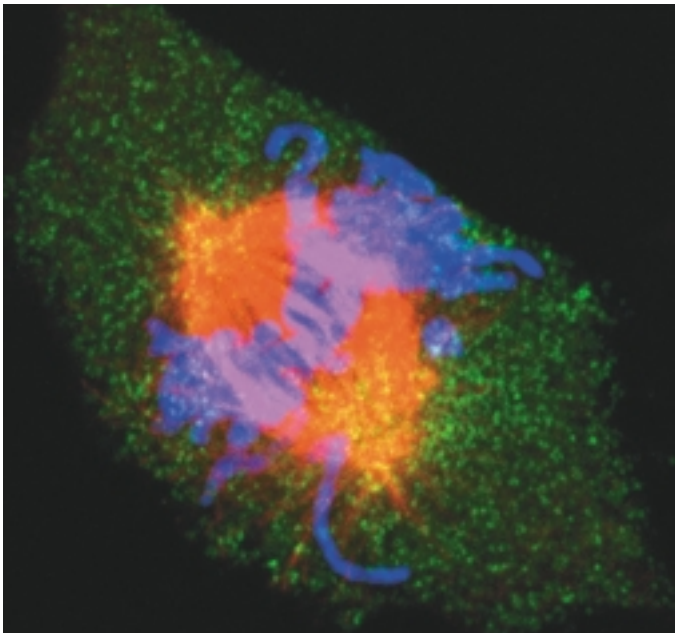
21

REGULATING THE EUKARYOTIC CELL CYCLE



This cultured rat kidney cell in metaphase shows condensed chromosomes (blue), microtubules of the spindle apparatus (red), and the inner nuclear envelope protein POM121 (green). The POM121 staining demonstrates that the inner nuclear envelope proteins retract into the ER during mitosis.
[Brian Burke and Jan Ellenberger, 2002, *Nature Rev. Mol. Cell Biol.* **3:**487.]

The **cell cycle** entails an ordered series of macromolecular events that lead to **cell division** and the production of two daughter cells each containing chromosomes identical to those of the parental cell. Duplication of the parental chromosomes occurs during the S phase of the cycle, and one of the resulting daughter chromosomes is distributed to each daughter cell during **mitosis** (see Figure 9-3). Precise temporal control of the events of the cell cycle ensures that the replication of chromosomes and their **segregation** to daughter cells occur in the proper order and with extraordinarily high fidelity. Regulation of the cell cycle is critical for the normal development of multicellular organisms, and loss of control ultimately leads to cancer, an all-too-familiar disease that kills one in every six people in the developed world (Chapter 23).

In the late 1980s, it became clear that the molecular processes regulating the two key events in the cell cycle—chromosome replication and segregation—are fundamentally similar in all eukaryotic cells. Because of this similarity, research with diverse organisms, each with its own particular experimental advantages, has contributed to a growing understanding of how these events are coordinated and controlled. Biochemical and genetic techniques, as well as recombinant DNA technology, have been employed in studying various aspects of the eukaryotic cell cycle. These studies have revealed that cell replication is primarily controlled by regulating the timing of nuclear DNA replication and mitosis. The master controllers of these events are a small number of *heterodimeric protein kinases* that contain a regulatory subunit (cyclin) and catalytic subunit (cyclin-dependent kinase). These kinases regulate the activities of multiple proteins involved in DNA replication and mitosis by phosphorylating them at specific regulatory sites, activating some and inhibiting others to coordinate their activities. In this chapter we focus on how the cell cycle is regulated and the experimental systems that have led to our current understanding of these crucial regulatory mechanisms.

OUTLINE

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21.3 Genetic Studies with *S. pombe*
21.4 Molecular Mechanisms for Regulating Mitotic Events
21.5 Genetic Studies with *S. cerevisiae*
21.6 Cell-Cycle Control in Mammalian Cells
21.7 Checkpoints in Cell-Cycle Regulation
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21.1 Overview of the Cell Cycle and Its Control

We begin our discussion by reviewing the stages of the eukaryotic cell cycle, presenting a summary of the current model of how the cycle is regulated, and briefly describing key experimental systems that have provided revealing information about cell-cycle regulation.

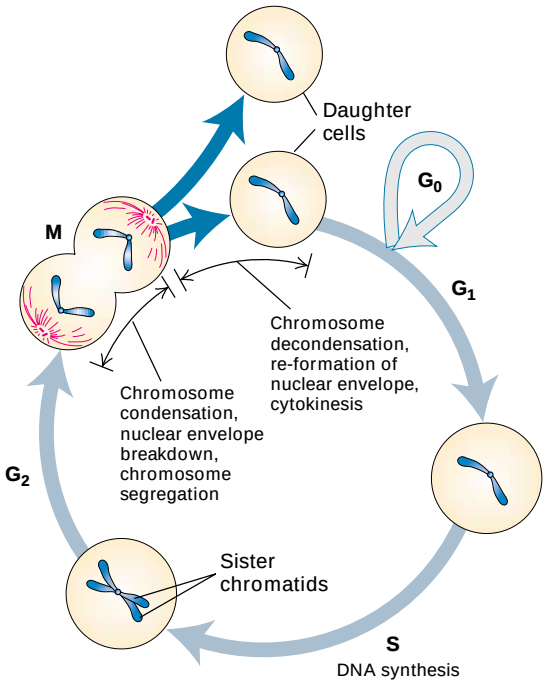
The Cell Cycle Is an Ordered Series of Events Leading to Cell Replication

As illustrated in Figure 21-1, the cell cycle is divided into four major phases. In cycling (replicating) somatic cells, cells synthesize RNAs and proteins during the **G_1 phase,** preparing for DNA synthesis and chromosome replication during the **S (synthesis) phase.** After progressing through the **G_2 phase,** cells begin the complicated process of mitosis, also called the **M (mitotic) phase,** which is divided into several stages (see Figure 20-29).

In discussing mitosis, we commonly use the term chromosome for the *replicated* structures that condense and become visible in the light microscope during the **prophase** period of mitosis. Thus each chromosome is composed of the two daughter DNA molecules resulting from DNA replication plus the histones and other chromosomal proteins associated with them (see Figure 10-27). The identical daughter DNA molecules and associated chromosomal proteins that form one chromosome are referred to as sister **chromatids.** Sister chromatids are attached to each other by protein cross-links along their lengths. In vertebrates, these become confined to a single region of association called the **centromere** as chromosome condensation progresses.

During **interphase,** the portion of the cell cycle between the end of one M phase and the beginning of the next, the outer nuclear membrane is continuous with the endoplasmic reticulum (see Figure 5-19). With the onset of mitosis in prophase, the nuclear envelope retracts into the endoplasmic reticulum in most cells from higher eukaryotes, and Golgi membranes break down into vesicles. As described in Chapter 20, cellular microtubules disassemble and reassemble into the **mitotic apparatus** consisting of a football-shaped bundle of microtubules (the spindle) with a star-shaped cluster of microtubules radiating from each end, or spindle pole. During the **metaphase** period of mitosis, a multiprotein complex, the **kinetochore,** assembles at each centromere. The kinetochores of sister chromatids then associate with microtubules coming from opposite spindle poles (see Figure 20-31). During the **anaphase** period of mitosis, sister chromatids separate. They initially are pulled by motor proteins along the spindle microtubules toward the opposite poles and then are further separated as the mitotic spindle elongates (see Figure 20-40).

Once chromosome separation is complete, the mitotic spindle disassembles and chromosomes decondense during **telophase.** The nuclear envelope re-forms around the segregated chromosomes as they decondense. The physical division of the cytoplasm, called **cytokinesis,** then yields two daughter cells as the Golgi complex re-forms in each daughter cell. Following mitosis, cycling cells enter the G_1 phase, embarking on another turn of the cycle. In yeasts and other fungi, the nuclear envelope does not break down during mitosis. In these organisms, the mitotic spindle forms within the nuclear envelope, which then pinches off, forming two nuclei at the time of cytokinesis.



▲ FIGURE 21-1 **Summary of major events in the eukaryotic cell cycle and the fate of a single parental chromosome.** In proliferating cells, G_1 is the period between "birth" of a cell following mitosis and the initiation of DNA synthesis, which marks the beginning of the S phase. At the end of the S phase, a replicated chromosome consists of two daughter DNA molecules and associated chromosomal proteins. Each of the individual daughter DNA molecules and their associated chromosomal proteins (not shown) is called a sister chromatid. The end of G_2 is marked by the onset of mitosis, during which the mitotic spindle (red lines) forms and pulls apart sister chromatids, followed by division of the cytoplasm (cytokinesis) to yield two daughter cells. The G_1, S, and G_2 phases are collectively referred to as interphase, the period between one mitosis and the next. Most nonproliferating cells in vertebrates leave the cell cycle in G_1, entering the G_0 state.

In vertebrates and diploid yeasts, cells in G_1 have a **diploid** number of chromosomes ($2n$), one inherited from each parent. In haploid yeasts, cells in G_1 have one of each chromosome ($1n$), the **haploid** number. Rapidly replicating human cells progress through the full cell cycle in about 24 hours: mitosis takes ≈30 minutes; G_1, 9 hours; the S phase, 10 hours; and G_2, 4.5 hours. In contrast, the full cycle takes only ≈90 minutes in rapidly growing yeast cells.

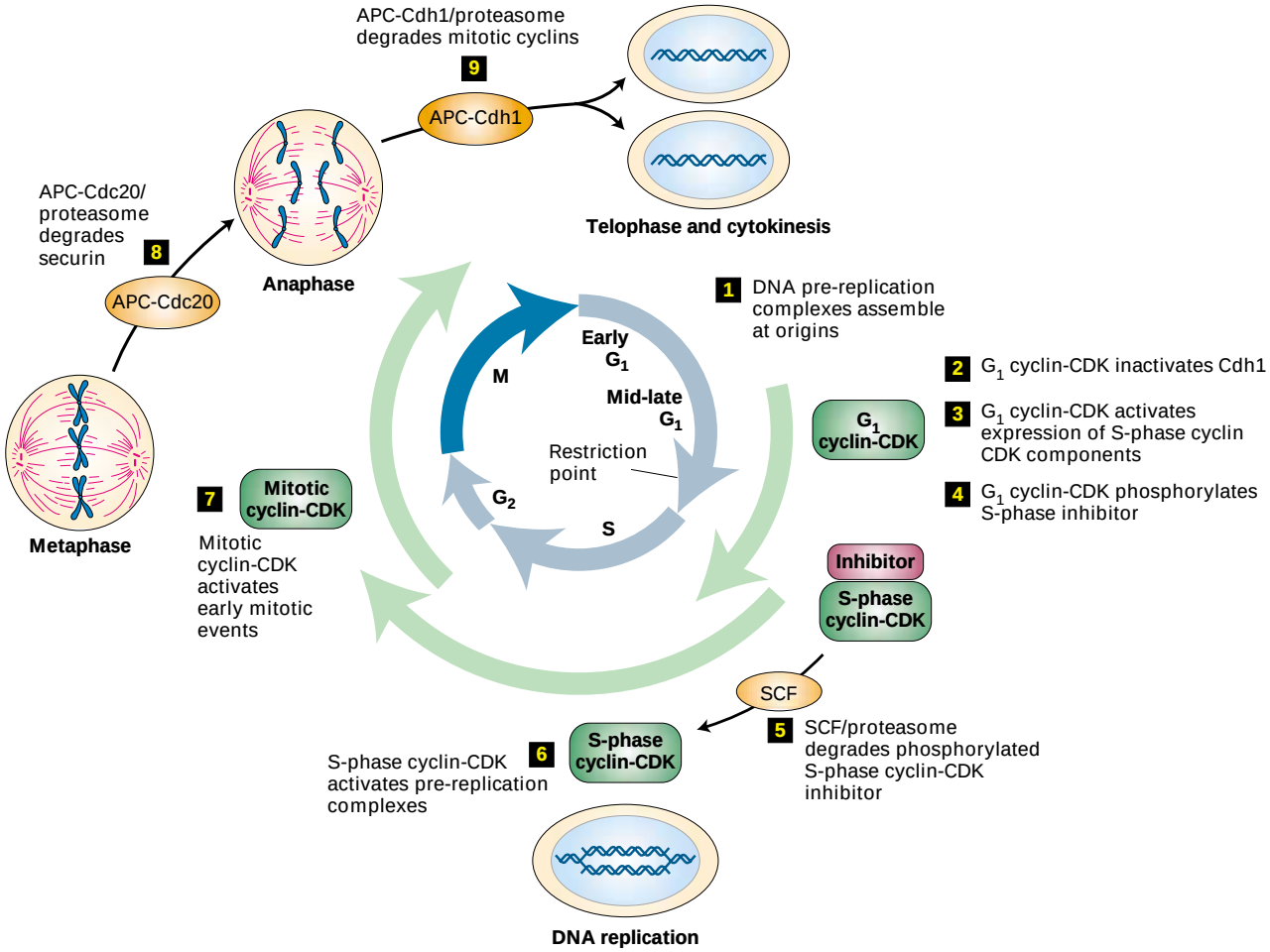
In multicellular organisms, most differentiated cells "exit" the cell cycle and survive for days, weeks, or in some

cases (e.g., nerve cells and cells of the eye lens) even the lifetime of the organism without dividing again. Such *postmitotic* cells generally exit the cell cycle in G_1, entering a phase called G_0 (see Figure 21-1). Some G_0 cells can return to the cell cycle and resume replicating; this reentry is regulated, thereby providing control of cell proliferation.

Regulated Protein Phosphorylation and Degradation Control Passage Through the Cell Cycle

The concentrations of the **cyclins,** the regulatory subunits of the heterodimeric protein kinases that control cell-cycle events, increase and decrease as cells progress through the cell cycle. The catalytic subunits of these kinases, called **cyclin-dependent kinases (CDKs),** have no kinase activity unless they are associated with a cyclin. Each CDK can associate with different cyclins, and the associated cyclin determines which proteins are phosphorylated by a particular cyclin-CDK complex.

Figure 21-2 outlines the role of the three major classes of cyclin-CDK complexes that control passage through the cell cycle: the G_1, S-phase, and mitotic cyclin-CDK complexes. When cells are stimulated to replicate, G_1 cyclin-CDK complexes are expressed first. These prepare the cell for the S phase by activating transcription factors that promote



▲ FIGURE 21-2 **Overview of current model for regulation of the eukaryotic cell cycle.** Passage through the cycle is controlled by G_1, S-phase, and mitotic cyclin-dependent kinase complexes (green). These are composed of a regulatory cyclin subunit and a catalytic cyclin-dependent kinase (CDK) subunit. Two ubiquitin ligase complexes (orange), SCF and APC, polyubiquitinate specific substrates including S-phase inhibitors (step 5), securin (step 8), and mitotic cyclins (step 9), marking these substrates for degradation by proteasomes. Proteolysis of the S-phase inhibitor activates S-phase cyclin-CDK complexes, leading to chromosome replication. Proteolysis of securin results in degradation of protein complexes that connect sister chromatids at metaphase, thereby initiating anaphase, the mitotic period in which sister chromatids are separated and moved to the opposite spindle poles. Reduction in the activity of mitotic cyclin-CDK complexes caused by proteolysis of mitotic cyclins permits late mitotic events and cytokinesis to occur. These proteolytic cleavages drive the cycle in one direction because of the irreversibility of protein degradation. See text for further discussion.

MEDIA CONNECTIONS
Overview Animation: Cell-Cycle Control

transcription of genes encoding enzymes required for DNA synthesis and the genes encoding S-phase cyclins and CDKs. The activity of S-phase cyclin-CDK complexes is initially held in check by inhibitors. Late in G_1, the G_1 cyclin-CDK complexes induce degradation of the S-phase inhibitors by phosphorylating them and consequently stimulating their polyubiquitination by the multiprotein SCF ubiquitin ligase (step 5). Subsequent degradation of the polyubiquitinated S-phase inhibitor by proteasomes releases active S-phase cyclin-CDK complexes.

Once activated, the S-phase cyclin-CDK complexes phosphorylate regulatory sites in the proteins that form DNA pre-replication complexes, which are assembled on *replication origins* during G_1 (Chapter 4). Phosphorylation of these proteins not only activates initiation of DNA replication but also prevents reassembly of new pre-replication complexes. Because of this inhibition, each chromosome is replicated just once during passage through the cell cycle, ensuring that the proper chromosome number is maintained in the daughter cells.

Mitotic cyclin-CDK complexes are synthesized during the S phase and G_2, but their activities are held in check by phosphorylation at inhibitory sites until DNA synthesis is completed. Once activated by dephosphorylation of the inhibitory sites, mitotic cyclin-CDK complexes phosphorylate multiple proteins that promote chromosome condensation, retraction of the nuclear envelope, assembly of the mitotic spindle apparatus, and alignment of condensed chromosomes at the metaphase plate. During mitosis, the anaphase-promoting complex (APC), a multisubunit ubiquitin ligase, polyubiquitinates key regulatory proteins marking them for proteasomal degradation. One important substrate of the APC is securin, a protein that inhibits degradation of the cross-linking proteins between sister chromatids. The polyubiquitination of securin by the APC is inhibited until the kinetochores assembled at the centromeres of all chromosomes have become attached to spindle microtubules, causing chromosomes to align at the metaphase plate. Once all the chromosomes are aligned, the APC polyubiquitinates securin, leading to its proteasomal degradation and the subsequent degradation of the cross-linking proteins connecting sister chromatids (see Figure 21-2, step 8). This sequence of events initiates anaphase by freeing sister chromatids to segregate to opposite spindle poles.

Late in anaphase, the APC also directs polyubiquitination and subsequent proteasomal degradation of the mitotic cyclins. Polyubiquitination of the mitotic cyclins by APC is inhibited until the segregating chromosomes have reached the proper location in the dividing cell (see Figure 21-2, step 9). Degradation of the mitotic cyclins leads to inactivation of the protein kinase activity of the mitotic CDKs. The resulting decrease in mitotic CDK activity permits constitutively active protein phosphatases to remove the phosphates that were added to specific proteins by the mitotic cyclin-CDK complexes. As a result, the now separated chromosomes decondense, the nuclear envelope re-forms around daughter-cell nuclei, and the Golgi apparatus reassembles during telophase; finally, the cytoplasm divides at cytokinesis, yielding the two daughter cells.

During early G_1 of the next cell cycle, phosphatases dephosphorylate the proteins that form pre-replication complexes. These proteins had been phosphorylated by S-phase cyclin-CDK complexes during the previous S phase, and their phosphorylation was maintained during mitosis by mitotic cyclin-CDK complexes. As a result of their dephosphorylation in G_1, new pre-replication complexes are able to reassemble at replication origins in preparation for the next S phase (see Figure 21-2, step 1). Phosphorylation of Cdh1 by G_1 cyclin-CDK complexes in late G_1 inactivates it, allowing accumulation of S-phase and mitotic cyclins during the ensuing cycle.

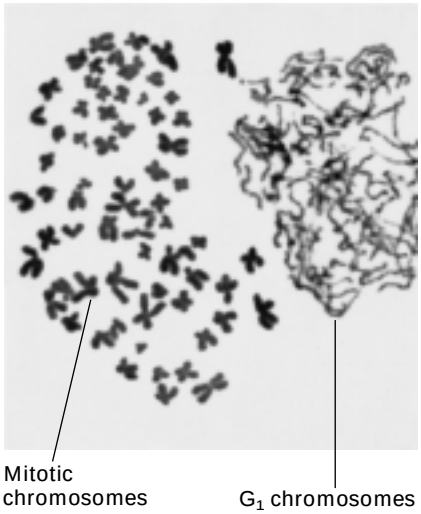
Passage through three critical cell-cycle transitions—$G_1 \rightarrow$ S phase, metaphase $\rightarrow$ anaphase, and anaphase $\rightarrow$ telophase and cytokinesis—is irreversible because these transitions are triggered by the regulated degradation of proteins, an irreversible process. As a consequence, cells are forced to traverse the cell cycle in one direction only.

In higher organisms, control of the cell cycle is achieved primarily by regulating the synthesis and activity of G_1 cyclin-CDK complexes. Extracellular **growth factors** function as **mitogens** by inducing synthesis of G_1 cyclin-CDK complexes. The activity of these and other cyclin-CDK complexes is regulated by phosphorylation at specific inhibitory and activating sites in the catalytic subunit. Once mitogens have acted for a sufficient period, the cell cycle continues through mitosis even when they are removed. The point in late G_1 where passage through the cell cycle becomes independent of mitogens is called the restriction point (see Figure 21-2).

Diverse Experimental Systems Have Been Used to Identify and Isolate Cell-Cycle Control Proteins

The first evidence that diffusible factors regulate the cell cycle came from cell-fusion experiments with cultured mammalian cells. When interphase cells in the G_1, S, or G_2 phase of the cell cycle were fused to cells in mitosis, their nuclear envelopes retracted and their chromosomes condensed (Figure 21-3). This finding indicates that some diffusible component or components in the cytoplasm of the mitotic cells forced interphase nuclei to undergo many of the processes associated with early mitosis. We now know that these factors are the mitotic cyclin-CDK complexes.

