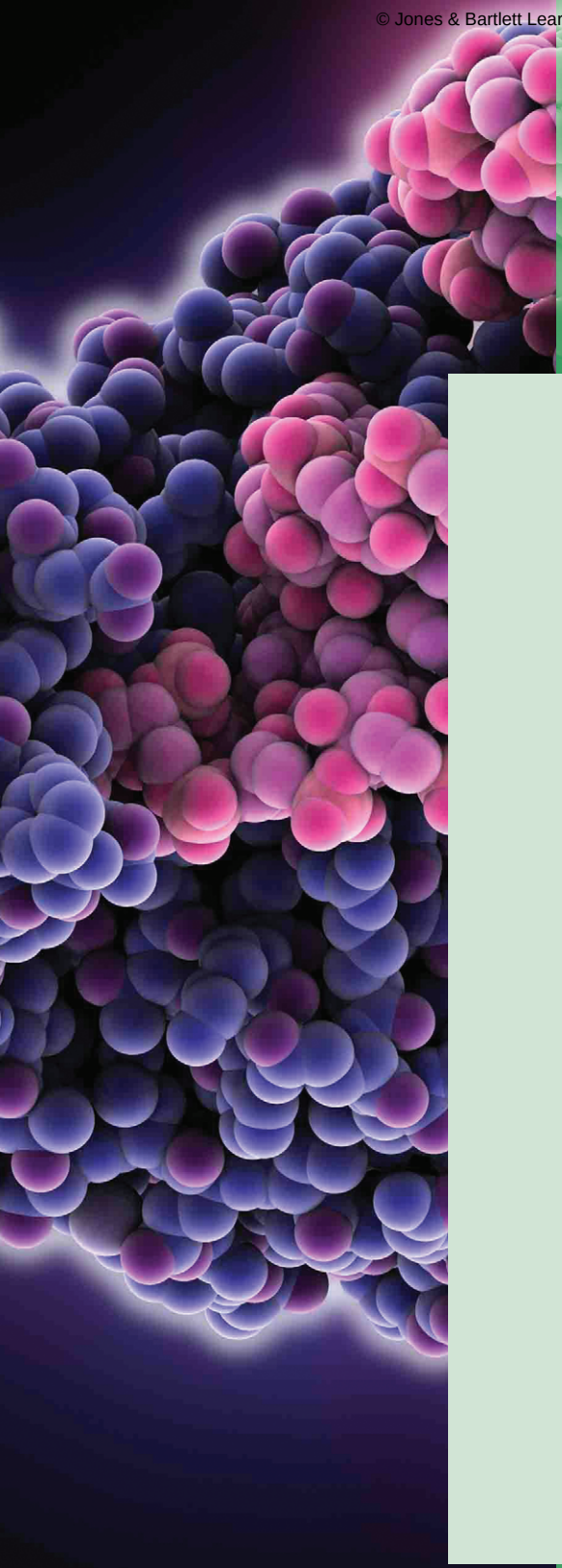
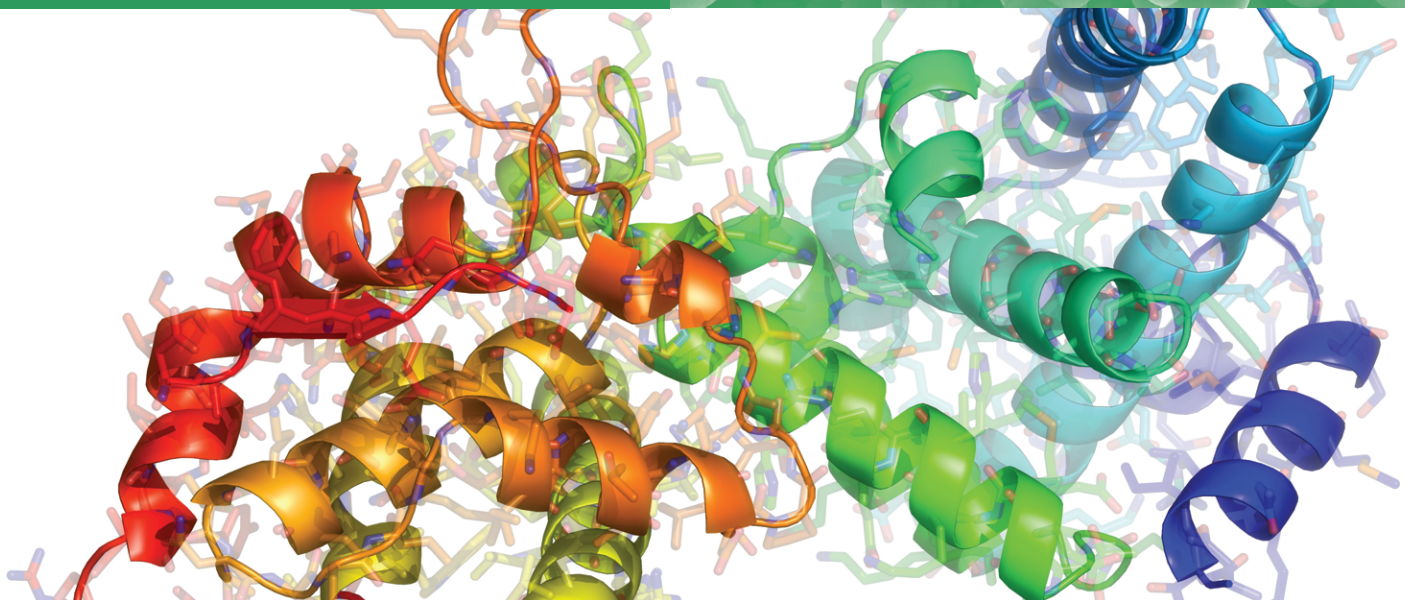


PART II

DNA Replication and Recombination

CHAPTER 9



Replication Is Connected to the Cell Cycle

Edited by Barbara Funnell

CHAPTER OUTLINE

9.1 Introduction

A major difference between prokaryotes and eukaryotes is the way in which replication is controlled and linked to the cell cycle.

In eukaryotes, the following are true:

- Chromosomes reside in the nucleus.
- Each chromosome consists of many units of replication called replicons.
- Replication requires coordination of these replicons to reproduce DNA during a discrete period of the cell cycle.
- The decision about whether to replicate is determined by a complex pathway that regulates the cell cycle.
- Duplicated chromosomes are segregated to daughter cells during mitosis by means of a special apparatus.

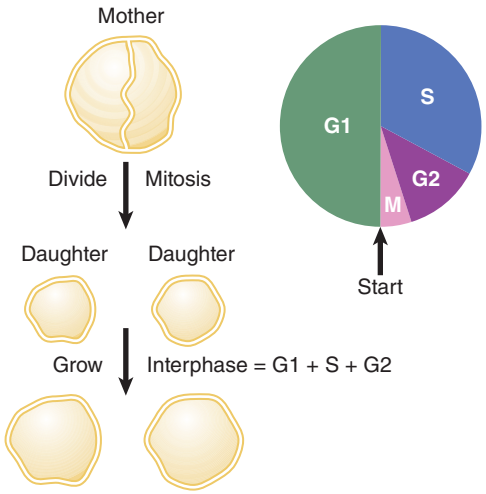


FIGURE 9.1 A growing cell alternates between cell division of a mother cell into two daughter cells and growth back to the original size.

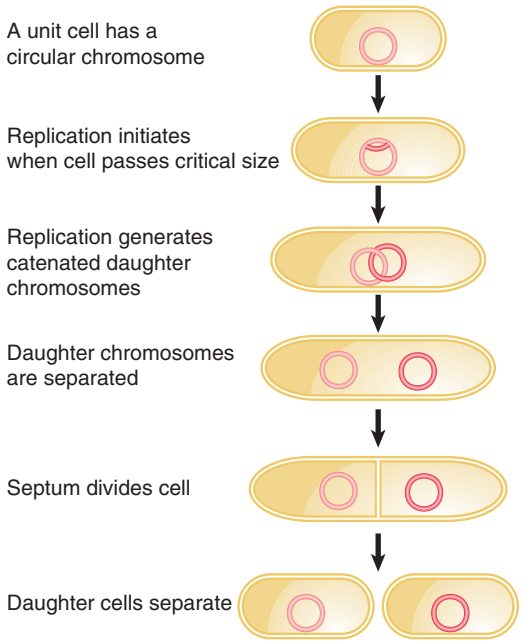


FIGURE 9.2 Replication initiates at the bacterial origin when a cell passes a critical threshold of size. Completion of replication produces daughter chromosomes that might be linked by recombination or that might be catenated. They are separated and moved to opposite sides of the septum before the bacterium is divided into two.

In eukaryotic cells, replication of DNA is confined to the second part of the cell cycle called **S phase**, which follows G1 phase (see **FIGURE 9.1**). The eukaryotic cell cycle is composed of alternating rounds of growth followed by DNA replication and then cell division. After the cell divides into two daughter cells, each has the option to continue dividing or stop and enter G0. If the decision is to continue to divide, the cell must grow back to the size of the original parent cell before division can occur again.

The G1 phase of the cell cycle is concerned primarily with growth (although G1 is an abbreviation for *first gap* because the early cytologists could not see any activity). In G1 everything except DNA begins to be doubled: RNA, protein, lipids, and carbohydrates. The progression from G1 into S is very tightly regulated and is controlled by a **checkpoint**. For a cell to be allowed to progress into S phase, there must be a certain minimum amount of growth that is biochemically monitored. In addition, there must not be any damage to the DNA. Damaged DNA or too little growth prevents the cell from progressing into S phase. When S phase is complete, G2 phase commences; there is no control point and no sharp demarcation.

