely to DNA repair functions. The importance of DNA repair is also demonstrated by the increased rate of mutation that follows the inactivation of a DNA repair gene. Many DNA repair pathways and the genes that encode them—which we now know operate in a wide variety of organisms, including humans—were originally identified in bacteria by the isolation and characterization of mutants that displayed an increased mutation rate or an increased sensitivity to DNA-damaging agents.

Recent studies of the consequences of a diminished capacity for DNA repair in humans have linked a variety of human diseases with decreased repair (Table 5–2). Thus, we saw previously that defects in a human gene that normally functions to repair the mismatched base pairs in DNA resulting from replication errors can lead to an inherited predisposition to certain cancers, reflecting an increased mutation rate. In another human disease, *xeroderma pigmentosum* (XP), the afflicted individuals have an extreme sensitivity to ultraviolet radiation because they are unable to repair certain DNA photoproducts. This repair defect results in an increased mutation rate that leads to serious skin lesions and an increased susceptibility to certain cancers.

Without DNA Repair, Spontaneous DNA Damage Would Rapidly Change DNA Sequences

Although DNA is a highly stable material, as required for the storage of genetic information, it is a complex organic molecule that is susceptible, even under

TABLE 5–2 Inherited Syndromes with Defects in DNA Repair

NAME	PHENOTYPE	ENZYME OR PROCESS AFFECTED
MSH2, 3, 6, MLH1, PMS2	colon cancer	mismatch repair
Xeroderma pigmentosum (XP) groups A–G	skin cancer, cellular UV sensitivity, neurological abnormalities	nucleotide excision-repair
XP variant	cellular UV sensitivity	translesion synthesis by DNA polymerase δ
Ataxia–telangiectasia (AT)	leukemia, lymphoma, cellular γ-ray sensitivity, genome instability	ATM protein, a protein kinase activated by double-strand breaks
BRCA-2	breast and ovarian cancer	repair by homologous recombination
Werner syndrome	premature aging, cancer at several sites, genome instability	accessory 3′-exonuclease and DNA helicase
Bloom syndrome	cancer at several sites, stunted growth, genome instability	accessory DNA helicase for replication
Fanconi anemia groups A–G	congenital abnormalities, leukemia, genome instability	DNA interstrand cross-link repair
46 BR patient	hypersensitivity to DNA-damaging agents, genome instability	DNA ligase I

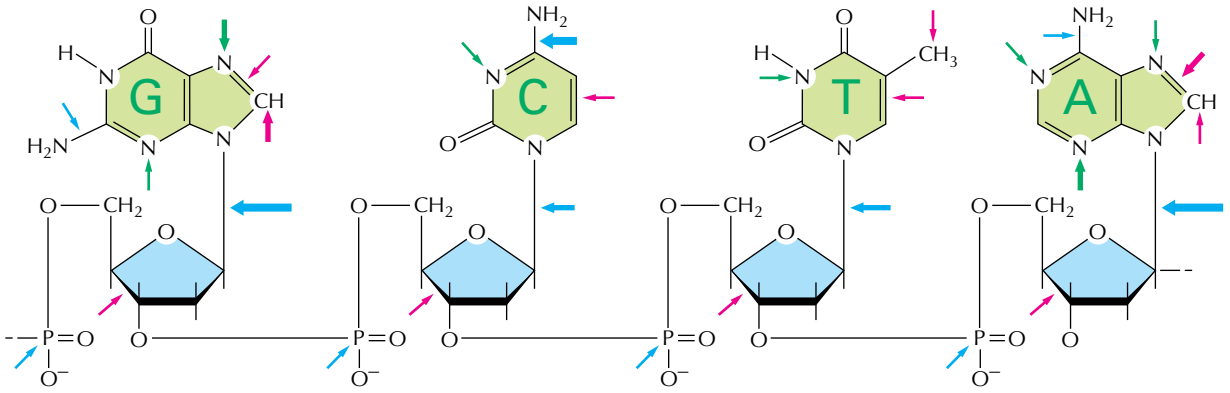


Figure 5–46 A summary of spontaneous alterations likely to require DNA repair. The sites on each nucleotide that are known to be modified by spontaneous oxidative damage *(red arrows)*, hydrolytic attack *(blue arrows)*, and uncontrolled methylation by the methyl group donor S-adenosylmethionine *(green arrows)* are shown, with the width of each arrow indicating the relative frequency of each event. (After T. Lindahl, *Nature* 362:709–715, 1993. © Macmillan Magazines Ltd.)

normal cellular conditions, to spontaneous changes that would lead to mutations if left unrepaired (Figure 5–46). DNA undergoes major changes as a result of thermal fluctuations: for example, about 5000 purine bases (adenine and guanine) are lost every day from the DNA of each human cell because their N-glycosyl linkages to deoxyribose hydrolyze, a spontaneous reaction called *depurination*. Similarly, a spontaneous *deamination* of cytosine to uracil in DNA occurs at a rate of about 100 bases per cell per day (Figure 5–47). DNA bases are also occasionally damaged by an encounter with reactive metabolites (including reactive forms of oxygen) or environmental chemicals. Likewise, ultraviolet radiation from the sun can produce a covalent linkage between two adjacent pyrimidine bases in DNA to form, for example, thymine dimers (Figure 5–48). If left uncorrected when the DNA is replicated, most of these changes would be expected to lead either to the deletion of one or more base pairs or to a base-pair substitution in the daughter DNA chain (Figure 5–49). The mutations would then be propagated throughout subsequent cell generations as the DNA is replicated. Such a high rate of random changes in the DNA sequence would have disastrous consequences for an organism.

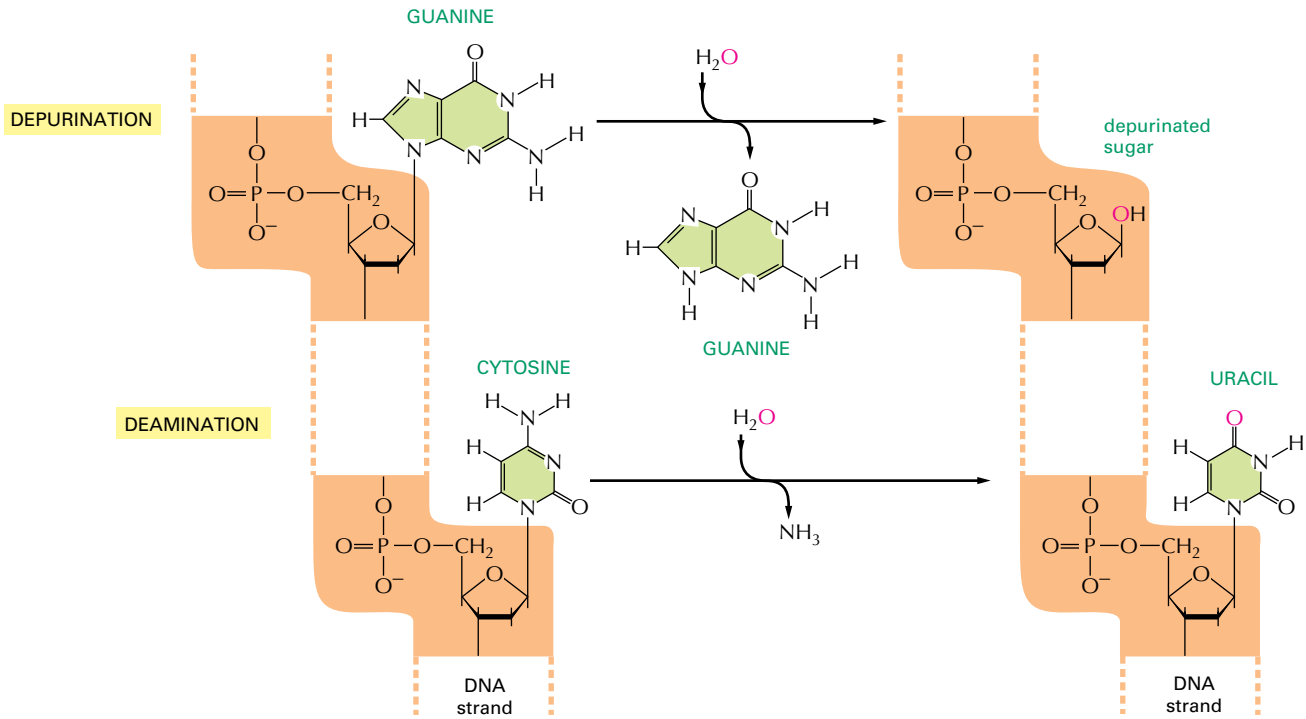


Figure 5–47 Depurination and deamination. These two reactions are the most frequent spontaneous chemical reactions known to create serious DNA damage in cells. Depurination can release guanine (shown here), as well as adenine, from DNA. The major type of deamination reaction (shown here) converts cytosine to an altered DNA base, uracil, but deamination occurs on other bases as well. These reactions take place on double-helical DNA; for convenience, only one strand is shown.

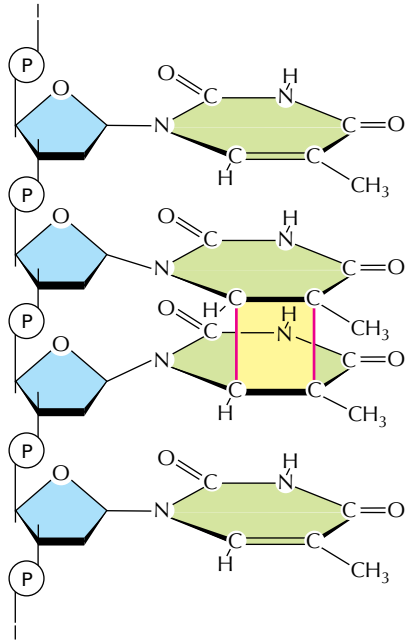


Figure 5–48 The thymine dimer. This type of damage is introduced into DNA in cells that are exposed to ultraviolet irradiation (as in sunlight). A similar dimer will form between any two neighboring pyrimidine bases (C or T residues) in DNA.

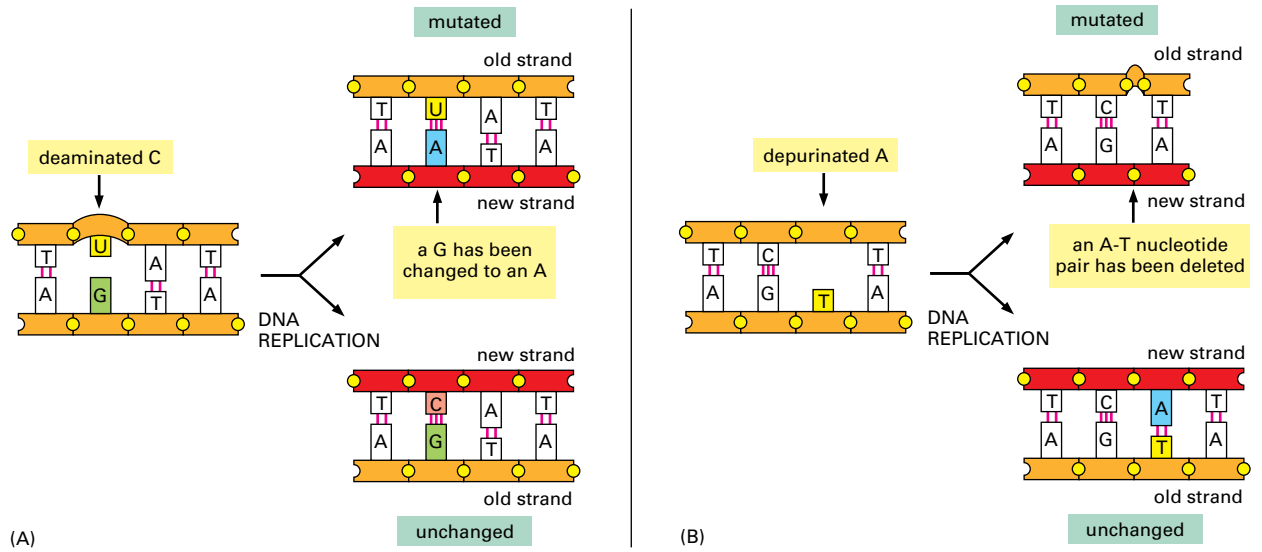
The DNA Double Helix Is Readily Repaired

The double-helical structure of DNA is ideally suited for repair because it carries two separate copies of all the genetic information—one in each of its two strands. Thus, when one strand is damaged, the complementary strand retains an intact copy of the same information, and this copy is generally used to restore the correct nucleotide sequences to the damaged strand.

An indication of the importance of a double-stranded helix to the safe storage of genetic information is that all cells use it; only a few small viruses use single-stranded DNA or RNA as their genetic material. The types of repair processes described in this section cannot operate on such nucleic acids, and the chance of a permanent nucleotide change occurring in these single-stranded genomes of viruses is thus very high. It seems that only organisms with tiny genomes can afford to encode their genetic information in any molecule other than a DNA double helix.

Each cell contains multiple DNA repair systems, each with its own enzymes and preferences for the type of damage recognized. As we see in the rest of this section, most of these systems use the undamaged strand of the double helix as a template to repair the damaged strand.

Figure 5–49 How chemical modifications of nucleotides produce mutations. (A) Deamination of cytosine, if uncorrected, results in the substitution of one base for another when the DNA is replicated. As shown in Figure 5–47, deamination of cytosine produces uracil. Uracil differs from cytosine in its base-pairing properties and preferentially base-pairs with adenine. The DNA replication machinery therefore adds an adenine when it encounters a uracil on the template strand. (B) Depurination, if uncorrected, can lead to either the substitution or the loss of a nucleotide pair. When the replication machinery encounters a missing purine on the template strand, it may skip to the next complete nucleotide as illustrated here, thus producing a nucleotide deletion in the newly synthesized strand. Many other types of DNA damage (see Figure 5–46) also produce mutations when the DNA is replicated if left uncorrected.



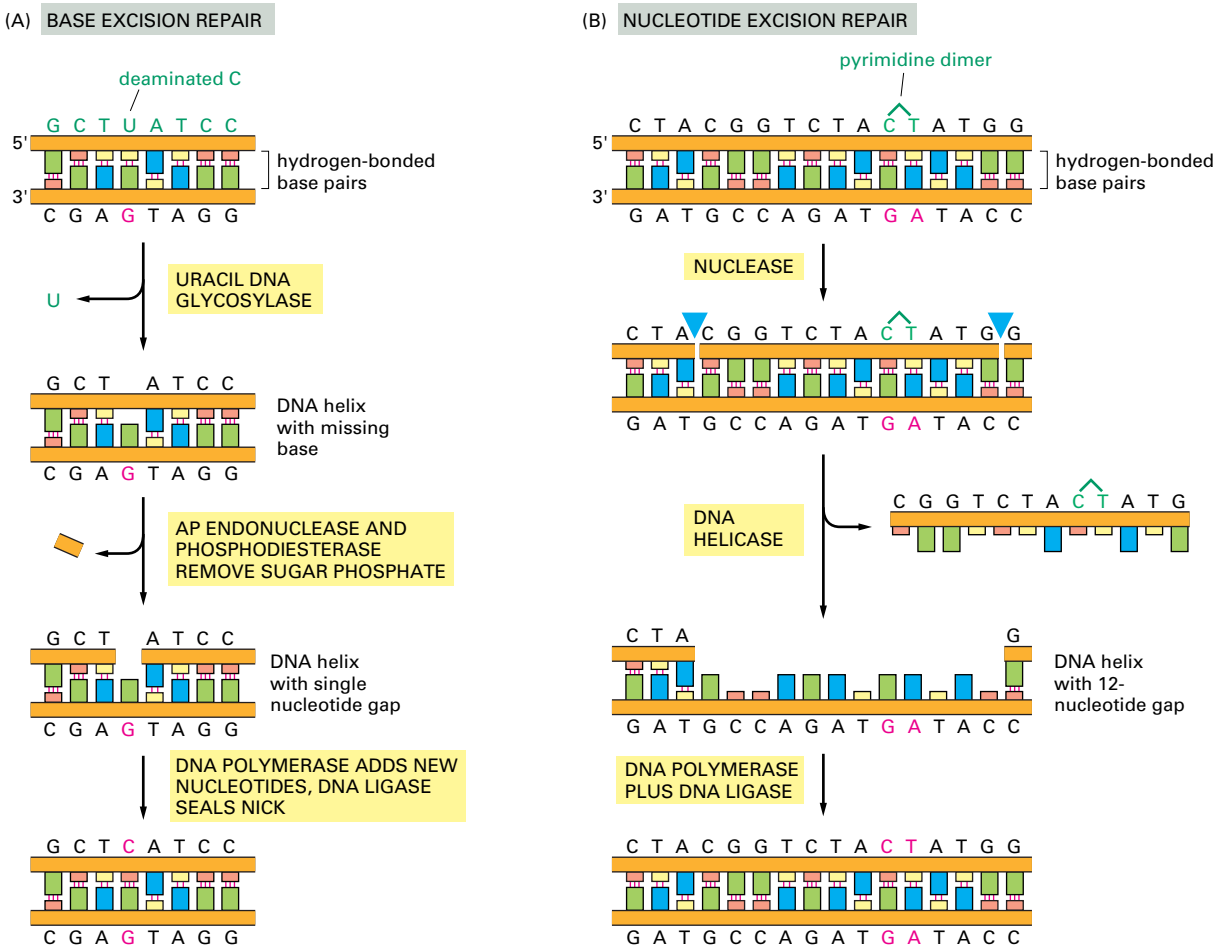


Figure 5–50 A comparison of two major DNA repair pathways. (A) *Base excision repair.* This pathway starts with a DNA glycosylase. Here the enzyme uracil DNA glycosylase removes an accidentally deaminated cytosine in DNA. After the action of this glycosylase (or another DNA glycosylase that recognizes a different kind of damage), the sugar phosphate with the missing base is cut out by the sequential action of AP endonuclease and a phosphodiesterase. (These same enzymes begin the repair of depurinated sites directly.) The gap of a single nucleotide is then filled by DNA polymerase and DNA ligase. The net result is that the U that was created by accidental deamination is restored to a C. The AP endonuclease derives its name from the fact that it recognizes any site in the DNA helix that contains a deoxyribose sugar with a missing base; such sites can arise either by the loss of a purine (*ap*urinic sites) or by the loss of a pyrimidine (*ap*yrimidinic sites). (B) *Nucleotide excision repair.* After a multienzyme complex has recognized a bulky lesion such as a pyrimidine dimer (see Figure 5–48), one cut is made on each side of the lesion, and an associated DNA helicase then removes the entire portion of the damaged strand. The multienzyme complex in bacteria leaves the gap of 12 nucleotides shown; the gap produced in human DNA is more than twice this size. The nucleotide excision repair machinery can recognize and repair many different types of DNA damage.