Similarly, when cells in G_1 were fused to cells in S phase and the fused cells exposed to radiolabeled thymidine, the label was incorporated into the DNA of the G_1 nucleus as well as the S-phase nucleus, indicating that DNA synthesis began in the G_1 nucleus shortly after fusion. However, when cells in G_2 were fused to S-phase cells, no incorporation of labeled thymidine occurred in the G_2 nuclei. Thus diffusible factors in an S-phase cell can enter the nucleus of a G_1 cell and stimulate DNA synthesis, but these factors cannot in-

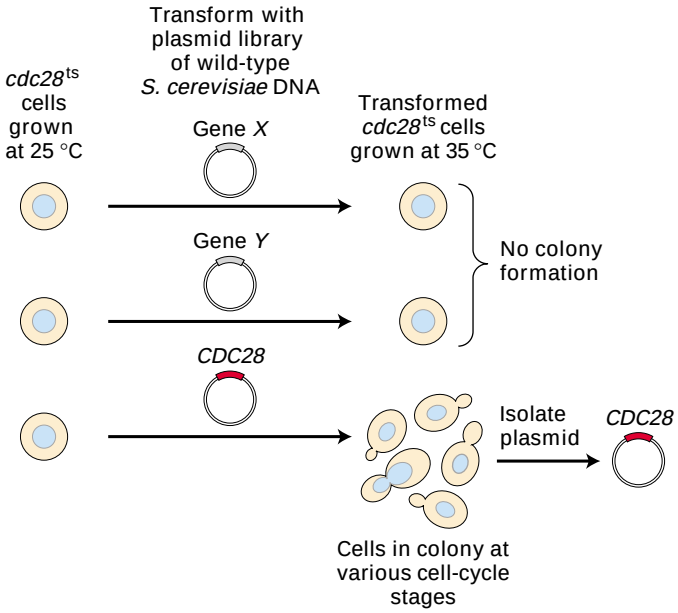


▲ EXPERIMENTAL FIGURE 21-3 **A diffusable factor in mitotic cells can induce mitosis in an interphase cell.** In unfused interphase cells, the nuclear envelope is intact and the chromosomes are not condensed, so individual chromosomes cannot be distinguished (see Figures 1-2b and 5-25). In mitotic cells, the nuclear envelope is absent and the individual replicated chromosomes are highly condensed. This micrograph shows a hybrid cell resulting from fusion of a mitotic cell (left side) with an interphase cell in G_1 (right side). A factor from the mitotic cell cytoplasm has caused the nuclear envelope of the G_1 cell to retract into the endoplasmic reticulum, so that it is not visible. The factor has also caused the G_1 cell chromosomes to partially condense. The mitotic chromosomes can be distinguished because the two sister chromatids are joined at the centromere. [From R. T. Johnson and P. N. Rao, 1970, *Biol. Rev.* **46**:97.]

duce DNA synthesis in a G_2 nucleus. We now know that these factors are S-phase cyclin-CDK complexes, which can activate the pre-replication complexes assembled on DNA replication origins in early G_1 nuclei. Although these cell-fusion experiments demonstrated that diffusible factors control entry into the S and M phases of the cell cycle, genetic and biochemical experiments were needed to identify these factors.

The budding yeast *Saccharomyces cerevisiae* and the distantly related fission yeast *Schizosaccharomyces pombe* have been especially useful for isolation of mutants that are blocked at specific steps in the cell cycle or that exhibit altered regulation of the cycle. In both of these yeasts, **temperature-sensitive mutants** with defects in specific proteins required to progress through the cell cycle are readily recognized microscopically and therefore easily isolated (see Figure 9-6). Such cells are called *cdc* (*c*ell-*d*ivision *c*ycle) mutants. The wild-type alleles of recessive temperature-sensitive *cdc* mutant alleles can be isolated readily by transforming haploid mutant cells with a plasmid library prepared from wild-type cells and then plating the transformed cells at the nonpermissive temperature (Figure 21-4). When plated out, the haploid mutant cells cannot form colonies at the nonpermissive temperature. However, complementation of the recessive mutation by the wild-type allele carried by one of the plasmid clones in the library allows a transformed mutant cell to grow into a colony; the plasmids bearing the wild-type allele can then be recovered from those cells. Because many of the proteins that regulate the cell cycle are highly conserved, human cDNAs cloned into yeast expression vectors often can complement yeast cell-cycle mutants, leading to the rapid isolation of human genes encoding cell-cycle control proteins.

Biochemical studies require the preparation of cell extracts from many cells. For biochemical studies of the cell cycle, the eggs and early embryos of amphibians and marine invertebrates are particularly suitable. In these organisms, multiple synchronous cell cycles follow fertilization of a large egg. By isolating large numbers of eggs from females and



▲ EXPERIMENTAL FIGURE 21-4 **Wild-type cell-division cycle *(CDC)* genes can be isolated from a *S. cerevisiae* genomic library by functional complementation of *cdc* mutants.** Mutant cells with a temperature-sensitive mutation in a *CDC* gene are transformed with a genomic library prepared from wild-type cells and plated on nutrient agar at the nonpermissive temperature (35 °C). Each transformed cell takes up a single plasmid containing one genomic DNA fragment. Most such fragments include genes (e.g., *X* and *Y*) that do not encode the defective Cdc protein; transformed cells that take up such fragments do not form colonies at the nonpermissive temperature. The rare cell that takes up a plasmid containing the wild-type version of the mutant gene (in this case *CDC28*) is complemented, allowing the cell to replicate and form a colony at the nonpermissive temperature. Plasmid DNA isolated from this colony carries the wild-type *CDC* gene corresponding to the gene that is defective in the mutant cells. The same procedure is used to isolate wild-type *CDC* genes in *S. pombe*. See Figures 9-19 and 9-20 for more detailed illustrations of the construction and screening of a yeast genomic library.

TABLE 21-1 Selected Cyclins and Cyclin-Dependent Kinases (CDKs) *

Organism/Protein	Name
S. POMBE	
CDK (one only)	Cdc2
Mitotic cyclin (one only)	Cdc13
S. CEREVISIAE	
CDK (one only)	Cdc28
Mid G_1 cyclin	Cln3
Late G_1 cyclins	Cln1, Cln2
Early S-phase cyclins	Clb5, Clb6
Late S-phase and early mitotic cyclins	Clb3, Clb4
Late mitotic cyclins	Clb1, Clb2
VERTEBRATES	
Mid G_1 CDKs	CDK4, CDK6
Late G_1 and S-phase CDK	CDK2
Mitotic CDK	CDK1
Mid G_1 cyclins	D-type cyclins
Late G_1 and S-phase cyclin	Cyclin E
S-phase and mitotic cyclin	Cyclin A
Mitotic cyclin	Cyclin B

* Those cyclins and CDKs discussed in this chapter are listed and classified by the period in the cell cycle in which they function. A heterodimer composed of a mitotic cyclin and CDK is commonly referred to as a mitosis-promoting factor (MPF).

fertilizing them simultaneously by addition of sperm (or treating them in ways that mimic fertilization), researchers can obtain extracts from cells at specific points in the cell cycle for analysis of proteins and enzymatic activities.

In the following sections we describe critical experiments that led to the current model of eukaryotic cell-cycle regulation summarized in Figure 21-2 and present further details of the various regulatory events. As we will see, results obtained with different experimental systems and approaches have provided insights about each of the key transition points in the cell cycle. For historical reasons, the names of various cyclins and cyclin-dependent kinases from yeasts and vertebrates differ. Table 21-1 lists the names of those that we discuss in this chapter and indicates when in the cell cycle they are active.

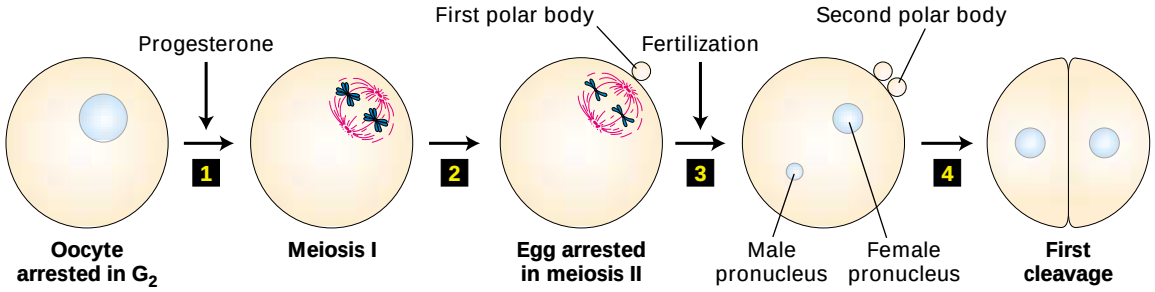
KEY CONCEPTS OF SECTION 21.1

Overview of the Cell Cycle and Its Control

- The eukaryotic cell cycle is divided into four phases: M (mitosis), G_1 (the period between mitosis and the initiation of nuclear DNA replication), S (the period of nuclear DNA replication), and G_2 (the period between the completion of nuclear DNA replication and mitosis) (see Figure 21-1).
- Cyclin-CDK complexes, composed of a regulatory cyclin subunit and a catalytic cyclin-dependent kinase subunit, regulate progress of a cell through the cell cycle (see Figure 21-2). Large multisubunit ubiquitin ligases also polyubiquitinate key cell-cycle regulators, marking them for degradation by proteasomes.
- Diffusible mitotic cyclin-CDK complexes cause chromosome condensation and disassembly of the nuclear envelope in G_1 and G_2 cells when they are fused to mitotic cells. Similarly, S-phase cyclin-CDK complexes stimulate DNA replication in the nuclei of G_1 cells when they are fused to S-phase cells.
- The isolation of yeast cell-division cycle *(cdc)* mutants led to the identification of genes that regulate the cell cycle (see Figure 21-4).
- Amphibian and invertebrate eggs and early embryos from synchronously fertilized eggs provide sources of extracts for biochemical studies of cell-cycle events.

21.2 Biochemical Studies with Oocytes, Eggs, and Early Embryos

A breakthrough in identification of the factor that induces mitosis came from studies of oocyte maturation in the frog *Xenopus laevis*. To understand these experiments, we must first lay out the events of oocyte maturation, which can be duplicated in vitro. As **oocytes** develop in the frog ovary, they replicate their DNA and become arrested in G_2 for 8 months during which time they grow in size to a diameter of 1 mm, stockpiling all the materials needed for the multiple cell divisions required to generate a swimming, feeding tadpole. When stimulated by a male, an adult female's ovarian cells secrete the steroid hormone progesterone, which induces the G_2-arrested oocytes to enter meiosis I and progress through meiosis to the second meiotic metaphase (Figure 21-5). At this stage the cells are called *eggs*. When fertilized by sperm, the egg nucleus is released from its metaphase II arrest and completes meiosis. The resulting haploid egg pronucleus then fuses with the haploid sperm pronucleus, producing a diploid **zygote** nucleus. DNA replication follows and the first mitotic division of early embryogenesis begins. The resulting embryonic cells then proceed through 11 more rapid, synchronous cell cycles generating a hollow sphere, the blastula. Cell division then



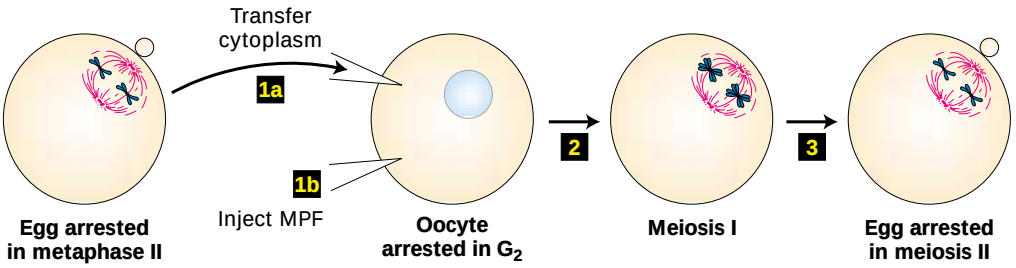
▲ EXPERIMENTAL FIGURE 21-5 **Progesterone stimulates meiotic maturation of *Xenopus* oocytes in vitro.** Step 1: Treatment of G_2-arrested *Xenopus* oocytes surgically removed from the ovary of an adult female with progesterone causes the oocytes to enter meiosis I. Two pairs of synapsed homologous chromosomes (blue) connected to mitotic spindle microtubules (red) are shown schematically to represent cells in metaphase of meiosis I. Step 2: Segregation of homologous chromosomes and a highly asymmetrical cell division expels half the chromosomes into a small cell called the first polar body. The oocyte immediately commences meiosis II and arrests in metaphase to yield an egg. Two chromosomes connected to spindle microtubules are shown schematically to represent egg cells arrested in metaphase of meiosis II. Step 3: Fertilization by sperm releases eggs from their metaphase arrest, allowing them to proceed through anaphase of meiosis II and undergo a second highly asymmetrical cell division that eliminates one chromatid of each chromosome in a second polar body. Step 4: The resulting haploid female pronucleus fuses with the haploid sperm pronucleus to produce a diploid zygote, which undergoes DNA replication and the first mitosis of 12 synchronous early embryonic cleavages.

slows, and subsequent divisions are nonsynchronous with cells at different positions in the blastula dividing at different times.

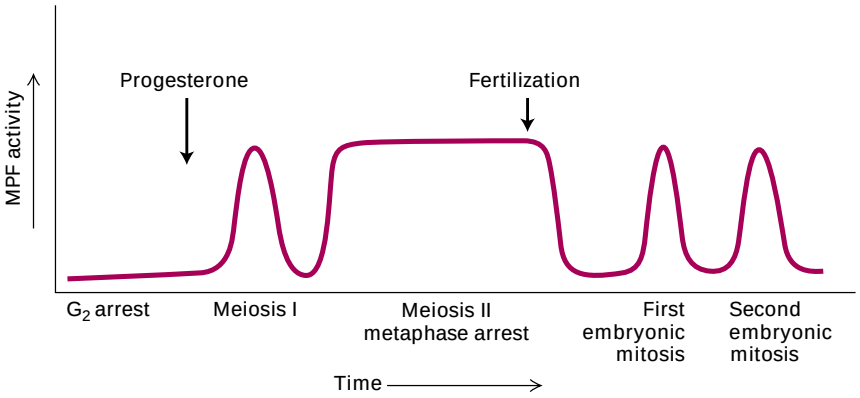
Maturation-Promoting Factor (MPF) Stimulates Meiotic Maturation of Oocytes and Mitosis in Somatic Cells

When G_2-arrested *Xenopus* oocytes are removed from the ovary of an adult female frog and treated with progesterone, they undergo *meiotic maturation*, the process of oocyte maturation from a G_2-arrested oocyte to the egg arrested in metaphase of meiosis II (see Figure 21-5). Microinjection of cytoplasm from eggs arrested in metaphase of meiosis II into G_2-arrested oocytes stimulates the oocytes to mature into eggs in the absence of progesterone (Figure 21-6). This system not only led to the initial identification of a factor in egg cytoplasm that stimulates maturation of oocytes in vitro but also provided an assay for this factor, called *maturation-promoting factor (MPF)*. As we will see shortly, MPF turned out to be the key factor that regulates the initiation of mitosis in all eukaryotic cells.

Using the microinjection system to assay MPF activity at different times during oocyte maturation in vitro, researchers found that untreated G_2-arrested oocytes have low levels of MPF activity; treatment with progesterone induces MPF activity as the cells enter meiosis I (Figure 21-7). MPF activity falls as the cells enter the interphase between meiosis I and II, but then rises again as the cells enter meiosis II and remains high in the egg cells arrested in metaphase II. Following



▲ EXPERIMENTAL FIGURE 21-6 **A diffusible factor in arrested *Xenopus* eggs promotes meiotic maturation.** When ≈5 percent of the cytoplasm from an unfertilized *Xenopus* egg arrested in metaphase of meiosis II is microinjected into a G_2-arrested oocyte (step 1a), the oocyte enters meiosis I (step 2) and proceeds to metaphase of meiosis II (step 3), generating a mature egg in the absence of progesterone. This process can be repeated multiple times without further addition of progesterone, showing that egg cytoplasm contains an oocyte maturation-promoting factor (MPF). Microinjection of G_2-arrested oocytes provided the first assay for MPF activity (step 1b) at different stages of the cell cycle and in different organisms. [See Y. Masui and C. L. Markert, 1971, *J. Exp. Zool.* **177**:129.]



◀ EXPERIMENTAL FIGURE 21-7 **MPF activity in *Xenopus* oocytes, eggs, and early embryos peaks as cells enter meiosis and mitosis.** Diagrams of the cell structures corresponding to each stage are shown in Figure 21-5. MPF activity was determined by the microinjection assay shown in Figure 21-6 and quantitated by making dilutions of cell extracts. See text for discussion. [See J. Gerhart et al., 1984, *J. Cell Biol.* **98**:1247; adapted from A. Murray and M. W. Kirschner, 1989, *Nature* **339**:275.]

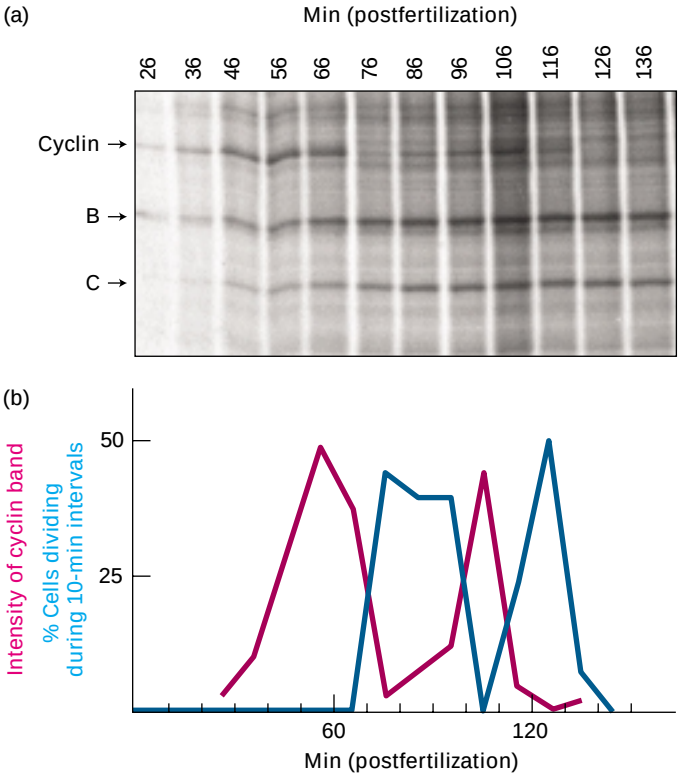
fertilization, MPF activity falls again until the zygote (fertilized egg) enters the first mitosis of embryonic development. Throughout the following 11 synchronous cycles of mitosis in the early embryo, MPF activity is low in the interphase periods between mitoses and then rises as the cells enter mitosis.

Although initially discovered in frogs, MPF activity has been found in mitotic cells from all species assayed. For example, cultured mammalian cells can be arrested in mitosis by treatment with compounds (e.g., colchicine) that inhibit assembly of microtubules. When cytoplasm from such mitotically arrested mammalian cells was injected into G_2-arrested *Xenopus* oocytes, the oocytes matured into eggs; that is, the mammalian somatic mitotic cells contained a cytosolic factor that exhibited frog MPF activity. This finding suggested that MPF controls the entry of mammalian somatic cells into mitosis as well as the entry of frog oocytes into meiosis. When cytoplasm from mitotically arrested mammalian somatic cells was injected into interphase cells, the interphase cells entered mitosis; that is, their nuclear membranes disassembled and their chromosomes condensed. Thus MPF is the diffusible factor, first revealed in cell-fusion experiments (see Figure 21-3), that promotes entry of cells into mitosis. Conveniently, the acronym MPF also can stand for **mitosis-promoting factor,** a name that denotes the more general activity of this factor.

Because the oocyte injection assay initially used to measure MPF activity is cumbersome, several years passed before MPF was purified by column chromatography and characterized. MPF is in fact one of the heterodimeric complexes composed of a cyclin and CDK now known to regulate the cell cycle. Each MPF subunit initially was recognized through different experimental approaches. First we discuss how the regulatory cyclin subunit was identified and then describe how yeast genetic experiments led to discovery of the catalytic CDK subunit.

Mitotic Cyclin Was First Identified in Early Sea Urchin Embryos

Experiments with inhibitors showed that new protein synthesis is required for the increase in MPF during the mitotic phase of each cell cycle in early frog embryos. As in early



▲ EXPERIMENTAL FIGURE 21-8 **Experimental detection of cyclical synthesis and destruction of mitotic cyclin in sea urchin embryos.** A suspension of sea urchin eggs was synchronously fertilized by the addition of sea urchin sperm, and ^{35}S-methionine was added. At 10-minute intervals beginning 16 minutes after fertilization, samples were taken for protein analysis on an SDS polyacrylamide gel and for detection of cell cleavage by microscopy. (a) Autoradiograms of the SDS gels at each sampling time. Most proteins, such as X and Y, continuously increased in intensity. In contrast, cyclin suddenly decreased in intensity at 76 minutes after fertilization and then began increasing again at 86 minutes. The cyclin band peaked again at 106 min and decreased again at 126 min. (b) Plot of the intensity of the cyclin band (red line) and the fraction of cells that had undergone cleavage during the previous 10-minute interval (cyan line). Note that the amount of cyclin fell precipitously just before cell cleavage. [From T. Evans et al., 1983, *Cell* **33**:389; courtesy of R. Timothy Hunt, Imperial Cancer Research Fund.]

frog embryos, the initial cell cycles in the early sea urchin embryo occur synchronously, with all the embryonic cells entering mitosis simultaneously. Biochemical studies with sea urchin eggs and embryos led to identification of the cyclin component of MPF. These studies with synchronously fertilized sea urchin eggs revealed that the concentration of one protein peaked early in mitosis, fell abruptly just before cell cleavage, and then accumulated during the following interphase to peak early in the next mitosis and fall abruptly just before the second cleavage (Figure 21-8). Careful analysis showed that this protein, named *cyclin B*, is synthesized continuously during the embryonic cell cycles but is abruptly destroyed following anaphase. Since its concentration peaks in mitosis, cyclin B functions as a *mitotic cyclin*.

In subsequent experiments, a cDNA clone encoding sea urchin cyclin B was used as a probe to isolate a homologous cyclin B cDNA from *Xenopus laevis*. Pure *Xenopus* cyclin B, obtained by expression of the cDNA in *E. coli*, was used to produce antibody specific for cyclin B. Using this antibody, a polypeptide was detected in Western blots that copurified with MPF activity from *Xenopus* eggs, demonstrating that one subunit of MPF is indeed cyclin B.