The start of S phase is signaled by the activation of the first replicon—usually in euchromatin—in areas of active genes. Over the next few hours, initiation events occur at other replicons in an ordered manner.

However, replication in bacteria, as shown in **FIGURE 9.2**, is triggered at a single origin when the cell mass increases past a threshold level, and the segregation of the daughter chromosomes is accomplished by ensuring that they find themselves on opposite sides of the septum that grows to divide the bacterium into two.

How does the cell know when to initiate the replication cycle? The initiation event occurs once in each cell cycle and at the same time in every cell cycle. How is this timing set? An initiator protein could be synthesized continuously throughout the cell cycle; accumulation of a critical amount would trigger initiation. This is consistent with the fact that protein synthesis is needed for the initiation event. Another possibility is that an inhibitor protein might be synthesized or activated at a fixed point and then diluted below an effective level by the increase in cell volume. Current models suggest that variations of both possibilities operate to turn initiation on and then off precisely in each cell cycle. Synthesis of active DnaA protein, the bacterial initiator protein, reaches a threshold that turns on initiation, and the activity of inhibitors turns subsequent initiations off for the rest of the cell cycle. This is described in the *The Replicon: Initiation of Replication* chapter.

Bacterial chromosomes are specifically compacted and arranged inside the cell, and this organization is important for proper segregation, or partition, of daughter chromosomes at cell division. Some of the events in partitioning the daughter chromosomes are consequences of the circularity of the bacterial chromosome. Circular chromosomes are said to be catenated when one passes through another, connecting them. **Catenation** is a consequence of incomplete removal of topological links during DNA replication, and **topoisomerases** are required to remove these links and separate the chromosomes. An alternative type of structure is formed when a recombination event occurs: A single recombination between two monomers converts them into a single dimer. This is resolved by a specialized recombination system that recreates the independent monomers.

The key goals in the chapters that follow are to define the DNA sequences that function in replication and to determine how they are recognized by appropriate proteins of

the replication apparatus. In subsequent chapters, we examine the unit of replication and how that unit is regulated to start replication; the biochemistry and mechanism of DNA synthesis; and autonomously replicating units in bacteria, mitochondria, and chloroplasts.

9.2 Bacterial Replication Is Connected to the Cell Cycle

KEY CONCEPTS

- The doubling time of *Escherichia coli* can vary over a range of up to 10 times, depending on growth conditions.
- It requires 40 minutes to replicate the bacterial chromosome (at normal temperature).
- Completion of a replication cycle triggers a bacterial division 20 minutes later.
- If the doubling time is approximately 60 minutes, a replication cycle is initiated before the division resulting from the previous replication cycle.
- Fast rates of growth therefore produce multiforked chromosomes.

Bacteria have two links between replication and cell growth:

- The frequency of initiation of cycles of replication is adjusted to fit the rate at which the cell is growing.
- The completion of a replication cycle is connected with division of the cell.

The rate of bacterial growth is assessed by the **doubling time**, the period required for the number of cells to double. The shorter the doubling time, the faster the bacteria are growing. *E. coli* growth rates can range from doubling times as fast as 18 minutes to slower than 180 minutes. The bacterial chromosome is a single replicon; thus, the frequency of replication cycles is controlled by the number of initiation events at the single origin. Researchers can define the replication cycle in terms of two constants:

- *C* is the fixed time of approximately 40 minutes required to replicate the entire *E. coli* chromosome. Its duration corresponds to a rate of replication fork movement of approximately 50,000 bp/minute. (The rate of DNA synthesis is more or less invariant at a constant temperature; it proceeds at the same speed unless and until the supply of precursors becomes limiting.)
- *D* is the fixed time of approximately 20 minutes that elapses between the completion of a round of replication and the cell division with which it is connected. This period might represent the time required to assemble the components needed for division.

The constants *C* and *D* can be viewed as representing the maximum speed with which the bacterium is capable of completing these processes. They apply for all growth rates between doubling times of 18 and 60 minutes, but both constant phases become longer when the cell cycle occupies more than 60 minutes.

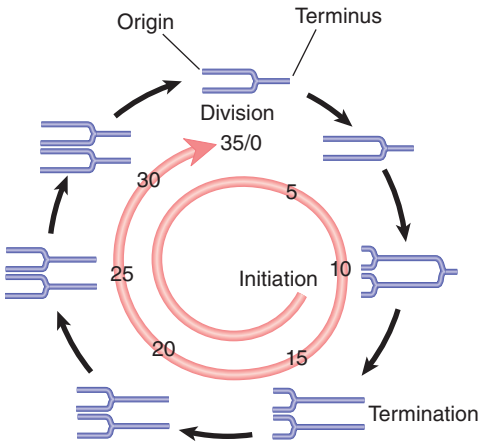


FIGURE 9.3 The fixed interval of 60 minutes between initiation of replication and cell division produces multiforked chromosomes in rapidly growing cells. Note that only the replication forks moving in one direction are shown; the chromosome actually is replicated symmetrically by two sets of forks moving in opposite directions on circular chromosomes.

A cycle of chromosome replication must be initiated at a fixed time of $C + D = 60$ minutes before cell division. For bacteria dividing more frequently than every 60 minutes, a cycle of replication must be initiated before the end of the preceding division cycle. You might say that a cell is born "already pregnant" with the next generation.

Consider the example of cells dividing every 35 minutes. The cycle of replication connected with a division must have been initiated 25 minutes before the preceding division. This situation is illustrated in **FIGURE 9.3**, which shows the chromosomal complement of a bacterial cell at 5-minute intervals throughout the cycle.

At division (35/0 minutes), the cell receives a partially replicated chromosome. The replication fork continues to advance. At 10 minutes, when this "old" replication fork has not yet reached the terminus, initiation occurs at both origins on the partially replicated chromosome. The start of these "new" replication forks creates a **multiforked chromosome**.

At 15 minutes—that is, at 20 minutes before the next division—the old replication fork reaches the terminus. Its arrival allows the two daughter chromosomes to separate; each of them has already been partially replicated by the new replication forks (which now are the only replication forks). These forks continue to advance.

At the point of division, the two partially replicated chromosomes segregate. This recreates the point at which we started. The single replication fork becomes "old," it terminates at 15 minutes, and 20 minutes later, there is a division. We see that the initiation event occurs $1^{25}/_{35}$ cell cycles before the division event with which it is associated.

The general principle of the link between initiation and the cell cycle is that as cells grow more rapidly (the cycle is shorter), the initiation event occurs at an increasing number of cycles before the related division. There are correspondingly more chromosomes in the individual bacterium. This relationship can be viewed as the cell's response to its inability to reduce the periods of *C* and *D* to keep pace with the shorter cycle.

9.3 The Shape and Spatial Organization of a Bacterium Are Important During Chromosome Segregation and Cell Division

KEY CONCEPTS

- Bacterial chromosomes are specifically arranged and positioned inside cells.
- A rigid peptidoglycan cell wall surrounds the cell and gives it its shape.
- The rod shape of *E. coli* is dependent on MreB, PBP2, and RodA.
- Septum formation is initiated mid-cell, 50% of the distance from the septum to each end of the bacterium.

The shape of bacterial cells varies among different species, but many, including *E. coli* cells, are shaped like cylindrical rods that end in two curved poles. Bacterial cells have an internal cytoskeleton that is similar to what is found in eukaryotes. There are low homology homologs of actin, tubulin, and intermediate filaments. The bacterial chromosome is compacted into a dense protein–DNA structure called the *nucleoid,* which takes up most of the space inside the cell. It is not a disorganized mass of DNA; instead, specific DNA regions are localized to specific regions in the cell, and this positioning depends on the cell cycle and on the bacterial species. The movement apart of newly replicated bacterial chromosomes—that is, the segregation of the chromosomes—occurs concurrently with DNA replication. **FIGURE 9.4** summarizes the arrangement in *E. coli*. In newborn cells, the origin and terminus regions of the chromosome are at mid-cell. Following initiation, the new origins move toward the poles, or the one-quarter and three-quarters positions, and the terminus remains at mid-cell. Following cell division, the origins and termini reorient to mid-cell.

The shape of a bacterial cell is established by a rigid layer of peptidoglycan in the cell wall, which surrounds the inner membrane. The peptidoglycan is made by polymerization of tri- or pentapeptide-disaccharide units in a reaction involving connections between both types of subunit (transpeptidation and transglycosylation). Three proteins that are required to maintain the rodlike shape of bacteria are MreB, PBP2, and RodA. Mutations in any one of their genes and/or depletion of one of these proteins cause the bacterium to lose its extended shape and become round.