DNA Damage Can Be Removed by More Than One Pathway

There are multiple pathways for DNA repair, using different enzymes that act upon different kinds of lesions. Two of the most common pathways are shown in Figure 5–50. In both, the damage is excised, the original DNA sequence is restored by a DNA polymerase that uses the undamaged strand as its template, and the remaining break in the double helix is sealed by DNA ligase (see Figure 5–14).

The two pathways differ in the way in which the damage is removed from DNA. The first pathway, called **base excision repair**, involves a battery of enzymes called *DNA glycosylases,* each of which can recognize a specific type of altered base in DNA and catalyze its hydrolytic removal. There are at least six types of these enzymes, including those that remove deaminated Cs, deaminated As, different types of alkylated or oxidized bases, bases with opened rings, and bases in which a carbon–carbon double bond has been accidentally converted to a carbon–carbon single bond.

As an example of the general mechanism of base excision repair, the removal of a deaminated C by uracil DNA glycosylase is shown in Figure 5–50A. How is the altered base detected within the context of the double helix? A key step is an enzyme-mediated "flipping-out" of the altered nucleotide from the helix, which allows the enzyme to probe all faces of the base for damage (Figure 5–51). It is thought that DNA glycosylases travel along DNA using base-flipping to evaluate the status of each base pair. Once a damaged base is recognized, the DNA glycosylase reaction creates a deoxyribose sugar that lacks its base. This "missing tooth" is recognized by an enzyme called *AP endonuclease,* which cuts the phosphodiester backbone, and the damage is then removed and repaired (see Figure 5–50A). Depurination, which is by far the most frequent type of damage suffered by DNA, also leaves a deoxyribose sugar with a missing base. Depurinations are directly repaired beginning with AP endonuclease, following the bottom half of the pathway in Figure 5–50A.

The second major repair pathway is called **nucleotide excision repair**. This mechanism can repair the damage caused by almost any large change in the structure of the DNA double helix. Such "bulky lesions" include those created by the covalent reaction of DNA bases with large hydrocarbons (such as the carcinogen benzopyrene), as well as the various pyrimidine dimers (T-T, T-C, and C-C) caused by sunlight. In this pathway, a large multienzyme complex scans the DNA for a distortion in the double helix, rather than for a specific base change. Once a bulky lesion has been found, the phosphodiester backbone of the abnormal strand is cleaved on both sides of the distortion, and an oligonucleotide containing the lesion is peeled away from the DNA double helix by a DNA helicase enzyme. The large gap produced in the DNA helix is then repaired by DNA polymerase and DNA ligase (Figure 5–50B).

The Chemistry of the DNA Bases Facilitates Damage Detection

The DNA double helix seems to be optimally constructed for repair. As noted above, it contains a backup copy of the genetic information, so that if one strand is damaged, the other undamaged strand can be used as a template for repair. The nature of the bases also facilitates the distinction between undamaged and damaged bases. Thus, every possible deamination event in DNA yields an unnatural base, which can therefore be directly recognized and removed by a specific DNA glycosylase. Hypoxanthine, for example, is the simplest purine base capable of pairing specifically with C, but hypoxanthine is the direct deamination product of A (Figure 5–52A). The addition of a second amino group to hypoxanthine produces G, which cannot be formed from A by spontaneous deamination, and whose deamination product is likewise unique.

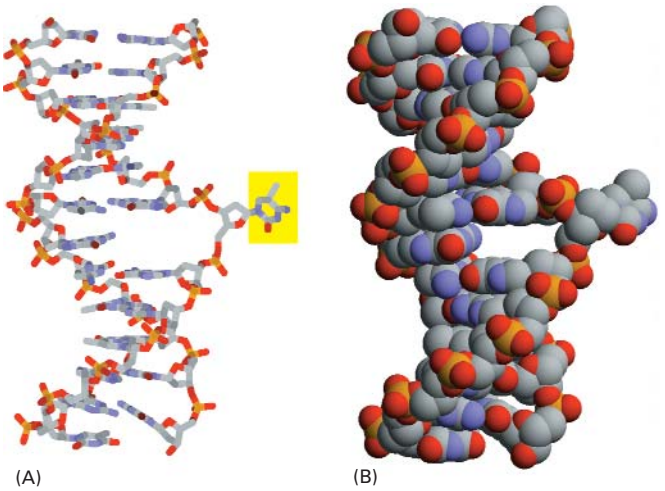


Figure 5–51 The recognition of an unusual nucleotide in DNA by base-flipping. The DNA glycosylase family of enzymes recognizes specific bases in the conformation shown. Each of these enzymes cleaves the glycosyl bond that connects a particular recognized base *(yellow)* to the backbone sugar, removing it from the DNA. (A) Stick model; (B) space-filling model.

As discussed in Chapter 6, RNA is thought, on an evolutionary time-scale, to have served as the genetic material before DNA, and it seems likely that the genetic code was initially carried in the four nucleotides A, C, G, and U. This raises the question of why the U in RNA was replaced in DNA by T (which is 5-methyl U). We have seen that the spontaneous deamination of C converts it to U, but that this event is rendered relatively harmless by uracil DNA glycosylase. However, if DNA contained U as a natural base, the repair system would be unable to distinguish a deaminated C from a naturally occuring U.

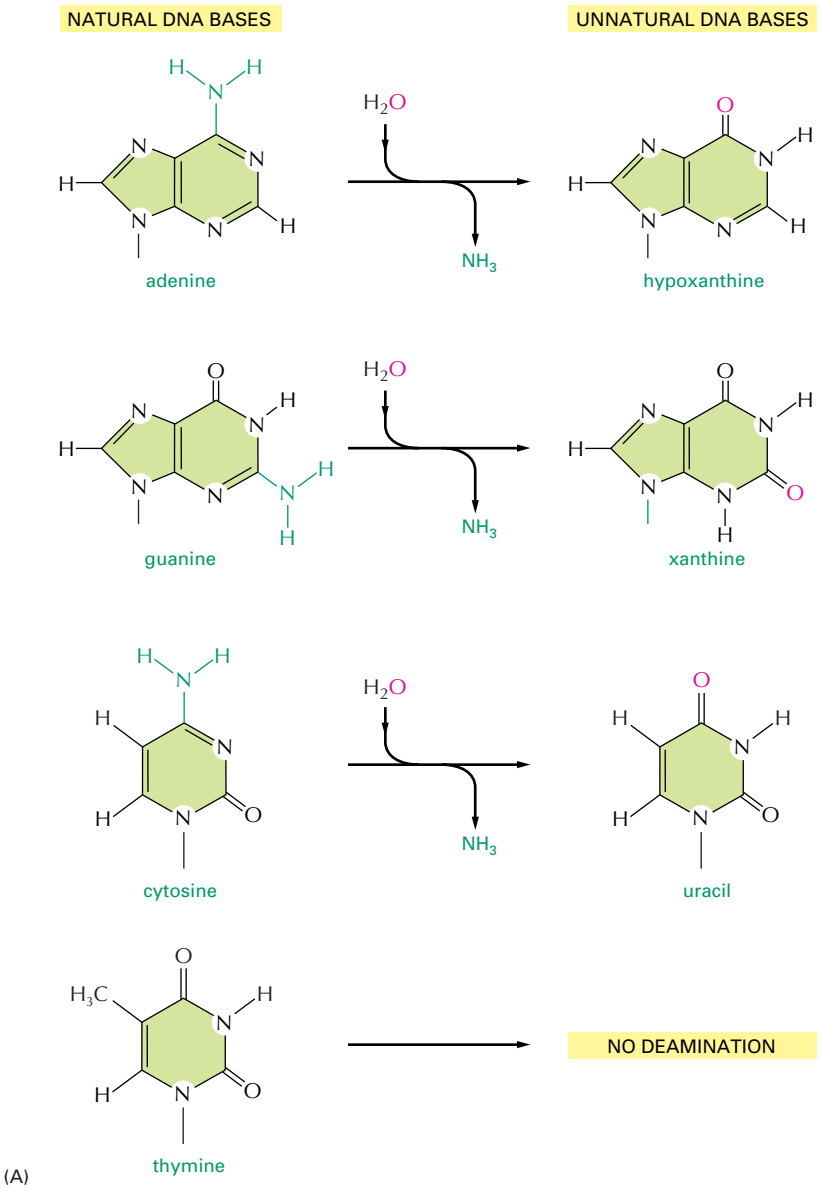


Figure 5–52 The deamination of DNA nucleotides. In each case the oxygen atom that is added in this reaction with water is colored *red*. (A) The spontaneous deamination products of A and G are recognizable as unnatural when they occur in DNA and thus are readily recognized and repaired. The deamination of C to U was previously illustrated in Figure 5–47; T has no amino group to deaminate. (B) About 3% of the C nucleotides in vertebrate DNAs are methylated to help in controlling gene expression (discussed in Chapter 7). When these 5-methyl C nucleotides are accidentally deaminated, they form the natural nucleotide T. This T would be paired with a G on the opposite strand, forming a mismatched base pair.

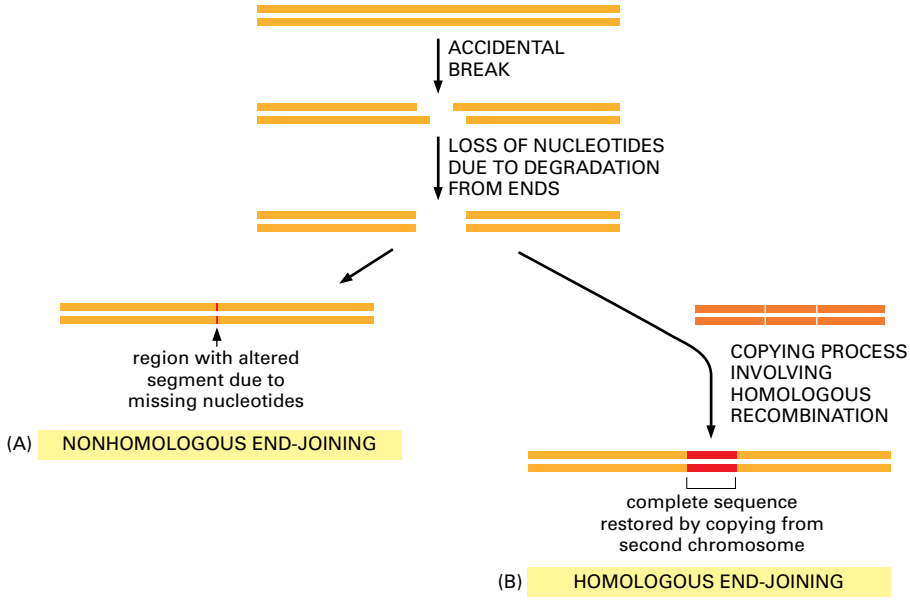


Figure 5–53 Two different types of end-joining for repairing double-strand breaks. (A) Nonhomologous end-joining alters the original DNA sequence when repairing broken chromosomes. These alterations can be either deletions (as shown) or short insertions. (B) Homologous end-joining is more difficult to accomplish, but is much more precise.

A special situation occurs in vertebrate DNA, in which selected C nucleotides are methylated at specific C-G sequences that are associated with inactive genes (discussed in Chapter 7). The accidental deamination of these methylated C nucleotides produces the natural nucleotide T (Figure 5–52B) in a mismatched base pair with a G on the opposite DNA strand. To help in repairing deaminated methylated C nucleotides, a special DNA glycosylase recognizes a mismatched base pair involving T in the sequence T-G and removes the T. This DNA repair mechanism must be relatively ineffective, however, because methylated C nucleotides are common sites for mutations in vertebrate DNA. It is striking that, even though only about 3% of the C nucleotides in human DNA are methylated, mutations in these methylated nucleotides account for about one-third of the single-base mutations that have been observed in inherited human diseases.

Double-Strand Breaks are Efficiently Repaired

A potentially dangerous type of DNA damage occurs when both strands of the double helix are broken, leaving no intact template strand for repair. Breaks of this type are caused by ionizing radiation, oxidizing agents, replication errors, and certain metabolic products in the cell. If these lesions were left unrepaired, they would quickly lead to the breakdown of chromosomes into smaller fragments. However, two distinct mechanisms have evolved to ameliorate the potential damage. The simplest to understand is *nonhomologous end-joining*, in which the broken ends are juxtaposed and rejoined by DNA ligation, generally with the loss of one or more nucleotides at the site of joining (Figure 5–53A). This end-joining mechanism, which can be viewed as an emergency solution to the repair of double-strand breaks, is a common outcome in mammalian cells. Although a change in the DNA sequence (a mutation) results at the site of breakage, so little of the mammalian genome codes for proteins that this mechanism is apparently an acceptable solution to the problem of keeping chromosomes intact. As previously discussed, the specialized structure of telomeres prevents the ends of chromosomes from being mistaken for broken DNA, thereby preserving natural DNA ends.

An even more effective type of double-strand break repair exploits the fact that cells that are diploid contain two copies of each double helix. In this second repair pathway, called *homologous end-joining*, general recombination mechanisms are called into play that transfer nucleotide sequence information from the intact DNA double helix to the site of the double-strand break in the broken helix. This type of reaction requires special recombination proteins that recognize

areas of DNA sequence matching between the two chromosomes and bring them together. A DNA replication process then uses the undamaged chromosome as the template for transferring genetic information to the broken chromosome, repairing it with no change in the DNA sequence (Figure 5–53B). In cells that have replicated their DNA but not yet divided, this type of DNA repair can readily take place between the two sister DNA molecules in each chromosome; in this case, there is no need for the broken ends to find the matching DNA sequence in the homologous chromosome. The molecular details of the homologous end-joining reaction are considered later in this chapter because they require a general understanding of the way in which cells carry their genetic recombination events. Although present in humans, this type of DNA double-strand break repair predominates in bacteria, yeasts, and *Drosophila*—all organisms in which little nonhomologous DNA end-joining is observed.

Cells Can Produce DNA Repair Enzymes in Response to DNA Damage

Cells have evolved many mechanisms that help them survive in an unpredictably hazardous world. Often an extreme change in a cell's environment activates the expression of a set of genes whose protein products protect the cell from the deleterious effects of this change. One such mechanism shared by all cells is the *heat-shock response,* which is evoked by the exposure of cells to unusually high temperatures. The induced "heat-shock proteins" include some that help stabilize and repair partly denatured cell proteins, as discussed in Chapter 6.

Cells also have mechanisms that elevate the levels of DNA repair enzymes, as an emergency response to severe DNA damage. The best-studied example is the so-called **SOS response** in *E. coli*. In this bacterium, any block to DNA replication caused by DNA damage produces a signal that induces an increase in the transcription of more than 15 genes, many of which code for proteins that function in DNA repair. The signal (thought to be an excess of single-stranded DNA) first activates the *E. coli* RecA protein (see Figure 5–58), so that it destroys a gene regulatory protein that normally represses the transcription of a large set of SOS response genes.

Studies of mutant bacteria deficient in different parts of the SOS response demonstrate that the newly synthesized proteins have two effects. First, as would be expected, the induction of these additional DNA repair enzymes increases cell survival after DNA damage. Second, several of the induced proteins transiently increase the mutation rate by increasing the number of errors made in copying DNA sequences. The errors are caused by the production of low-fidelity DNA polymerases that can efficiently use damaged DNA as a template for DNA synthesis. While this "error-prone" DNA repair can be harmful to individual bacterial cells, it is presumed to be advantageous in the long term because it produces a burst of genetic variability in the bacterial population that increases the likelihood of a mutant cell arising that is better able to survive in the altered environment.