Cyclin B Levels and Kinase Activity of Mitosis-Promoting Factor (MPF) Change Together in Cycling *Xenopus* Egg Extracts

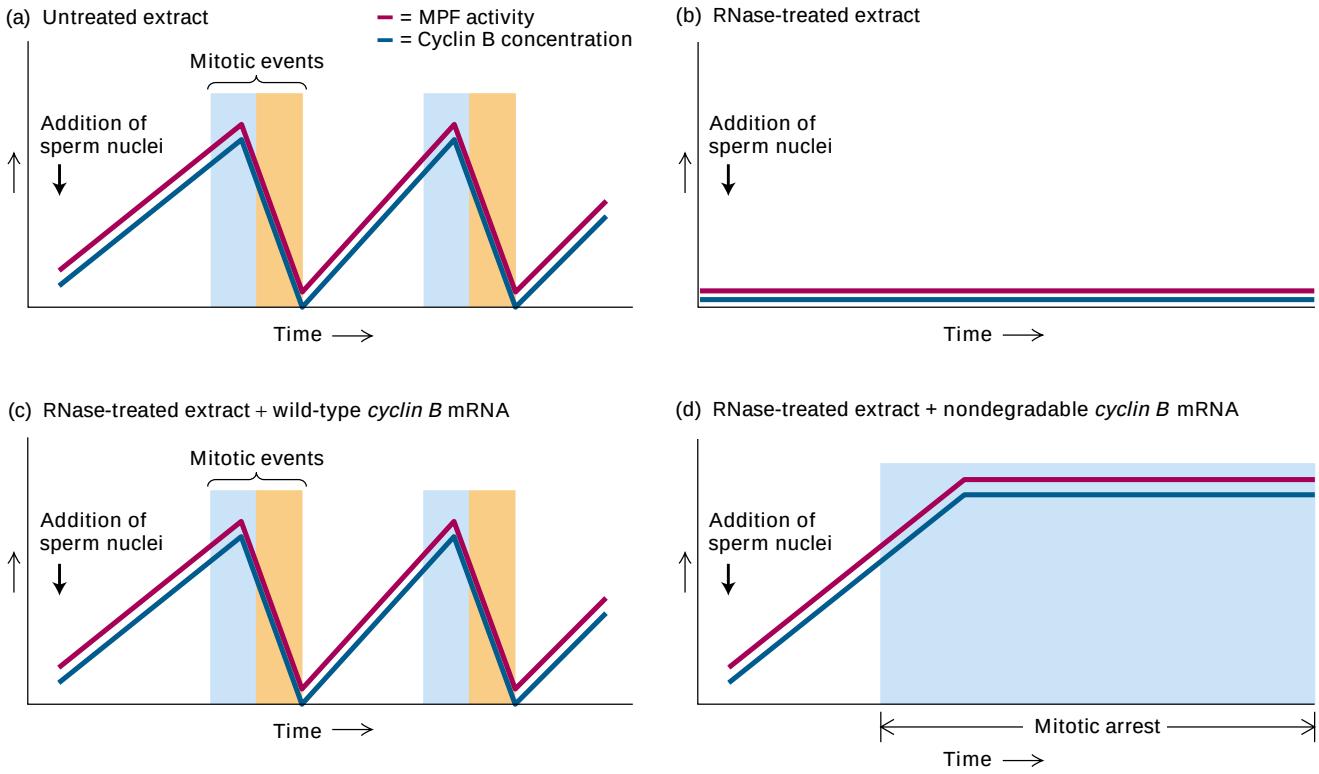
Some unusual aspects of the synchronous cell cycles in early *Xenopus* embryos provided a way to study the role of mitotic cyclin in controlling MPF activity. First, following fertilization of *Xenopus* eggs, the G_1 and G_2 periods are minimized during the initial 12 synchronous cell cycles. That is, once mitosis is complete, the early embryonic cells proceed immediately into the next S phase, and once DNA replication is complete, the cells progress almost immediately into the next mitosis. Second, the oscillation in MPF activity that occurs as early frog embryos enter and exit mitosis is observed in the cytoplasm of a fertilized frog egg even when the nucleus is removed and no transcription can occur. This finding indicates that all the cellular components required for progress through the truncated cell cycles in early *Xenopus* embryos are stored in the unfertilized egg. In somatic cells generated later in development and in yeasts considered in later sections, specific mRNAs must be produced at particular points in the cell cycle for progress through the cycle to proceed. But in early *Xenopus* embryos, all the mRNAs necessary for the early cell divisions are present in the unfertilized egg.

Extracts prepared from unfertilized *Xenopus* eggs thus contain all the materials required for multiple cell cycles, including the enzymes and precursors needed for DNA replication, the histones and other chromatin proteins involved in assembling the replicated DNA into chromosomes, and the proteins and phospholipids required in formation of the nuclear envelope. These egg extracts also synthesize proteins encoded by mRNAs in the extract, including cyclin B.

When nuclei prepared from *Xenopus* sperm are added to such a *Xenopus* egg extract, the highly condensed sperm chromatin decondenses, and the sperm DNA replicates one time. The replicated sperm chromosomes then condense and the nuclear envelope disassembles, just as it does in intact cells entering mitosis. About 10 minutes after the nuclear envelope disassembles, all the cyclin B in the extract suddenly is degraded, as it is in intact cells following anaphase. Following cyclin B degradation, the sperm chromosomes decondense and a nuclear envelope re-forms around them, as in an intact cell at the end of mitosis. After about 20 minutes, the cycle begins again. DNA within the nuclei formed after the first mitotic period (now 2*n*) replicates, forming 4*n* nuclei. Cyclin B, synthesized from the cyclin B mRNA present in the extract, accumulates. As cyclin B approaches peak levels, the chromosomes condense once again, the nuclear envelopes break down, and about 10 minutes later cyclin B is once again suddenly destroyed. These remarkable *Xenopus* egg extracts can mediate several of these cycles, which mimic the rapid synchronous cycles of an early frog embryo.

Studies with this egg extract experimental system were aided by development of a new assay for MPF activity. Using MPF purified with the help of the oocyte injection assay (see Figure 21-6), researchers had found that MPF phosphorylates histone H1. This H1 kinase activity provided a much simpler and more easily quantitated assay for MPF activity than the oocyte injection assay. Armed with a convenient assay, researchers tracked the MPF activity and concentration of cyclin B in cycling *Xenopus* egg extracts. These studies showed that MPF activity rises and falls in synchrony with the concentration of cyclin B (Figure 21-9a). The early events of mitosis—chromosome condensation and nuclear envelope disassembly—occurred when MPF activity reached its highest levels in parallel with the rise in cyclin B concentration. Addition of cycloheximide, an inhibitor of protein synthesis, prevented cyclin B synthesis and also prevented the rise in MPF activity, chromosome condensation, and nuclear envelope disassembly.

To test the functions of cyclin B in these cell-cycle events, all mRNAs in the egg extract were degraded by digestion with a low concentration of RNase, which then was inactivated by addition of a specific inhibitor. This treatment destroys mRNAs without affecting the tRNAs and rRNAs required for protein synthesis, since their degradation requires much higher concentrations of RNase. When sperm nuclei were added to the RNase-treated extracts, the 1*n* nuclei replicated their DNA, but the increase in MPF activity and the early mitotic events (chromosome condensation and nuclear envelope disassembly), which the untreated extract supports, did not occur (Figure 21-9b). Addition of *cyclin B* mRNA, produced in vitro from cloned *cyclin B* cDNA, to the RNase-treated egg extract and sperm nuclei restored the parallel oscillations in the cyclin B concentration and MPF activity and the characteristic early and late mitotic events as observed with the untreated egg extract (Figure 21-9c). Since cyclin B is the only protein synthesized under these conditions, these results demonstrate that it is the



▲ EXPERIMENTAL FIGURE 21-9 **Cycling of MPF activity and mitotic events in *Xenopus* egg extracts depend on synthesis and degradation of cyclin B.** In all cases, MPF activity and cyclin B concentration were determined at various times after addition of sperm nuclei to a *Xenopus* egg extract treated as indicated in each panel. Microscopic observations determined the occurrence of early mitotic events (blue shading), including chromosome condensation and nuclear envelope disassembly, and of late events (orange shading), including chromosome decondensation and nuclear envelope reassembly. See text for discussion. [See A. W. Murray et al., 1989, *Nature* **339**:275; adapted from A. Murray and T. Hunt, 1993, *The Cell Cycle: An Introduction,* W. H. Freeman and Company.]

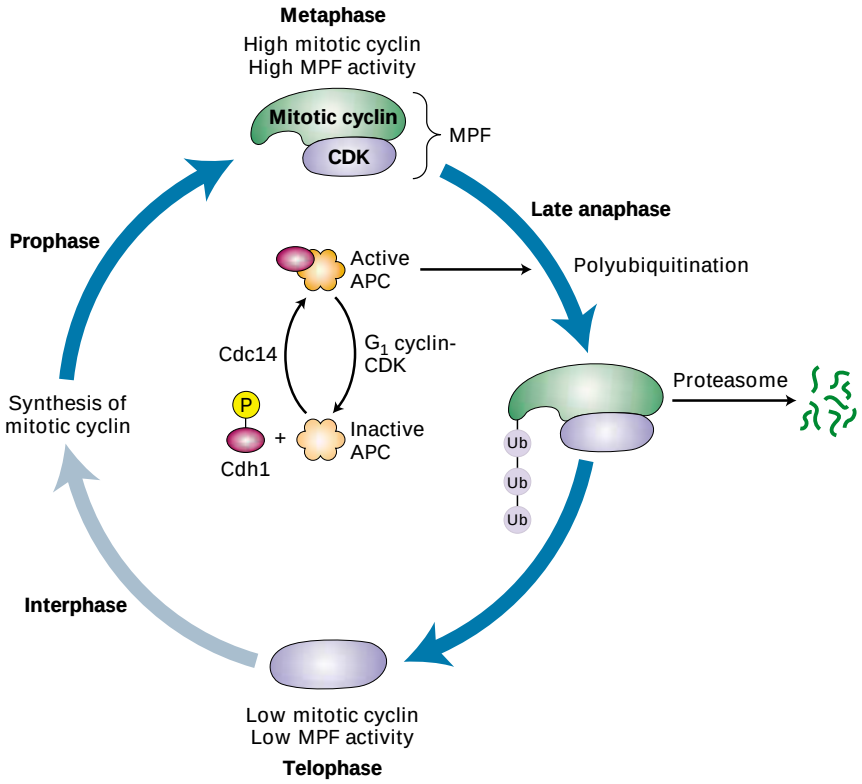
crucial protein whose synthesis is required to regulate MPF activity and the cycles of chromosome condensation and nuclear envelope breakdown mediated by cycling *Xenopus* egg extracts.

In these experiments, chromosome decondensation and nuclear envelope assembly (late mitotic events) coincided with decreases in the cyclin B level and MPF activity. To determine whether degradation of cyclin B is required for exit from mitosis, researchers added a mutant mRNA encoding a nondegradable cyclin B to a mixture of RNase-treated *Xenopus* egg extract and sperm nuclei. As shown in Figure 21-9d, MPF activity increased in parallel with the level of the mutant cyclin B, triggering condensation of the sperm chromatin and nuclear envelope disassembly (early mitotic events). However, the mutant cyclin B produced in this reaction never was degraded. As a consequence, MPF activity remained elevated, and the late mitotic events of chromosome decondensation and nuclear envelope formation were both blocked. This experiment demonstrates that the fall in MPF activity and exit from mitosis depend on degradation of cyclin B.

The results of the two experiments with RNase-treated extracts show that entry into mitosis requires the accumulation of cyclin B, the *Xenopus* mitotic cyclin, to high levels, and that exit from mitosis requires the degradation of this mitotic cyclin. Since MPF kinase activity varied in parallel with the concentration of the mitotic cyclin, the results implied that high MPF kinase activity results in entry into mitosis and that a fall in MPF kinase activity is required to exit mitosis.

Anaphase-Promoting Complex (APC) Controls Degradation of Mitotic Cyclins and Exit from Mitosis

Further studies revealed that vertebrate cells contain three proteins that can function like cyclin B to stimulate *Xenopus* oocyte maturation: two closely related cyclin Bs and cyclin A. Collectively called *B-type cyclins,* these proteins exhibit regions of high sequence homology. (B-type cyclins are distinguished from G_1 cyclins described in Section 21.5.) In intact cells, degradation of all the B-type cyclins begins after the onset of anaphase, the period of mitosis when sister chromatids are separated and pulled toward opposite spindle poles.



◀ FIGURE 21-10 **Regulation of mitotic cyclin levels in cycling *Xenopus* early embryonic cells.** In late anaphase, the anaphase-promoting complex (APC) polyubiquitinates mitotic cyclins. As the cyclins are degraded by proteasomes, MPF kinase activity declines precipitously, triggering the onset of telophase. APC activity is directed toward mitotic cyclins by a specificity factor, called Cdh1, that is phosphorylated and thereby inactivated by G_1 cyclin-CDK complexes. A specific phosphatase called Cdc14 removes the regulatory phosphate from the specificity factor late in anaphase. Once the specificity factor is inhibited in G_1, the concentration of mitotic cyclin increases, eventually reaching a high enough level to stimulate entry into the subsequent mitosis.

Biochemical studies with *Xenopus* egg extracts showed that at the time of their degradation, wild-type B-type cyclins are modified by covalent addition of multiple **ubiquitin** molecules. This process of polyubiquitination marks proteins for rapid degradation in eukaryotic cells by **proteasomes,** multiprotein cylindrical structures containing numerous proteases. Addition of a ubiquitin chain to a B-type cyclin or other target protein requires three types of enzymes (see Figure 3-13). Ubiquitin is first activated at its carboxyl-terminus by formation of a thioester bond with a cysteine residue of *ubiquitin-activating enzyme,* E1. Ubiquitin is subsequently transferred from E1 to a cysteine of one of a class of related enzymes called *ubiquitin-conjugating enzymes,* E2. The specific E2 determines, along with a third protein, *ubiquitin ligase* (E3), the substrate protein to which a ubiquitin chain will be covalently linked. Many ubiquitin ligases are multisubunit proteins.

Sequencing of cDNAs encoding several B-type cyclins from various eukaryotes showed that all contain a homologous nine-residue sequence near the N-terminus called the *destruction box*. Deletion of this destruction box, as in the mutant mRNA used in the experiment depicted in Figure 21-8, prevents the polyubiquitination of B-type cyclins and thus makes them nondegradable. The ubiquitin ligase that recognizes the mitotic cyclin destruction box is a multisubunit protein called the *anaphase-promoting complex (APC),* which we introduced earlier in the chapter (see Figure 21-2, steps 8 and 9).

Figure 21-10 depicts the current model that best explains the changes in mitotic cyclin levels seen in cycling *Xenopus* early embryonic cells. Cyclin B, the primary mitotic cyclin in multicellular animals (metazoans), is synthesized throughout the cell cycle from a stable mRNA. The observed fall in its concentration in late anaphase results from its APC-stimulated degradation at this point in the cell cycle. As we discuss in Section 21.5, genetic studies with yeast led to identification of an *APC specificity factor,* called *Cdh1,* that binds to APC and directs it to polyubiquitinate mitotic cyclins. This specificity factor is active only in late anaphase when the segregating chromosomes have moved far enough apart in the dividing cell to assure that both daughter cells will contain one complete set of chromosomes. Phosphorylation of Cdh1 by other cyclin-CDK complexes during G_1 inhibits its association with the APC and thus degradation of mitotic cyclin (see Figure 21-2, step 2). This inhibition permits the gradual rise in mitotic cyclin levels observed throughout interphase of the next cell cycle.

KEY CONCEPTS OF SECTION 21.2

Biochemical Studies with Oocytes, Eggs, and Early Embryos

- MPF is a protein kinase that requires a mitotic cyclin for activity. The protein kinase activity of MPF stimulates the onset of mitosis by phosphorylating multiple specific protein substrates, most of which remain to be identified.
- In the synchronously dividing cells of early *Xenopus* and sea urchin embryos, the concentration of mitotic cyclins

(e.g., cyclin B) and MPF activity increase as cells enter mitosis and then fall as cells exit mitosis (see Figures 21-7 and 21-8).

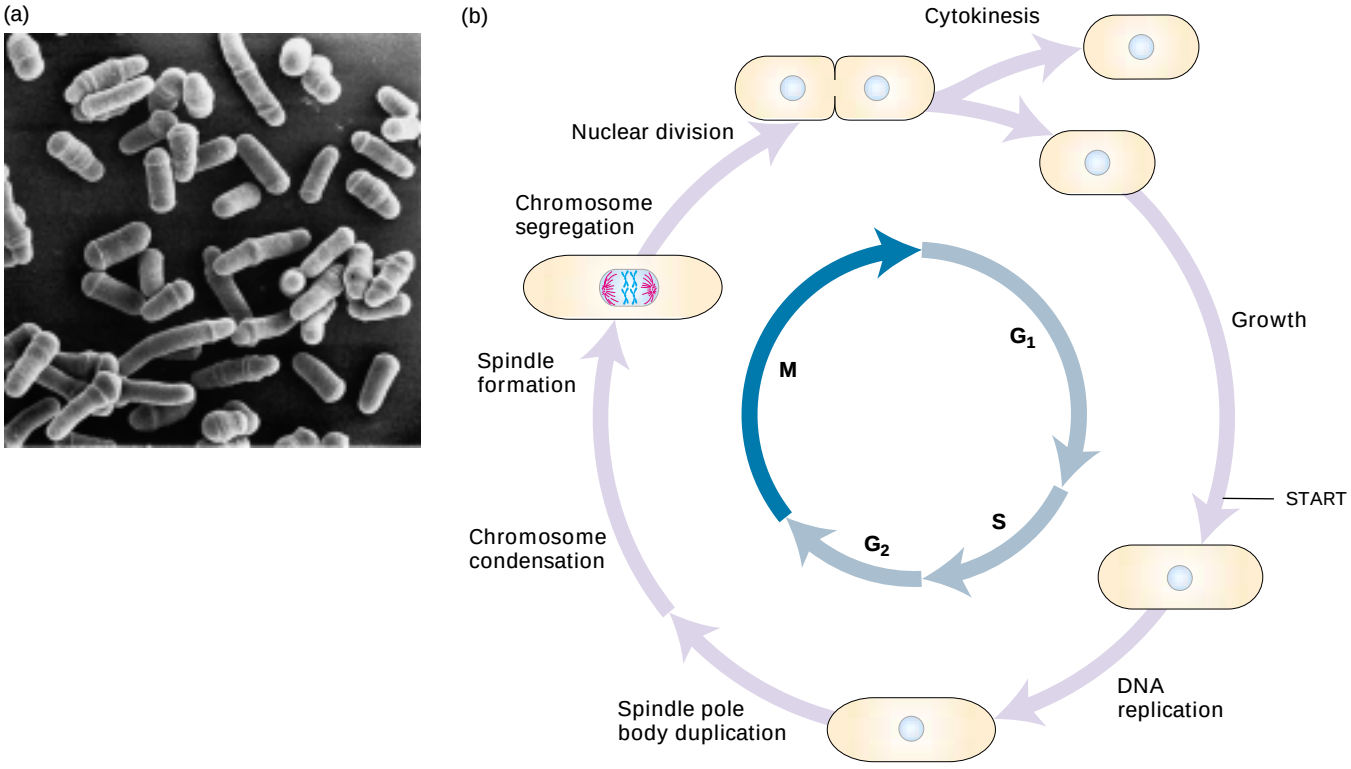
- The rise and fall in MPF activity during the cell cycle result from concomitant synthesis and degradation of mitotic cyclin (see Figure 21-9).
- The multisubunit anaphase-promoting complex (APC) is a ubiquitin ligase that recognizes a conserved destruction box sequence in mitotic cyclins and promotes their polyubiquitination, marking the proteins for rapid degradation by proteasomes. The resulting decrease in MPF activity leads to completion of mitosis.
- The ubiquitin ligase activity of APC is controlled so that mitotic cyclins are polyubiquitinated only during late anaphase (see Figure 21-10). Deactivation of APC in G_1 permits accumulation of mitotic cyclins during the next cell cycle. This results in the cyclical increases and decreases in MPF activity that cause the entry into and exit from mitosis.

21.3 Genetic Studies with *S. pombe*

The studies with *Xenopus* egg extracts described in the previous section showed that continuous synthesis of a mitotic cyclin followed by its periodic degradation at late anaphase is required for the rapid cycles of mitosis observed in early *Xenopus* embryos. Identification of the catalytic protein kinase subunit of MPF and further insight into its regulation came from genetic analysis of the cell cycle in the fission yeast *S. pombe*. This yeast grows as a rod-shaped cell that increases in length as it grows and then divides in the middle during mitosis to produce two daughter cells of equal size (Figure 21-11).

In wild-type *S. pombe*, entry into mitosis is carefully regulated in order to properly coordinate cell division with cell growth. Temperature-sensitive mutants of *S. pombe* with conditional defects in the ability to progress through the cell cycle are easily recognized because they cause characteristic changes in cell length at the nonpermissive temperature. The many such mutants that have been isolated fall into two groups. In the first group are *cdc* mutants, which fail to progress through one of the phases of the cell cycle at the nonpermissive temperature; they form extremely long cells because they continue to grow in length, but fail to divide. In contrast, *wee* mutants form smaller-than-normal cells because they are defective in the proteins that normally prevent cells from dividing when they are too small.

In *S. pombe* wild-type genes are indicated in italics with a superscript plus sign (e.g., $cdc2^+$); genes with a recessive



▲ FIGURE 21-11 **The fission yeast *S. pombe*.** (a) Scanning electron micrograph of *S. pombe* cells at various stages of the cell cycle. Long cells are about to enter mitosis; short cells have just passed through cytokinesis. (b) Main events in the *S. pombe* cell cycle. Note that the nuclear envelope does not disassemble during mitosis in *S. pombe* and other yeasts. [Part (a) courtesy of N. Hajibagheri.]

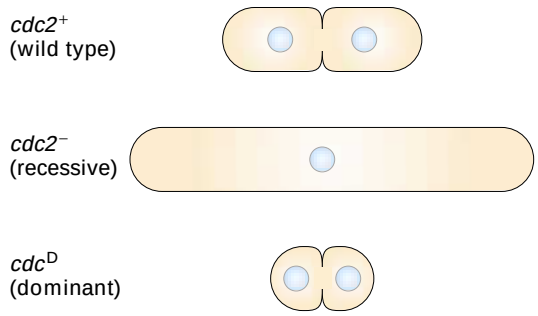
MEDIA CONNECTIONS
Video: Mitosis and Cell Division in *S. pombe*

mutation, in italics with a superscript minus sign (e.g., *cdc2*$^-$). The protein encoded by a particular gene is designated by the gene symbol in Roman type with an initial capital letter (e.g., Cdc2).