The structure of MreB protein resembles that of the eukaryotic protein actin, which polymerizes to form

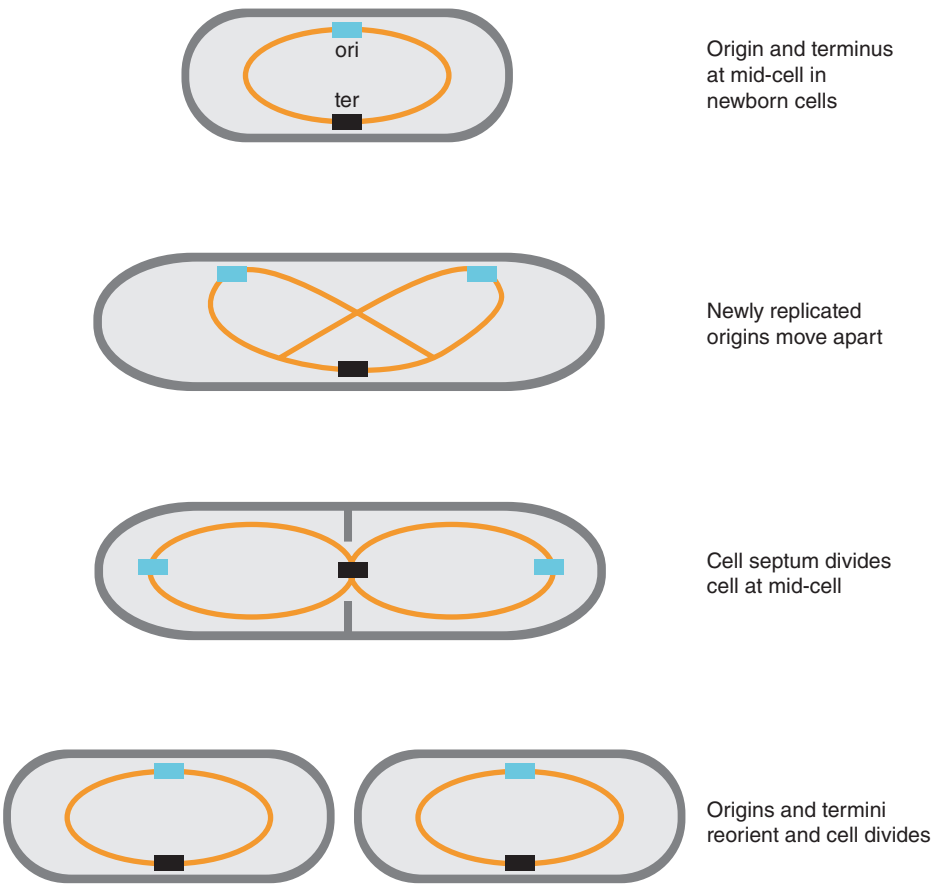


FIGURE 9.4 Attachment of bacterial DNA to the membrane could provide a mechanism for segregation.

cytoskeletal filaments in eukaryotic cells. In bacteria, MreB polymerizes and appears to move dynamically around the circumference of the cell attached to the peptidoglycan synthesis machinery, including PBP2. These interactions are necessary for the lateral integrity of the cell walls, because the lack of MreB results in round, rather than rod-shaped, cells. RodA is a member of the SEDS (*s*hape, *e*longation, *d*ivision, and *s*porulation) family present in all bacteria that have a peptidoglycan cell wall. Each SEDS protein functions together with a specific transpeptidase, which catalyzes the formation of the crosslinks in the peptidoglycan. PBP2 (penicillin-binding protein 2) is the transpeptidase that interacts with RodA. This demonstrates the important principle that shape and rigidity can be determined by the simple extension of a polymeric structure.

The end of the cell cycle in a bacterium is defined by the division of a mother cell into two daughter cells. Bacteria divide in the center of the cell by the formation of a **septum**, a structure that forms in the center of the cell as an invagination from the surrounding envelope. The septum forms an impenetrable barrier between the two parts of the cell and provides the site at which the two daughter cells eventually separate entirely. The septum then becomes the new pole of each daughter cell. The septum consists of the same components as the cell envelope. The septum initially forms as a double layer of peptidoglycan, and the protein EnvA is required to split the covalent links between the layers so that the daughter cells can separate. Two related questions address the role of the septum in division: "What determines the location at which it forms?" and "What ensures that the daughter chromosomes lie on opposite sides of it?"

9.4 Mutations in Division or Segregation Affect Cell Shape

KEY CONCEPTS

- *fts* mutants form long filaments because the septum that divides the daughter bacteria fails to form.
- Minicells form in mutants that produce too many septa; they are small and lack DNA.
- Anucleate cells of normal size are generated by partition mutants, in which the duplicate chromosomes fail to separate.

A difficulty in isolating mutants that affect cell division is that mutations in the critical functions might be lethal and/or pleiotropic. Most mutations in the division apparatus have been identified as conditional mutants (whose division is affected under nonpermissive conditions; typically, they are temperature sensitive). Mutations that affect cell division or chromosome segregation cause striking phenotypic changes. **FIGURE 9.5** and **FIGURE 9.6** illustrate the opposite consequences of failure in the division process and failure in segregation:

- Long filaments form when septum formation is inhibited, but chromosome replication is unaffected. The bacteria continue to grow—and even continue to segregate their daughter chromosomes—but septa do not form. Thus, the cell consists of a very long filamentous structure, with the nucleoids (bacterial chromosomes) regularly distributed along the length of the cell. This phenotype is displayed by *fts* mutants (named for temperature-sensitive filamentation), which identify a defect or multiple defects that lie in the division process itself.
- **Minicells** form when septum formation occurs too frequently or in the wrong place, with the result that one of the new daughter cells lacks a chromosome. The minicell has a rather small size and lacks DNA, but otherwise appears morphologically normal. Anucleate cells form when segregation is aberrant; like minicells, they lack a chromosome, but because septum formation is normal, their size is unaltered. This phenotype is caused by *par* (partition) mutants (named because they are defective in chromosome segregation).

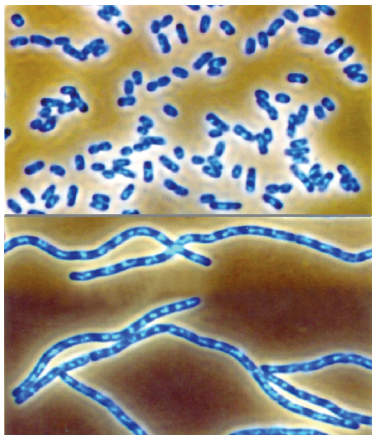


FIGURE 9.5 *Top panel:* Wild-type cells. *Bottom panel:* Failure of cell division under nonpermissive temperatures generates multinucleated filaments.

Photos courtesy of Sota Hiraga, Kyoto University.

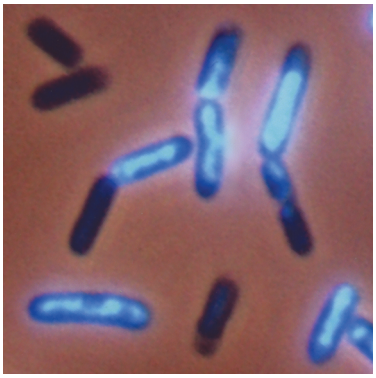


FIGURE 9.6 *E. coli* generate anucleate cells when chromosome segregation fails. Cells with chromosomes stain blue; daughter cells lacking chromosomes have no blue stain. This field shows cells of the *mukB* mutant; both normal and abnormal divisions can be seen.

Photo courtesy of Sota Hiraga, Kyoto University.

9.5 FtsZ Is Necessary for Septum Formation

KEY CONCEPTS

- The product of *ftsZ* is required for septum formation.
- FtsZ is a GTPase that resembles tubulin, and polymerizes to form a ring on the inside of the bacterial envelope. It is required to recruit the enzymes needed to form the septum.

The gene *ftsZ* plays a central role in division. Mutations in *ftsZ* block septum formation and generate filaments. Overexpression induces minicells by causing an increased number of septation events per unit cell mass. FtsZ (the protein) recruits a battery of cell division proteins that are responsible for synthesis of the new septum.

FtsZ functions at an early stage of septum formation. Early in the division cycle, FtsZ is localized throughout the cytoplasm, but prior to cell division FtsZ becomes localized in a ring around the circumference at the mid-cell position. The structure is called the **Z-ring**, which is shown in **FIGURE 9.7**. The formation of the Z-ring is the rate-limiting step in septum formation, and its assembly defines the position of the septum. In a typical division cycle, it forms in the center of the cell 1 to 5 minutes after division, remains for 15 minutes, and then quickly constricts to pinch the cell into two.

The structure of FtsZ resembles tubulin, suggesting that assembly of the ring could resemble the formation of microtubules in eukaryotic cells. FtsZ has GTPase activity, and GTP cleavage is used to support the oligomerization of FtsZ monomers into the ring structure. The Z-ring is a dynamic structure, in which there is continuous exchange of subunits with a cytoplasmic pool.

Two other proteins needed for division, ZipA and FtsA, interact directly and independently with FtsZ. ZipA is an integral membrane protein that is located in the inner bacterial membrane. It provides the means for linking FtsZ to the membrane. FtsA is a cytosolic protein, but is often found associated with the membrane. The Z-ring can form in the absence of either ZipA or FtsA, but it cannot form if both are absent. Both are needed for subsequent steps. This suggests that they have overlapping roles in stabilizing the Z-ring and perhaps in linking it to the membrane.

The products of several other *fts* genes join the Z-ring in a defined order after FtsA has been incorporated. They are all transmembrane proteins. The final structure is sometimes called the **septal ring**. It consists of a multiprotein complex that is presumed to have the ability to constrict the membrane. One of the last components to be incorporated into the septal ring is FtsW, which is a protein belonging to the SEDS family. The *ftsW* gene is expressed as part of an operon with *ftsI*, which encodes a transpeptidase (also called PBP3 for penicillin-binding protein 3), a membrane-bound protein that has its catalytic site in the periplasm. FtsW is responsible for incorporating FtsI into the septal ring. This suggests a model for septum formation in which the transpeptidase activity then causes the peptidoglycan to grow inward, thus pushing the inner membrane and pulling the outer membrane.

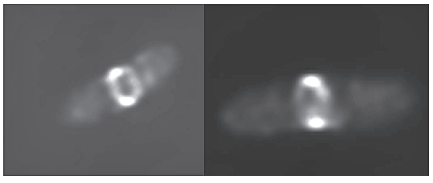


FIGURE 9.7 Immunofluorescence with an antibody against FtsZ shows that it is localized at the mid-cell.