Human cells contain more than ten minor DNA polymerases, many of which are specifically called into play, as a last resort, to copy over unrepaired lesions in the DNA template. These enzymes can recognize a specific type of DNA damage and add the nucleotides that restore the initial sequence. Each such polymerase molecule is given a chance to add only one or a few nucleotides, because these enzymes are extremely error-prone when they copy a normal DNA sequence. Although the details of these fascinating reactions are still being worked out, they provide elegant testimony to the care with which organisms maintain their DNA sequences.

DNA Damage Delays Progression of the Cell Cycle

We have just seen that cells contain multiple enzyme systems that can recognize DNA damage and promote the repair of these lesions. Because of the importance

of maintaining intact, undamaged DNA from generation to generation, cells have an additional mechanism that helps them respond to DNA damage: they delay progression of the cell cycle until DNA repair is complete. For example, one of the genes expressed in response to the *E. coli* SOS signal is *sulA*, which encodes an inhibitor of cell division. Thus, when the SOS functions are turned on in response to DNA damage, a block to cell division extends the time for repair. When DNA repair is complete, the expression of the SOS genes is repressed, the cell cyle resumes, and the undamaged DNA is segregated to the daughter cells.

Damaged DNA also generates signals that block cell-cycle progression in eucaryotes. As discussed in detail in Chapter 17, the orderly progression of the cell cycle is maintained through the use of *checkpoints* that ensure the completion of one step before the next step can begin. At several of these cell-cycle checkpoints, the cycle stops if damaged DNA is detected. Thus, in yeast, the presence of DNA damage can block entry into the G_1 phase; it can slow DNA replication once begun; and it can block the transition from S phase to M phase. The DNA damage results in an increased synthesis of some DNA repair enzymes, and the delays further facilitate repair by providing the time needed for repair to reach completion.

The importance of the special signaling mechanisms that respond to DNA damage is indicated by the phenotype of humans who are born with defects in the gene that encodes the *ATM protein,* a large protein kinase. These individuals have the disease *ataxia–telangiectasia (AT),* whose symptoms include neurodegeneration, a predisposition to cancer, and genome instability. In both humans and yeasts, the ATM protein is needed to generate the initial intracellular signals that produce a response to oxygen-inflicted DNA damage, and individual organisms with defects in this protein are hypersensitive to agents that cause such damage, such as ionizing radiation.

Summary

Genetic information can be stored stably in DNA sequences only because a large set of DNA repair enzymes continuously scan the DNA and replace any damaged nucleotides. Most types of DNA repair depend on the presence of a separate copy of the genetic information in each of the two strands of the DNA double helix. An accidental lesion on one strand can therefore be cut out by a repair enzyme and a corrected strand resynthesized by reference to the information in the undamaged strand.

Most of the damage to DNA bases is excised by one of two major DNA repair pathways. In base excision repair, the altered base is removed by a DNA glycosylase enzyme, followed by excision of the resulting sugar phosphate. In nucleotide excision repair, a small section of the DNA strand surrounding the damage is removed from the DNA double helix as an oligonucleotide. In both cases, the gap left in the DNA helix is filled in by the sequential action of DNA polymerase and DNA ligase, using the undamaged DNA strand as the template.

Other critical repair systems—based on either nonhomologous or homologous end-joining mechanisms—reseal the accidental double-strand breaks that occur in the DNA helix. In most cells, an elevated level of DNA damage causes both an increased synthesis of repair enzymes and a delay in the cell cycle. Both factors help to ensure that DNA damage is repaired before a cell divides.

GENERAL RECOMBINATION

In the two preceding sections, we discussed the mechanisms that allow the DNA sequences in cells to be maintained from generation to generation with very little change. However, it is also clear that these DNA sequences can occasionally be rearranged. The particular combination of genes present in any individual genome, as well as the timing and the level of expression of these genes, is often altered by such DNA rearrangements. In a population, this type of genetic

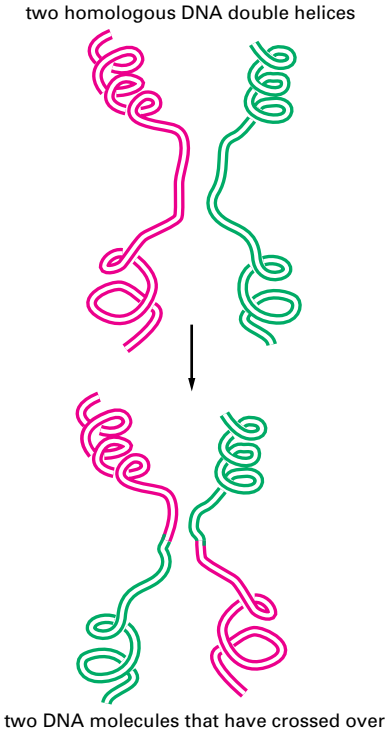


Figure 5–54 General recombination. The breaking and rejoining of two homologous DNA double helices creates two DNA molecules that have "crossed over." In meiosis, this process causes each chromosome in a germ cell to contain a mixture of maternally and paternally inherited genes.

variation is crucial to allow organisms to evolve in response to a changing environment. The DNA rearrangements are caused by a set of mechanisms that are collectively called **genetic recombination**. Two broad classes are commonly recognized—*general recombination* and *site-specific recombination*. In this part of the chapter we discuss the first of these two mechanisms; in the next part, we consider the second mechanism.

In **general recombination** (also known as *homologous recombination),* genetic exchange takes place between a pair of homologous DNA sequences. These are usually located on two copies of the same chromosome, although other types of DNA molecules that share the same nucleotide sequence can also participate. The general recombination reaction is essential for every proliferating cell, because accidents occur during nearly every round of DNA replication that interrupt the replication fork and require general recombination mechanisms to repair. The details of the intimate interplay between replication and recombination are still incompletely understood, but they include using variations of the homologous end-joining reaction (see Figure 5–53) to restart replication forks that have run into a break in the parental DNA template.

General recombination is also essential for the accurate chromosome segregation that occurs during meiosis in fungi, plants, and animals (see Figure 20–11). The crossing-over of chromosomes that results causes bits of genetic information to be exchanged to create new combinations of DNA sequences in each chromosome. The evolutionary benefit of this type of gene mixing is apparently so great that the reassortment of genes by general recombination is not confined to multicellular organisms; it is also widespread in single-celled organisms.

The central features that lie at the heart of the general recombination mechanism seem to be the same in all organisms. Most of what we know about the biochemistry of genetic recombination was originally derived from studies of bacteria, especially of *E. coli* and its viruses, as well as from experiments with simple eucaryotes such as yeasts. For these organisms with short generation times and relatively small genomes, it was possible to isolate a large set of mutants with defects in their recombination processes. The identification of the protein altered in each mutant then allowed the collection of proteins that catalyze general recombination to be identified and characterized. More recently, close relatives of these proteins have been discovered and extensively characterized in *Drosophila,* mice, and humans as well.

General Recombination Is Guided by Base-pairing Interactions Between Two Homologous DNA Molecules

The abundant general recombination observed in meiosis has the following characteristics: (1) Two homologous DNA molecules that were originally part of different chromosomes "cross over;" that is, their double helices break and the two broken ends join to their opposite partners to re-form two intact double helices, each composed of parts of the two initial DNA molecules (Figure 5–54). (2) The site of exchange (that is, where a *red* double helix is joined to a *green* double helix in Figure 5–54) can occur anywhere in the homologous nucleotide sequences of the two participating DNA molecules. (3) At the site of exchange, a strand of one DNA molecule has become base-paired to a strand of the second DNA molecule to create a *heteroduplex joint* that links the two double helices (Figure 5–55). This heteroduplex region can be thousands of base pairs long; we explain later how it forms. (4) No nucleotide sequences are altered at the site of exchange; some DNA replication usually takes place, but the cleavage and rejoining events occur so precisely that not a single nucleotide is lost or gained. Despite its precision, general recombination creates DNA molecules of novel sequence: the heteroduplex joint can tolerate a small number of mismatched

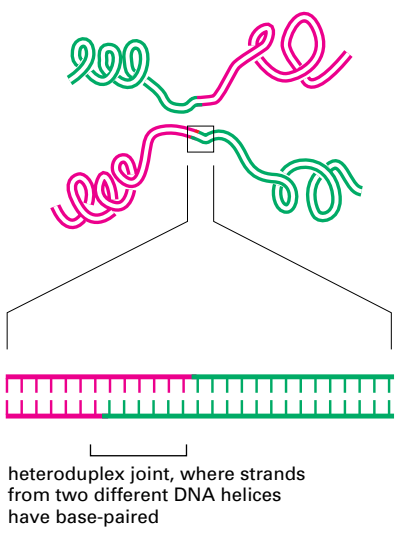


Figure 5–55 A heteroduplex joint. This structure unites two DNA molecules where they have crossed over. Such a joint is often thousands of nucleotides long.

base pairs, and, more importantly, the two DNA molecules that cross over are usually not exactly the same on either side of the joint. As a result, new recombinant DNA molecules (recombinant chromosomes) are generated.

The mechanism of general recombination ensures that two DNA double helices undergo an exchange reaction only if they contain an extensive region of sequence similarity (homology). The formation of a long heteroduplex joint requires such homology because it involves a long region of complementary base-pairing between a strand from one of the two original double helices and a complementary strand from the other double helix. But how does this heteroduplex joint arise, and how do the two homologous regions of DNA at the site of crossing-over recognize each other? As we shall see, recognition takes place during a process called **DNA synapsis**, in which base pairs form between complementary strands from the two DNA molecules. This base-pairing is then extended to guide the general recombination process, allowing it to occur only between DNA molecules that contain long regions of matching (or nearly matching) DNA sequence.

Meiotic Recombination Is Initiated by Double-strand DNA Breaks

Extensive base-pair interactions cannot occur between two intact DNA double helices. Thus, the DNA synapsis that is critical for general recombination in meiosis can begin only after a DNA strand from one DNA helix has been exposed and its nucleotides have been made available for pairing with another DNA helix. In the absence of direct experimental evidence, theoretical models were proposed based on the idea that a break needed to be made in just one of the two strands of a DNA helix to produce the exposed DNA strand required for DNA synapsis. This break in the phosphodiester backbone was thought to allow one of the nicked strand ends to separate from its base-paired partner strand, freeing it to form a short heteroduplex with a second intact DNA helix—thereby beginning synapsis. Models of this type are reasonable in theory, and they have been described in textbooks for nearly 30 years.

In the early 1990s, sensitive biochemical techniques became available for determining the actual structure of the recombination intermediates that form in yeast chromosomes at various stages of meiosis. These studies revealed that general recombination is initiated by a special endonuclease that simultaneously cuts *both* strands of the double helix, creating a complete break in the DNA molecule. The 5′ ends at the break are then chewed back by an exonuclease, creating protruding single-stranded 3′ ends. It is these single strands that search for a homologous DNA helix with which to pair—leading to the formation of a "joint molecule" between a maternal and a paternal chromosome (Figure 5–56).

In the next section, we begin to explain how a DNA single strand can "find" a homologous double-stranded DNA molecule to begin DNA synapsis.

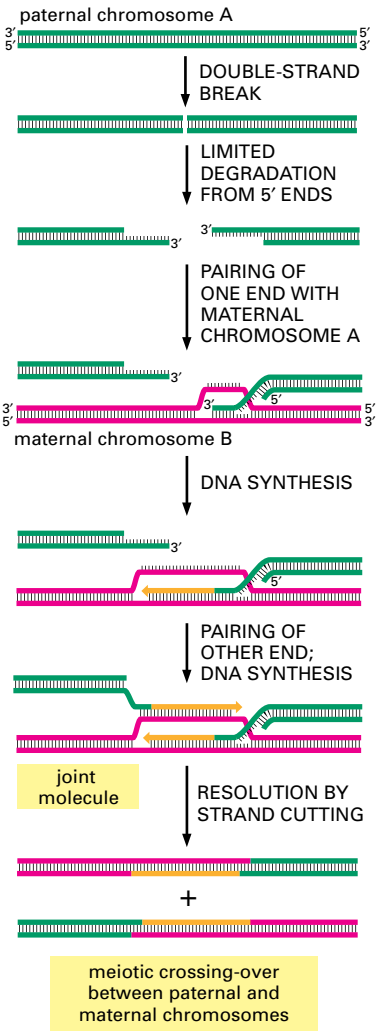
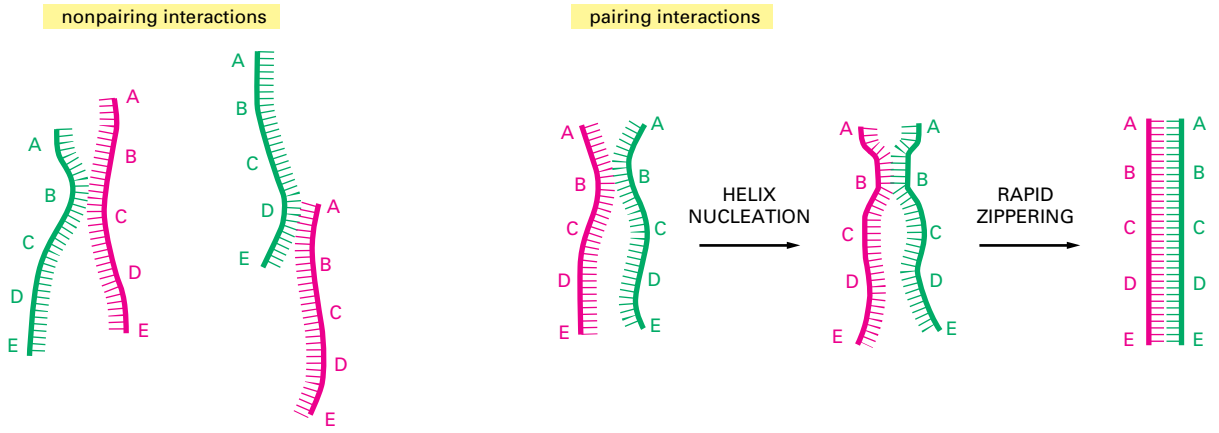


Figure 5–56 General recombination in meiosis. As indicated, the process begins when an endonuclease makes a double-strand break in a chromosome. An exonuclease then creates two protruding 3′ single-stranded ends, which find the homologous region of a second chromosome to begin DNA synapsis. The joint molecule formed can eventually be resolved by selective strand cuts to produce two chromosomes that have crossed over, as shown.

DNA Hybridization Reactions Provide a Simple Model for the Base-pairing Step in General Recombination

In its simplest form, the type of base-pairing interaction central to the synapsis step of general recombination can be mimicked in a test tube by allowing a DNA double helix to re-form from its separated single strands. This process, called *DNA renaturation* or **hybridization**, occurs when a rare random collision juxtaposes complementary nucleotide sequences on two matching DNA single strands, allowing the formation of a short stretch of double helix between them. This relatively slow helix nucleation step is followed by a very rapid "zippering" step, as the region of double helix is extended to maximize the number of base-pairing interactions (Figure 5–57).

Formation of a new double helix in this way requires that the annealing strands be in an open, unfolded conformation. For this reason, *in vitro* hybridization reactions are performed at either high temperature or in the presence of an organic solvent such as formamide; these conditions "melt out" the



short hairpin helices that result from the base-pairing interactions that occur within a single strand that folds back on itself. Most cells cannot survive such harsh conditions and instead use a single-strand DNA-binding (SSB) protein (see p. 246) to melt out the hairpin helices and help anneal their complementary single strands. This protein is essential for DNA replication (as described earlier) as well as for general recombination; it binds tightly and cooperatively to the sugar-phosphate backbone of all single-stranded DNA regions of DNA, holding them in an extended conformation with the bases exposed (see Figures 5–17 and 5–18). In this extended conformation, a DNA single strand can base-pair efficiently either with a nucleoside triphosphate molecule (in DNA replication) or with a complementary section of another DNA single strand (as part of a genetic recombination process).

The partner that a DNA single-strand needs to find in the synapsis step of general recombination is a DNA double helix, rather than a second single strand of DNA (see Figure 5–56). In the next section we see how the critical event that allows DNA hybridization to begin during recombination—the initial invasion of a single-stranded DNA into a DNA double helix—is achieved by the cell.

Figure 5–57 DNA hybridization. DNA double helices re-form from their separated strands in a reaction that depends on the random collision of two complementary DNA strands. The vast majority of such collisions are not productive, as shown on the left, but a few result in a short region where complementary base pairs have formed (helix nucleation). A rapid zippering then leads to the formation of a complete double helix. Through this trial-and-error process, a DNA strand will find its complementary partner even in the midst of millions of nonmatching DNA strands. A related, highly efficient trial-and-error recognition of a complementary partner DNA sequence seems to initiate all general recombination events.

The RecA Protein and its Homologs Enable a DNA Single Strand to Pair with a Homologous Region of DNA Double Helix

General recombination is more complex than the simple hybridization reactions just described involving single-stranded DNA, and it requires several types of specialized proteins. In particular, the *E. coli* **RecA protein** has a central role in the recombination between chromosomes; it and its homologs in yeast, mice, and humans make synapsis possible (Figure 5–58).