A Highly Conserved MPF-like Complex Controls Entry into Mitosis in *S. pombe*

Temperature-sensitive mutations in *cdc2*, one of several different *cdc* genes in *S. pombe*, produce opposite phenotypes depending on whether the mutation is recessive or dominant (Figure 21-12). Recessive mutations (*cdc2*$^-$) give rise to abnormally long cells, whereas dominant mutations (*cdc2*^{D}) give rise to abnormally small cells, the wee phenotype. As discussed in Chapter 9, recessive mutations generally cause a *loss* of the wild-type protein function; in diploid cells, both alleles must be mutant in order for the mutant phenotype to be observed. In contrast, dominant mutations generally result in a *gain* in protein function, either because of overproduction or lack of regulation; in this case, the presence of only one mutant allele confers the mutant phenotype in diploid cells. The finding that a loss of Cdc2 activity (*cdc2*$^-$ mutants) prevents entry into mitosis and a gain of Cdc2 activity (*cdc2*^{D} mutants) brings on mitosis earlier than normal identified Cdc2 as a key regulator of entry into mitosis in *S. pombe*.

The wild-type *cdc2*$^+$ gene contained in a *S. pombe* plasmid library was identified and isolated by its ability to complement *cdc2*$^-$ mutants (see Figure 21-4). Sequencing showed that *cdc2*$^+$ encodes a 34-kDa protein with homology to eukaryotic protein kinases. In subsequent studies, researchers identified cDNA clones from other organisms that could complement *S. pombe cdc2*$^-$ mutants. Remarkably, they isolated a human cDNA encoding a protein identical to *S. pombe* Cdc2 in 63 percent of its residues.



▲ EXPERIMENTAL FIGURE 21-12 **Recessive and dominant *S. pombe cdc2* mutants have opposite phenotypes.** Wild-type cell (*cdc2*$^+$) is schematically depicted just before cytokinesis with two normal-size daughter cells. A recessive *cdc2*$^-$ mutant cannot enter mitosis at the nonpermissive temperature and appears as an elongated cell with a single nucleus, which contains duplicated chromosomes. A dominant *cdc2*^{D} mutant enters mitosis prematurely before reaching normal size in G_2; thus, the two daughter cells resulting from cytokinesis are smaller than normal, that is, they have the wee phenotype.

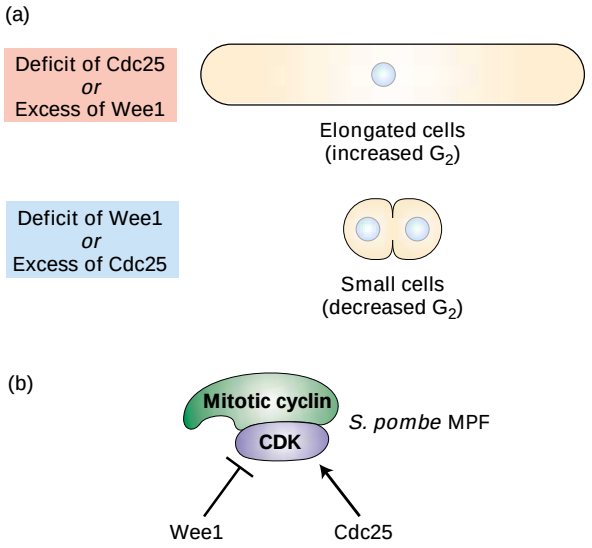
Isolation and sequencing of another *S. pombe cdc* gene (*cdc13*$^+$), which also is required for entry into mitosis, revealed that it encodes a protein with homology to sea urchin and *Xenopus* cyclin B. Further studies showed that a heterodimer of Cdc13 and Cdc2 forms the *S. pombe* MPF; like *Xenopus* MPF, this heterodimer has protein kinase activity that will phosphorylate histone H1. Moreover, the H1 protein kinase activity rises as *S. pombe* cells enter mitosis and falls as they exit mitosis in parallel with the rise and fall in the Cdc13 level. These findings, which are completely analogous to the results obtained with *Xenopus* egg extracts (see Figure 21-9a), identified Cdc13 as the mitotic cyclin in *S. pombe*. Further studies showed that the isolated Cdc2 protein and its homologs in other eukaryotes have little protein kinase activity until they are bound by a cyclin. Hence, this family of protein kinases became known as cyclin-dependent kinases, or CDKs.

Researchers soon found that antibodies raised against a highly conserved region of Cdc2 recognize a polypeptide that co-purifies with MPF purified from *Xenopus* eggs. Thus *Xenopus* MPF is also composed of a mitotic cyclin (cyclin B) and a CDK (called CDK1). This convergence of findings from biochemical studies in an invertebrate (sea urchin) and a vertebrate (*Xenopus*) and from genetic studies in a yeast indicated that entry into mitosis is controlled by analogous mitotic cyclin-CDK complexes in all eukaryotes (see Figure 21-2, step 7). Moreover, most of the participating proteins have been found to be highly conserved during evolution.

Phosphorylation of the CDK Subunit Regulates the Kinase Activity of MPF

Analysis of additional *S. pombe cdc* mutants revealed that proteins encoded by other genes regulate the protein kinase activity of the mitotic cyclin-CDK complex (MPF) in fission yeast. For example, temperature-sensitive *cdc25*$^-$ mutants are delayed in entering mitosis at the nonpermissive temperature, producing elongated cells. On the other hand, overexpression of Cdc25 from a plasmid present in multiple copies per cell decreases the length of G_2 causing premature entry into mitosis and small (wee) cells (Figure 21-13a). Conversely, loss-of-function mutations in the *wee1*$^+$ gene causes premature entry into mitosis resulting in small cells, whereas overproduction of Wee1 protein increases the length of G_2 resulting in elongated cells. A logical interpretation of these findings is that Cdc25 protein stimulates the kinase activity of *S. pombe* MPF, whereas Wee1 protein inhibits MPF activity (Figure 21-13b).

In subsequent studies, the wild-type *cdc25*$^+$ and *wee1*$^+$ genes were isolated, sequenced, and used to produce the encoded proteins with suitable expression vectors. The deduced sequences of Cdc25 and Wee1 and biochemical studies of the proteins demonstrated that they regulate the kinase activity of *S. pombe* MPF by phosphorylating and dephosphorylating specific regulatory sites in Cdc2, the CDK subunit of MPF.



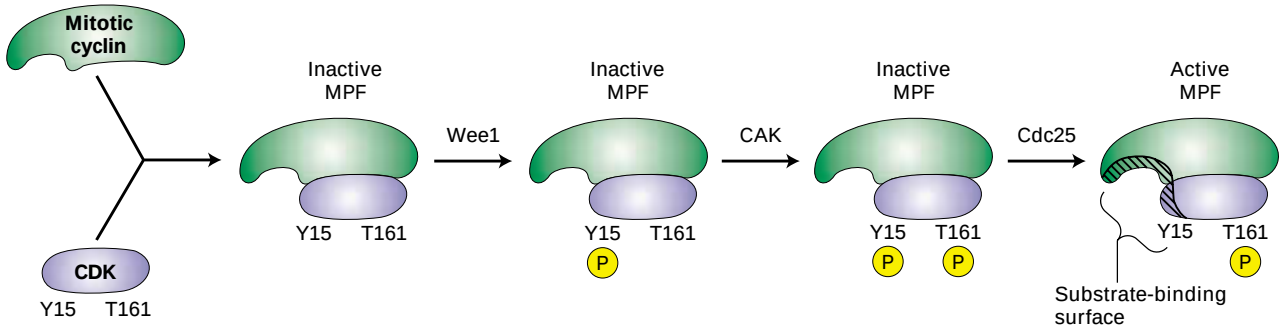
▲ EXPERIMENTAL FIGURE 21-13 **Cdc25 and Wee1 have opposing effects on *S. pombe* MPF activity.** (a) Cells that lack Cdc25 or Wee1 activity, as a result of recessive temperature-sensitive mutations in the corresponding genes, have the opposite phenotype. Likewise, cells with multiple copies of plasmids containing wild-type *cdc25*$^+$ or *wee1*$^+$, and which thus produce an excess of the encoded proteins, have opposite phenotypes. (b) These phenotypes imply that the mitotic cyclin-CDK complex is activated (→) by Cdc25 and inhibited (—l) by Wee1. See text for further discussion.

Figure 21-14 illustrates the functions of four proteins that regulate the protein kinase activity of the *S. pombe* CDK. First is Cdc13, the mitotic cyclin of *S. pombe* (equivalent to cyclin B in metazoans), which associates with the CDK to form MPF with extremely low activity. Second is the **Wee1 protein-tyrosine kinase,** which phosphorylates an inhibitory tyrosine residue (Y15) in the CDK subunit. Third is another kinase, designated CDK-activating kinase (CAK), which phosphorylates an activating threonine residue (T161). When both residues are phosphorylated, MPF is inactive. Finally, the *Cdc25 phosphatase* removes the phosphate from Y15, yielding highly active MPF. Site-specific mutagenesis that changed the Y15 in *S. pombe* CDK to a phenylalanine, which cannot be phosphorylated, produced mutants with the wee phenotype, similar to that of *wee1*$^-$ mutants. Both mutations prevent the inhibitory phosphorylation at Y15, resulting in the inability to properly regulate MPF activity, and, consequently, premature entry into mitosis.

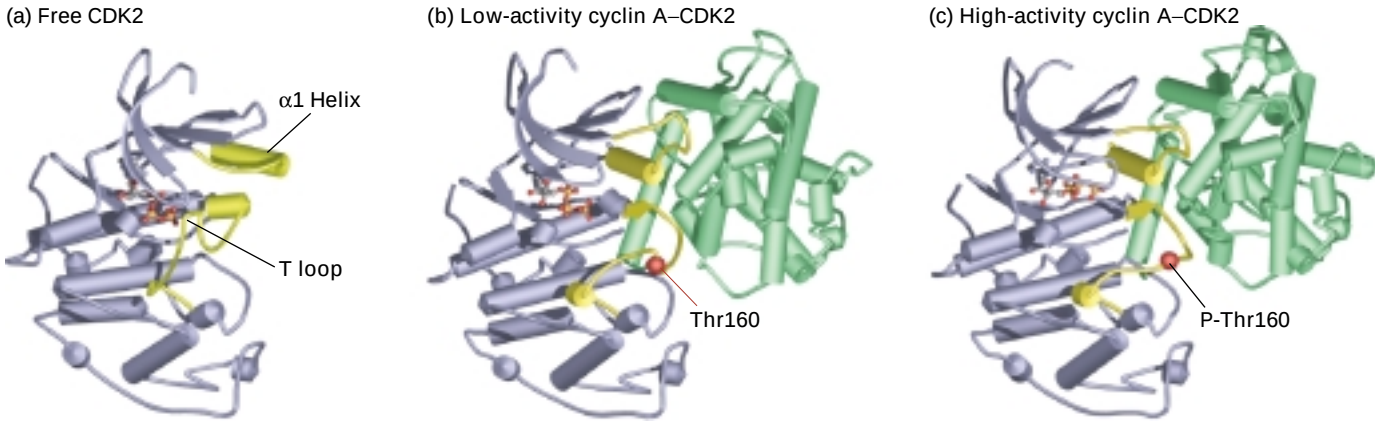
Conformational Changes Induced by Cyclin Binding and Phosphorylation Increase MPF Activity

Unlike both fission and budding yeasts, each of which produce just one CDK, vertebrates produce several CDKs (see Table 21-1). The three-dimensional structure of one human cyclin-dependent kinase (CDK2) has been determined and provides insight into how cyclin binding and phosphorylation of CDKs regulate their protein kinase activity. Although the three-dimensional structures of the *S. pombe* CDK and most other cyclin-dependent kinases have not been determined, their extensive sequence homology with human CDK2 suggests that all these CDKs have a similar structure and are regulated by a similar mechanism.

Unphosphorylated, inactive CDK2 contains a flexible region, called the T-loop, that blocks access of protein substrates to the active site where ATP is bound (Figure 21-15a). Steric blocking by the T-loop largely explains why free CDK2, unbound to cyclin, has no protein kinase activity. Unphosphorylated CDK2 bound to one of its cyclin partners, cyclin A, has minimal but detectable protein kinase activity in vitro, although it may be essentially inactive in vivo. Extensive interactions between cyclin A and the T-loop cause a dramatic shift in the position of the T-loop, thereby exposing the CDK2 active site (Figure 21-15b). Binding of cyclin A also shifts the position of the α1 helix in CDK2, modifying its substrate-binding surface. Phosphorylation of the activating threonine, located



▲ FIGURE 21-14 **Regulation of the kinase activity of *S. pombe* mitosis-promoting factor (MPF).** Interaction of mitotic cyclin (Cdc13) with cyclin-dependent kinase (Cdc2) forms MPF. The CDK subunit can be phosphorylated at two regulatory sites: by Wee1 at tyrosine-15 (Y15) and by CDK-activating kinase (CAK) at threonine-161 (T161). Removal of the phosphate on Y15 by Cdc25 phosphatase yields active MPF in which the CDK subunit is monophosphorylated at T161. The mitotic cyclin subunit contributes to the specificity of substrate binding by MPF, probably by forming part of the substrate-binding surface (cross-hatch), which also includes the inhibitory Y15 residue.



▲ FIGURE 21-15 **Structural models of human CDK2, which is homologous to the *S. pombe* cyclin-dependent kinase (CDK).** (a) Free, inactive CDK2 unbound to cyclin A. In free CDK2, the T-loop blocks access of protein substrates to the γ-phosphate of the bound ATP, shown as a ball-and-stick model. The conformations of the regions highlighted in yellow are altered when CDK is bound to cyclin A. (b) Unphosphorylated, low-activity cyclin A–CDK2 complex. Conformational changes induced by binding of a domain of cyclin A (green) cause the T-loop to pull away from the active site of CDK2, so that substrate proteins can bind. The α1 helix in CDK2, which interacts extensively with cyclin A, moves several angstroms into the catalytic cleft, repositioning key catalytic side chains required for the phosphorotransfer reaction to substrate specificity. The red ball marks the position equivalent to threonine 161 in *S. pombe* Cdc2. (c) Phosphorylated, high-activity cyclin A–CDK2 complex. The conformational changes induced by phosphorylation of the activating threonine (red ball) alter the shape of the substrate-binding surface, greatly increasing the affinity for protein substrates. [Courtesy of P. D. Jeffrey. See A. A. Russo et al., 1996, *Nature Struct. Biol.* **3**:696.]

in the T-loop, causes additional conformational changes in the cyclin A–CDK2 complex that greatly increase its affinity for protein substrates (Figure 21-15c). As a result, the kinase activity of the phosphorylated complex is a hundredfold greater than that of the unphosphorylated complex.

The inhibitory tyrosine residue (Y15) in the *S. pombe* CDK is in the region of the protein that binds the ATP phosphates. Vertebrate CDK2 proteins contain a second inhibitory residue, threonine-14 (T14), that is located in the same region of the protein. Phosphorylation of Y15 and T14 in these proteins prevents binding of ATP because of electrostatic repulsion between the phosphates linked to the protein and the phosphates of ATP. Thus these phosphorylations inhibit protein kinase activity even when the CDK protein is bound by a cyclin and the activating residue is phosphorylated.

Other Mechanisms Also Control Entry into Mitosis by Regulating MPF Activity

So far we have discussed two mechanisms for controlling entry into mitosis: (a) regulation of the concentration of mitotic cyclins as outlined in Figure 21-10 and (b) regulation of the kinase activity of MPF as outlined in Figure 21-14. Further studies of *S. pombe* mutants with altered cell cycles have revealed additional genes whose encoded proteins directly or indirectly influence MPF activity. At present it is clear that MPF activity in *S. pombe* is regulated in a complex fashion in order to control precisely the timing of mitosis and therefore the size of daughter cells.

Enzymes with activities equivalent to *S. pombe* Wee1 and Cdc25 have been found in cycling *Xenopus* egg extracts. The *Xenopus* Wee1 tyrosine kinase activity is high and Cdc25 phosphatase activity is low during interphase. As a result, MPF assembled from *Xenopus* CDK1 and newly synthesized mitotic cyclin is inactive. As the extract initiates the events of mitosis, Wee1 activity diminishes and Cdc25 activity increases so that MPF is converted into its active form. Although cyclin B is the only protein whose synthesis is required for the cycling of early *Xenopus* embryos, the activities of other proteins, including *Xenopus* Wee1 and Cdc25, must be properly regulated for cycling to occur. In its active form, Cdc25 is phosphorylated. Its activity is also controlled by additional protein kinases and phosphatases.

MPF activity also can be regulated by controlling transcription of the genes encoding the proteins that regulate MPF activity. For example, after the initial rapid synchronous cell divisions of the early *Drosophila* embryo, all the mRNAs are degraded, and the cells become arrested in G_2. This arrest occurs because the *Drosophila* homolog of Cdc25, called String, is unstable. The resulting decrease in String phosphatase activity maintains MPF in its inhibited state, preventing entry into mitosis. The subsequent regulated entry into mitosis by specific groups of cells is then triggered by the regulated transcription of the *string* gene.

KEY CONCEPTS OF SECTION 21.3

Genetic Studies with *S. pombe*

■ In the fission yeast *S. pombe*, the $cdc2^+$ gene encodes a cyclin-dependent protein kinase (CDK) that associates with a mitotic cyclin encoded by the $cdc13^+$ gene. The resulting

mitotic cyclin-CDK heterodimer is equivalent to *Xenopus* MPF. Mutants that lack either the mitotic cyclin or the CDK fail to enter mitosis and form elongated cells.

- The protein kinase activity of the mitotic cyclin-CDK complex (MPF) depends on the phosphorylation state of two residues in the catalytic CDK subunit (see Figure 21-14). The activity is greatest when threonine-161 is phosphorylated and is inhibited by Wee1-catalyzed phosphorylation of tyrosine-15, which interferes with correct binding of ATP. This inhibitory phosphate is removed by the Cdc25 protein phosphatase.
- A decrease in Wee1 activity and increase in Cdc25 activity, resulting in activation of the mitotic cyclin-CDK complex, results in the onset of mitosis.
- The human cyclin A–CDK2 complex is similar to MPF from *Xenopus* and *S. pombe*. Structural studies with the human proteins reveal that cyclin binding to CDK2 and phosphorylation of the activating threonine (equivalent to threonine-161 in the *S. pombe* CDK) cause conformational changes that expose the active site and modify the substrate-binding surface so that it has high activity and affinity for protein substrates (see Figure 21-15).

21.4 Molecular Mechanisms for Regulating Mitotic Events

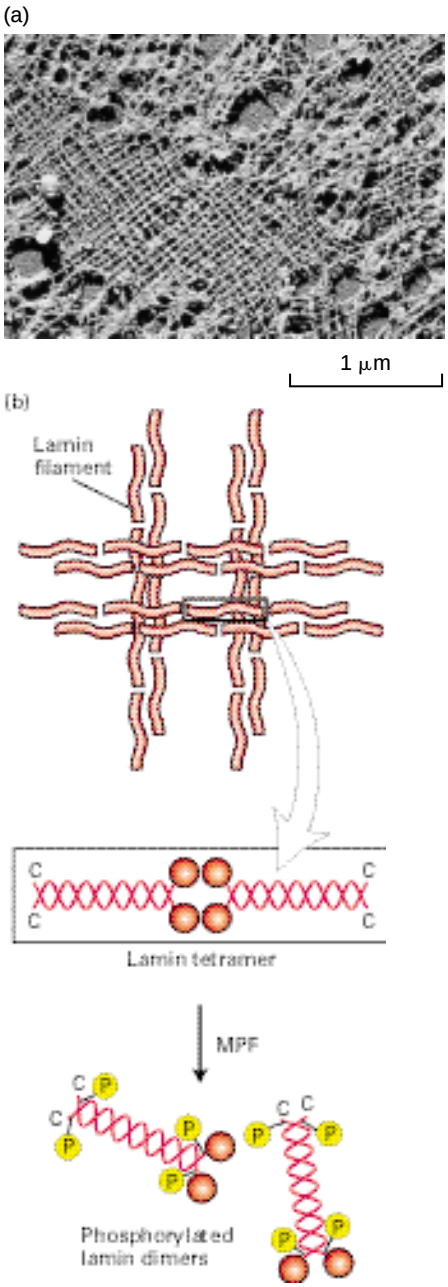
In the previous sections, we have seen that a regulated increase in MPF activity induces entry into mitosis. Presumably, the entry into mitosis is a consequence of the phosphorylation of specific proteins by the protein kinase activity of MPF. Although many of the critical substrates of MPF remain to be identified, we now know of examples that show how regulation by MPF phosphorylation mediates many of the early events of mitosis leading to metaphase: chromosome condensation, formation of the mitotic spindle, and disassembly of the nuclear envelope (see Figure 20-29).

Recall that a decrease in mitotic cyclins and the associated inactivation of MPF coincides with the later stages of mitosis (see Figure 21-9a). Just before this, in early anaphase, sister chromatids separate and move to opposite spindle poles. During telophase, microtubule dynamics return to interphase conditions, the chromosomes decondense, the nuclear envelope re-forms, the Golgi complex is remodeled, and cytokinesis occurs. Some of these processes are triggered by dephosphorylation; others, by protein degradation.

In this section, we discuss the molecular mechanisms and specific proteins associated with some of the events that characterize early and late mitosis. These mechanisms illustrate how cyclin-CDK complexes together with ubiquitin ligases control passage through the mitotic phase of the cell cycle.