Photo courtesy of William Margolin, University of Texas Medical School at Houston.

9.6 *min* and *noc/slm* Genes Regulate the Location of the Septum

KEY CONCEPTS

- The location of the septum is controlled by *minC, -D,* and *-E,* and by *noc/slmA.*
- The number and location of septa are determined by the ratio of MinE/MinCD.
- Dynamic movement of the Min proteins in the cell sets up a pattern in which inhibition of Z-ring assembly is highest at the poles and lowest at mid-cell.
- SlmA/Noc proteins prevent septation from occurring in the space occupied by the bacterial chromosome.

Clues to the localization of the septum were first provided by minicell mutants. The original minicell mutation lies in the locus *minB.* Deletion of *minB* generates minicells by allowing septation to occur near the poles instead of at mid-cell, and therefore the role of the wild-type *minB* locus is to suppress septation at the poles. The *minB* locus consists of three genes, *minC, -D,* and *-E.* The products of *minC* and *minD* form a division inhibitor. MinD is required to activate MinC, which prevents FtsZ from polymerizing into the Z-ring.

Expression of MinCD in the absence of MinE, or overexpression even in the presence of MinE, causes a generalized inhibition of division. The resulting cells grow as long filaments without septa. Expression of MinE at levels comparable to MinCD confines the inhibition to the polar regions, thus restoring normal growth. The determinant of septation at the proper (mid-cell) site is, therefore, the ratio of MinCD to MinE.

The localization activities of the Min system are due to a remarkable dynamic behavior of MinD and MinE, which is illustrated in **FIGURE 9.8**. MinD, an ATPase, oscillates from one end of the cell to the other on a rapid time scale. MinD-ATP binds to and accumulates at the bacterial lipid membrane at one pole of the cell, is released, and then rebinds to the opposite pole. The periodicity of this process takes about 30 seconds, so that multiple oscillations occur within one

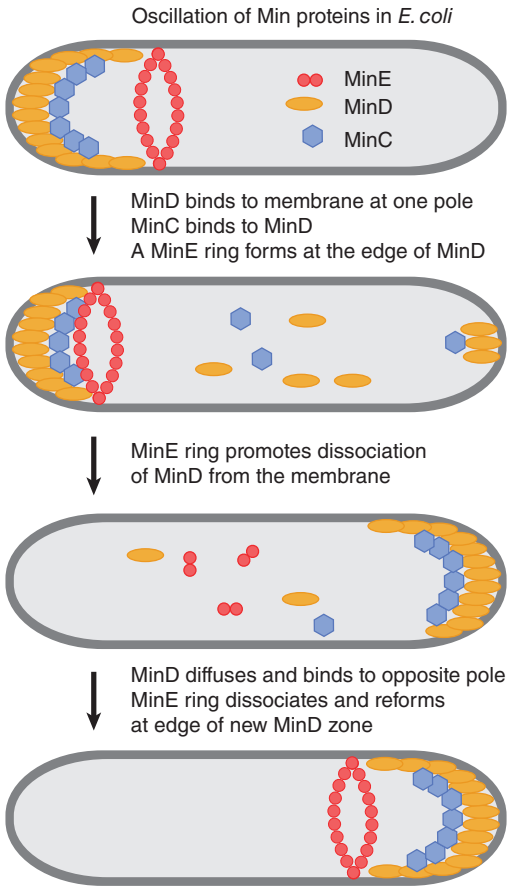


FIGURE 9.8 MinCD is a division inhibitor whose action is confined to the polar sites by MinE.

bacterial cell generation. MinC, which cannot move on its own, oscillates as a passenger protein bound to MinD. MinE forms a ring around the cell at the edge of the zone of MinD. The MinE ring moves toward MinD at the poles and is necessary for ATP hydrolysis and the release of MinD from the membrane. The MinE ring then disassembles and reforms at the edge of the MinD zone that forms at the opposite pole. MinD and MinE are each required for the dynamics of the other. The consequence of this dynamic behavior is that the concentration of the MinC inhibitor is lowest at mid-cell and highest at the poles, which directs FtsZ assembly at mid-cell and inhibits its assembly at the poles.

Another process, called nucleoid occlusion, prevents Z-ring formation over the bacterial chromosome and thus prevents the septum from bisecting an individual chromosome at cell division. A protein called SlmA, which interacts with FtsZ, is necessary for nucleoid occlusion in *E. coli*. SlmA binds specifically to at least 24 sites on the bacterial chromosome. DNA binding activates SlmA to antagonize the polymerization of FtsZ, which prevents septum formation in this region of the cell. In *Bacillus subtilis*, a DNA-binding protein called Noc performs a similar nucleoid occlusion role, but by a different mechanism. Noc interacts directly with the membrane, rather than with FtsZ, and this interaction interferes with the assembly of the cell division machinery. The bacterial nucleoid takes up a large volume of the cell, and as a result this process restricts Z-ring assembly to the limited nucleoid-free spaces at the poles and mid-cell. The combination of nucleoid occlusion and the Min system promotes the Z-rings to form, and thus cell division to occur, at mid-cell.

9.7 Partition Involves Separation of the Chromosomes

KEY CONCEPTS

- Daughter chromosomes are disentangled from each other by topoisomerases.
- Chromosome segregation occurs concurrently with DNA replication; that is, it begins before DNA replication is finished.
- Condensation of the chromosome by MukBEF or SMC proteins is necessary for proper chromosome orientation and segregation.

Partition is the process by which the two daughter chromosomes find themselves on either side of the position at which the septum forms. Two types of event are required for proper partition:

- The two daughter chromosomes must be released from one another so that they can segregate following termination. This requires disentangling of DNA regions that are coiled around each other in the vicinity of the terminus. Mutations affecting partition map in genes coding for topoisomerases—enzymes with the ability to pass DNA strands through one another. The mutations prevent the daughter chromosomes from segregating, with the result that the DNA is located in a single, large mass at mid-cell. Septum formation then releases an anucleate cell and a cell containing both daughter chromosomes. This tells us that the bacterium must be able to disentangle its chromosomes topologically in order to be able to segregate them into different daughter cells.
- The two daughter chromosomes must move apart during partition. The original models for chromosome segregation suggested that the cell envelope grows by insertion of material between membrane-attachment sites of the two chromosomes, thus pushing them apart. In fact, the cell wall and membrane grow heterogeneously over the whole cell surface. Current models of bacterial chromosome segregation do not require attachment to the membrane, although the confinement that is provided by the membrane is thought to be necessary to help push chromosomes apart. Some of the machinery and forces that drive segregation have been identified but the picture is still incomplete. The first important step is to promote separation of the newly replicated origin regions of the chromosome. As new origins move to new cellular locations (Figure 9.4), the rest of the chromosomes follow after they are replicated. The replicated chromosomes are capable of abrupt movements, which

indicates that some regions are held together for an interval of time before they rapidly separate. The final step is to separate newly replicated terminus regions of the chromosome.

Mutations that affect the partition process itself are rare. Segregation is interrupted by mutations of the *muk* class in *E. coli*, which give rise to anucleate progeny at a much increased frequency: Both daughter chromosomes remain on the same side of the septum instead of segregating. Mutations in the *muk* genes are not lethal, and they identify components of the apparatus that segregate the chromosomes. The gene *mukB* encodes a large (180-kD) protein, which has the same general type of organization as the two groups of structural maintenance of chromosomes (SMC) proteins that are involved in condensing and in holding together eukaryotic chromosomes. SMC-like proteins have also been found in other bacteria and mutations in their genes also increase the frequency of anucleate cells. Another phenotype of *mukB* mutants is that the organization of the chromosome is altered from that shown in Figure 9.4; origins and termini are reoriented toward the poles for the entire cell cycle. Therefore, MukB also acts to properly orient and position the origin regions of the chromosome during segregation.

Initial insight into the role of MukB was the discovery that some mutations in *mukB* can be suppressed by mutations in *topA*, the gene that encodes topoisomerase I. MukB forms a complex with two other proteins, MukE and MukF, and the MukBEF complex is considered to be a condensin analogous to eukaryotic condensins. A defect in this function can be compensated for by preventing topoisomerases from relaxing negative supercoils; the resulting increase in supercoil density helps to restore the proper state of condensation and allow segregation. **FIGURE 9.9** shows one model for the role of condensation. The parental genome is centrally positioned. It must be decondensed in order to pass through the replication apparatus. The daughter chromosomes emerge from replication, are disentangled by topoisomerases, and then passed in an uncondensed state to MukBEF, which causes them to form condensed masses at the positions that will become the centers of the daughter cells.

It is likely that MukBEF (or SMC in other bacteria) works with other factors to promote the initial steps in segregation of the origin region of the chromosome. Researchers have identified some of these factors in other bacteria, such as partition genes, called *parA* and *parB*, that resemble those necessary for partition of low-copy-number plasmids. These discoveries and analyses in current research will lead to a better understanding of how genomes are positioned in the cell.

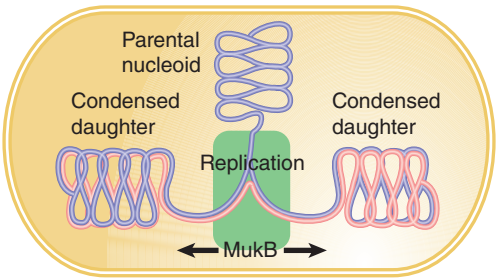


FIGURE 9.9 The DNA of a single parental nucleoid becomes decondensed during replication. MukB is an essential component of the apparatus that recondenses the daughter nucleoids.