Like a single-strand DNA-binding protein, the RecA type of protein binds tightly and in long cooperative clusters to single-stranded DNA to form a

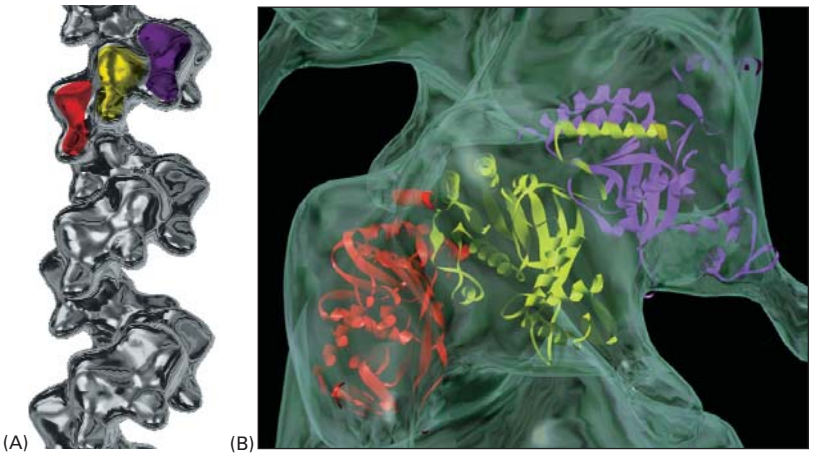


Figure 5–58 The structure of the RecA and Rad51 protein–DNA filaments. (A) The Rad51 protein bound to a DNA single strand. Rad51 is a human homolog of the bacterial RecA protein; three successive monomers in this helical filament are colored. (B) A short section of the RecA filament, with the three-dimensional structure of the protein fitted to the image of the filament determined by electron microscopy. The two DNA–protein filaments appear to be quite similar. There are about six RecA monomers per turn of the helix, holding 18 nucleotides of single-stranded DNA that is stretched out by the protein. The exact path of the DNA in this structure is not known. (A, courtesy of Edward Egelman; B, from X. Yu et al., *J. Mol. Biol.* 283:985–992, 1998.)

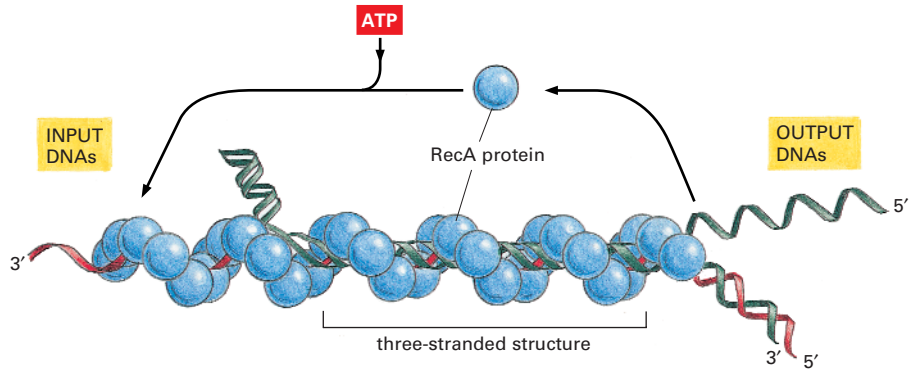


Figure 5–59 DNA synapsis catalyzed by the RecA protein. *In vitro* experiments show that several types of complex are formed between a DNA single strand covered with RecA protein *(red)* and a DNA double helix *(green)*. First a non-base-paired complex is formed, which is converted through transient base-flipping (see Figure 5–51) to a three-stranded structure as soon as a region of homologous sequence is found. This complex is unstable because it involves an unusual form of DNA, and it spins out a DNA heteroduplex (one strand *green* and the other strand *red)* plus a displaced single strand from the original helix *(green)*. Thus the structure shown in this diagram migrates to the left, reeling in the "input DNAs" while producing the "output DNAs." The net result is a DNA strand exchange identical to that diagrammed earlier in Figure 5–56. (Adapted from S.C. West, *Annu. Rev. Biochem*. 61:603–640, 1992.)

nucleoprotein filament. Because each RecA monomer has more than one DNA-binding site, a RecA filament can hold a single strand and a double helix together. This allows it to catalyze a multistep DNA synapsis reaction between a DNA double helix and a homologous region of single-stranded DNA. The region of homology is identified before the duplex DNA target has been opened up, through a three-stranded intermediate in which the DNA single strand forms transient base pairs with bases that flip out from the helix in the major groove of the double-stranded DNA molecule (Figure 5–59). This reaction begins the pairing shown previously in Figure 5–56, and it thereby initiates the exchange of strands between two recombining DNA double helices.

Once DNA synapsis has occurred, the short heteroduplex region where the strands from two different DNA molecules have begun to pair is enlarged through a process called *branch migration*. Branch migration can take place at any point where two single DNA strands with the same sequence are attempting to pair with the same complementary strand; in this reaction, an unpaired region of one of the single strands displaces a paired region of the other single strand, moving the branch point without changing the total number of DNA base pairs. Although spontaneous branch migration can occur, it proceeds equally in both directions, so it makes little progress and is unlikely to complete recombination efficiently (Figure 5–60A). The RecA protein catalyzes unidirectional branch migration, readily producing a region of heteroduplex DNA that is thousands of base pairs long (Figure 5–60B).

The catalysis of directional branch migration depends on a further property of the RecA protein. In addition to having two DNA-binding sites, the RecA protein is a DNA-dependent ATPase, with an additional site for binding and hydrolyzing ATP. The protein associates much more tightly with DNA when it has ATP bound than when it has ADP bound. Moreover, new RecA molecules

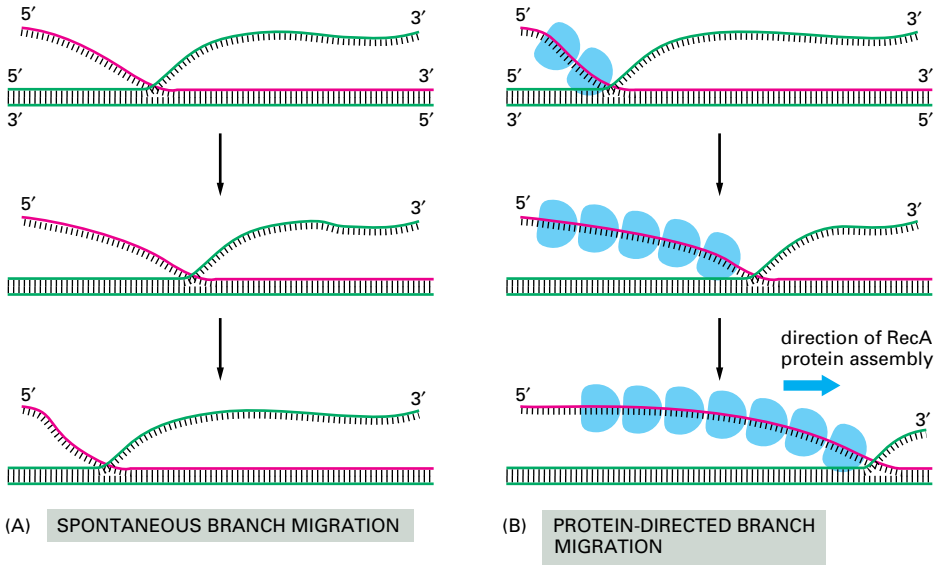


Figure 5–60 Two types of DNA branch migration observed in experiments *in vitro*. (A) Spontaneous branch migration is a back-and-forth, random-walk process, and it therefore makes little progress over long distances. (B) RecA-protein-directed branch migration proceeds at a uniform rate in one direction and may be driven by the polarized assembly of the RecA protein filament on a DNA single strand, which occurs in the direction indicated. Special DNA helicases that catalyze branch migration even more efficiently are also involved in recombination (for example see Figure 5–63).

with ATP bound are preferentially added at one end of the RecA protein filament, and the ATP is then hydrolyzed to ADP. The RecA protein filaments that form on DNA may therefore share some of the dynamic properties displayed by the cytoskeletal filaments formed from actin or tubulin (discussed in Chapter 16); an ability of the protein to "treadmill" unidirectionally along a DNA strand, for example, could drive the branch migration reaction shown in Figure 5–60B.

There Are Multiple Homologs of the RecA Protein in Eucaryotes, Each Specialized for a Specific Function

When one compares the proteins that catalyze the basic genetic functions in eucaryotes with those in bacteria such as *E. coli*, one generally finds that evolutionarily related proteins are present that catalyze similar reactions. In many cases, however, multiple eucaryotic homologs take the place of a particular bacterial protein, each specialized for a specific aspect of the bacterial protein's function.

This generalization applies to the *E. coli* RecA protein: humans and mice contain at least seven RecA homologs. Each homolog is presumed to have special catalytic activities and its own set of accessory proteins. The *Rad51 protein* is a particularly important RecA homolog in yeast, mice, and humans; it catalyzes a synaptic reaction between a DNA single strand and a DNA double helix in experiments *in vitro*. Genetic studies in which the Rad51 protein is mutated suggest that this protein is critical for the health of all three organisms, being required to repair replication forks that have been accidentally broken during the normal course of each S phase. Its proper function requires multiple accessory proteins. Two of these, the *Brca1* and *Brca2* proteins, were first discovered because mutations in their genes are inherited in a subset of human families with a greatly increased frequency of breast cancer. Whereas the removal of the Rad51 protein kills a cell, less drastic changes in its function caused by an alteration in such an accessory protein is thought to lead to an accumulation of DNA damage that often, in a small proportion of cells, gives rise to a cancer (see Figure 23–11).

Different RecA homologs in eucaryotes are specialized for meiosis, or for other unique types of DNA synaptic events that are less well understood. It is likely that each eucaryotic RecA homolog loads onto a DNA single strand to begin a general recombination event only when a particular DNA structure or cell condition allows the protein to bind there.

General Recombination Often Involves a Holliday Junction

The synapsis that exchanges the first single strand between two different DNA double helices is presumed to be the slow and difficult step in a general recombination event (see Figure 5–56). After this step, extending the region of pairing and establishing further strand exchanges between the two DNA helices is thought to proceed rapidly. In most cases, a key recombination intermediate, the *Holliday junction* (also called a *cross-strand exchange)* forms as a result.

In a **Holliday junction**, the two homologous DNA helices that have initially paired are held together by the reciprocal exchange of two of the four strands present, one originating from each of the helices. As shown in Figure 5–61A, a

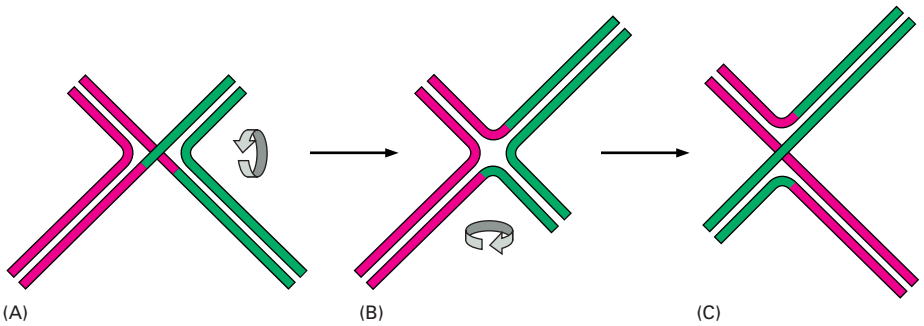


Figure 5–61 A Holliday junction and its isomerization. As described in the text, the synapsis step in general recombination is catalyzed by a RecA type of protein bound to a DNA single strand. This step is often followed by a reciprocal exchange of strands between two DNA double helices that have thereby paired with each other. This exchange produces a unique DNA structure known as a Holliday junction, named after the scientist who first proposed its formation. (A) The initially formed structure contains two crossing (inside) strands and two noncrossing (outside) strands. (B) An isomerization of the Holliday junction produces an open, symmetrical structure. (C) Further isomerization can interconvert the crossing and noncrossing strands, producing a structure that is otherwise the same as that in (A).

Holliday junction can be considered to contain two pairs of strands: one pair of crossing strands and one pair of noncrossing strands. The structure can *isomerize,* however, by undergoing a series of rotational movements, catalyzed by specialized proteins, to form a more open structure in which both pairs of strands occupy equivalent positions (Figures 5–61B and 5–62). This structure can, in turn, isomerize to a conformation that closely resembles the original junction, except that the crossing strands have been converted into noncrossing strands, and vice versa (Figure 5–61C).

Once the Holliday junction has formed an open structure, a special set of proteins can engage with the junction: one of these proteins uses the energy of ATP hydrolysis to move the crossover point (the point at which the two DNA helices are joined) rapidly along the two helices, extending the region of heteroduplex DNA (Figure 5–63).

To regenerate two separate DNA helices, and thus end the exchange process, the strands connecting the two helices in a Holliday junction must eventually be cut, a process referred to as *resolution*. There are two ways in which a Holliday junction can be resolved. In one, the original pair of crossing strands is cut (the invading, or inside, strands in Figure 5–61A). In this case, the two original DNA helices separate from each other nearly unaltered, exchanging the single-stranded DNA that formed the heteroduplex. In the other way, the original pair of noncrossing strands is cut (the inside strands in Figure 5–61C). Now the outcome is far more profound: two recombinant chromosomes are formed, having reciprocally exchanged major segments of double-stranded DNA with each other through a crossover event (Figure 5–64).

Genetic analyses reveal that heteroduplex regions of several thousand base pairs are readily formed during recombination. As described next, the processing of these heteroduplexes—which generally consist of nearly identical paired complementary strands—can further change the information in each resulting DNA helix.

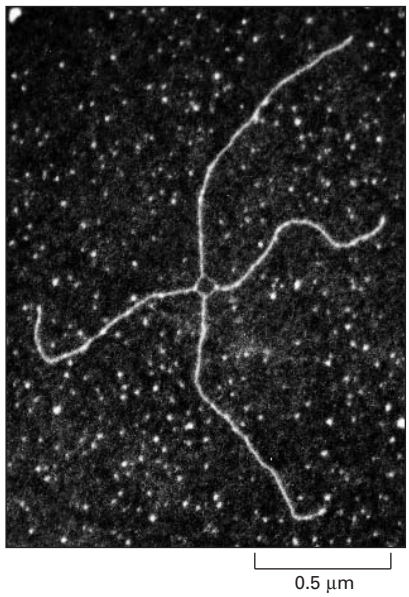


Figure 5–62 Electron micrograph of a Holliday junction. This view of the junction corresponds to the open structure illustrated in Figure 5–61B. (Courtesy of Huntington Potter and David Dressler.)

General Recombination Can Cause Gene Conversion

In sexually reproducing organisms, it is a fundamental law of genetics that each parent makes an equal genetic contribution to an offspring, which inherits one complete set of genes from the father and one complete set from the mother. Thus, when a diploid cell undergoes meiosis to produce four haploid cells (discussed in Chapter 20), exactly half of the genes in these cells should be maternal (genes that the diploid cell inherited from its father) and the other half paternal (genes that the diploid cell inherited from its father). In some organisms (fungi,

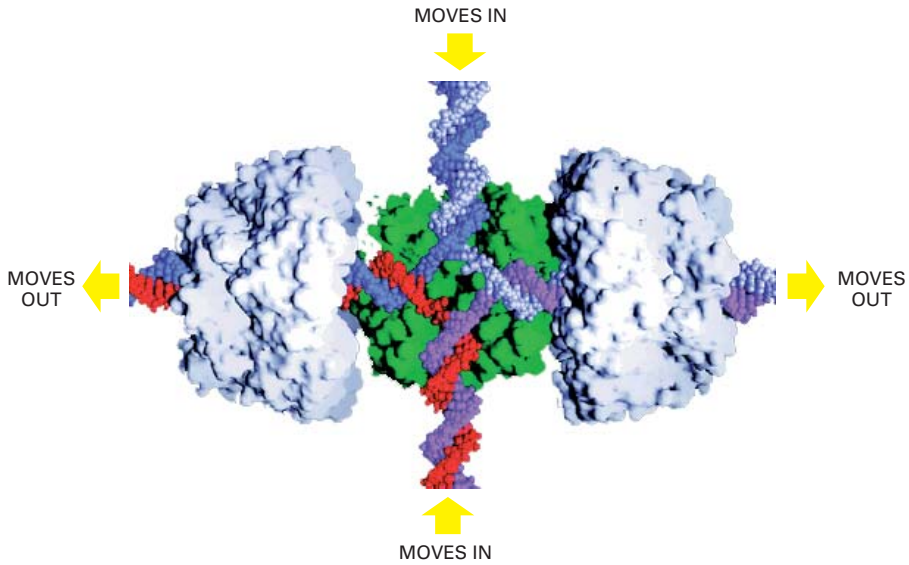
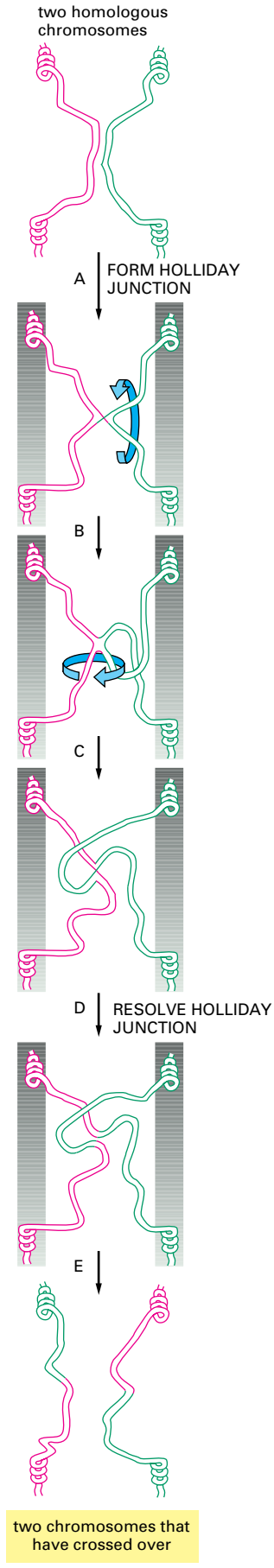


Figure 5–63 Enzyme-catalyzed double branch migration at a Holliday junction. In *E. coli*, a tetramer of the RuvA protein *(green)* and two hexamers of the RuvB protein *(pale gray)* bind to the open form of the junction. The RuvB protein uses the energy of ATP hydrolysis to move the crossover point rapidly along the paired DNA helices, extending the heteroduplex region as shown. There is evidence that similar proteins perform this function in vertebrate cells. (Image courtesy of P. Artymiuk; modified from S.C. West, *Cell* 94:699–701, 1998.)