Phosphorylation of Nuclear Lamins and Other Proteins Promotes Early Mitotic Events

The nuclear envelope is a double-membrane extension of the rough endoplasmic reticulum containing many nuclear pore

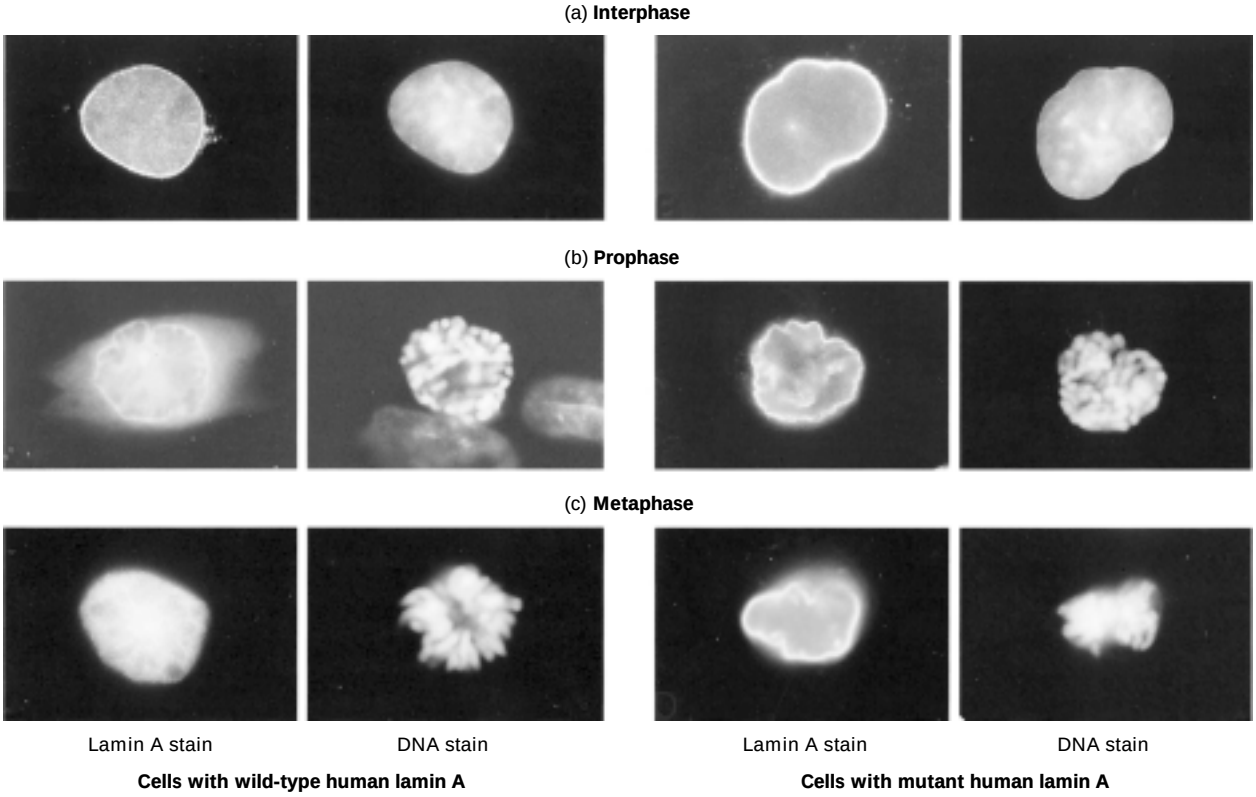


▲ FIGURE 21-16 **The nuclear lamina and its depolymerization.** (a) Electron micrograph of the nuclear lamina from a *Xenopus* oocyte. Note the regular meshlike network of lamin intermediate filaments. This structure lies adjacent to the inner nuclear membrane (see Figure 19-31). (b) Schematic diagrams of the structure of the nuclear lamina. Two orthogonal sets of 10-nm-diameter filaments built of lamins A, B, and C form the nuclear lamina (*top*). Individual lamin filaments are formed by end-to-end polymerization of lamin tetramers, which consist of two lamin dimers (*middle*). The red circles represent the globular N-terminal domains. Phosphorylation of specific serine residues near the ends of the coiled-coil rodlike central section of lamin dimers causes the tetramers to depolymerize (*bottom*). As a result, the nuclear lamina disintegrates. [Part (a) from U. Aebi et al., 1986, *Nature* **323**:560; courtesy of U. Aebi. Part (b) adapted from A. Murray and T. Hunt, 1993, *The Cell Cycle: An Introduction,* W. H. Freeman and Company.]

complexes (see Figure 5-19). The lipid bilayer of the inner nuclear membrane is supported by the **nuclear lamina,** a meshwork of lamin filaments located adjacent to the inside face of the nuclear envelope (Figure 21-16a). The three nuclear **lamins** (A, B, and C) present in vertebrate cells belong to the class of cytoskeletal proteins, the intermediate filaments, that are critical in supporting cellular membranes (Chapter 19).

Lamins A and C, which are encoded by the same transcription unit and produced by alternative splicing of a single pre-mRNA, are identical except for a 133-residue region at the C-terminus of lamin A, which is absent in lamin C. Lamin B, encoded by a different transcription unit, is modified post-transcriptionally by the addition of a hydrophobic isoprenyl group near its carboxyl-terminus. This fatty acid becomes embedded in the inner nuclear membrane, thereby anchoring the nuclear lamina to the membrane (see Figure 5-15). All three nuclear lamins form dimers containing a rodlike α-helical coiled-coil central section and globular head and tail domains; polymerization of these dimers through head-to-head and tail-to-tail associations generates the intermediate filaments that compose the nuclear lamina (see Figure 19-33).

Early in mitosis, MPF phosphorylates specific serine residues in all three nuclear lamins, causing depolymerization of the lamin intermediate filaments (Figure 21-16b). The phosphorylated lamin A and C dimers are released into solution, whereas the phosphorylated lamin B dimers remain associated with the nuclear membrane via their isoprenyl anchor. Depolymerization of the nuclear lamins leads to disintegration of the nuclear lamina meshwork and contributes to disassembly of the nuclear envelope. The experiment summarized in Figure 21-17 shows that disassembly of the nuclear envelope, which normally occurs early in mitosis, depends on phosphorylation of lamin A.



▲ EXPERIMENTAL FIGURE 21-17 **Transfection experiments demonstrate that phosphorylation of human lamin A is required for lamin depolymerization.** Site-directed mutagenesis was used to prepare a mutant human *lamin A* gene encoding a protein in which alanines replace the serines that normally are phosphorylated in wild-type lamin A (see Figure 21-16b). As a result, the mutant lamin A cannot be phosphorylated. Expression vectors carrying the wild-type or mutant human gene were separately transfected into cultured hamster cells. Because the transfected *lamin* genes are expressed at much higher levels than the endogenous hamster *lamin* gene, most of the lamin A produced in transfected cells is human lamin A. Transfected cells at various stages in the cell cycle then were stained with a fluorescent-labeled monoclonal antibody specific for human lamin A and with a fluorescent dye that binds to DNA. The bright band of fluorescence around the perimeter of the nucleus in interphase cells stained for human lamin A represents polymerized (unphosphorylated) lamin A (a). In cells expressing the wild-type human lamin A, the diffuse lamin staining throughout the cytoplasm in prophase and metaphase (b and c) and the absence of the bright peripheral band in metaphase (c) indicate depolymerization of lamin A. In contrast, no lamin depolymerization occurred in cells expressing the mutant lamin A. DNA staining showed that the chromosomes were fully condensed by metaphase in cells expressing either wild-type or mutant lamin A. [From R. Heald and F. McKeon, 1990, *Cell* **61**:579.]

In addition, MPF-catalyzed phosphorylation of specific nucleoporins (see Chapter 12) causes nuclear pore complexes to dissociate into subcomplexes during prophase. Similarly, phosphorylation of integral membrane proteins of the inner nuclear membrane is thought to decrease their affinity for chromatin and contribute to disassembly of the nuclear envelope. The weakening of the associations between the inner nuclear membrane and the nuclear lamina and chromatin may allow sheets of inner nuclear membrane to retract into the endoplasmic reticulum, which is continuous with the outer nuclear membrane.

Several lines of evidence indicate that MPF-catalyzed phosphorylation also plays a role in chromosome condensation and formation of the mitotic spindle apparatus. For instance, genetic experiments in the budding yeast *S. cerevisiae* identified a family of SMC (structural *m*aintenance of *c*hromosomes) proteins that are required for normal chromosome segregation. These large proteins (≈1200 amino acids) contain characteristic ATPase domains at their C-terminus and long regions predicted to participate in coiled-coil structures.

Immunoprecipitation studies with antibodies specific for *Xenopus* SMC proteins revealed that in cycling egg extracts some SMC proteins are part of a multiprotein complex called *condensin*, which becomes phosphorylated as cells enter mitosis. When the anti-SMC antibodies were used to deplete condensin from an egg extract, the extract lost its ability to condense added sperm chromatin. Other in vitro experiments showed that phosphorylated purified condensin binds to DNA and winds it into supercoils (see Figure 4-7), whereas unphosphorylated condensin does not. These results have lead to the model that individual condensin complexes are activated by phosphorylation catalyzed by MPF or another protein kinase regulated by MPF. Once activated, condensin complexes bind to DNA at intervals along the chromosome scaffold. Self-association of the bound complexes via their coiled-coil domains and supercoiling of the DNA segments between them is proposed to cause chromosome condensation.

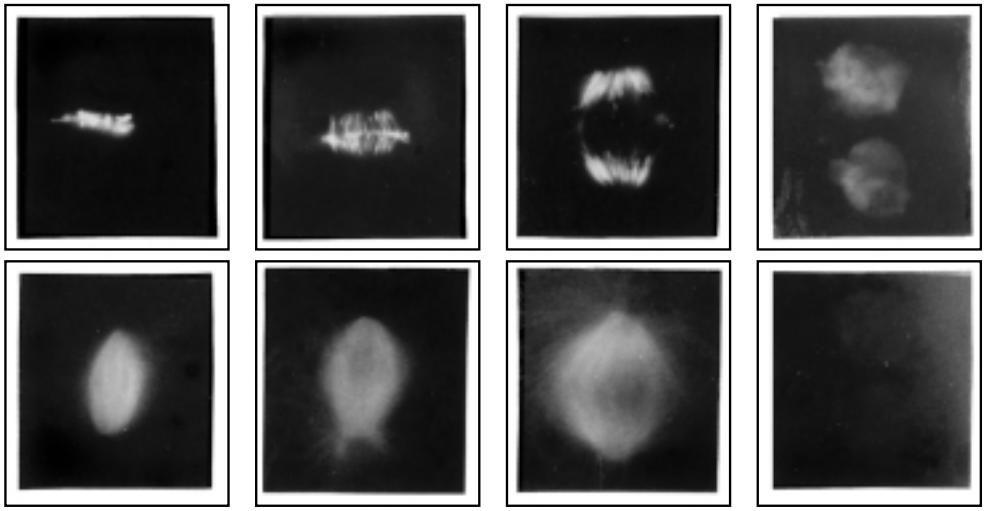
Phosphorylation of microtubule-associated proteins by MPF probably is required for the dramatic changes in microtubule dynamics that result in the formation of the mitotic spindle and asters (Chapter 20). In addition, phosphorylation of proteins associated with the endoplasmic reticulum (ER) and Golgi complex, by MPF or other protein kinases activated by MPF-catalyzed phosphorylation, is thought to alter the trafficking of vesicles between the ER and Golgi to favor trafficking in the direction of the ER during prophase. As a result, vesicular traffic from the ER through the Golgi to the cell surface (Chapter 17), seen in interphase cells, does not occur during mitosis.

Unlinking of Sister Chromatids Initiates Anaphase

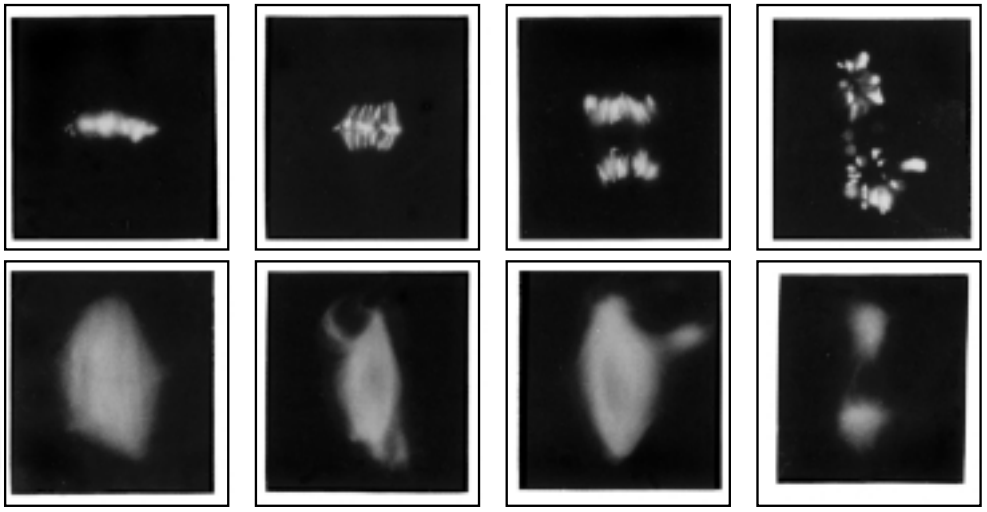
We saw earlier that in late anaphase, polyubiquitination of mitotic cyclin by the anaphase-promoting complex (APC) leads to the proteasomal destruction of this cyclin (see Figure 21-10). Additional experiments with *Xenopus* egg extracts provided evidence that degradation of cyclin B, the *Xenopus* mitotic cyclin, and the resulting decrease in MPF activity are required for chromosome decondensation but not for chromosome segregation (Figure 21-18a, b).

▶ EXPERIMENTAL FIGURE 21-18 **Onset of anaphase depends on polyubiquitination of proteins other than cyclin B in cycling *Xenopus* egg extracts.** The reaction mixtures contained an untreated or RNase-treated *Xenopus* egg extract and isolated *Xenopus* sperm nuclei, plus other components indicated below. Chromosomes were visualized with a fluorescent DNA-binding dye. Fluorescent rhodamine-labeled tubulin in the reactions was incorporated into microtubules, permitting observation of the mitotic spindle apparatus. (a, b) After the egg extract was treated with RNase to destroy endogenous mRNAs, an RNase inhibitor was added. Then mRNA encoding either wild-type cyclin B or a mutant nondegradable cyclin B was added. The time at which the condensed chromosomes and assembled spindle apparatus became visible after addition of sperm nuclei is designated 0 minutes. In the presence of wild-type cyclin B (a), condensed chromosomes attached to the spindle microtubules and segregated toward the poles of the spindle. By 40 minutes, the spindle had depolymerized (thus is not visible), and the chromosomes had decondensed (diffuse DNA staining) as cyclin B was degraded. In the presence of nondegradable cyclin B (b), chromosomes segregated to the spindle poles by 15 minutes, as in (a), but the spindle microtubules did not depolymerize and the chromosomes did not decondense even after 80 minutes. These observations indicate that degradation of cyclin B is not required for chromosome segregation during anaphase, although it is required for depolymerization of spindle microtubules and chromosome decondensation during telophase. (c) Various concentrations of a cyclin B peptide containing the destruction box were added to extracts that had not been treated with RNase; the samples were stained for DNA at 15 or 35 minutes after formation of the spindle apparatus. The two lowest peptide concentrations delayed chromosome segregation, and the higher concentrations completely inhibited chromosome segregation. In this experiment, the added cyclin B peptide is thought to competitively inhibit APC-mediated polyubiquitination of cyclin B as well as another target protein whose degradation is required for chromosome segregation. [From S. L. Holloway et al., 1993, *Cell* **73**:1393; courtesy of A. W. Murray.]

(a) RNase treated extract + mRNA encoding wild-type cyclin B



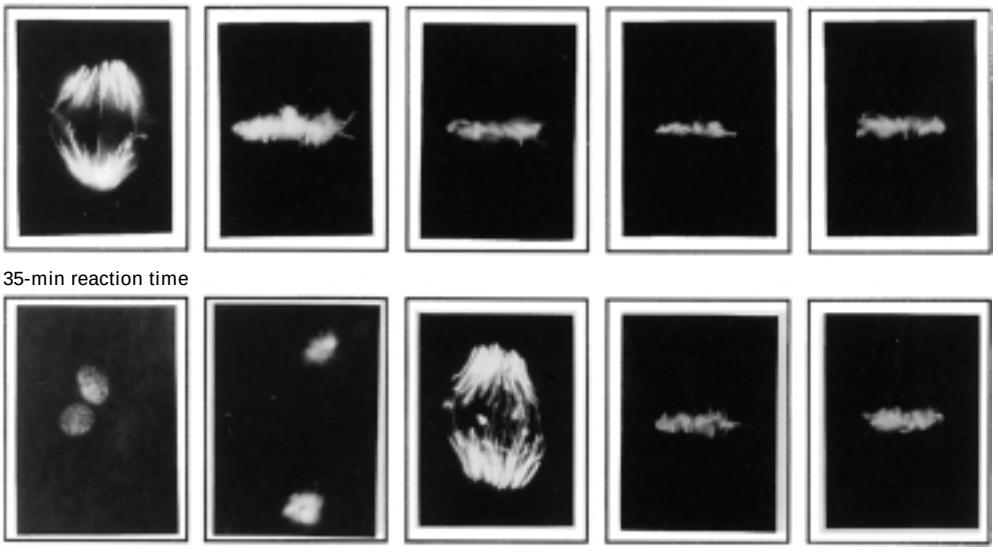
(b) RNase treated extract + mutant mRNA encoding nondegradable cyclin B



(c) Untreated extract + cylin B destruction-box peptide

15-min reaction time

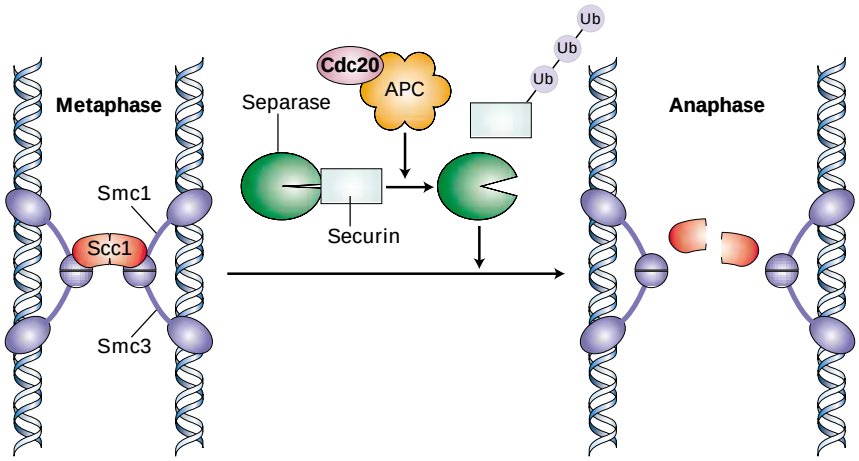
35-min reaction time



To determine if ubiquitin-dependent degradation of another protein is required for chromosome segregation, researchers prepared a peptide containing the cyclin destruction-box sequence and the site of polyubiquitination. When this peptide was added to a reaction mixture containing untreated egg extract and sperm nuclei, decondensation of the chromosomes and, more interestingly, movement of chromosomes toward the spindle poles were greatly delayed at peptide concentrations of 20–40 μg/ml and blocked altogether at higher concentrations (Figure 21-18c). The added excess destruction-box peptide is thought to act as a substrate for the APC-directed polyubiquitination system, competing with the normal endogenous target proteins and thereby delaying or preventing their degradation by proteasomes. Competition for cyclin B accounts for the observed inhibition of chromosome decondensation. The observation that chromosome segregation also was inhibited in this experiment but not in the experiment with mutant nondegradable cyclin B (see Figure 21-18b) indicated that segregation depends on proteasomal degradation of a different target protein.

As mentioned earlier, each sister chromatid of a metaphase chromosome is attached to microtubules via its kinetochore, a complex of proteins assembled at the centromere. The opposite ends of these kinetochore microtubules associate with one of the spindle poles (see Figure 20-31). At metaphase, the spindle is in a state of tension with forces pulling the two kinetochores toward the opposite spindle poles balanced by forces pushing the spindle poles apart. Sister chromatids do not separate because they are held together at their centromeres and multiple positions along the chromosome arms by multiprotein complexes called *cohesins.* Among the proteins composing the cohesin complexes are members of the SMC protein family discussed in the previous section. When *Xenopus* egg extracts were depleted of cohesin by treatment with antibodies specific for the cohesin SMC proteins, the depleted extracts were able to replicate the DNA in added sperm nuclei, but the resulting sister chromatids did not associate properly with each other. These findings demonstrate that cohesin is necessary for cross-linking sister chromatids.

Recent genetic studies in the budding yeast *S. cerevisiae* have led to the model depicted in Figure 21-19 for how the APC regulates sister chromatid separation to initiate anaphase. Cohesin SMC proteins bind to each sister chromatid; other subunits of cohesin, including *Scc1*, then link the SMC proteins, firmly associating the two chromatids. The cross-linking activity of cohesin depends on *securin*, which is found in all eukaryotes. Prior to anaphase, securin binds to and inhibits separase, a ubiquitous protease related to the caspase proteases that regulate programmed cell death (Chapter 22). Once all chromosome kinetochores have attached to spindle microtubules, the APC is directed by a specificity factor called *Cdc20* to polyubiquitinate securin, leading to the onset of anaphase. (This specificity factor is distinct from Cdh1, which directs the APC to polyubiquitinate B-type cyclins.) Polyubiquitinated securin is rapidly degraded by proteasomes, thereby releasing separase. Free from its inhibitor, separase cleaves Scc1, breaking the protein cross-link between sister chromatids. Once this link is bro-



▲ FIGURE 21-19 **Model for control of entry into anaphase by APC-regulated degradation of the cohesin link between sister chromatids.** (*Left*) The multiprotein cohesin complex contains SMC1 and SMC3 (purple), dimeric proteins that bind DNA of each sister chromatid through globular domains at one end. Scc1 (orange) and two other cohesin subunits (not shown) bind to the SMC proteins associated with each chromatid, thus cross-linking the chromatids. (*Center*) Once all chromosome kinetochores have bound to spindle microtubules, the APC specificity factor Cdc20 targets APC to polyubiquitinate securin which is then degraded by the proteasome (not shown). (*Right*) The released separase then cleaves Scc1, severing the cross-link between sister chromatids. See text for discussion. [Adapted from F. Uhlmann, 2001, *Curr. Opin. Cell Biol.* **13**:754; and A. Tomans, *Nature Milestones,* http://www.nature.com/celldivision/milestones/full/milestone23.html.]

ken, the poleward force exerted on kinetochores can move sister chromatids toward the opposite spindle poles.