9.8 Chromosomal Segregation Might Require Site-Specific Recombination

KEY CONCEPTS

- The Xer site-specific recombination system acts on a target sequence near the chromosome terminus to recreate monomers if a generalized recombination event has converted the bacterial chromosome to a dimer.
- FtsK acts at the terminus of replication to promote the final separation of chromosomes and their transport through the growing septum.

After replication has created duplicate copies of a bacterial chromosome or plasmid, the copies can recombine. **FIGURE 9.10** demonstrates the consequences. A single intermolecular recombination event between two circles generates a dimeric circle; further recombination can generate higher multimeric forms. Such an event reduces the number of physically segregating units. In the extreme case of a single-copy plasmid that has just replicated, formation of a dimer by recombination means that the cell only has one unit to segregate, and the plasmid therefore must inevitably be lost from one daughter cell. To counteract this effect, plasmids often have **site-specific recombination** systems that act upon particular sequences to sponsor an intramolecular recombination that restores the monomeric condition. For example, plasmid P1 encodes the Cre protein-*lox* site recombination system for this purpose. Scientists have further exploited the *Cre-lox* system extensively for genetic engineering in many different

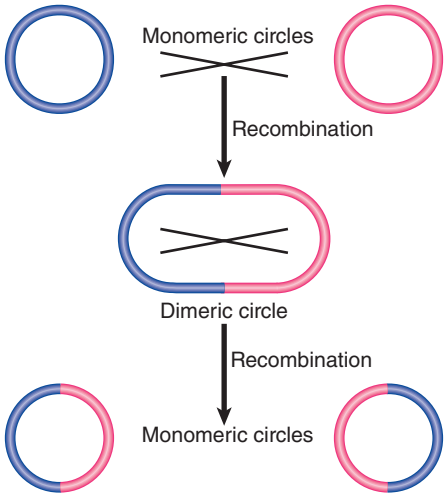


FIGURE 9.10 Intermolecular recombination merges monomers into dimers, and intramolecular recombination releases individual units from oligomers.

organisms. These systems are also discussed in the chapter titled *Homologous and Site-Specific Recombination*.

The same type of events can occur with the bacterial chromosome; **FIGURE 9.11** shows how such an event affects its segregation. If no recombination occurs, there is no problem, and the separate daughter chromosomes can segregate to the daughter cells. A dimer will be produced, however, if homologous recombination occurs between the daughter chromosomes produced by a replication cycle. If there has been such a recombination event, the daughter chromosomes cannot separate. In this case, a second recombination is required to achieve resolution in the same way as a plasmid dimer.

Most bacteria with circular chromosomes possess the Xer site-specific recombination system. In *E. coli*, this consists of two recombinases, XerC and XerD, which act on a 28-base-pair (bp) target site called *dif* that is located in the terminus region of the chromosome. The use of the Xer system is related to cell division in an interesting way. The relevant events are summarized in **FIGURE 9.12**. XerC can bind to a pair of *dif* sequences and form a Holliday junction between them. The complex might form soon after the replication fork passes over the *dif* sequence, which explains how the two copies of the target sequence can find each other consistently. Resolution of the junction to give recombinants, however, occurs only in the presence of FtsK, a protein located in the septum that is required for chromosome segregation

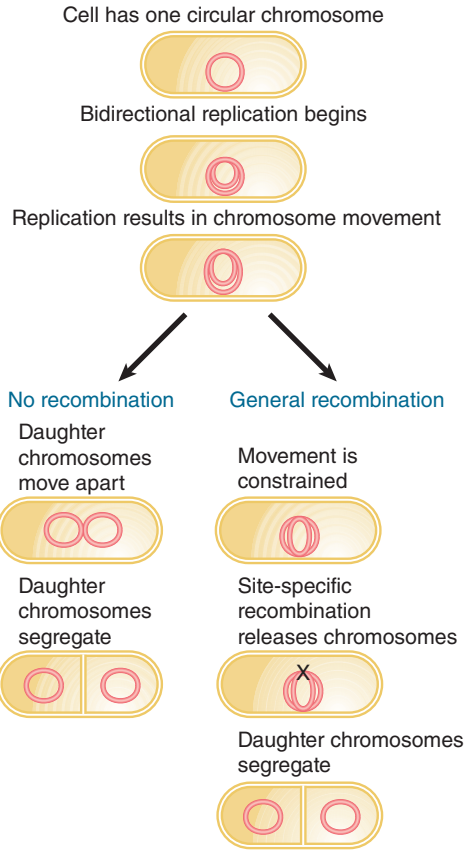


FIGURE 9.11 A circular chromosome replicates to produce two monomeric daughters that segregate to daughter cells. A generalized recombination event, however, generates a single dimeric molecule. This can be resolved into two monomers by a site-specific recombination.

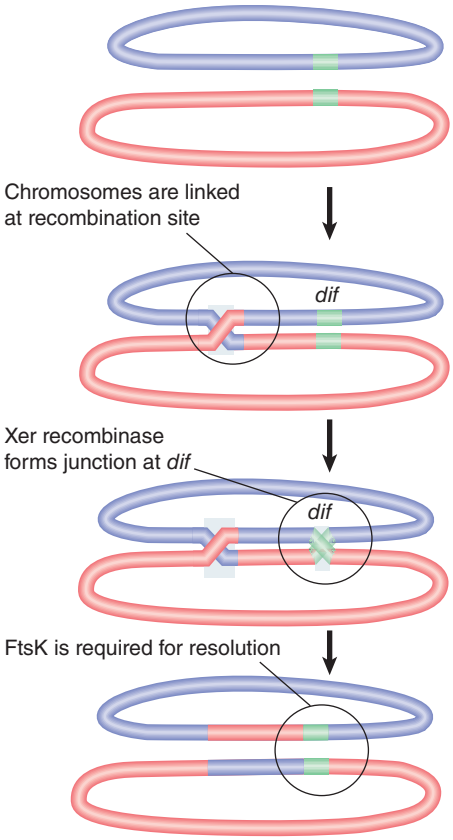


FIGURE 9.12 A recombination event creates two linked chromosomes. Xer creates a Holliday junction at the *dif* site, but can resolve it only in the presence of FtsK.

and cell division. In addition, the *dif* target sequence must be located in a region of approximately 30 kb; if it is moved outside of this region, it cannot support the reaction. Remember that the terminus region of the chromosome is located near the septum prior to cell division as discussed in the section *The Shape and Spatial Organization of a Bacterium Are Important During Chromosome Segregation and Cell Division* earlier in this chapter.

The bacterium, however, should have site-specific recombination at *dif* only when there has already been a general recombination event to generate a dimer. (Otherwise, the site-specific recombination would create the dimer!) How does the system know whether the daughter chromosomes exist as independent monomers or have been recombined into a dimer? One answer is the timing of chromosome segregation. Remember that the terminus is the last region of the chromosome to be segregated. If there has been no recombination, the two chromosomes move apart from one another shortly after they are replicated. The ability to move apart from one another, however, will be constrained if a dimer has been formed. This forces the terminus region to remain in the vicinity of the septum, where sites are exposed to the Xer system.

Another factor that promotes separation of the terminus is the FtsK protein. Bacteria that have the Xer system always have an FtsK homolog, and vice versa, which suggests that the system has evolved so that resolution is connected to the septum. FtsK is a large transmembrane protein. Its N-terminal domain is associated with the membrane and

causes it to be localized to the septum. Its C-terminal domain has two functions. One is to cause Xer to resolve a dimer into two monomers. It also has an ATPase activity, which it uses to pump DNA through the septum.

A special type of chromosome segregation occurs during sporulation in *B. subtilis*. One daughter chromosome must be segregated into the forespore compartment. This is an unusual process that involves transfer of the chromosome across the nascent septum. One of the sporulation genes, *spoIIIE,* is required for this process. The SpoIIIE protein resembles FtsK, is located at the septum, and has a translocation function that pumps DNA through to the forespore compartment.

9.9 The Eukaryotic Growth Factor Signal Transduction Pathway Promotes Entry to S Phase

KEY CONCEPTS

- The function of a growth factor is to stabilize dimerization of its receptor and subsequent phosphorylation of the cytoplasmic domain of the receptor.
- The function of the growth factor receptor is to recruit the exchange factor SOS to the membrane to activate RAS.
- The function of activated RAS is to recruit RAF to the membrane to become activated.
- The function of RAF is to initiate a phosphorylation cascade leading to the phosphorylation of a set of transcription factors that can enter the nucleus and begin S phase.

The vast majority of eukaryotic cells in a multicellular individual are not growing; that is, they are in the cell cycle stage of G0, as we saw in the beginning of this chapter. Stem cells and most embryonic cells, however, are actively growing. A growing cell exiting mitosis has two choices—it can enter G1 and begin a new round of cell division or it can stop dividing and enter G0, a quiescent stage and, if so programmed, begin differentiation. This decision is controlled by the developmental history of the cell and the presence or absence of growth factors and their receptors.

For a cell to begin the cell cycle from G0, or continue to divide after M phase, it must be programmed to express the proper **growth factor receptor** gene. Elsewhere in the organism, typically in a master gland (but can also occur in neighboring cells), the gene for the proper *growth factor* must be expressed. The **signal transduction pathway** is the biochemical mechanism by which the growth factor signal to grow is communicated from its source outside of the cell into the nucleus to ultimately cause that cell to begin replication and growth. The pathway that we describe in this section is universal in eukaryotes, ranging from yeast to humans.