Figure 5–64 The resolution of a Holliday junction to produce crossed-over chromosomes. In this example, homologous regions of a *red* and a *green* chromosome have formed a Holliday junction by exchanging two strands. Cutting these two strands would terminate the exchange without crossing-over. With isomerization of the Holliday junction (steps B and C), the original noncrossing strands become the two crossing strands; cutting them now creates two DNA molecules that have crossed over *(bottom)*. This type of isomerization may be involved in the breaking and rejoining of two homologous DNA double helices in meiotic general recombination. The grey bars in the central panels have been drawn to make it clear that the isomerization events shown can occur without disturbing the rest of the two chromosomes.



for example), it is possible to recover and analyze all four of the haploid gametes produced from a single cell by meiosis. Studies in such organisms have revealed rare cases in which the standard rules of genetics have been violated. Occasionally, for example, meiosis yields three copies of the maternal version of the gene and only one copy of the paternal allele (alternative versions of the same gene are called **alleles**). This phenomenon is known as **gene conversion** (Figure 5–65). Gene conversion often occurs in association with homologous genetic recombination events in meiosis (and more rarely in mitosis), and it is believed to be a straightforward consequence of the mechanisms of general recombination and DNA repair. Genetic studies show that only small sections of DNA typically undergo gene conversion, and in many cases only a part of a gene is changed.

In the process of gene conversion, DNA sequence information is transferred from one DNA helix that remains unchanged (a donor sequence) to another DNA helix whose sequence is altered (an acceptor sequence). There are several different ways this might happen, all of which involve the following two processes: (1) a homologous recombination event that juxtaposes two homologous DNA double helices, and (2) a limited amount of localized DNA synthesis, which is necessary to create an extra copy of one allele. In the simplest case, a general recombination process forms a heteroduplex joint (see Figure 5–55), in which the two paired DNA strands are not identical in sequence and therefore contain some mismatched base pairs. If the mispaired nucleotides in one of the two strands are recognized and removed by the DNA repair enzyme that catalyzes mismatch repair, an extra copy of the DNA sequence on the opposite strand is produced (Figure 5–66). The same gene conversion process can occur without crossover events, since it simply requires that a single DNA strand invade a

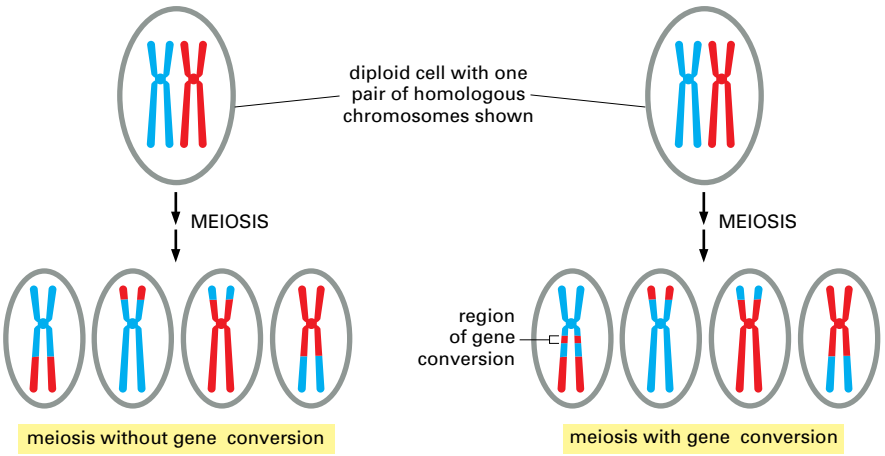


Figure 5–65 Gene conversion in meiosis. As described in Chapter 20, meiosis is the process through which a diploid cell gives rise to four haploid cells. Germ cells (eggs and sperm, for example) are produced by meiosis.

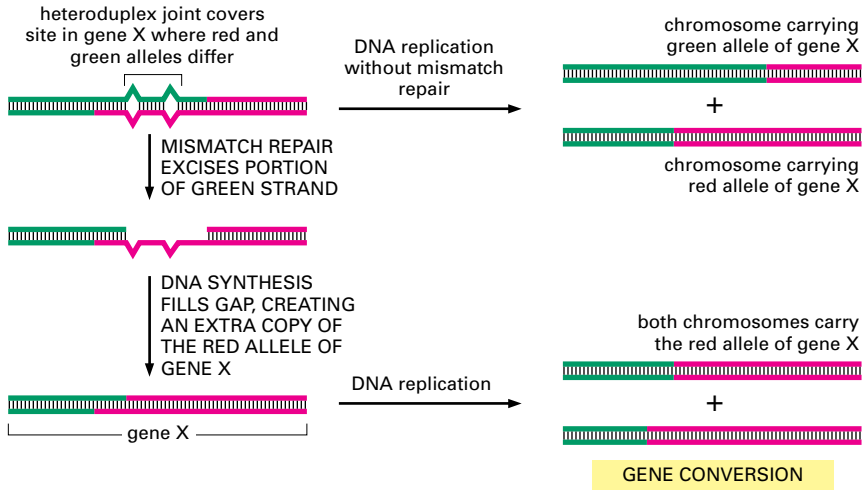


Figure 5–66 Gene conversion by mismatch correction. In this process, heteroduplex joints are formed at the sites of the crossing-over between homologous maternal and paternal chromosomes. If the maternal and paternal DNA sequences are slightly different, the heteroduplex joint will include some mismatched base pairs. The resulting mismatch in the double helix may then be corrected by the DNA mismatch repair machinery (see Figure 5–23), which can erase nucleotides on either the paternal or the maternal strand. The consequence of this mismatch repair is gene conversion, dectected as a deviation from the segregation of equal copies of maternal and paternal alleles that normally occurs in meiosis.

double helix to form a short heteroduplex region. The latter type of gene conversion is thought to be responsible for the unusually facile transfer of genetic information that is often observed between the different gene copies in a tandem array of repeated genes.

General Recombination Events Have Different Preferred Outcomes in Mitotic and Meiotic Cells

We have seen that meiotic recombination starts with a very bold stroke—the breakage of both strands of the double helix in one of the recombining chromosomes. How does the meiotic process that follows differ from the mechanism, also based on general recombination, that cells use for the precise repair of the accidental double-strand breaks that occur in chromosomes (the homologous end-joining reaction in Figure 5–53)? In both cases, the two new chromosome ends produced by a double-strand break are subjected to a degradative process, which exposes a single strand with an overhanging 3′ end. Moreover, in both cases, this strand seeks out a region of unbroken DNA double helix with the same nucleotide sequence and undergoes a synaptic reaction with it that is catalyzed by a RecA type of protein.

For double-strand break repair, DNA synthesis extends the invading 3′ end by thousands of nucleotides, using one of the strands of the recipient DNA helix as a template. If the second broken end becomes similarly engaged in the synaptic reaction, a joint molecule will be formed (see Figure 5–56). Depending on subsequent events, the final outcome can either be restoration of the two original DNA helices with repair of the double-strand break (the predominant reaction in mitotic cells), or a crossover event that leaves heteroduplex joints holding two different DNA helices together (the predominant reaction in meiotic cells). It is thought that the crossover events are created by a set of specific proteins that guide these reactions cells undergoing meiosis. These proteins not only ensure that a joint molecule with two Holliday junctions is formed but also cause a different pair of strands at each of the two junctions, thereby causing a crossover event (Figure 5–67).

With either outcome of general recombination, the DNA synthesis involved converts some of the genetic information at the site of the double-stranded break to that of the homologous chromosome. If these regions represent different alleles of the same gene, the nucleotide sequence in the broken helix is converted to that of the unbroken helix, causing a gene conversion. The yeast *Saccharomyces cerevisiae* exploits the gene conversion that accompanies double-strand break repair to switch from one mating type to another (discussed in Chapter 7). In this case, a double-strand break is intentionally induced by cleavage of a specific DNA sequence at the yeast *mating type locus* by an enzyme

called HO endonuclease. After DNA degradation at the site of the break has removed the old sequence, the missing genetic information is restored by a synapsis of the broken ends with a "mating-type cassette" DNA sequence of the opposite mating type (a or α), followed by local DNA synthesis in the manner previously indicated to reseal the broken region of the chromosome. In fact, it is through a detailed study of this precisely positioned form of double-strand break repair that the general mechanism of homologous end-joining was revealed.

Mismatch Proofreading Prevents Promiscuous Recombination Between Two Poorly Matched DNA Sequences

As previously discussed, a critical step in recombination occurs when two DNA strands of complementary sequence pair to form a heteroduplex joint between two double helices. Experiments *in vitro* with purified RecA protein show that pairing can occur efficiently even when the sequences of the two DNA strands do not match well—when, for example, only four out of every five nucleotides on average can form base pairs. If recombination proceeded from these mismatched sequences, it would create havoc in cells, especially in those that contain a series of closely related DNA sequences in their genomes. How do cells prevent crossing over between these sequences?

Although the complete answer is not known, studies with bacteria and yeasts demonstrate that components of the same mismatch proofreading system that removes replication errors (see Figure 5–23) have the additional role of interrupting genetic recombination events between poorly matched DNA sequences. It has long been known, for example, that homologous genes in two closely related bacteria, *E. coli* and *Salmonella typhimurium*, generally will not recombine, even though their nucleotide sequences are 80% identical. However, when the mismatch proofreading system is inactivated by mutation, there is a 1000-fold increase in the frequency of such interspecies recombination events. It is thought that the mismatch proofreading system normally recognizes the mispaired bases in an initial strand exchange, and—if there are a significant number of mismatches—the subsequent steps required to break and rejoin the two paired DNA helices are prevented. This mechanism protects the bacterial genome from the sequence changes that would otherwise be caused by recombination with the foreign DNA molecules that occasionally enter the cell. In vertebrate cells, which contain many closely related DNA sequences, the same type of recombinational proofreading is thought to help prevent promiscuous recombination events that would otherwise scramble the genome (Figure 5–68).

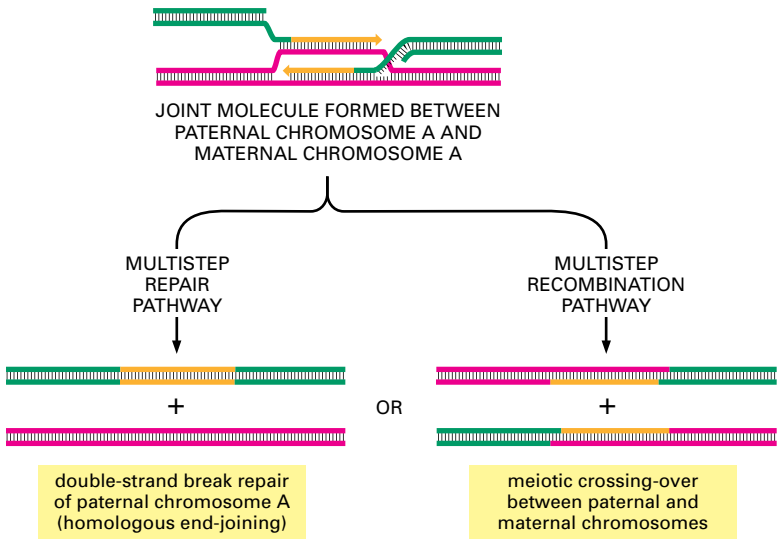


Figure 5–67 The different resolutions of a general recombination intermediate in mitotic and meiotic cells. As shown previously in Figure 5–56, general recombination begins when a double-strand break is generated in one double helix *(green),* followed by DNA degradation and strand invasion into a homologous DNA duplex *(red)*. New DNA synthesis *(orange)* follows to generate the joint molecule shown. Depending on subsequent events, resolution of the joint molecule can lead either to a precise repair of the initial double-strand break *(left)* or to chromosome crossing-over *(right)*. The experimental induction of double-strand breaks at specific DNA sites has allowed the outcome of general recombination to be quantified in both mitotic and meiotic cells. More than 99% of these events fail to produce a crossover in mitotic cells, whereas crossovers are often the outcome in meiotic cells. In either case, if the maternal and paternal chromosomes differ in DNA sequence in the region of new DNA synthesis shown here, the sequence of the *green* DNA duplex in the region of new DNA synthesis is converted to that of the *red* duplex (a gene conversion).

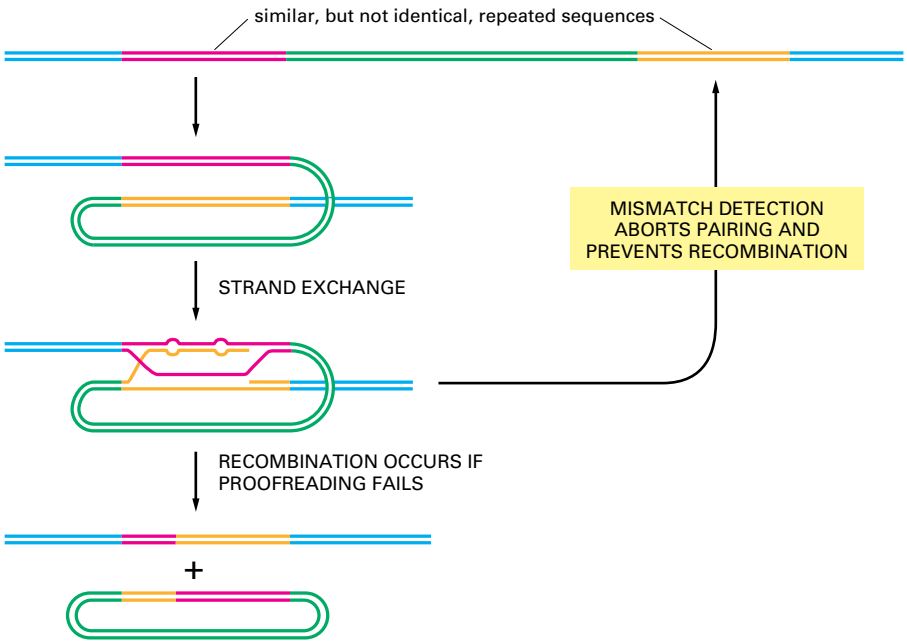


Figure 5-68 The mechanism that prevents general recombination from destabilizing a genome that contains repeated sequences. Studies with bacterial and yeast cells suggest that components of the mismatch proofreading system, diagrammed previously in Figure 5–23, have the additional function shown here.

Summary

General recombination (also called homologous recombination) allows large sections of the DNA double helix to move from one chromosome to another, and it is responsible for the crossing-over of chromosomes that occurs during meiosis in fungi, animals, and plants. General recombination is essential for the maintenance of chromosomes in all cells, and it usually begins with a double-strand break that is processed to expose a single-stranded DNA end. Synapsis between this single strand and a homologous region of DNA double helix is catalyzed by the bacterial RecA protein and its eucaryotic homologs, and it often leads to the formation of a four-stranded structure known as a Holliday junction. Depending on the pattern of strand cuts made to resolve this junction into two separate double helices, the products can be either a precisely repaired double-strand break or two chromosomes that have crossed over.

Because general recombination relies on extensive base-pairing interactions between the strands of the two DNA double helices that recombine, it occurs only between homologous DNA molecules. Gene conversion, the nonreciprocal transfer of genetic information from one chromosome to another, results from the mechanisms of general recombination, which involve a limited amount of associated DNA synthesis.

SITE-SPECIFIC RECOMBINATION

In general recombination, DNA rearrangements occur between DNA segments that are very similar in sequence. Although these rearrangements can result in the exchange of alleles between chromosomes, the order of the genes on the interacting chromosomes typically remains the same. A second type of recombination, called **site-specific recombination**, can alter gene order and also add new information to the genome. Site-specific recombination moves specialized nucleotide sequences, called *mobile genetic elements,* between nonhomologous sites within a genome. The movement can occur between two different positions in a single chromosome, as well as between two different chromosomes.