Because Cdc20—the specificity factor that directs APC to securin—is activated before Cdh1—the specificity factor that directs APC to mitotic cyclins—MPF activity does not decrease until after the chromosomes have segregated (see Figure 21-2, steps 8 and 9). As a result of this temporal order in the activation of the two APC specificity factors (Cdc20 and Cdh1), the chromosomes remain in the condensed state and reassembly of the nuclear envelope does not occur until chromosomes are moved to the proper position. We consider how the timing of Cdh1 activation is regulated in a later section.

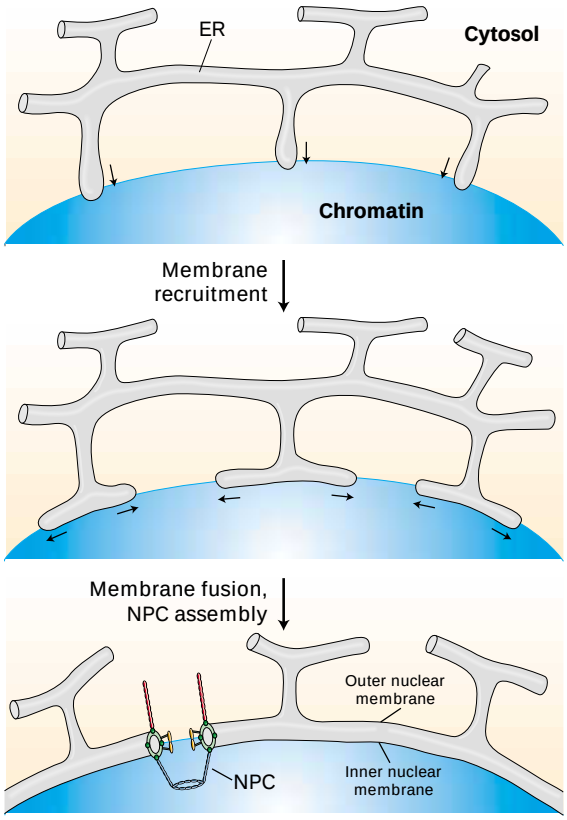
Reassembly of the Nuclear Envelope and Cytokinesis Depend on Unopposed Constitutive Phosphatase Activity

Earlier we discussed how MPF-mediated phosphorylation of nuclear lamins, nucleoporins, and proteins in the inner nuclear membrane contributes to the dissociation of nuclear pore complexes and retraction of the nuclear membrane into the reticular ER. Once MPF is inactivated in late anaphase by the degradation of mitotic cyclins, the unopposed action of phosphatases reverses the action of MPF. The dephosphorylated inner nuclear membrane proteins are thought to bind to chromatin once again. As a result, multiple projections of regions of the ER membrane containing these proteins are thought to associate with the surface of the decondensing chromosomes and then fuse with each other to form a continuous double membrane around each chromosome (Figure 21-20). Dephosphorylation of nuclear pore subcomplexes is thought to allow them to reassemble nuclear pore complexes traversing the inner and outer membranes soon after fusion of the ER projections (see Figure 12-20).

The fusion of ER projections depicted in Figure 21-20 occurs by a mechanism similar to that described for the fusion of vesicles and target membranes in the secretory pathway (see Figure 17-11). Proteins with activities similar to those of NSF and α-SNAP in the secretory pathway have been shown to function in the fusion of experimentally produced nuclear envelope vesicles in vitro and are thought to mediate the fusion of ER projections around chromosomes during telophase in the intact cell. These same proteins also function in the fusion events that reassemble the Golgi apparatus. The membrane-associated **SNARE** proteins that direct the fusion of nuclear envelope extensions from the ER have not been identified, but syntaxin 5 has been shown to function as both the V-SNARE and T-SNARE during reassembly of the Golgi. During fusion of nuclear envelope vesicles in vitro, the same Ran GTPase that functions in transport through nuclear pore complexes (Chapter 12) functions similarly to Rab GTPases in vesicle fusion in the secretory pathway. Because the Ran-specific guanosine nucleotide–exchange factor (Ran-GEF) is associated with chromatin, a high local concentration of Ran · GTP is produced around the chromosomes, directing membrane fusions at the chromosome surface.

The reassembly of nuclear envelopes containing nuclear pore complexes around each chromosome forms individual mininuclei called *karyomeres* (see Figure 21-20). Subsequent fusion of the karyomeres associated with each spindle pole generates the two daughter-cell nuclei, each containing a full set of chromosomes. Dephosphorylated lamins A and C appear to be imported through the reassembled nuclear pore complexes during this period and reassemble into a new nuclear lamina. Reassembly of the nuclear lamina in the daughter nuclei probably is initiated on lamin B molecules, which remain associated with the ER membrane via their isoprenyl anchors throughout mitosis and become localized to the inner membrane of the reassembled nuclear envelopes of karyomeres.

During cytokinesis, the final step in cell division, the actin and myosin filaments composing the contractile ring slide past each other to form a cleavage furrow of steadily decreasing



▲ FIGURE 21-20 **Model for reassembly of the nuclear envelope during telophase.** Extensions of the endoplasmic reticulum (ER) associate with each decondensing chromosome and then fuse with each other, forming a double membrane around the chromosome. Nuclear pore subcomplexes reassemble into nuclear pores, forming individual mininuclei called karyomeres. The enclosed chromosome further decondenses, and subsequent fusion of the nuclear envelopes of all the karyomeres at each spindle pole forms a single nucleus containing a full set of chromosomes. Reassembly of the nuclear lamina is not shown. [Adapted from B. Burke and J. Ellenberg, 2002, *Nature Rev. Mol. Cell Biol.* **3**:487.]

MEDIA CONNECTIONS
Video: Nuclear Envelope Dynamics During Mitosis

diameter (see Figure 19-20). As MPF activity rises early in mitosis, it phosphorylates the regulatory myosin light chain, thereby inhibiting the ability of myosin to associate with actin filaments. The inactivation of MPF at the end of anaphase permits protein phosphatases to dephosphorylate myosin light chain (see Figure 20-42). As a result, the contractile machinery is activated, the cleavage furrow can form, and cytokinesis proceeds. This regulatory mechanism assures that cytokinesis does not occur until the daughter chromosomes have segregated sufficiently toward the opposite poles to assure that each daughter cell receives the proper number of chromosomes.

KEY CONCEPTS OF SECTION 21.4

Molecular Mechanisms for Regulating Mitotic Events

- Early in mitosis, MPF-catalyzed phosphorylation of lamins A, B, and C and of nucleoporins and inner nuclear envelope proteins causes depolymerization of lamin filaments (see Figure 21-16) and dissociation of nuclear pores into pore subcomplexes, leading to disassembly of the nuclear envelope and its retraction into the ER.
- Phosphorylation of condensin complexes by MPF or a kinase regulated by MPF promotes chromosome condensation early in mitosis.
- Sister chromatids formed by DNA replication in the S phase are linked at the centromere by cohesin complexes that contain DNA-binding SMC proteins and other proteins.
- At the onset of anaphase, the APC is directed by Cdc20 to polyubiquitinate securin, which subsequently is degraded by proteasomes. This activates separase, which cleaves a subunit of cohesin, thereby unlinking sister chromatids (see Figure 21-19).
- After sister chromatids have moved to the spindle poles, the APC is directed by Cdh1 to polyubiquitinate mitotic cyclins, leading to their destruction and causing the decrease in MPF activity that marks the onset of telophase.
- The fall in MPF activity in telophase allows constitutive protein phosphatases to remove the regulatory phosphates from condensin, lamins, nucleoporins, and other nuclear membrane proteins, permitting the decondensation of chromosomes and the reassembly of the nuclear membrane, nuclear lamina, and nuclear pore complexes.
- The association of Ran-GEF with chromatin results in a high local concentration of Ran · GTP near the decondensing chromosomes, promoting the fusion of nuclear envelope extensions from the ER around each chromosome. This forms karyomeres that then fuse to form daughter cell nuclei (see Figure 21-20).
- The fall in MPF activity also removes its inhibition of myosin light chain, allowing the cleavage furrow to form and cytokinesis to proceed.

21.5 Genetic Studies with *S. cerevisiae*

In most vertebrate cells the key decision determining whether or not a cell will divide is the decision to enter the S phase. In most cases, once a vertebrate cell has become committed to entering the S phase, it does so a few hours later and progresses through the remainder of the cell cycle until it completes mitosis. *S. cerevisiae* cells regulate their proliferation similarly, and much of our current understanding of the molecular mechanisms controlling entry into the S phase and the control of DNA replication comes from genetic studies of *S. cerevisiae.*

S. cerevisiae cells replicate by budding (Figure 21-21). Both mother and daughter cells remain in the G_1 period of the cell cycle while growing, although it takes the initially larger mother cells a shorter time to reach a size compatible with cell division. When *S. cerevisiae* cells in G_1 have grown sufficiently, they begin a program of gene expression that leads to entry into the S phase. If G_1 cells are shifted from a rich medium to a medium low in nutrients before they reach a critical size, they remain in G_1 and grow slowly until they are large enough to enter the S phase. However, once G_1 cells reach the critical size, they become committed to completing the cell cycle, entering the S phase and proceeding through G_2 and mitosis, even if they are shifted to a medium low in nutrients. The point in late G_1 of growing *S. cerevisiae* cells when they become irrevocably committed to entering the S phase and traversing the cell cycle is called **START.** As we shall see in Section 21.6, a comparable phenomenon occurs in replicating mammalian cells.

A Cyclin-Dependent Kinase (CDK) Is Critical for S-Phase Entry in *S. cerevisiae*

All *S. cerevisiae* cells carrying a mutation in a particular *cdc* gene arrest with the same size bud at the nonpermissive temperature (see Figure 9-6b). Each type of mutant has a terminal phenotype with a particular bud size: no bud (*cdc28*), intermediate-sized buds, or large buds (*cdc7*). Note that in *S. cerevisiae* wild-type genes are indicated in italic capital letters (e.g., *CDC28*) and recessive mutant genes in italic lowercase letters (e.g., *cdc28*); the corresponding wild-type

▶ FIGURE 21-21 **The budding yeast *S. cerevisiae.*** (a) Scanning electron micrograph of *S. cerevisiae* cells at various stages of the cell cycle. The larger the bud, which emerges at the end of the G_1 phase, the further along in the cycle the cell is. (b) Main events in *S. cerevisiae* cell cycle. Daughter cells are born smaller than mother cells and must grow to a greater extent in G_1 before they are large enough to enter the S phase. As in *S. pombe,* the nuclear envelope does not break down during mitosis. Unlike *S. pombe* chromosomes, the small *S. cerevisiae* chromosomes do not condense sufficiently to be visible by light microscopy. [Part (a) courtesy of E. Schachtbach and I. Herskowitz.]

protein is written in Roman letters with an initial capital (e.g., Cdc28), similar to *S. pombe* proteins.

The phenotypic behavior of temperature-sensitive *cdc28* mutants indicates that Cdc28 function is critical for entry into the S phase. When these mutants are shifted to the nonpermissive temperature, they behave like wild-type cells suddenly deprived of nutrients. That is, *cdc28* mutant cells that have grown large enough to pass START at the time of the temperature shift continue through the cell cycle normally and undergo mitosis, whereas those that are too small to have passed START when shifted to the nonpermissive temperature do not enter the S phase even though nutrients are plentiful. Even though *cdc28* cells blocked in G_1 continue to grow in size at the nonpermissive temperature, they cannot pass START and enter the S phase. Thus they appear as large cells with no bud.

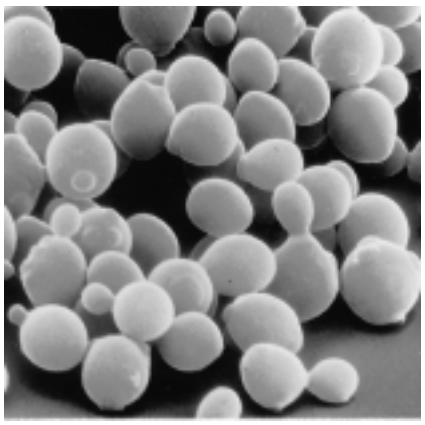
The wild-type *CDC28* gene was isolated by its ability to complement mutant *cdc28* cells at the nonpermissive temperature (see Figure 21-4). Sequencing of *CDC28* showed that the encoded protein is homologous to known protein kinases, and when Cdc28 protein was expressed in *E. coli*, it exhibited protein kinase activity. Actually, Cdc28 from *S. cerevisiae* was the first cell-cycle protein shown to be a protein kinase. Subsequently, the wild-type *S. pombe* $cdc2^+$ gene was found to be highly homologous to the *S. cerevisiae* *CDC28* gene, and the two encoded proteins—Cdc2 and Cdc28—are functionally analogous. Each type of yeast contains a *single* cyclin-dependent protein kinase (CDK), which can substitute for each other: Cdc2 in *S. pombe* and Cdc28 in *S. cerevisiae* (see Table 21-1).

The difference in the mutant phenotypes of $cdc2^-$ *S. pombe* cells and *cdc28 S. cerevisiae* cells can be explained in terms of the physiology of the two yeasts. In *S. pombe* cells growing in rich media, cell-cycle control is exerted primarily at the $G_2 \rightarrow M$ transition (i.e., entry to mitosis). In many $cdc2^-$ mutants, including those isolated first, enough Cdc2 activity is maintained at the nonpermissive temperature to permit cells to enter the S phase, but not enough to permit entry into mitosis. Such mutant cells are observed to be elongated cells arrested in G_2. At the nonpermissive temperature, cultures of completely defective $cdc2^-$ mutants include some cells arrested in G_1 and some arrested in G_2, depending on their location in the cell cycle at the time of the temperature shift. Conversely, cell-cycle regulation in *S. cerevisiae* is exerted primarily at the $G_1 \rightarrow S$ transition (i.e., entry to the S phase). Therefore, partially defective *cdc28* cells are arrested in G_1, but completely defective *cdc28* cells are arrested in either G_1 or G_2. These observations demonstrate that both the *S. pombe* and the *S. cerevisiae* CDKs are required for entry into both the S phase and mitosis.

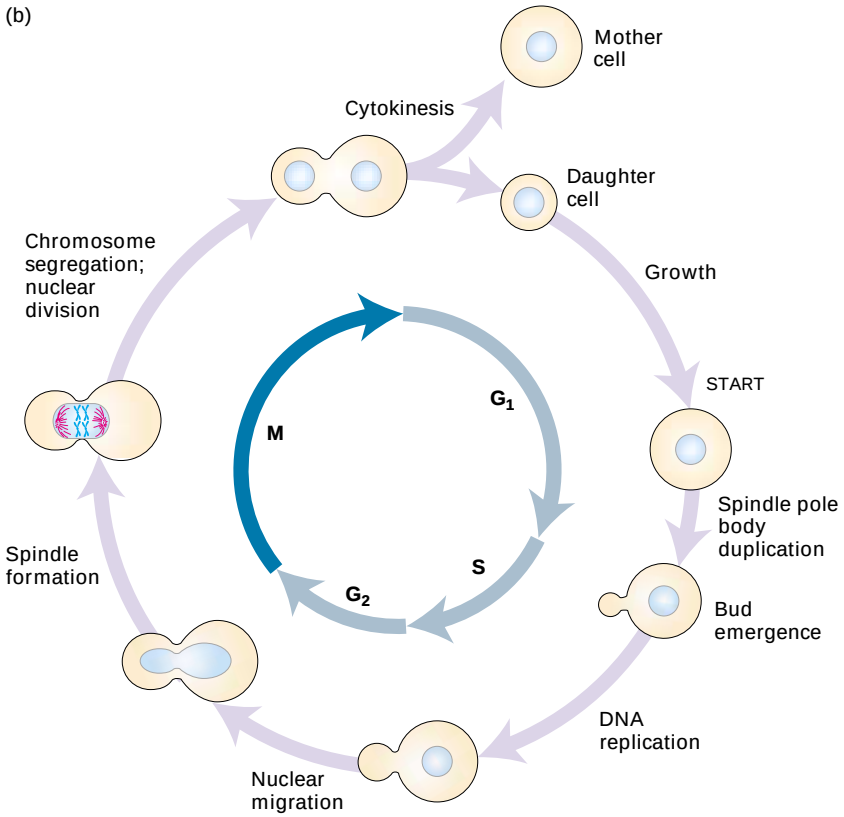
Three G_1 Cyclins Associate with *S. cerevisiae* CDK to form S Phase–Promoting Factors

By the late 1980s, it was clear that mitosis-promoting factor (MPF) is composed of two subunits: a CDK and a mitotic

(a)



(b)



MEDIA CONNECTIONS

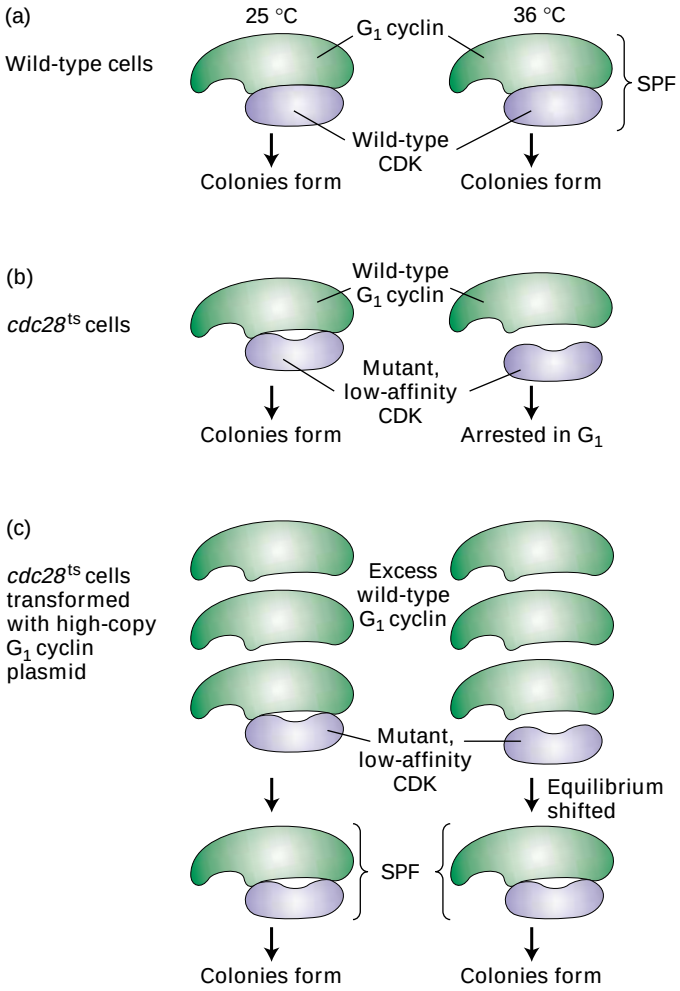
Video: Mitosis and Budding in *S. cerevisiae*

B-type cyclin required to activate the catalytic subunit. By analogy, it seemed likely that *S. cerevisiae* contains an **S phase–promoting factor (SPF)** that phosphorylates and regulates proteins required for DNA synthesis. Similar to MPF, SPF was proposed to be a heterodimer composed of the *S. cerevisiae* CDK and a cyclin, in this case one that acts in G_1 (see Figure 21-2, steps 2–4).

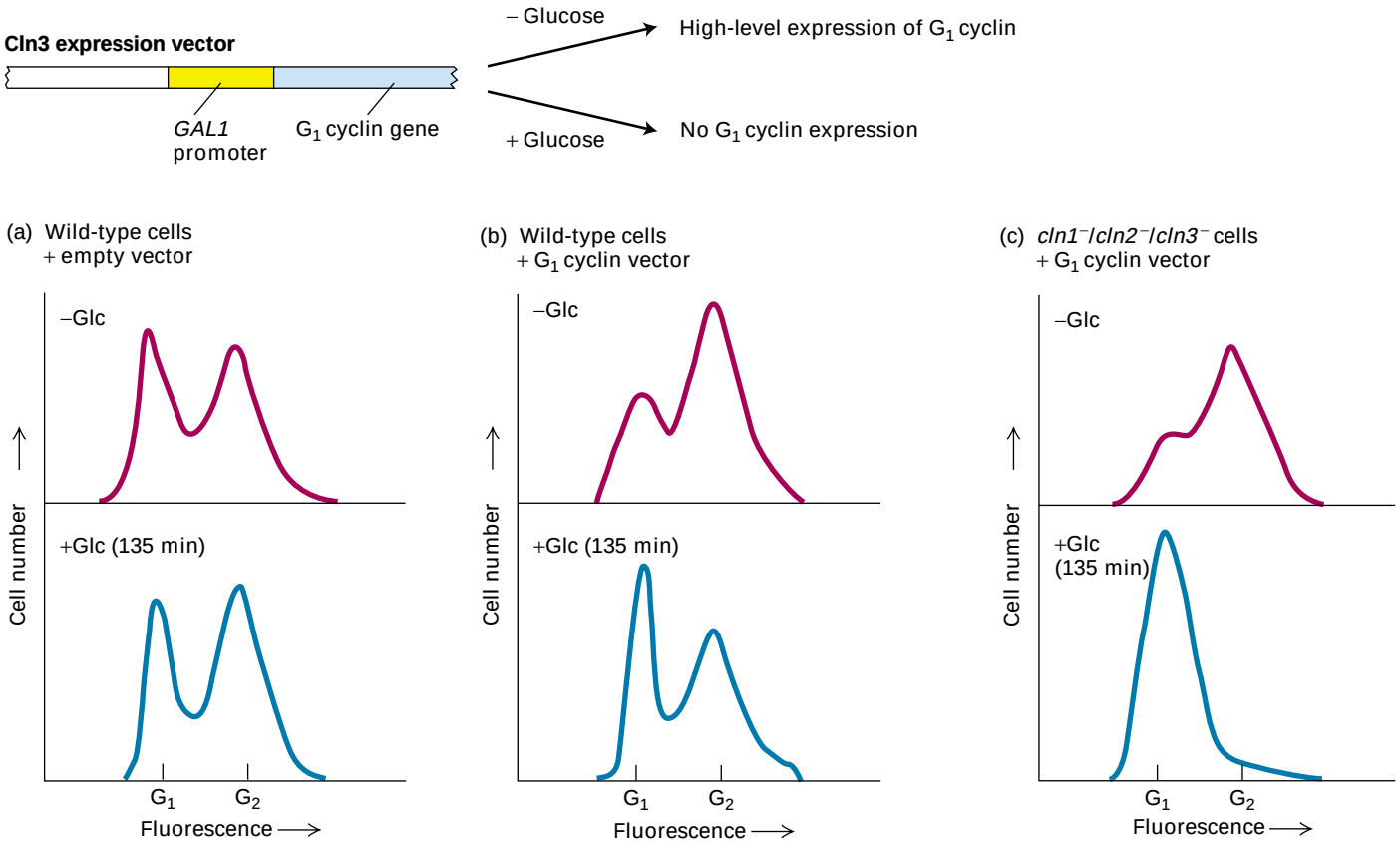
To identify this putative G_1 cyclin, researchers looked for genes that, when expressed at high concentration, could suppress certain temperature-sensitive mutations in the *S. cerevisiae* CDK. The rationale of this approach is illustrated in Figure 21-22. Researchers isolated two such genes designated *CLN1* and *CLN2*. Using a different approach, researchers identified a dominant mutation in a third gene called *CLN3*. Sequencing of the three *CLN* genes showed that they encoded related proteins each of which includes an ≈100-residue region exhibiting significant homology with B-type cyclins from sea urchin, *Xenopus*, human, and *S. pombe*. This region encodes the cyclin domain that interacts with CDKs and is included in the domain of human cyclin A shown in Figure 21-15b,c. The finding that the three Cln proteins contain this region of homology with mitotic cyclins suggested that they were the sought-after *S. cerevisiae* G_1 cyclins. (Note that the homologous CDK-binding domain found in various cyclins differs from the destruction box mentioned earlier, which is found only in B-type cyclins.)