The genes that encode elements of the signal transduction pathway are **proto-oncogenes**, genes that when altered can cause cancer. As an example of this pathway, we examine **Epidermal Growth Factor (EGF)** and its receptor, **EGFR**—a member of the erbB family of four related receptors. These two proteins, EGF and EGFR, and the genes that encode them are the first two elements in the pathway. EGF is a peptide hormone (as opposed to a steroid hormone such as estrogen). The EGFR specifically binds EGF in a lock-and-key type of mechanism. EGFR is a one-pass membrane protein in the family known as receptor tyrosine kinases (RTK), as shown in **FIGURE 9.13a**. The receptor has an external domain (that is outside the cell) that binds EGF, a single membrane-spanning domain, and an internal cytoplasmic domain with intrinsic tyrosine kinase activity. The local membrane composition (e.g., cholesterol) can modulate the dynamics of the signal transduction pathway.

Hormone binding to receptor stabilizes receptor dimerization (usually homodimerization, but heterodimers with other erbB family members can occur), which leads to multiple cross-phosphorylation events of each receptor's cytoplasmic domain. *The only function of the hormone is to stabilize receptor dimerization.* Each receptor phosphorylates the other on a set of five tyrosine amino acid residues in the cytoplasmic domain, as shown in **FIGURE 9.13b**. Each phosphorylated tyrosine (Tyr-P) serves as a docking site for a specific adaptor protein to bind to the receptor, as shown in **FIGURE 9.13c**. We will examine a single pathway, but it is important to keep in mind that cells contain many different receptors that are active at the same time, and each receptor has multiple docking sites for multiple proteins. The reality is that it is not a pathway but rather an information network.

Paradoxically, hormone binding to the receptor also causes clathrin-mediated endocytosis of the hormone receptor complex to the lysosomal complex, where it is targeted for destruction, and thus turnover. This trafficking is regulated by microtubule deacetylation, which controls the proportion of receptors that are returned to the surface. This is part of an important attenuation mechanism to prevent accidental triggering of the pathway and it means that growth factor must be continually present to propagate a sustained signal.

The third member of the signal transduction pathway is the RAS protein (encoded by the *ras* gene). RAS is a member of a large family of G-proteins, proteins that bind a guanosine nucleotide, either GTP (for the active form of RAS) or GDP (for the inactive form). RAS is connected to the membrane by a prenylated (lipid) tail, and typically found in nanoclusters on the cytoplasmic side of the membrane to enhance downstream signaling. To continue the flow of information through the signal transduction pathway communicating that a growth factor is present, inactive RAS must be converted from RAS-GDP to RAS-GTP by a protein called Son of Sevenless (SOS), a *g*uanosine nucleotide *e*xchange *f*actor (GEF) that exchanges GTP for GDP. Its function is to remove the GDP from RAS and replace it with GTP, as shown in **FIGURE 9.13d**. RAS also has a weak intrinsic phosphatase (GTPase) activity that slowly converts GTP to GDP. Again, this provides a mechanism to ensure that growth factor must be present continually for the signal to propagate.

To activate RAS, SOS must be specifically recruited to the membrane in order to interact with RAS-GDP. It is the

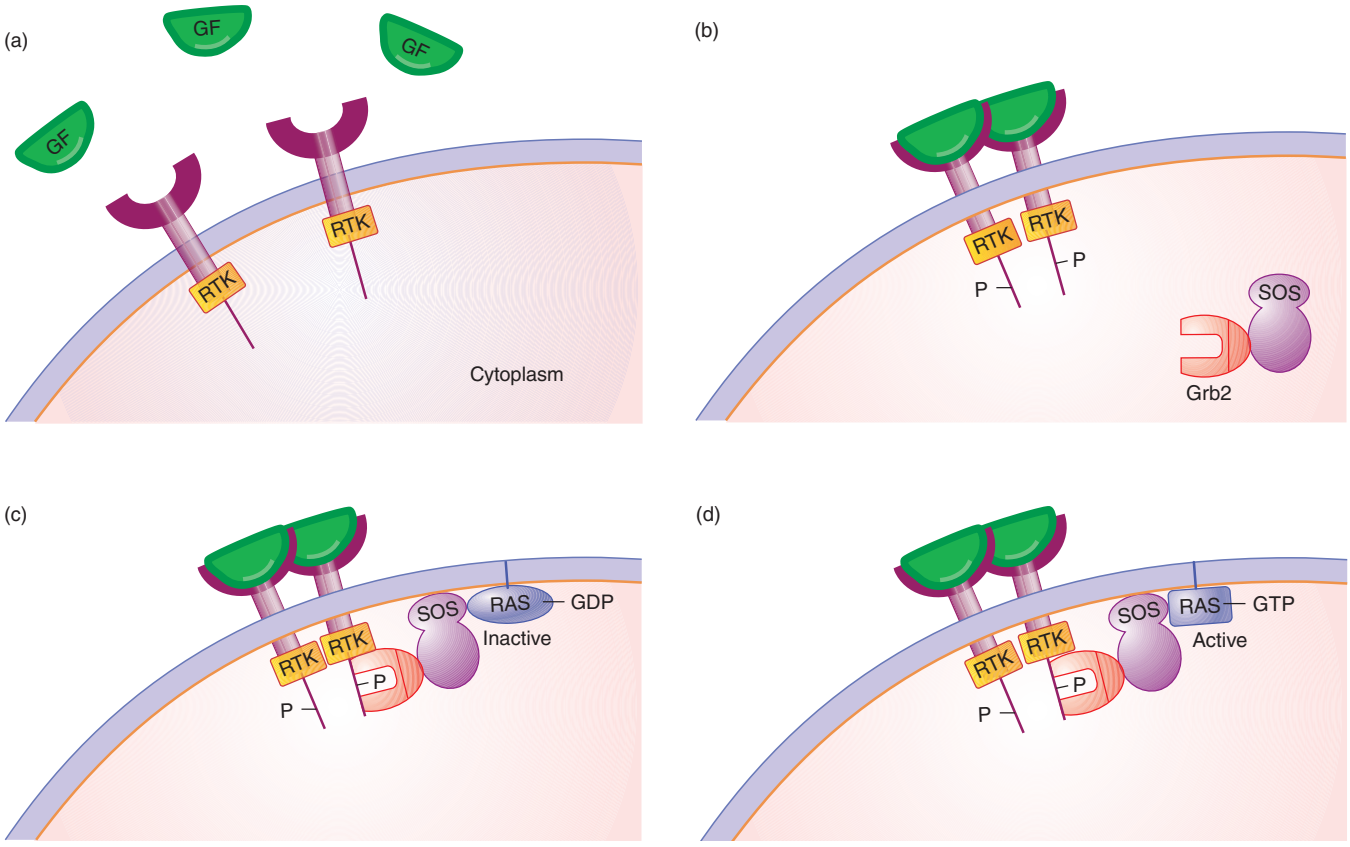


FIGURE 9.13 The signal transduction pathway. **(a)** Growth factors and growth factor receptors: The growth factor extracellular domain will bind the growth factor in a lock-and-key fashion. The growth factor receptor intracellular domain contains an intrinsic protein kinase domain called RTK. **(b)** Growth factor binding to its receptor will stabilize receptor dimerization, leading to phosphorylation of each cytoplasmic domain on tyrosine. The phosphotyrosine residues can serve as binding sites for proteins such as Grb2, shown here. **(c)** Grb2 binds the Tyr-P so that its binding partner SOS, a guanosine nucleotide exchange factor, is brought to the membrane and can activate the inactive RAS-GDP. **(d)** SOS removes the GDP, replacing it with GTP, activating RAS.

membrane phospholipids themselves that serve to unlock an auto-inhibitory domain so that SOS can bind to RAS. SOS is in a complex with an adaptor protein called Grb2, an interesting protein with two domains: an SH2 domain that binds Tyr-P, and an SH3 domain that binds proteins containing another SH3 domain. The specificity for binding to the receptor lies in the amino acids surrounding each Tyr-P. *The only function of the growth factor is to stabilize dimerization of the receptor, which leads to its phosphorylation, which in turn leads to recruitment of SOS to the membrane to activate RAS.*

Inactive RAS-GDP and active RAS-GTP are in a dynamic equilibrium controlled by the exchange factor GEF and another set of proteins that stimulate the intrinsic GTPase of RAS, such as RAS GAP (*G*TPase *a*ctivating *p*rotein).

ras oncogenic mutations that constitutively activate RAS are among the most frequent oncogenic mutations found in tumors. The most common mutation is a single nucleotide change that causes a single amino acid change, resulting in altered function. RAS^{ONC} has a key altered property: It binds GTP with a higher affinity than GDP. The consequence is that it no longer requires a growth factor to trigger activation; it is constitutively active. This kind of mutation is referred to as a **dominant gain-of-function mutation**.

Activated RAS, RAS-GTP, now itself serves as a docking site to recruit the fourth member of the pathway to be activated: a structurally inactive form of RAF (also known as MAPKKK or *m*itogen-*a*ctivated *p*rotein *k*inase *k*inase *k*inase), a serine/threonine protein kinase. The activation of RAF on the membrane has been one of the most baffling steps, with researchers having proposed many models over the years. *The only function of RAS-GTP is to recruit RAF to the membrane for activation; it does nothing else.* The most recent model is the dimer model for RAS-mediated activation of a dimer of RAF (see **FIGURE 9.14**). This activation is facilitated by the fact that RAS is present in the membrane in high concentration in nanoclusters. This high concentration of RAS leads to the formation of a dimer of RAS-GTP which facilitates the next step. RAF activation on the membrane involves its dimerization leading to the RAS-assisted unfolding of the autoinhibitory domains of the RAF dimer. This then allows phosphorylation by another membrane associated kinase, SRC, and release of the RAF dimer from the platform.