Mobile genetic elements range in size from a few hundred to tens of thousands of nucleotide pairs, and they have been identified in virtually all cells that have been examined. Some of these elements are viruses in which site-specific recombination is used to move their genomes into and out of the chromosomes

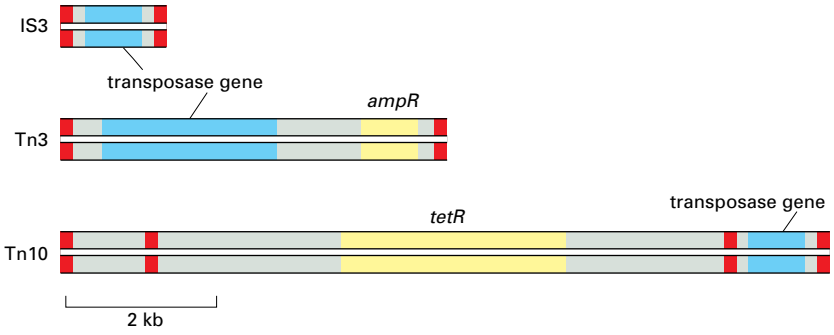


Figure 5–69 Three of the many types of mobile genetic elements found in bacteria. Each of these DNA elements contains a gene that encodes a *transposase*, an enzyme that conducts at least some of the DNA breakage and joining reactions needed for the element to move. Each mobile element also carries short DNA sequences (indicated in *red)* that are recognized only by the transposase encoded by that element and are necessary for movement of the element. In addition, two of the three mobile elements shown carry genes that encode enzymes that inactivate the antibiotics ampicillin *(ampR)* and tetracycline *(tetR)*. The transposable element Tn10, shown in the bottom diagram, is thought to have evolved from the chance landing of two short mobile elements on either side of a tetracyclin-resistance gene; the wide use of tetracycline as an antibiotic has aided the spread of this gene through bacterial populations. The three mobile elements shown are all examples of *DNA-only transposons* (see text).

of their host cell. A virus can package its nucleic acid into viral particles that can move from one cell to another through the extracellular environment. Many other mobile elements can move only within a single cell (and its descendents), lacking any intrinsic ability to leave the cell in which they reside.

The relics of site-specific recombination events can constitute a considerable fraction of a genome. The abundant repeated DNA sequences found in many vertebrate chromosomes are mostly derived from mobile genetic elements; in fact, these sequences account for more than 45% of the human genome (see Figure 4–17). Over time, the nucleotide sequences of these elements have been altered by random mutation. As a result, only a few of the many copies of these elements in our DNA are still active and capable of movement.

In addition to moving themselves, all types of mobile genetic elements occasionally move or rearrange neighboring DNA sequences of the host cell genome. These movements can cause deletions of adjacent nucleotide sequences, for example, or can carry these sequences to another site. In this way, site-specific recombination, like general recombination, produces many of the genetic variants upon which evolution depends. The translocation of mobile genetic elements gives rise to spontaneous mutations in a large range of organisms including humans; in some, such as the fruit fly *Drosophila*, these elements are known to produce most of the mutations observed. Over time, site-specific recombination has thereby been responsible for a large fraction of the important evolutionary changes in genomes.

Mobile Genetic Elements Can Move by Either Transpositional or Conservative Mechanisms

Unlike general recombination, site-specific recombination is guided by recombination enzymes that recognize short, specific nucleotide sequences present on one or both of the recombining DNA molecules. Extensive DNA homology is not required for a recombination event. Each type of mobile element generally encodes the enzyme that mediates its own movement and contains special sites upon which the enzyme acts. Many elements also carry other genes. For example, viruses encode coat proteins that enable them to exist outside cells, as well as essential viral enzymes. The spread of mobile elements that carry antibiotic resistance genes is a major factor underlying the widespread dissemination of antibiotic resistance in bacterial populations (Figure 5–69).

Site-specific recombination can proceed via either of two distinct mechanisms, each of which requires specialized recombination enzymes and specific DNA sites. (1) **Transpositional site-specific recombination** usually involves breakage reactions at the ends of the mobile DNA segments embedded in chromosomes and the attachment of those ends at one of many different nonhomologous target DNA sites. It does not involve the formation of heteroduplex DNA. (2) **Conservative site-specific recombination** involves the production of a very short heteroduplex joint, and it therefore requires a short DNA sequence that is the same on both donor and recipient DNA molecules. We first discuss transpositional site-specific recombination *(transposition* for short), returning to conservative site-specific recombination at the end of the chapter.

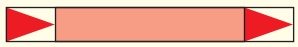
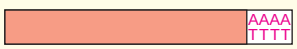
Transpositional Site-specific Recombination Can Insert Mobile Genetic Elements into Any DNA Sequence

Transposons, also called **transposable elements**, are mobile genetic elements that generally have only modest target site selectivity and can thus insert themselves into many different DNA sites. In transposition, a specific enzyme, usually encoded by the transposon and called a *transposase,* acts on a specific DNA sequence at each end of the transposon—first disconnecting it from the flanking DNA and then inserting it into a new target DNA site. There is no requirement for homology between the ends of the element and the insertion site.

Most transposons move only very rarely (once in 10^5 cell generations for many elements in bacteria), and for this reason it is often difficult to distinguish them from nonmobile parts of the chromosome. In most cases, it is not known what suddenly triggers their movement.

On the basis of their structure and transposition mechanisms, transposons can be grouped into three large classes (Table 5–3), each of which is discussed in detail in subsequent sections. Those in the first two of these classes use virtually identical DNA breakage and DNA joining reactions to translocate. However, for the *DNA-only transposons,* the mobile element exists as DNA throughout its life cycle: the translocating DNA segment is directly cut out of the donor DNA and joined to the target site by a transposase. In contrast, *retroviral-like retrotransposons* move by a less direct mechanism. An RNA polymerase first transcribes the DNA sequence of the mobile element into RNA. The enzyme reverse transcriptase then transcribes this RNA molecule back into DNA using the RNA as a template, and it is this DNA copy that is finally inserted into a new site in the genome. For historical reasons, the transposase-like enzyme that catalyzes this insertion reaction is called an *integrase* rather than a transposase. The third type of transposon in Table 5–3 also moves by making a DNA copy of an RNA

TABLE 5–3 Three Major Classes of Transposable Elements

CLASS DESCRIPTION AND STRUCTURE	GENES IN COMPLETE ELEMENT	MODE OF MOVEMENT	EXAMPLES
DNA-only transposons			
			
short inverted repeats at each end	encodes transposase	moves as DNA, either excising or following a replicative pathway	P element (*Drosophila*) Ac-Ds (maize) Tn3 and IS1 (*E.coli*) Tam3 (snapdragon)
Retroviral-like retrotransposons			
			
directly repeated long terminal repeats (LTRs) at ends	encodes reverse transcriptase and resembles retrovirus	moves via an RNA intermediate produced by promoter in LTR	Copia (*Drosophila*) Ty1 (yeast) THE-1 (human) Bs1 (maize)
Nonretroviral retrotransposons			
			
Poly A at 3′ end of RNA transcript; 5′ end is often truncated	encodes reverse transcriptase	moves via an RNA intermediate that is often produced from a neighboring promotor	F element (*Drosophila*) L1 (human) Cin4 (maize)

These elements range in length from 1000 to about 12,000 nucleotide pairs; each family contains many members, only a few of which are listed here. In addition to transposable elements, there are selected viruses that can move in and out of host cell chromosomes; these viruses are related to the first two classes of transposons.

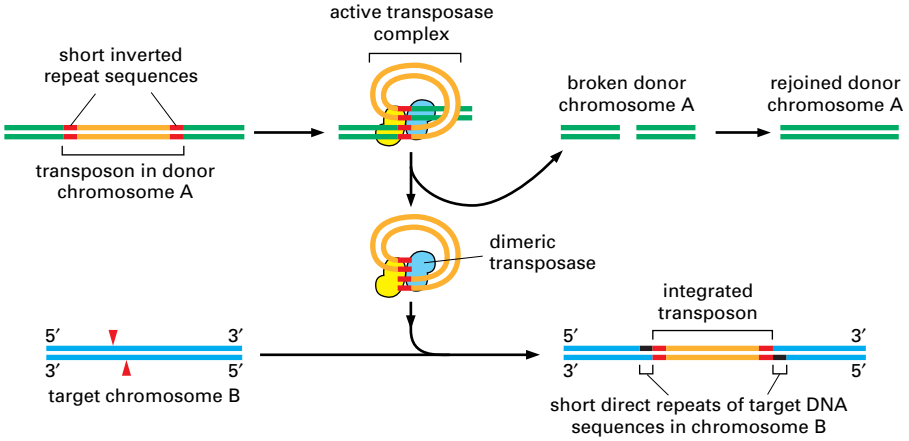


Figure 5–70 Cut-and-paste transposition. DNA-only transposons can be recognized in chromosomes by the "inverted repeat DNA sequences" *(red)* at their ends. Experiments show that these sequences, which can be as short as 20 nucleotides, are all that is necessary for the DNA between them to be transposed by the particular transposase enzyme associated with the element. The cut-and-paste movement of a DNA-only transposable element from one chromosomal site to another begins when the transposase brings the two inverted DNA sequences together, forming a DNA loop. Insertion into the target chromosome, catalyzed by the transposase, occurs at a random site through the creation of staggered breaks in the target chromosome *(red arrowheads)*. As a result, the insertion site is marked by a short direct repeat of the target DNA sequence, as shown. Although the break in the donor chromosome *(green)* is resealed, the breakage-and-repair process often alters the DNA sequence, causing a mutation at the original site of the excised transposable element (not shown).

molecule that is transcribed from it. However, the mechanism involved for these *nonretroviral retrotransposons* is distinct from that just described in that the RNA molecule is directly involved in the transposition reaction.

DNA-only Transposons Move By DNA Breakage and Joining Mechanisms

Many **DNA-only transposons** move from a donor site to a target site by **cut-and-paste transposition**, using the mechanism outlined in Figure 5–70. Each subunit of a transposase recognizes the same specific DNA sequence at an end of the element; the joining together of these two subunits to form a dimeric transposase creates a DNA loop that brings the two ends of the element together. The transposase then introduces cuts at both ends of this DNA loop to expose the element termini and remove the element completely from its original chromosome (Figure 5–71). To complete the reaction, the transposase catalyses a direct attack of the element's two DNA termini on a target DNA molecule, breaking two phosphodiester bonds in the target molecule as it joins the element and target DNAs together.

Because the breaks made in the two target DNA strands are staggered *(red arrowheads* in Figure 5–70), two short, single-stranded gaps are initially formed in the product DNA molecule, one at each end of the inserted transposon. These gaps are filled-in by a host cell DNA polymerase and DNA ligase to complete the recombination process, producing a short duplication of the adjacent target DNA sequence. These flanking direct repeat sequences, whose length is different for different transposons, serve as convenient markers of a prior transpositional site-specific recombination event.

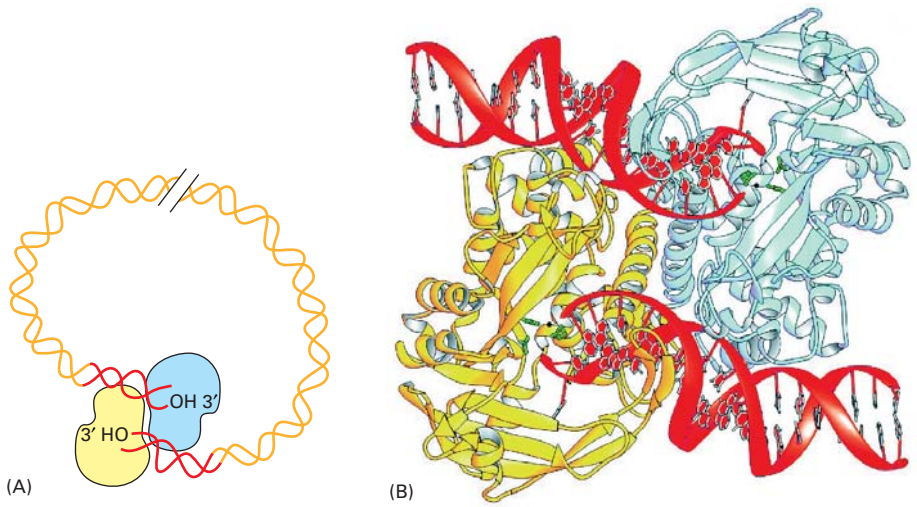


Figure 5–71 The structure of the central intermediate formed by a cut-and-paste transposase. (A) Schematic view of the overall structure. (B) The detailed structure of a transposase holding the two DNA ends, whose 3′-OH groups are poised to attack a target chromosome. (B, from D.R. Davies et al., *Science* 289:77–85, 2000. © AAAS.)

When a cut-and-paste DNA-only transposon is excised from the donor chromosome, a double-strand break is created in the vacated chromosome. This break can be perfectly "healed" by a homologous end-joining reaction. Alternatively, the break can be resealed by a nonhomologous end-joining reaction; in this case, the DNA sequence that flanked the transposon is often altered, producing a mutation at the chromosomal site from which the transposon was excised (see Figure 5–53).

Some DNA-only transposons move using a variation of the cut-and-paste mechanism called *replicative transposition*. In this case, the transposon DNA is replicated and a copy is inserted at a new chromosomal site, leaving the original chromosome intact (Figure 5–72). Although the mechanism used is more complex, it is closely related to the cut-and-paste mechanism just described; indeed, some transposons can move by either pathway.

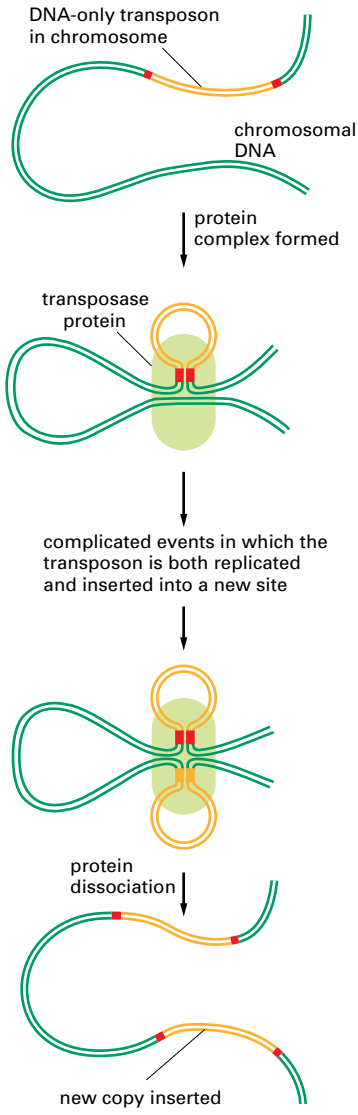


Figure 5–72 Replicative transposition. In the course of replicative transposition, the DNA sequence of the transposon is copied by DNA replication. The end products are a DNA molecule that is identical to the original donor and a target DNA molecule that has a transposon inserted into it. In general, a particular DNA-only transposon moves either by the cut-and-paste pathway shown in Figure 5–70 or by the replicative pathway outlined here. However, the two mechanisms have many enzymatic similarities, and a few transposons can move by either pathway.

Some Viruses Use Transpositional Site-specific Recombination to Move Themselves into Host Cell Chromosomes

Certain viruses are considered mobile genetic elements because they use transposition mechanisms to integrate their genomes into that of their host cell. However, these viruses also encode proteins that package their genetic information into virus particles that can infect other cells. Many of the viruses that insert themselves into a host chromosome do so by employing one of the first two mechanisms listed in Table 5–3. Indeed, much of our knowledge of these mechanisms has come from studies of particular viruses that employ them.

A virus that infects a bacterium is known as a **bacteriophage**. The *bacteriophage Mu* not only uses DNA-based transposition to integrate its genome into its host cell chromosome, it also uses the transposition process to initiate its viral DNA replication. The Mu transposase was the first to be purified in active form and characterized; it recognizes the sites of recombination at each end of the viral DNA by binding specifically to this DNA, and closely resembles the transposases just described.

Transposition also has a key role in the life cycle of many other viruses. Most notable are the **retroviruses**, which include the AIDS virus, called HIV, that infects human cells. Outside the cell, a retrovirus exists as a single-stranded RNA genome packed into a protein capsid along with a virus-encoded **reverse transcriptase** enzyme. During the infection process, the viral RNA enters a cell and is converted to a double-stranded DNA molecule by the action of this crucial enzyme, which is able to polymerize DNA on either an RNA or a DNA template (Figures 5–73 and 5–74). The term *retrovirus* refers to the fact that these viruses reverse the usual flow of genetic information, which is from DNA to RNA (see Figure 1–5).

Specific DNA sequences near the two ends of the double-stranded DNA product produced by reverse transcriptase are then held together by a virus-encoded integrase enzyme. This integrase creates activated 3′-OH viral DNA ends that can directly attack a target DNA molecule through a mechanism very similar to that used by the cut-and-paste DNA-only transposons (Figure 5–75). In fact, detailed analyses of the three-dimensional structures of bacterial transposases and HIV integrase have revealed remarkable similarities in these enzymes, even though their amino acid sequences have diverged considerably.