Gene knockout experiments showed that *S. cerevisiae* cells can grow in rich medium if they carry any one of the three G_1 cyclin genes. As the data presented in Figure 21-23 indicate, overproduction of one G_1 cyclin decreases the fraction of cells in G_1, demonstrating that high levels of the G_1 cyclin-CDK complex drive cells through START prematurely. Moreover, in the absence of any of the G_1 cyclins, cells become arrested in G_1, indicating that a G_1 cyclin–CDK heterodimer, or SPF, is required for *S. cerevisiae* cells to enter the S phase. These findings are reminiscent of the results for the *S. pombe* mitotic cyclin (Cdc13) with regard to passage through G_2 and entry into mitosis. Overproduction of the mitotic cyclin caused a shortened G_2 and premature entry into mitosis, whereas inhibition of the mitotic cyclin by mutation resulted in a lengthened G_2 (see Figure 21-12). Thus these results confirmed that the *S. cerevisiae* Cln proteins are *G_1 cyclins* that regulate passage through the G_1 phase of the cell cycle.

In wild-type yeast cells, *CLN3* mRNA is produced at a nearly constant level throughout the cell cycle, but its translation is regulated in response to nutrient levels. The *CLN3* mRNA contains a short upstream open-reading frame that inhibits initiation of translation of this mRNA. This inhibition is diminished when nutrients and hence translation initiation factors are in abundance. Since Cln3 is a highly unstable protein, its concentration fluctuates with the translation rate of *CLN3* mRNA. Consequently, the amount and activity of Cln3-CDK complexes, which depends on the concentration of Cln3 protein, is largely regulated by the nutrient level.



▲ EXPERIMENTAL FIGURE 21-22 **Genes encoding two *S. cerevisiae* G_1 cyclins were identified by their ability to supress a temperature-sensitive mutant CDK.** This genetic screen is based on differences in the interactions between G_1 cyclins and wild-type and temperature-sensitive (ts) *S. cerevisiae* CDKs. (a) Wild-type cells produce a normal CDK that associates with G_1 cyclins, forming the active S phase–promoting factor (SPF), at both the permissive and nonpermissive temperature (i.e., 25 ° and 36 °C). (b) Some *cdc28*^{ts} mutants express a mutant CDK with low affinity for G_1 cyclin at 36 °C. These mutants produce enough G_1 cyclin-CDK (SPF) to support growth and colony development at 25 °C, but not at 36 °C. (c) When *cdc28*^{ts} cells were transformed with a *S. cerevisiae* genomic library cloned in a high-copy plasmid, three types of colonies formed at 36 °C: one contained a plasmid carrying the wild-type *CDC28* gene; the other two contained plasmids carrying either the *CLN1* or *CLN2* gene. In transformed cells carrying the *CLN1* or *CLN2* gene, the concentration of the encoded G_1 cyclin is high enough to offset the low affinity of the mutant CDK for a G_1 cyclin at 36 °C, so that enough SPF forms to support entry into the S phase and subsequent mitosis. Untransformed *cdc28*^{ts} cells and cells transformed with plasmids carrying other genes are arrested in G_1 and do not form colonies. [See J. A. Hadwiger et al., 1989, *Proc. Nat'l. Acad. Sci. USA* **86**:6255.]



▲ EXPERIMENTAL FIGURE 21-23 **Overexpression of G_1 cyclin prematurely drives *S. cerevisiae* cells into the S phase.** The yeast expression vector used in these experiments (*top*) carried one of the three *S. cerevisiae* G_1 cyclin genes linked to the strong *GAL1* promoter, which is turned off when glucose is present in the medium. To determine the proportion of cells in G_1 and G_2, cells were exposed to a fluorescent dye that binds to DNA and then were passed through a fluorescence-activated cell sorter (see Figure 5-34). Since the DNA content of G_2 cells is twice that of G_1 cells, this procedure can distinguish cells in the two cell-cycle phases. (a) Wild-type cells transformed with an empty expression vector displayed the normal distribution of cells in G_1 and G_2 in the absence of glucose (Glc) and after addition of glucose. (b) In the absence of glucose, wild-type cells transformed with the G_1 cyclin expression vector displayed a higher-than-normal percentage of cells in the S phase and G_2 because overexpression of the G_1 cyclin decreased the G_1 period (top curve). When expression of the G_1 cyclin from the vector was shut off by addition of glucose, the cell distribution returned to normal (bottom curve). (c) Cells with mutations in all three G_1 cyclin genes and transformed with the G_1 cyclin expression vector also showed a high percentage of cells in S and G_2 in the absence of glucose (top curve). Moreover, when expression of G_1 cyclin from the vector was shut off by addition of glucose, the cells completed the cell cycle and arrested in G_1 (bottom curve), indicating that a G_1 cyclin is required for entry into the S phase. [Adapted from H. E. Richardson et al., 1989, *Cell* **59:**1127.]

Once sufficient Cln3 is synthesized from its mRNA, the Cln3-CDK complex phosphorylates and activates two related transcription factors, SBF and MBF. These induce transcription of the *CLN1* and *CLN2* genes whose encoded proteins accelerate entry into the S phase. Thus regulation of *CLN3* mRNA translation in response to the concentration of nutrients in the medium is thought to be primarily responsible for controlling the length of G_1 in *S. cerevisiae*. SBF and MBF also stimulate transcription of several other genes required for DNA replication, including genes encoding DNA polymerase subunits, RPA subunits (the eukaryotic ssDNA-binding protein), DNA ligase, and enzymes required for deoxyribonucleotide synthesis.

One of the important substrates of the late G_1 cyclin–CDK complexes (Cln1-CDK and Cln2-CDK in *S. cerevisiae*) is Cdh1. Recall that this specificity factor directs the APC to polyubiquitinate B-type cyclins during late anaphase of the previous mitosis, marking these cyclins for proteolysis by proteasomes (see Figure 21-10). The MBF transcription factor activated by the Cln3-CDK complex also stimulates transcription of *CLB5* and *CLB6*, which encode *cyc*lins of the *B*-type, hence their name. Because the complexes formed between these B-type cyclins and the *S. cerevisiae* CDK are required for initiation of DNA synthesis, the Clb5 and Clb6 proteins are called *S-phase cyclins*. Inactivation of the APC earlier in G_1 allows the S-phase cyclin-CDK complexes to

accumulate in late G_1. The specificity factor Cdh1 is phosphorylated and inactivated by both late G_1 and B-type cyclin-CDK complexes, and thus remains inhibited throughout S, G_2, and M phase until late anaphase.

Degradation of the S-Phase Inhibitor Triggers DNA Replication

As the S-phase cyclin-CDK heterodimers accumulate in late G_1, they are immediately inactivated by binding of an inhibitor, called *Sic1*, that is expressed late in mitosis and in early G_1. Because Sic1 specifically inhibits B-type cyclin-CDK complexes, but has no effect on the G_1 cyclin-CDK complexes, it functions as an *S-phase inhibitor.*

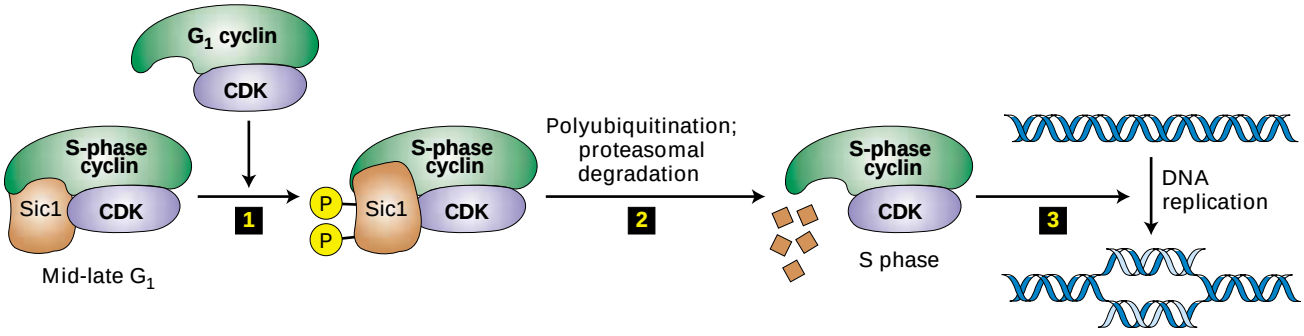
Entry into the S phase is defined by the initiation of DNA replication. In *S. cerevisiae* cells this occurs when the Sic1 inhibitor is precipitously degraded following its polyubiquitination by a distinct ubiquitin ligase called *SCF* (Figure 21-24; see also Figure 21-2, step 5). Once Sic1 is degraded, the S-phase cyclin-CDK complexes induce DNA replication by phosphorylating multiple proteins bound to replication origins. This mechanism for activating the S-phase cyclin-CDK complexes—that is, inhibiting them as the cyclins are synthesized and then precipitously degrading the inhibitor—permits the sudden activation of large numbers of complexes, as opposed to the gradual increase in kinase activity that would result if no inhibitor were present during synthesis of the S-phase cyclins.

We can now see that regulated proteasomal degradation directed by two ubiquitin ligase complexes, SCF and APC, controls three major transitions in the cell cycle: onset of the S phase through degradation of Sic1, the beginning of anaphase through degradation of securin, and exit from mitosis through degradation of B-type cyclins. The APC is directed to polyubiquitinate securin, which functions as an anaphase inhibitor, by the Cdc20 specificity factor (see Figure 21-19). Another specificity factor, Cdh1, targets APC to B-type cyclins (see Figure 21-10). The SCF is directed to polyubiquitinate Sic1 by a different mechanism, namely, phosphorylation of Sic1 by a G_1 cyclin-CDK (see Figure 21-24). This difference in strategy probably occurs because the APC has several substrates, including securin and B-type cyclins, which must be degraded at different times in the cycle. In contrast, entry into the S phase requires the degradation of only a single protein, the Sic1 inhibitor. An obvious advantage of proteolysis for controlling passage through these critical points in the cell cycle is that protein degradation is an irreversible process, ensuring that cells proceed irreversibly in one direction through the cycle.

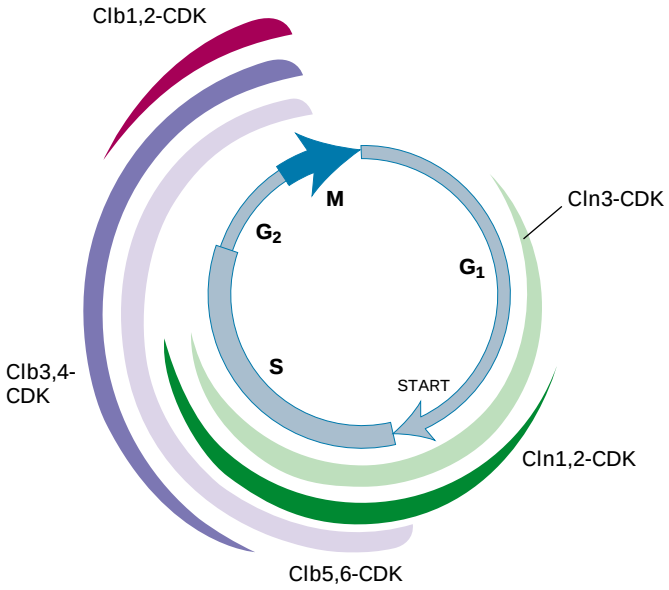
Multiple Cyclins Direct the Kinase Activity of *S. cerevisiae* CDK During Different Cell-Cycle Phases

As budding yeast cells progress through the S phase, they begin transcribing genes encoding two additional B-type cyclins, Clb3 and Clb4. These form heterodimeric cyclin-CDK complexes that, together with complexes including Clb5 and Clb6, activate DNA replication origins throughout the remainder of the S phase. The Clb3-CDK and Clb4-CDK complexes also initiate formation of the mitotic spindle at the beginning of mitosis. When *S. cerevisiae* cells complete chromosome replication and enter G_2, they begin expressing two more B-type cyclins, Clb1 and Clb2. These function as mitotic cyclins, associating with the CDK to form complexes that are required for mediating the events of mitosis.

Each group of cyclins thus directs the *S. cerevisiae* CDK to specific functions associated with various cell-cycle phases, as outlined in Figure 21-25. Cln3-CDK induces expression of Cln1, Cln2, and other proteins in mid-late G_1 by phosphorylating and activating the SBF and MBF transcription factors. Cln1-CDK and Cln2-CDK inhibit the APC, allowing B-type cyclins to accumulate; these G_1 cyclin-CDKs also activate degradation of the S-phase inhibitor Sic1. The S-phase CDK complexes containing Clb5, Clb6, Clb3, and Clb4 then trigger DNA synthesis. Clb3 and Clb4 also function as mitotic



▲ FIGURE 21-24 **Control of the $G_1 \rightarrow$ S phase transition in *S. cerevisiae* by regulated proteolysis of the S-phase inhibitor Sic1.** The S-phase cyclin-CDK complexes (Clb5-CDK and Clb6-CDK) begin to accumulate in G_1, but are inhibited by Sic1. This inhibition prevents initiation of DNA replication until the cell is fully prepared. G_1 cyclin-CDK complexes assembled in late G_1 (Cln1-CDK and Cln2-CDK) phosphorylate Sic1 (step 1), marking it for polyubiquitination by the SCF ubiquitin ligase, and subsequent proteasomal degradation (step 2). The active S-phase cyclin-CDK complexes then trigger initiation of DNA synthesis (step 3) by phosphorylating substrates that remain to be identified. [Adapted from R. W. King et al., 1996, *Science* **274**:1652.]



▲ FIGURE 21-25 **Activity of *S. cerevisiae* cyclin-CDK complexes through the course of the cell cycle.** The width of the colored bands is approximately proportional to the demonstrated or proposed protein kinase activity of the indicated cyclin-CDK complexes. *S. cerevisiae* produces a single cyclin-dependent kinase (CDK) whose activity is controlled by the various cyclins, which are expressed during different portions of the cell cycle.

cyclins in that the complexes they form with CDK trigger formation of mitotic spindles. The remaining two *S. cerevisiae* cyclins, Clb1 and Clb2, whose concentrations peak midway through mitosis, function exclusively as mitotic cyclins, forming complexes with CDK that trigger chromosome segregation and nuclear division (see Table 21-1).

Cdc14 Phosphatase Promotes Exit from Mitosis

Genetic studies with *S. cerevisiae* have provided insight into how B-type cyclin-CDK complexes are inactivated in late anaphase, permitting the various events constituting telophase and then cytokinesis to occur. These complexes are the *S. cerevisiae* equivalent of the mitosis-promoting factor (MPF) first identified in *Xenopus* oocytes and early embryos. As mentioned previously, the specificity factor Cdh1, which targets the APC to polyubiquitinate B-type cyclins, is phosphorylated by both late G_1 and B-type cyclin-CDK complexes, thereby inhibiting Cdh1 activity during late G_1, S, G_2, and mitosis before late anaphase.

When daughter chromosomes have segregated properly in late anaphase, the *Cdc14 phosphatase* is activated and dephosphorylates Cdh1, allowing it to bind to the APC. This interaction quickly leads to APC-mediated polyubiquitination and proteasomal degradation of B-type cyclins, and hence MPF inactivation (see Figure 21-10). Since MPF is still active when Cdc14 is first activated in late anaphase, it potentially could compete with Cdc14 by re-phosphorylating Cdh1. However, Cdc14 also induces expression of Sic1 by removing an inhibitory phosphate on a transcription factor that activates transcription of the *SIC1* gene. Sic1 can bind to and inhibit the activity of all B-type cyclin-CDK complexes. Thus, starting late in mitosis, the inhibition of MPF by Sic1 allows the Cdc14 phosphatase to get the upper hand; the B-type cyclin APC specificity factor Cdh1 is dephosphorylated and directs the precipitous degradation of all the *S. cerevisiae* B-type cyclins. In Section 21.7, we will see how the activity of Cdc14 itself is controlled to assure that a cell exits mitosis only when its chromosomes have segregated properly.

As discussed already, Sic1 also inhibits the S-phase cyclin-CDKs as they are formed in mid-G_1 (see Figure 21-24). This inhibitor of B-type cyclins thus serves a dual function in the cell cycle, contributing to the exit from mitosis and delaying entry into the S phase until the cell is ready.

Replication at Each Origin Is Initiated Only Once During the Cell Cycle

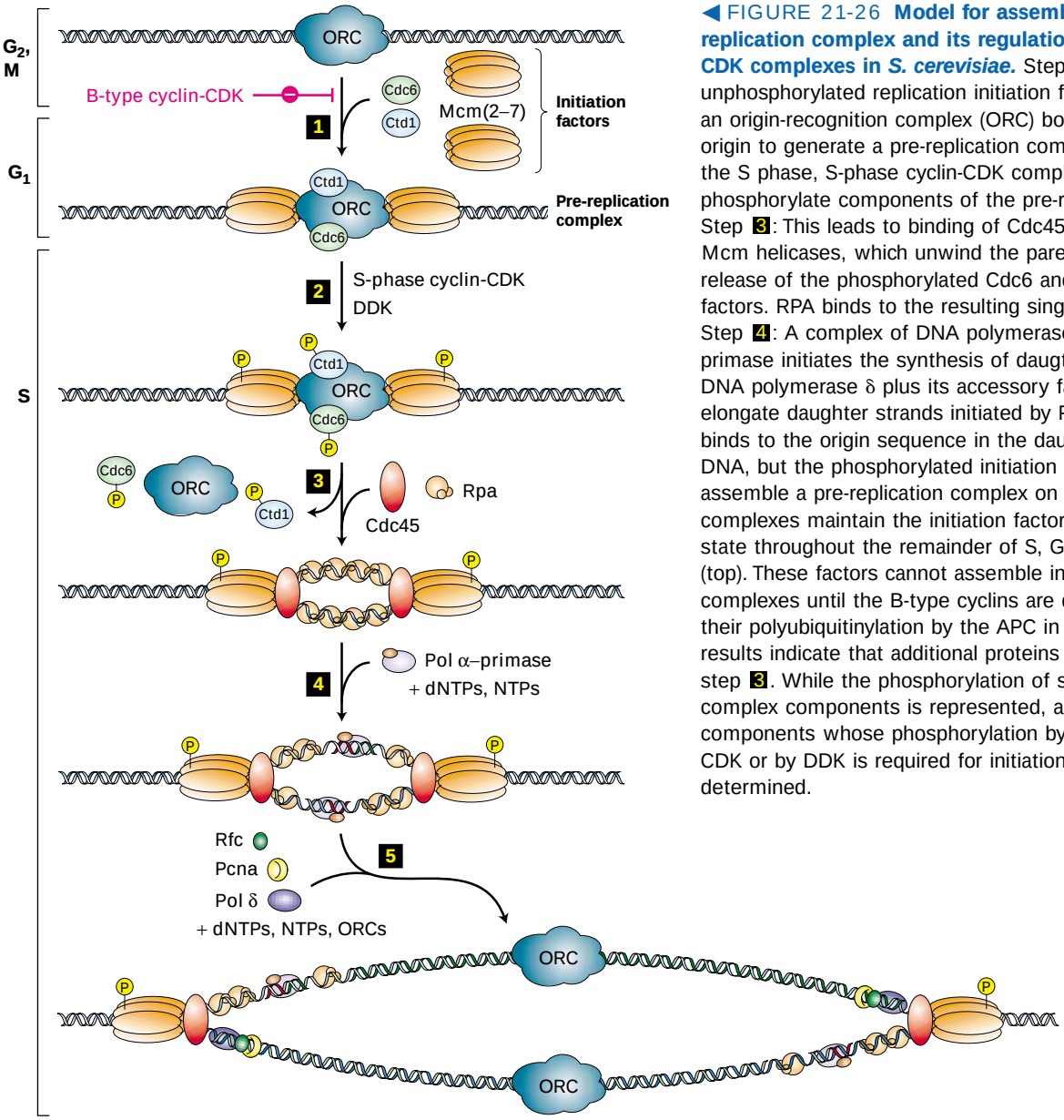
As discussed in Chapter 4, eukaryotic chromosomes are replicated from multiple origins. Some of these initiate DNA replication early in the S phase, some later, and still others toward the end. However, no eukaryotic origin initiates more than once per S phase. Moreover, the S phase continues until replication from all origins along the length of each chromosome results in replication of the chromosomal DNA in its entirety. These two factors ensure that the correct gene copy number is maintained as cells proliferate.