Activated RAF phosphorylates a second kinase, such as one of the mitogen-activated kinase (MEK) factors, which then phosphorylates a third kinase, such as one of the *e*xtracellular signal-*r*egulated *k*inase (ERK) factors, which can then phosphorylate and activate the set of transcription factors such as MYC, JUN, and FOS. This allows their entry into the nucleus to begin transcribing the genes to prepare for transit through G1 and entry into S phase. Again, note that this is

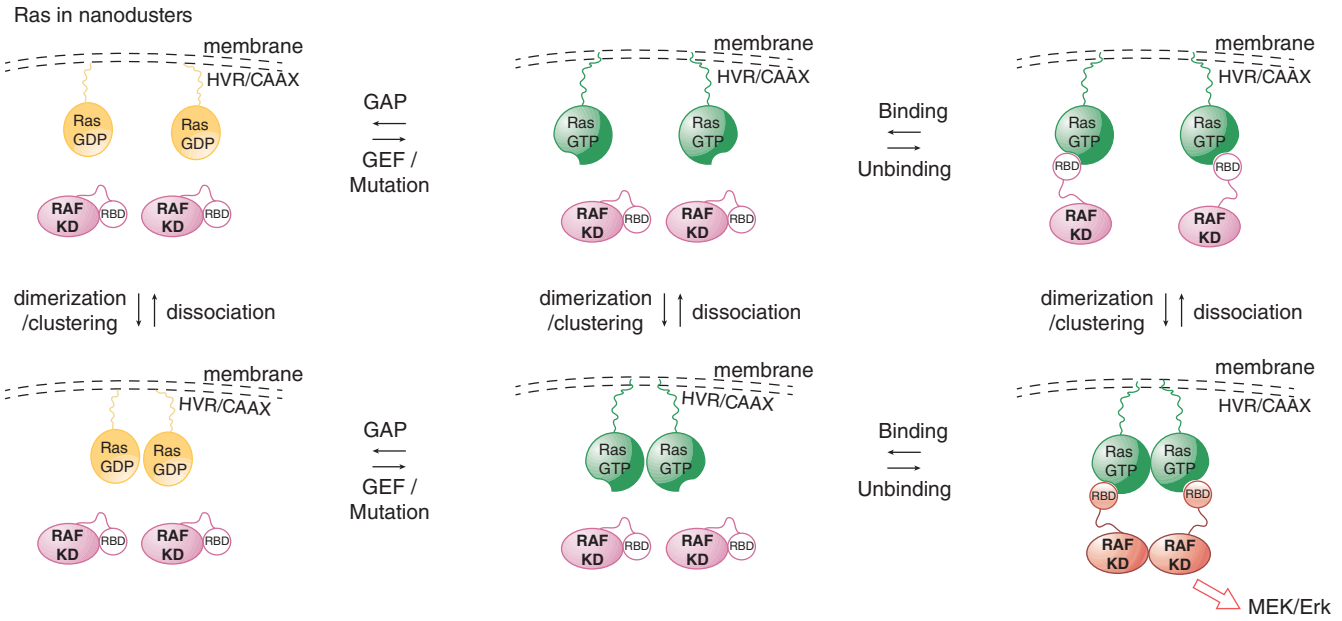


FIGURE 9.14 Dimer model for RAS-mediated activation of RAF. RAS-GTP forms dimers to cooperatively activate RAF.

a description of a single pathway within a network that has extensive crosstalk between members. In addition, this kinase cascade is modulated by an extensive network of phosphatases.

9.10 Checkpoint Control for Entry into S Phase: p53, a Guardian of the Checkpoint

KEY CONCEPTS

- The tumor suppressor proteins p53 and Rb act as guardians of cell integrity.
- A set of ser/thr protein kinases called cyclin-dependent kinases control cell cycle progression.
- Cyclin proteins are required to activate cyclin-dependent kinase proteins.
- Inhibitor proteins negatively regulate the cyclin/cyclin-dependent kinases.
- Activator proteins called CDK-activating kinases positively regulate the cyclin/cyclin-dependent kinases.

Progression through the cell cycle, after the initial activation by growth factor, requires continuous growth factor presence and is tightly controlled by a second set of ser/thr protein kinases called **cyclin-dependent kinases (CDKs**; and sometimes cell division–dependent kinases). The CDKs themselves are controlled in a very complex fashion as shown in **FIGURE 9.15**. They are inactive by themselves and are activated by the binding of cell cycle–specific proteins called **cyclins**. This means that the CDKs can be synthesized in advance and left in the cytoplasm. In addition to cyclins, the CDKs are regulated by multiple phosphorylation events. One set of kinases, the Wee1 family of ser/thr kinases, inhibits the CDKs, while another, the CDK-activating kinases (CAKs), activates them. (Wee1 kinases inhibit cell cycle progression, and if they are mutated, premature cell cycle progression results in wee, tiny cells.) This also means that *the balance of kinases and phosphatases regulates the activity of the CDKs.* We will focus on the G1 to S phase transition. (There is similar tight control at the G2 to M transition and within various stages of mitosis and meiosis.) The signal for entry into S phase is a positive signal controlled by negative regulators. The S to G2 transition occurs when replication is completed.

For a cell to be *allowed* to progress from G1 to S phase, two major requirements must be met. The cell must have grown a specific amount in size and there must be no DNA damage. The worst thing that a cell can do is to replicate damaged DNA. To ensure that both requirements are met, the CDK/cyclin complexes are controlled by checkpoint proteins. Two of the most important are the transcription factors p53 and Rb. These two proteins are in a class called **tumor suppressor** proteins. As guardians of the cell cycle, these proteins ensure that

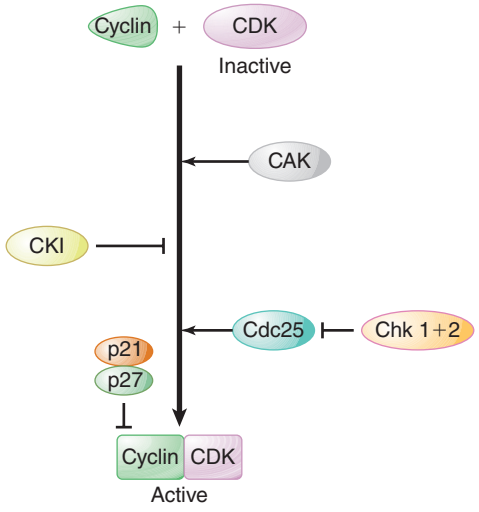


FIGURE 9.15 Formation of an active CDK requires binding to a cyclin. The process is regulated by positive and negative factors.

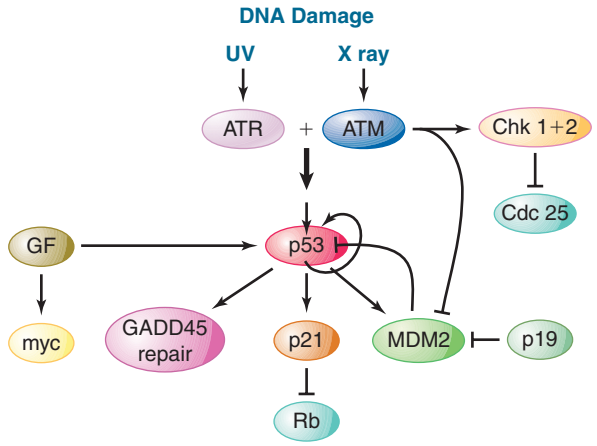


FIGURE 9.16 DNA damage pathway. p53 is activated by DNA damage. Activated p53 halts the cell cycle through Rb and stimulates DNA repair. p53 is regulated by a complex set of activators and inhibitors.

the cell size and absence of DNA damage criteria are met. Even in the presence of an oncogenic mutant RAS protein, tumor suppressors will prevent the cell from progressing from G1 to S; they are the brakes on the cell cycle. Mutations in tumor suppressor proteins allow damaged and undersized cells to replicate. These *recessive, loss-of-function* mutations, especially in p53 and Rb, are the most common tumor suppressor mutations in tumors; frequently both are seen together.

The DNA damage checkpoint controlled by p53 is the one that is best understood (**FIGURE 9.16**). The function of p53 is to relay information to the CDK/cyclins that damage has occurred to prevent entry into S phase; that is, it ultimately causes cell cycle arrest. In addition, in the event that damage is very extensive or otherwise unrepairable, p53 will initiate an alternate pathway, **apoptosis**, or **programmed cell death (PCD)**. p53 transcription is upregulated by growth factor stimulation, as the cell begins preparation for its trip through G1 and the important G1 to S transition.