Retroviral-like Retrotransposons Resemble Retroviruses, but Lack a Protein Coat

Retroviruses move themselves in and out of chromosomes by a mechanism that is identical to that used by a large family of transposons called **retroviral-like retrotransposons** (see Table 5–3). These elements are present in organisms as diverse as yeasts, flies, and mammals. One of the best understood is the *Ty1 element* found in yeast. As with a retrovirus, the first step in its transposition is the transcription of the entire transposon, producing an RNA copy of the element that is more than 5000 nucleotides long. This transcript, which is translated as a

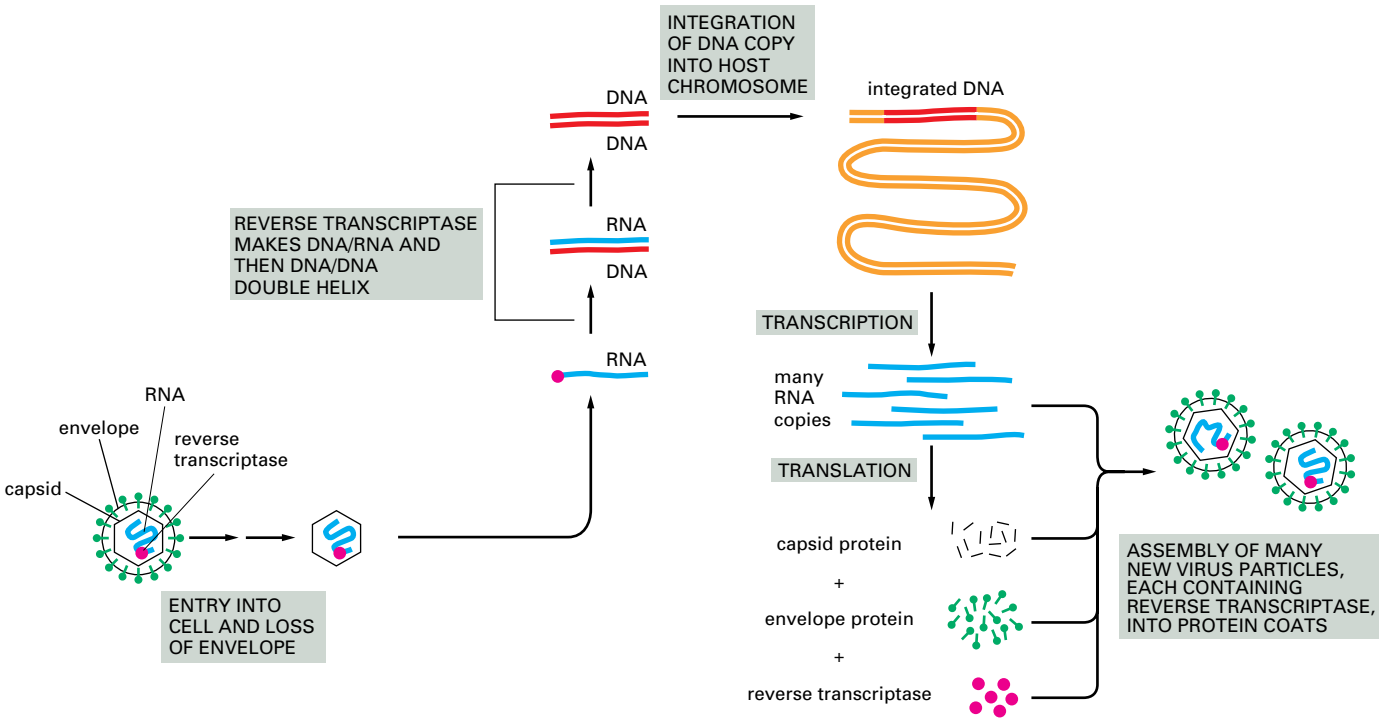
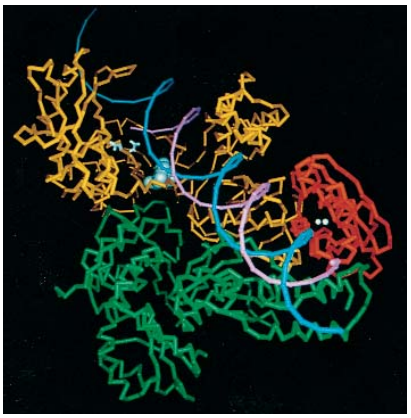
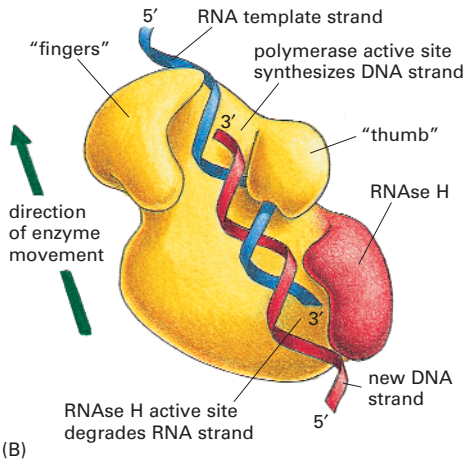


Figure 5–73 The life cycle of a retrovirus. The retrovirus genome consists of an RNA molecule of about 8500 nucleotides; two such molecules are packaged into each viral particle. The enzyme *reverse transcriptase* first makes a DNA copy of the viral RNA molecule and then a second DNA strand, generating a double-stranded DNA copy of the RNA genome. The integration of this DNA double helix into the host chromosome is then catalyzed by a virus-encoded *integrase* enzyme (see Figure 5–75). This integration is required for the synthesis of new viral RNA molecules by the host cell RNA polymerase, the enzyme that transcribes DNA into RNA (discussed in Chapter 6).

messenger RNA by the host cell, encodes a reverse transcriptase enzyme. This enzyme makes a double-stranded DNA copy of the RNA molecule via an RNA/DNA hybrid intermediate, precisely mimicking the early stages of infection by a retrovirus (see Figure 5–73). Like retroviruses, the linear double-stranded DNA molecule then integrates into a site on the chromosome by using an integrase enzyme that is also encoded by the *Ty1* DNA (see Figure 5–75). Although the resemblance to a retrovirus is striking, unlike a retrovirus, the *Ty1* element does not have a functional protein coat; it can therefore move only within a single cell and its descendants.



(A)



(B)

Figure 5–74 Reverse transcriptase. (A) The three-dimensional structure of the enzyme from HIV (the human AIDS virus) determined by x-ray crystallography. (B) A model showing the enzyme's activity on an RNA template. Note that the polymerase domain *(yellow* in B) has a covalently attached RNAse H (H for "Hybrid") domain *(red)* that degrades an RNA strand in an RNA/DNA helix. This activity helps the polymerase to convert the initial hybrid helix into a DNA double helix (A, courtesy of Tom Steitz; B, adapted from L.A. Kohlstaedt et al., *Science* 256:1783–1790, 1990.)

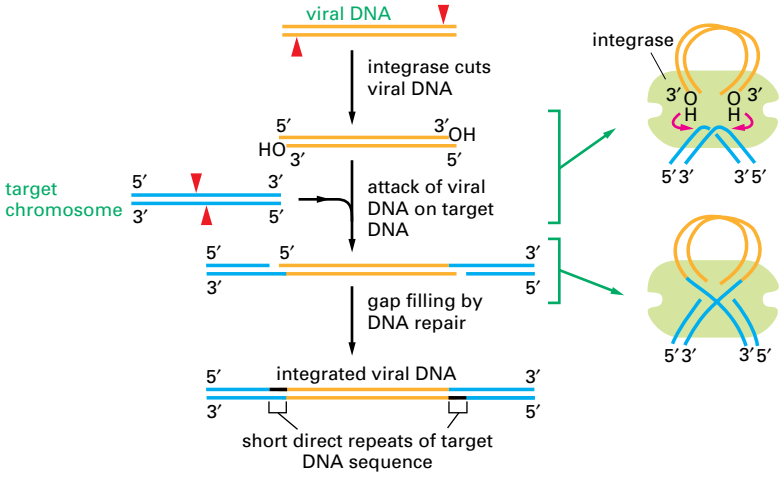


Figure 5–75 Transpositional site-specific recombination by a retrovirus or a retroviral-like retrotransposon. Outline of the strand-breaking and strand-rejoining events that lead to integration of the linear double-stranded DNA *(orange)* of a retrovirus (such as HIV) or a retroviral-like retrotransposon (such as *Ty1*) into the host cell chromosome *(blue)*. In an initial step, the integrase enzyme forms a DNA loop and cuts one strand at each end of the viral DNA sequence, exposing a protruding 3′-OH group. Each of these 3′-OH ends then directly attacks a phosphodiester bond on opposite strands of a randomly selected site on a target chromosome *(red arrowheads)*. This inserts the viral DNA sequence into the target chromosome, leaving short gaps on each side that are filled in by DNA repair processes. Because of the gap filling, this type of mechanism (like that of cut-and-paste transposons) leaves short repeats of target DNA sequence *(black)* on each side of the integrated DNA segment; these are 3–12 nucleotides long, depending on the integrase enzyme.

A Large Fraction of the Human Genome Is Composed of Nonretroviral Retrotransposons

A significant fraction of many vertebrate chromosomes is made up of repeated DNA sequences. In human chromosomes, these repeats are mostly mutated and truncated versions of a retrotransposon called an *L1 element* (sometimes referred to as a LINE or long interspersed nuclear element). Although most copies of the *L1* element are immobile, a few retain the ability to move. Translocations of the element have been identified, some of which result in human disease; for example, a particular type of hemophilia results from an *L1* insertion into the gene encoding a blood clotting factor, Factor VIII. Related mobile elements are found in other mammals and insects, as well as in yeast mitochondria. These **nonretroviral retrotransposons** (the third entry in Table 5–3) move via a distinct mechanism that requires a complex of an endonuclease and a reverse transcriptase. As illustrated in Figure 5–76, the RNA and reverse transcriptase have a much more direct role in the recombination event than for the mobile elements described above.

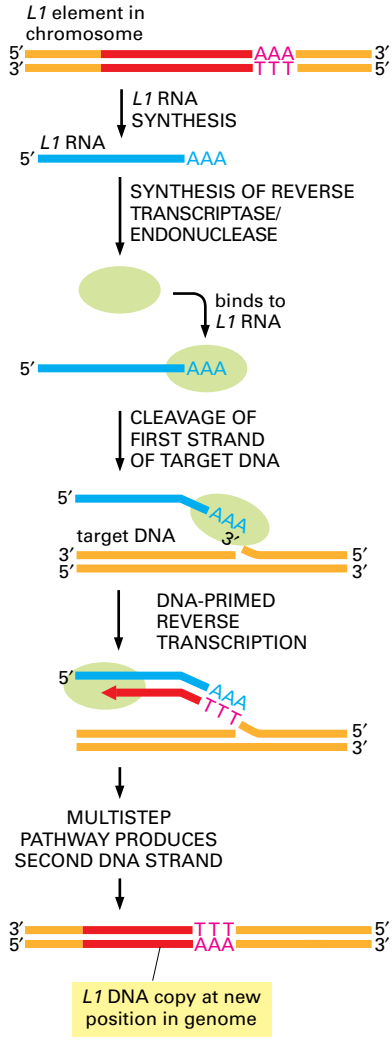


Figure 5–76 Transpositional site-specific recombination by a nonretroviral retrotransposon. Transposition by the *L1* element *(red)* begins when an endonuclease attached to the *L1* reverse transcriptase and the *L1* RNA *(blue)* makes a nick in the target DNA at the point at which insertion will occur. This cleavage releases a 3′-OH DNA end in the target DNA, which is then used as a primer for the reverse transcription step shown. This generates a single-stranded DNA copy of the element that is directly linked to the target DNA. In subsequent reactions, not yet understood in detail, further processing of the single-stranded DNA copy results in the generation of a new double-stranded DNA copy of the *L1* element that is inserted at the site where the initial nick was made.

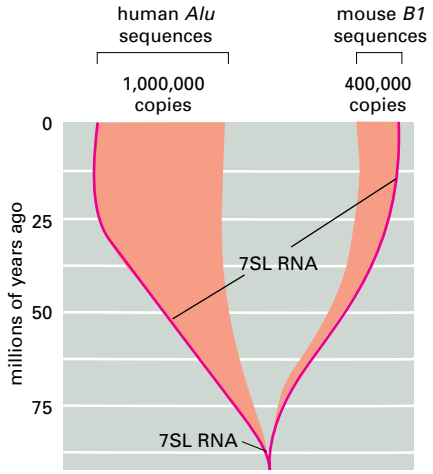


Figure 5–77 The proposed pattern of expansion of the abundant *Alu* and *B1* sequences found in the human and mouse genomes, respectively. Both of these transposable DNA sequences are thought to have evolved from the essential 7SL RNA gene which encodes the SRP RNA (see Figure 12–41). On the basis of the species distribution and sequence similarity of these highly repeated elements, the major expansion in copy numbers seems to have occurred independently in mice and humans (see Figure 5–78). (Adapted from P.L. Deininger and G.R. Daniels, *Trends Genet.* 2:76–80, 1986 and International Human Genome Sequencing Consortium, *Nature* 409:860–921, 2001.)

It is thought that other repeated DNAs that fail to encode an endonuclease or a reverse transcriptase in their own nucleotide sequence can multiply in chromosomes by a similar mechanism, using various endonucleases and reverse transcriptases present in the cell, including those encoded by *L1* elements. For example, the abundant *Alu* element lacks endonuclease or reverse transcriptase genes, yet it has amplified to become a major constituent of the human genome (Figure 5–77).

The *L1* and *Alu* elements seem to have multiplied in the human genome relatively recently. Thus, for example, the mouse contains sequences closely related to *L1* and *Alu*, but their placement in mouse chromosomes is very different from that in human chromosomes (Figure 5–78).

Different Transposable Elements Predominate in Different Organisms

We have described several types of transposable elements: (1) DNA-only transposons, the movement of which involves only DNA breakage and joining; (2) retroviral-like retrotransposons, which also move via DNA breakage and joining, but where RNA has a key role as a template to generate the DNA recombination substrate; and (3) nonretroviral retrotransposons, in which an RNA copy of the element is central to the incorporation of the element into the target DNA, acting as a direct template for a DNA target-primed reverse transcription event.

Interestingly, different types of transposons seem to predominate in different organisms. For example, the vast majority of bacterial transposons are DNA-only types, with a few related to the nonretroviral retrotransposons also present. In yeast, the main mobile elements that have been observed are retroviral-like retrotransposons. In *Drosophila*, DNA-based, retroviral, and nonretroviral transposons are all found. Finally, the human genome contains all three types of transposon, but as discussed below, their evolutionary histories are strikingly different.

Genome Sequences Reveal the Approximate Times when Transposable Elements Have Moved

The nucleotide sequence of the human genome provides a rich "fossil record" of the activity of transposons over evolutionary time spans. By carefully comparing the nucleotide sequences of the approximately 3 million transposable element

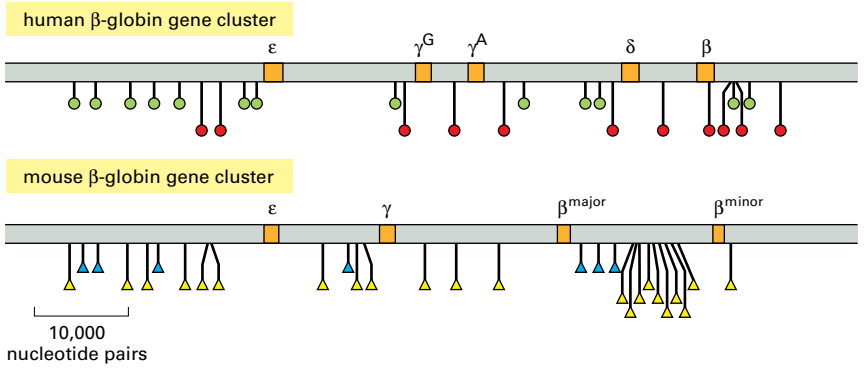


Figure 5–78 A comparison of the β-globin gene cluster in the human and mouse genomes, showing the location of transposable elements. This stretch of human genome contains five functional β-globin-like genes *(orange);* the comparable region from the mouse genome has only four. The positions of the human *Alu* sequence are indicated by *green circles*, and the human *L1* sequences by *red circles*. The mouse genome contains different but related transposable elements: the positions of *B1* elements (which are related to the human Alu sequences) are indicated by *blue triangles*, and the positions of the mouse *L1* elements (which are related to the human *L1* sequences) are indicated by *yellow triangles*. Because the DNA sequences and positions of the transposable elements found in the mouse and human β-globin gene clusters are so different, it is believed that they accumulated in each of these genomes independently, relatively recently in evolutionary time. (Courtesy of Ross Hardison and Webb Miller.)

remnants in the human genome, it has been possible to broadly reconstruct the movements of transposons in our ancestor's genomes over the past several hundred million years. For example, the DNA-only transposons appear to have been very active well before the divergence of humans and old world monkeys (25–35 million years ago); but, because they gradually accumulated inactivating mutations, they have been inactive in the human lineage since that time. Likewise, although our genome is littered with relics of retroviral-like transposons, none appear to be active today. Only a single family of retroviral-like retrotransposons is believed to have transposed in the human genome since the divergence of human and chimpanzee approximately 7 million years ago. The nonretroviral retrotransposons are also very ancient, but in contrast to other types, some are still moving in our genome. As mentioned previously, they are responsible for a fraction of new human mutations—perhaps 2 mutations in every thousand.