The p53 protein product is regulated by multiple complex pathways. The major regulator is a protein called MDM2, which works through a negative feedback loop. MDM2 transcription is increased by p53, and it in turn inhibits p53 in a positive feedback loop, by targeting it to the ubiquitin-dependent proteosomal degradation pathway, as described further in the section *Checkpoint Control for Entry into S Phase: Rb, a Guardian of the Checkpoint* coming up next. It also binds to p53 and prevents it from activating transcription. DNA damage leads to phosphorylation of MDM2, which inhibits its ability to promote p53 degradation, allowing p53 levels to increase. Growth factor stimulation of cell cycle progression also leads to an increase in transcription of the $p19^{ARF}$ protein (p14 in humans), which binds to and inhibits MDM2's ability to inhibit p53. The human $p14^{ARF}$ is transcribed from an interesting genetic locus, the *INK4a/ARF* locus, which gives rise to three proteins by alternative splicing and alternative promoter usage: $p15^{INK}$, $p16^{INK}$, and $p14^{ARF}$ (ARF stands for alternate reading frame).

p53 is activated by DNA damage or different kinds of stress through a protein kinase relay system from the nucleus that ultimately phosphorylates and stabilizes p53 from degradation. This leads to an increased level of p53 and activates its ability to serve as a transcription factor to turn on some genes and repress other genes. Among those genes turned on are *GADD45* to stimulate DNA repair; *p21/WAF-1*, whose product binds to and inhibits the CDK/cyclin complexes for G1 arrest (or promotes apoptosis if the DNA damage is too great); sets of large intergenic noncoding RNAs (lincRNAs) to mediate transcription repression; and miRNAs (as described in the chapter titled *Regulatory RNA*). A specific lincRNA, p21-lincRNA, mediates the repressive properties of p53 by binding to specific chromatin complexes.

DNA damage also independently activates a pair of protein kinases, Chk1 and Chk2, which phosphorylate and inhibit CDKs, and phosphorylate and inhibit the phosphatase Cdc25 (cell division cycle), which is required to activate the CDKs.

9.11 Checkpoint Control for Entry into S Phase: Rb, a Guardian of the Checkpoint

KEY CONCEPTS

- Rb is the major guardian of the cell cycle, integrating information about DNA damage and cell growth.
- Rb binds the activation domains of a set of essential transcription factors, the E2F family, in the cytoplasm to prevent them from turning on the genes required for cell cycle progression.
- When Rb is phosphorylated by a cyclin/CDK complex, it releases E2F to permit cell cycle progression.

Let's now examine how an undamaged cell progresses through G1 (**FIGURE 9.17**). A growth factor signal, executed through the signal transduction pathway, is required to turn on the gene for the first cyclin expressed, Cyclin D (humans have three different forms of this gene while *Drosophila* has one). Its partners, already in the cytoplasm, are CDK4 and -6. Cyclins are the positive regulators of the CDK protein kinases; by themselves CDKs are inactive. Cyclin D is required for entry into S phase. Growth factor must be continuously present for at least the first half of G1.

The key for cell cycle progression is the tumor suppressor protein Rb. Although Rb has multiple roles in the nucleus as direct regulator of chromatin structure and transcription, we focus in this section on its role in the cytoplasm as the major guardian of entry into S phase. Rb binds to the transcription factor E2F and inhibits its ability to enter the nucleus to turn on those genes required for progression through G1 and entry into S phase. Within G1 is a critical point controlled by Rb, called the **restriction point** or START point (different in different species), at which the cell becomes committed to continuing through the cell cycle. Ultimately, Rb integrates signals concerning both DNA damage as described in the section on p53, and cell size (or growth of the cell) pathways and is thus the key guardian of progression to S phase.

For cell cycle progression to occur, Rb must be phosphorylated by CDK/cyclin; phosphorylation of Rb releases E2F.

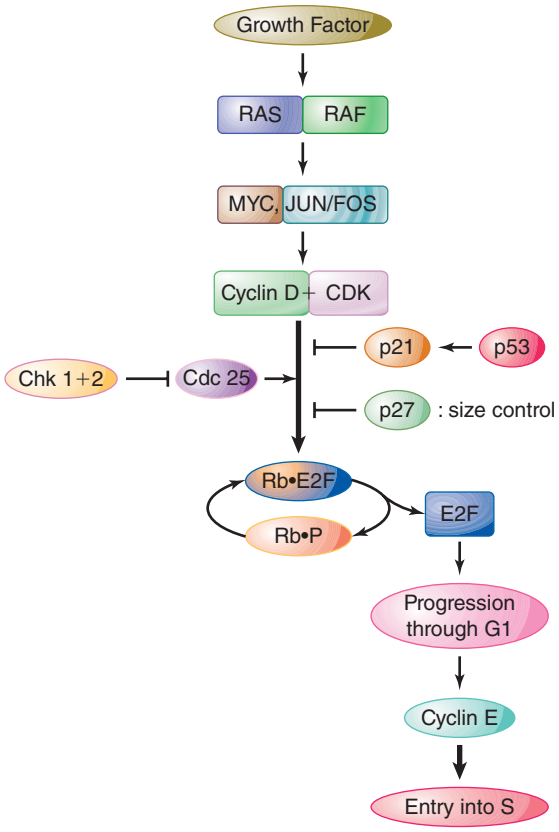


FIGURE 9.17 Growth factors are required to start the cell cycle and continue into S phase. The CDK-cyclin complex phosphorylates Rb to cause it to release the transcription factor E2F to go into the nucleus to turn on genes for progression through G1 and into S phase.

The ultimate control of cell cycle progression is thus the regulation of CDK activity by a set of inhibitor proteins, CKIs (cyclin kinase inhibitors). p21, induced by DNA damage through p53, is a CKI. It is the major link between the DNA damage checkpoint and Rb. Another major CKI is p27, a member of the Cip/Kip family. It is present in fairly high levels in G0 cells to prevent activation to G1. EGFR activation leads to its reduction. p27 is also activated in G1 by the cytokine TGF-3, a major growth inhibitor. p19/p16/INK/ARF is another major class of CKI proteins that control Cyclin D activity (these two different proteins, INK and ARF, are made from the same gene from alternate reading frames).

Cell size or growth of the cell is monitored by a titration mechanism. A cell entering G1 has a fixed set of different classes of CKI proteins to prevent cell cycle progression. For the cell to progress through G1, this inhibition must be overcome by the synthesis of more Cyclin D. *The length of G1 is determined by how long it takes to synthesize a sufficient level of cyclins to overcome the level of CKIs.*

During G1, three different cyclins are made. Cyclin D, as described earlier, is the first synthesized, activated by growth factor. As the cell continues to grow, the level of Cyclin D reaches a point of titrating out the CKIs, and the Cyclin D/cdk4/6 complex can begin phosphorylating Rb/E2F. This will cause Rb to begin to release E2F, which can then activate genes for progression through the cell cycle and ultimately S phase. Among the genes activated is the *E2F* gene to increase the abundance of the E2F protein and Cyclin E. Cyclin E is activated by the middle of G1, and it is also required for progression into S phase, adding to and amplifying the initial phosphorylation of Rb. Finally, just before S phase begins, Cyclin A is synthesized, and it is also required for entry and continuation through S phase.

Summary

- A fixed time of 40 minutes is required to replicate the *E. coli* chromosome, and an additional 20 minutes is required before the cell can divide. When cells divide more rapidly than every 60 minutes, a replication cycle is initiated before the end of the preceding division cycle. This generates multiforked chromosomes. The initiation event occurs once and at a specific time in each cell cycle. Initiation timing depends on accumulating the active initiator protein DnaA and on inhibitors that turn off newly synthesized origins until the next cell cycle.
- *E. coli* grows as a rod-shaped cell that divides into daughter cells by formation of a septum that forms at mid-cell. The shape is maintained by an envelope of peptidoglycan that surrounds the cell. The rod shape is dependent on the MreB actin-like protein that forms a scaffold for recruiting the enzymes necessary for peptidoglycan synthesis. The septum is dependent on FtsZ, which is a tubulin-like protein that can polymerize into a filamentous structure called a Z-ring. FtsZ recruits the enzymes necessary to make the septum. Absence of septum formation generates multinucleated filaments; an excess of septum formation generates anucleate minicells.
- Many transmembrane proteins interact to form the septum. ZipA is located in the inner bacterial membrane and binds to FtsZ. Several other *fts* products, most of which are transmembrane proteins, join the Z-ring in an ordered process that generates a septal ring. The last proteins to bind are the SEDS protein FtsW and the transpeptidase FtsI (PBP3), which together function to produce the peptidoglycans of the septum. Chromosome segregation involves several processes, including separation of catenated products by topoisomerases, site-specific recombination, and the action of MukB/SMC proteins in chromosome condensation following DNA replication. Plasmids and bacteria have site-specific recombination systems that regenerate pairs of monomers by resolving dimers created by general recombination. The Xer system acts on a target sequence located in the terminus region of the chromosome. The system is active only in the presence of the FtsK protein of the septum, which might ensure that it acts only when a dimer needs to be resolved.
- The eukaryotic cell cycle is governed by a complex set of regulatory factors. Licensing to begin the cell

cycle, as opposed to enter or remain in G0, requires a positive growth factor signal interacting with its receptor to initiate the signal transduction pathway. This biochemical relay of information from outside the cell through the RAS-GTP and RAF protein kinase ultimately results in the activation of a set of transcription factors in the cytoplasm. These can then enter the nucleus to begin the transcription of genes required for the progression through G1 and ultimate entry into S phase and replication of the chromosomes.

- The cell cycle—that is, progression from G1 to S phase and beyond—is regulated primarily by phosphorylation events carried out by a set of protein kinases, the CDKs, and balanced by phosphatases. The kinases are controlled by a set of cell cycle stage–specific proteins called cyclins that bind to the CDKs and convert an inactive CDK into an active kinase. Progression through G1 into S phase is allowed only if there is no DNA damage and the cell has grown a sufficient amount in size. These two requirements are enforced by a pair of tumor-suppressor proteins. p53 guards the DNA damage checkpoint to prevent the replication of damaged DNA. Rb is the guardian that integrates DNA damage and cell-size information to ultimately control whether the gene regulator E2F is allowed into the nucleus to begin transcription.

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