The situation in mice is significantly different. Although the mouse and human genomes contain roughly the same density of the three types of transposon, both types of retrotransposon are still actively transposing in the mouse genome, being responsible for approximately ten per cent of new mutations. Clearly we are only beginning to understand how the movement of transposons have shaped the genomes of present-day mammals. It has been proposed that bursts in transposition activity could have been involved in critical speciation events during the radiation of mammalian lineages from a common ancestor, a process that began approximately 170 million years ago. At this point, we can only wonder how many of our uniquely human qualities are due to the past activity of the many mobile genetic elements whose remnants are found today in our chromosomes.

Conservative Site-specific Recombination Can Reversibly Rearrange DNA

A different kind of site-specific recombination known as *conservative site-specific recombination* mediates the rearrangements of other types of mobile DNA elements. In this pathway, breakage and joining occur at two special sites, one on each participating DNA molecule. Depending on the orientation of the two recombination sites, DNA integration, DNA excision, or DNA inversion can occur (Figure 5–79).

Site-specific recombination enzymes that break and rejoin two DNA double helices at specific sequences on each DNA molecule often do so in a reversible way: the same enzyme system that joins two DNA molecules can take them apart again, precisely restoring the sequence of the two original DNA molecules. This type of recombination is therefore called "conservative" site-specific recombination to distinguish it from the mechanistically distinct, transpositional site-specific recombination just discussed.

A bacterial virus, *bacteriophage lambda*, was the first mobile DNA element to be understood in biochemical detail. When this virus enters a cell, a virus-

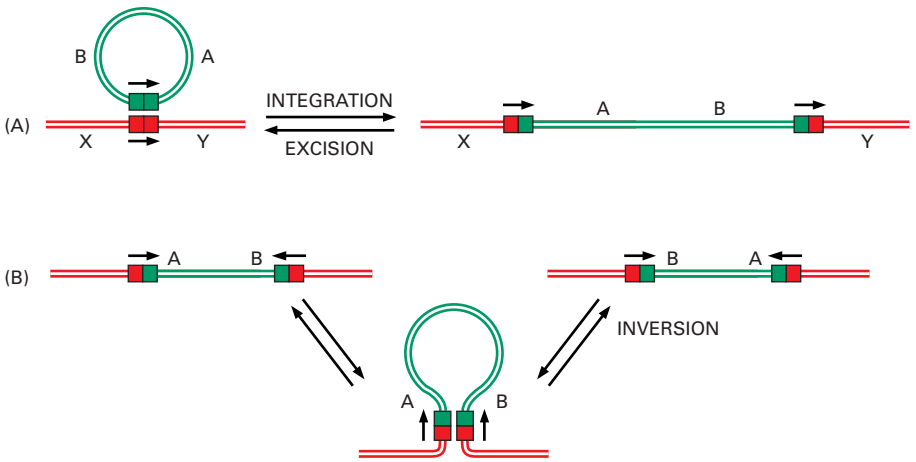


Figure 5–79 Two types of DNA rearrangement produced by conservative site-specific recombination. The only difference between the reactions in (A) and B) is the relative orientation of the two DNA sites (indicated by *arrows*) at which a site-specific recombination event occurs. (A) Through an integration reaction, a circular DNA molecule can become incorporated into a second DNA molecule; by the reverse reaction (excision), it can exit to reform the original DNA circle. Bacteriophage lambda and other bacterial viruses move in and out of their host chromosomes in precisely this way. (B) Conservative site-specific recombination can also invert a specific segment of DNA in a chromosome. A well-studied example of DNA inversion through site-specific recombination occurs in the bacterium *Salmonella typhimurium*, an organism that is a major cause of food poisoning in humans; the inversion of a DNA segment changes the type of flagellum that is produced by the bacterium (see Figure 7–64).

encoded enzyme called *lambda integrase* is synthesized. This enzyme mediates the covalent joining of the viral DNA to the bacterial chromosome, causing the virus to become part of this chromosome so that it is replicated automatically—as part of the host's DNA. A key feature of the lambda integrase reaction is that the site of recombination is determined by the recognition of two related but different DNA sequences—one on the bacteriophage chromosome and the other on the chromosome of the bacterial host. The recombination process begins when several molecules of the integrase protein bind tightly to a specific DNA sequence on the circular bacteriophage chromosome, along with several host proteins. This DNA–protein complex can now bind to an attachment site DNA sequence on the bacterial chromosome, bringing the bacterial and bacteriophage chromosomes together. The integrase then catalyzes the required cutting and resealing reactions that result in a site-specific strand exchange. Because of a short region of sequence homology in the two joined sequences, a tiny heteroduplex joint is formed at this point of exchange (Figure 5–80).

The lambda integrase resembles a DNA topoisomerase in forming a reversible covalent linkage to the DNA when it breaks a chain. Thus, this site-specific recombination event can occur in the absence of ATP and DNA ligase, which are normally required for phosphodiester bond formation.

The same type of site-specific recombination mechanism can also be used in reverse to promote the excision of a mobile DNA segment that is bounded by special recombination sites present as direct repeats. In bacteriophage lambda, excision enables it to exit from its integration site in the *E. coli* chromosome in

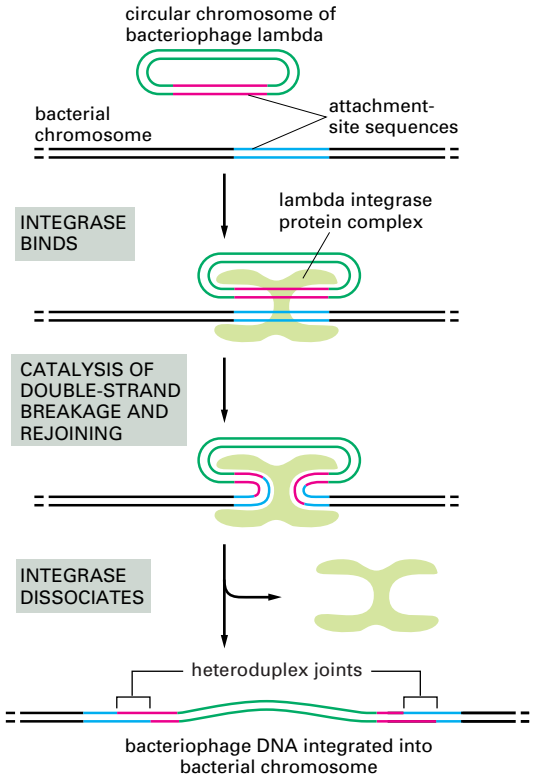


Figure 5–80 The insertion of a circular bacteriophage lambda DNA chromosome into the bacterial chromosome. In this example of site-specific recombination, the lambda integrase enzyme binds to a specific "attachment site" DNA sequence on each chromosome, where it makes cuts that bracket a short homologous DNA sequence. The integrase then switches the partner strands and reseals them to form a heteroduplex joint that is seven nucleotide pairs long. A total of four strand-breaking and strand-joining reactions is required; for each of them, the energy of the cleaved phosphodiester bond is stored in a transient covalent linkage between the DNA and the enzyme, so that DNA strand resealing occurs without a requirement for ATP or DNA ligase.

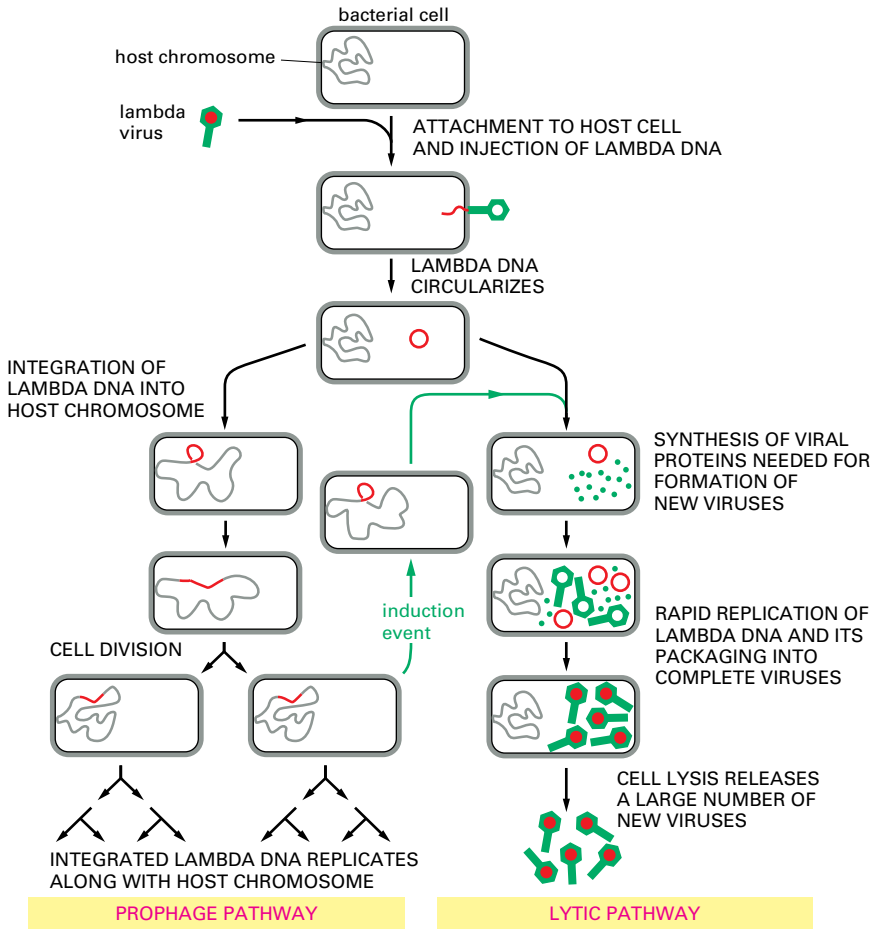


Figure 5–81 The life cycle of bacteriophage lambda. The double-stranded DNA lambda genome contains 50,000 nucleotide pairs and encodes 50–60 different proteins. When the lambda DNA enters the cell, the ends join to form a circular DNA molecule. This bacteriophage can multiply in *E. coli* by a lytic pathway, which destroys the cell, or it can enter a latent prophage state. Damage to a cell carrying a lambda prophage induces the prophage to exit from the host chromosome and shift to lytic growth *(green arrows)*. Both the entrance of the lambda DNA to, and its exit from, the bacterial chromosome are accomplished by a conservative site-specific recombination event, catalyzed by the lambda integrase enzyme (see Figure 5–80).

response to specific signals and multiply rapidly within the bacterial cell (Figure 5–81). Excision is catalyzed by a complex of integrase enzyme and host factors with a second bacteriophage protein, excisionase, which is produced by the virus only when its host cell is stressed—in which case, it is in the bacteriophage's interest to abandon the host cell and multiply again as a virus particle.

Conservative Site-Specific Recombination Can be Used to Turn Genes On or Off

When the special sites recognized by a conservative site-specific recombination enzyme are inverted in their orientation, the DNA sequence between them is inverted rather than excised (see Figure 5–79). Such inversion of a DNA sequence is used by many bacteria to control the gene expression of particular genes—for example, by assembling active genes from separated coding segments. This type of gene control has the advantage of being directly inheritable, since the new DNA arrangement is transferred to daughter chromosomes automatically when a cell divides.

These types of enzymes have also become powerful tools for cell and developmental biologists. To decipher the roles of specific genes and proteins in complex multicellular organisms, genetic engineering techniques can be used to introduce into a mouse a gene encoding a site-specific recombination enzyme and a carefully designed target DNA containing the DNA sites that are recognized by the enzyme. At an appropriate time, the gene encoding the enzyme can be activated to rearrange the target DNA sequence. This rearrangement is often used to cause the production of a specific protein in particular tissues of the mouse (Figure 5–82). By similar means, the technique can be used to turn off any specific gene in a tissue of interest. In this way, one can in principle determine the influence of any protein in any tissue of an intact animal.

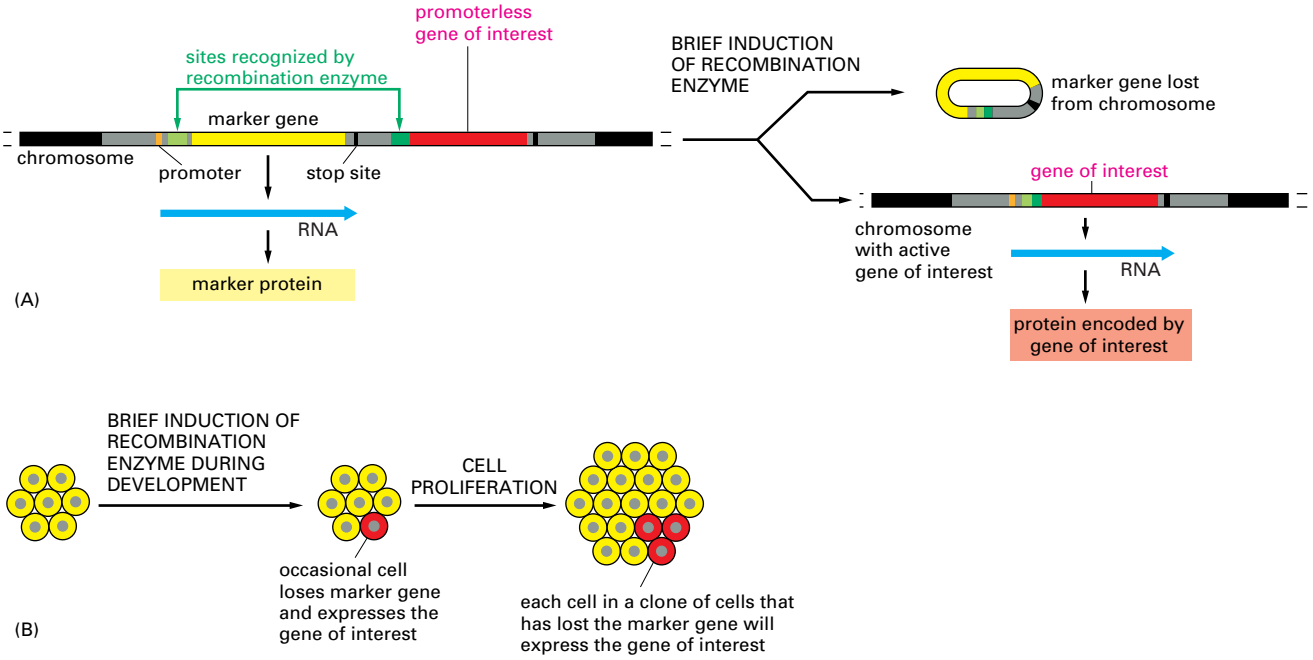


Figure 5–82 How a conservative site-specific recombination enzyme is used to turn on a specific gene in a group of cells in a transgenic animal. This technique requires the insertion of two specially engineered DNA molecules into the animal's germ line. (A) The DNA molecule shown has been engineered with specific recognition sites *(green)* so that the gene of interest *(red)* is transcribed only after a site-specific recombination enzyme that uses these sites is induced. As shown on the right, this induction removes a marker gene *(yellow)* and brings the promoter DNA *(orange)* adjacent to the gene of interest. The recombination enzyme is inducible, because it is encoded by a second DNA molecule (not shown) that has been engineered to ensure that the enzyme is made only when the animal is treated with a special small molecule or its temperature is raised. (B) Transient induction of the recombination enzyme causes a brief burst of synthesis of that enzyme, which in turn causes a DNA rearrangement in an occasional cell. For this cell and all its progeny, the marker gene is inactivated and the gene of interest is simultaneously activated (as shown in A). Those clones of cells in the developing animal that express the gene of interest can be identified by their loss of the marker protein. This technique is widely used in mice and *Drosophila*, because it allows one to study the effect of expressing any gene of interest in a group of cells in an intact animal. In one version of the technique, the Cre recombination enzyme of bacteriophage P1 is employed along with its loxP recognition sites (see pp. 542–543).

Summary

The genomes of nearly all organisms contain mobile genetic elements that can move from one position in the genome to another by either a transpositional or a conservative site-specific recombination process. In most cases this movement is random and happens at a very low frequency. Mobile genetic elements include transposons, which move only within a single cell (and its descendents), and those viruses whose genomes can integrate into the genome of their host cells.

There are three classes of transposons: the DNA-only transposons, the retroviral-like retrotransposons, and the nonretroviral retrotransposons. All but the last have close relatives among the viruses. Although viruses and transposable elements can be viewed as parasites, many of the new arrangements of DNA sequences that their site-specific recombination events produce have created the genetic variation crucial for the evolution of cells and organisms.

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