Yeast replication origins contain an 11-bp conserved core sequence to which is bound a hexameric protein, the *origin-recognition complex (ORC)*, required for initiation of DNA synthesis. DNase I footprinting analysis (Figure 11-15) and immunoprecipitation of chromatin proteins cross-linked to specific DNA sequences (Figure 11-40) during the various phases of the cell cycle indicate that the ORC remains associated with origins during all phases of the cycle. Several replication initiation factors required for the initiation of DNA synthesis were initially identified in genetic studies in *S. cerevisiae*. These include Cdc6, Cdt1, Mcm10, and the MCM hexamer, a complex of six additional, closely related Mcm proteins; these proteins associate with origins during G_1, but not during G_2 or M. During G_1 the various initiation factors assemble with the ORC into a *pre-replication complex* at each origin. The MCM hexamer is thought to act analogously to SV40 T-antigen hexamers, which function as a helicase to unwind the parental DNA strands at replication forks (see Figure 4-36). Cdc6, Cdt1, and Mcm10 are required to load opposing MCM hexamers on the origin.

The restriction of origin "firing" to once and only once per cell cycle in *S. cerevisiae* is enforced by the alternating cycle of B-type cyclin-CDK activities throughout the cell cycle: low in telophase through G_1 and high in S, G_2, and M through anaphase (see Figure 21-25). As we just discussed, S-phase cyclin-CDK complexes become active at the

beginning of the S phase when their specific inhibitor, Sic1, is degraded. In the current model for *S. cerevisiae* replication, pre-replication complexes assemble early in G_1 when B-type cyclin activity is low (Figure 21-26, step 1). Initiation of DNA replication requires an active S-phase cyclin-CDK complex and a second heterodimeric protein kinase, Dbf4-Cdc7, which is expressed in G_1 (step 2). By analogy with cyclin-dependent kinases (CDKs), which must be bound by a partner cyclin to activate their protein kinase activity, the *D*bf4-*d*ependent *k*inase Cdc7 is often called *DDK*. Although the complete set of proteins that must be phosphorylated to activate initiation of DNA synthesis has not yet been determined, there is evidence that phosphorylation of at least one subunit of the hexameric *MCM helicase* and of Cdc6 is required. Another consequence of S-phase cyclin-CDK activation is binding of the initiation factor Cdc45 to the pre-replication complex. Cdc45 is required for the subsequent binding of RPA, the heterotrimeric protein that binds single-stranded DNA generated when the MCM helicase unwinds the parental DNA duplex.

By stabilizing the unwound DNA, RPA promotes binding of the complex between primase and DNA polymerase α (Pol α) that initiates the synthesis of daughter strands (see Figure 21-26, step 3). Like the MCM helicase, Cdc45 remains associated with the replication forks as they extend in both directions from the origin. Presumably, it functions in the further cycles of RPA and primase–Pol α binding required to prime synthesis of the lagging daughter strand. Subsequent binding of DNA polymerase δ and its accessory cofactors Rfc and PCNA is thought to occur as it does in



◀ FIGURE 21-26 **Model for assembly of the pre-replication complex and its regulation by S-phase cyclin-CDK complexes in *S. cerevisiae*.** Step 1: During early G_1, unphosphorylated replication initiation factors assemble on an origin-recognition complex (ORC) bound to a replication origin to generate a pre-replication complex. Step 2: In the S phase, S-phase cyclin-CDK complexes and DDK phosphorylate components of the pre-replication complex. Step 3: This leads to binding of Cdc45, activation of the Mcm helicases, which unwind the parental DNA strands, and release of the phosphorylated Cdc6 and Ctd1 initiation factors. RPA binds to the resulting single-stranded DNA. Step 4: A complex of DNA polymerase α (Pol α) and primase initiates the synthesis of daugther strands. Step 5: DNA polymerase δ plus its accessory factors PCNA and Rfc elongate daughter strands initiated by Pol α-primase. ORC binds to the origin sequence in the daughter double-stranded DNA, but the phosphorylated initiation factors cannot assemble a pre-replication complex on it. B-type cyclin-CDK complexes maintain the initiation factors in a phosporylated state throughout the remainder of S, G_2, and early anaphase (top). These factors cannot assemble into new pre-replication complexes until the B-type cyclins are degraded following their polyubiquitinylation by the APC in late anaphase. Recent results indicate that additional proteins not shown function at step 3. While the phosphorylation of several pre-replication complex components is represented, all the critical components whose phosphorylation by an S phase cyclin-CDK or by DDK is required for initiation have not been determined.

the synthesis of SV40 DNA (see Figure 4-34); these proteins are required for continued synthesis of the leading strand and for synthesis of most of the lagging strand. An additional DNA polymerase, Pol ε, is also required for chromosomal DNA synthesis, but its function is not yet understood.

As the replication forks progress away from each origin, presumably phosphorylated forms of Cdc6, Cdt1, and Mcm10 are displaced from the chromatin. However, ORC complexes immediately bind to the origin sequence in the replicated daughter duplex DNAs and remain bound throughout the cell cycle (see Figure 21-26, step 4). Origins can fire only once during the S phase because the phosphorylated initiation factors cannot reassemble into a pre-replication complex. Consequently, phosphorylation of components of the pre-replication complex by S-phase cyclin-CDK complexes and the DDK complex simultaneously activates initiation of DNA replication at an origin and inhibits re-initiation of replication at that origin. As we have noted, B-type cyclin-CDK complexes remain active throughout the S phase, G_2, and early anaphase, maintaining the phosphorylated state of the replication initiation factors that prevents the assembly of new pre-replication complexes (step 5).

Only when the APC triggers degradation of all B-type cyclins in late anaphase and telophase does the then unopposed action of phosphatases remove the phosphates on the initiation factors (Cdc6, Cdt1, and Mcm10), allowing the reassembly of pre-replication complexes during G_1. As discussed previously, the inhibition of APC activity throughout G_1 sets the stage for accumulation of the S-phase cyclins needed for onset of the S phase. This regulatory mechanism has two consequences: (1) pre-replication complexes are assembled only during G_1, when the activity of B-type cyclin-CDK complexes is low, and (2) each origin initiates replication one time only during the S phase, when S phase cyclin-CDK complex activity is high. As a result, chromosomal DNA is replicated only one time each cell cycle.

KEY CONCEPTS OF SECTION 21.5

Genetic Studies with *S. cerevisiae*

- *S. cerevisiae* expresses a single cyclin-dependent protein kinase (CDK), encoded by *CDC28*, which interacts with several different cyclins during different phases of the cell cycle (see Figure 21-25).
- Three G_1 cyclins are active in G_1: Cln1, Cln2, and Cln3. The concentration of *CLN3* mRNA does not vary significantly through the cell cycle, but its translation is regulated by the availability of nutrients.
- Once active Cln3-CDK complexes accumulate in mid-late G_1, they phosphorylate and activate two transcription factors that stimulate expression of Cln1 and Cln2, of enzymes and other proteins required for DNA replication, and of the S-phase B-type cyclins Clb5 and Clb6.
- The late G_1 cyclin-CDK complexes (Cln1-CDK and Cln2-CDK) phosphorylate and inhibit Cdh1, the specificity factor that directs the anaphase-promoting complex (APC) to B-type cyclins, thus permitting accumulation of S-phase B-type cyclins.
- S-phase cyclin-CDK complexes initially are inhibited by Sic1. Polyubiquitination of Sic1 by the SCF ubiquitin ligase marks Sic1 for proteasomal degradation, releasing activated S-phase cyclin-CDK complexes that trigger onset of the S phase (see Figure 21-24).
- B-type cyclins Clb3 and Clb4, expressed later in the S phase, form heterodimers with the CDK that also promote DNA replication and initiate spindle formation early in mitosis.
- B-type cyclins Clb1 and Clb2, expressed in G_2, form heterodimers with the CDK that stimulate mitotic events.
- In late anaphase, the specificity factor Cdh1 is activated by dephosphorylation and then directs APC to polyubiquitinate all of the B-type cyclins (Clbs). Their subsequent proteasomal degradation inactivates MPF activity, permitting exit from mitosis (see Figure 21-10).
- DNA replication is initiated from pre-replication complexes assembled at origins during early G_1. S-phase cyclin-CDK complexes simultaneously trigger initiation from pre-replication complexes and inhibit assembly of new pre-replication complexes by phosphorylating components of the pre-replication complex (see Figure 21-26).
- Initiation of DNA replication occurs at each origin, but only once, until a cell proceeds through anaphase when activation of APC leads to the degradation of B-type cyclins. The block on reinitiation of DNA replication until replicated chromosomes have segregated assures that daughter cells contain the proper number of chromosomes per cell.

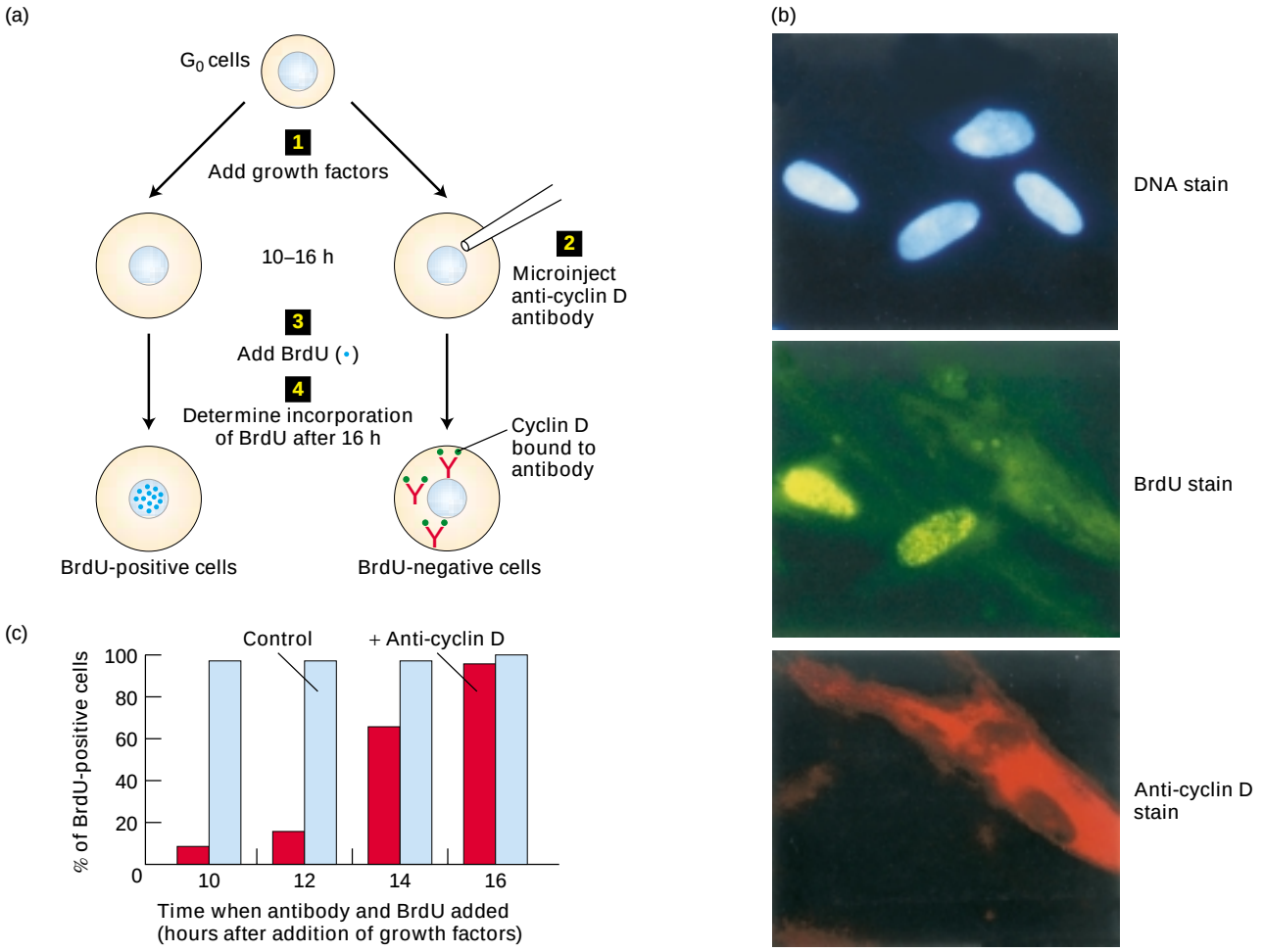
21.6 Cell-Cycle Control in Mammalian Cells

In multicellular organisms, precise control of the cell cycle during development and growth is critical for determining the size and shape of each tissue. Cell replication is controlled by a complex network of signaling pathways that integrate extracellular signals about the identity and numbers of neighboring cells and intracellular cues about cell size and developmental program (Chapter 15). Most differentiated cells withdraw from the cell cycle during G_1, entering the G_0 state (see Figure 21-1). Some differentiated cells (e.g., fibroblasts and lymphocytes) can be stimulated to reenter the cycle and replicate. Many postmitotic differentiated cells, however, never reenter the cell cycle to replicate again. As we discuss in this section, the cell-cycle regulatory mechanisms uncovered in yeasts and *Xenopus* eggs and early embryos also operate in the somatic cells of higher eukaryotes including humans and other mammals.

Mammalian Restriction Point Is Analogous to START in Yeast Cells

Most studies of mammalian cell-cycle control have been done with cultured cells that require certain polypeptide growth factors (mitogens) to stimulate cell proliferation. Binding of these growth factors to specific receptor proteins that span the plasma membrane initiates a cascade of signal transduction that ultimately influences transcription and cell-cycle control (Chapters 13–15).

Mammalian cells cultured in the absence of growth factors are arrested with a diploid complement of chromosomes



▲ EXPERIMENTAL FIGURE 21-27 **Microinjection experiments with anti-cyclin D antibody demonstrate that cyclin D is required for passage through the restriction point.** The G_0-arrested mammalian cells used in these experiments pass the restriction point 14–16 hours after addition of growth factors and enter the S phase 6–8 hours later. (a) Outline of experimental protocol. At various times 10–16 hours after addition of growth factors (1), some cells were microinjected with rabbit antibodies against cyclin D (2). Bromodeoxyuridine (BrdU), a thymidine analog, was then added to the medium (3), and the uninjected control cells (*left*) and microinjected experimental cells (*right*) were incubated for an additional 16 hours. Each sample then was analyzed to determine the percentage of cells that had incorporated BrdU (4), indicating that they had entered the S phase. (b) Analysis of control cells and experimental cells 8 hours after addition of growth factors. The three micrographs show the same field of cells stained 16 hours after addition of BrdU to the medium. Cells were stained with different fluorescent agents to visualize DNA (*top*), BrdU (*middle*), and anti-cyclin D antibody (*bottom*). Note that the two cells in this field injected with anti-cyclin D antibody (the red cells in the bottom micrograph) did not incorporate BrdU into nuclear DNA, as indicated by their lack of staining in the middle micrograph. (c) Percentage of control cells (blue bars) and experimental cells (red bars) that incorporated BrdU. Most cells injected with anti-cyclin D antibodies 10 or 12 hours after addition of growth factors failed to enter the S phase, indicated by the low level of BrdU incorporation. In contrast, anti-cyclin D antibodies had little effect on entry into the S phase and DNA synthesis when injected at 14 or 16 hours, that is, after cells had passed the restriction point. These results indicate that cyclin D is required to pass the restriction point, but once cells have passed the restriction point, they do not require cyclin D to enter the S phase 6–8 hours later. [Parts (b) and (c) adapted from V. Baldin et al., 1993, *Genes & Devel.* **7**:812.]

in the G_0 period of the cell cycle. If growth factors are added to the culture medium, these **quiescent cells** pass through the **restriction point** 14–16 hours later, enter the S phase 6–8 hours after that, and traverse the remainder of the cell cycle (see Figure 21-2). Like START in yeast cells, the restriction point is the point in the cell cycle at which mammalian cells become committed to entering the S phase and completing the cell cycle. If mammalian cells are moved from a medium containing growth factors to one lacking growth factors before they have passed the restriction point, the cells do not enter the S phase. But once cells have passed the restriction point, they are committed to entering the S phase and progressing through the entire cell cycle, which takes about 24 hours for most cultured mammalian cells.

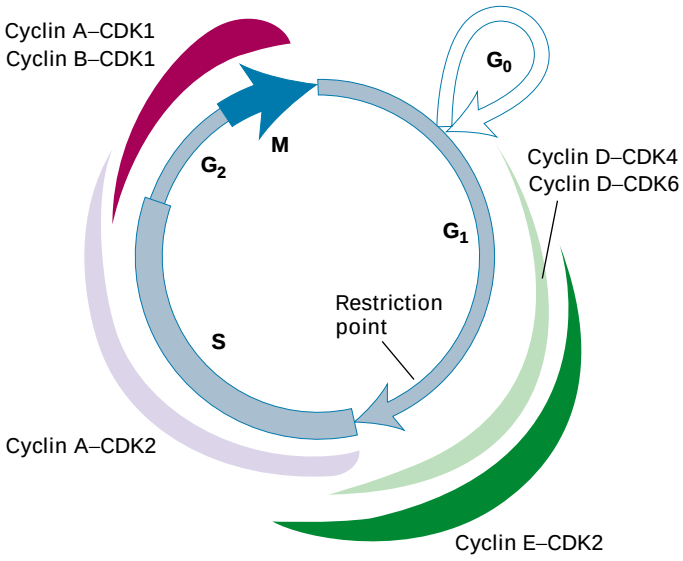
Multiple CDKs and Cyclins Regulate Passage of Mammalian Cells Through the Cell Cycle

Unlike *S. pombe* and *S. cerevisiae*, which each produce a single cyclin-dependent kinase (CDK) to regulate the cell cycle, mammalian cells use a small family of related CDKs to regulate progression through the cell cycle. Four CDKs are expressed at significant levels in most mammalian cells and play a role in regulating the cell cycle. Named CDK1, 2, 4, and 6, these proteins were identified by the ability of their cDNA clones to complement certain *cdc* yeast mutants or by their homology to other CDKs.

Like *S. cerevisiae*, mammalian cells express multiple cyclins. Cyclin A and cyclin B, which function in the S phase, G_2, and early mitosis, initially were detected as proteins whose concentration oscillates in experiments with synchronously cycling early sea urchin and clam embryos (see Figure 21-8). Homologous cyclin A and cyclin B proteins have been found in all multicellular animals examined. cDNAs encoding three related human D-type cyclins and cyclin E were isolated based on their ability to complement *S. cerevisiae* cells mutant in all three *CLN* genes encoding G_1 cyclins. The relative amounts of the three D-type cyclins expressed in various cell types (e.g., fibroblasts, hematopoietic cells) differ. Here we refer to them collectively as cyclin D. Cyclin D and E are the mammalian G_1 cyclins. Experiments in which cultured mammalian cells were microinjected with anti-cyclin D antibody at various times after addition of growth factors demonstrated that cyclin D is essential for passage through the restriction point (Figure 21-27).

Figure 21-28 presents a current model for the periods of the cell cycle in which different cyclin-CDK complexes act in G_0-arrested mammalian cells stimulated to divide by the addition of growth factors. In the absence of growth factors, cultured G_0 cells express neither cyclins nor CDKs; the absence of these critical proteins explains why G_0 cells do not progress through the cell cycle and replicate.

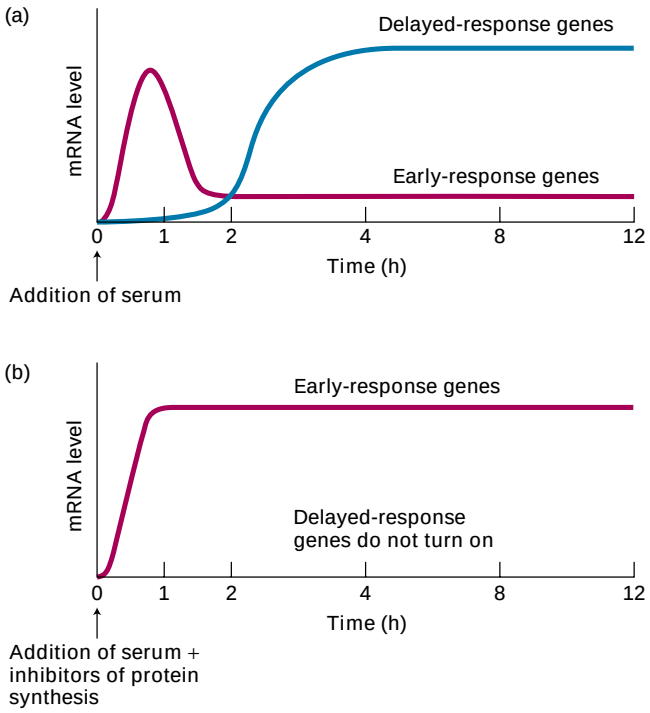
Table 21-1, presented early in this chapter, summarizes the various cyclins and CDKs that we have mentioned and the portions of the cell cycle in which they are active. The cyclins fall into two major groups, G_1 cyclins and B-type cyclins, which function in S, G_2, and M. Although it is not possible to draw a simple one-to-one correspondence between the functions of the several cyclins and CDKs in *S. pombe*, *S. cerevisiae*, and vertebrates, the various cyclin-CDK complexes they form can be broadly considered in terms of their functions in mid G_1, late G_1, S, and M phases. All B-type cyclins contain a conserved destruction box sequence that is recognized by the APC ubiquitin ligase, whereas G_1 cyclins lack this sequence. Thus the APC regulates the activity only of cyclin-CDK complexes that include B-type cyclins.



▲ FIGURE 21-28 **Activity of mammalian cyclin-CDK complexes through the course of the cell cycle in cultured G_0 cells induced to divide by treatment with growth factors.** The width of the colored bands is approximately proportional to the protein kinase activity of the indicated complexes. Cyclin D refers to all three D-type cyclins.

Regulated Expression of Two Classes of Genes Returns G_0 Mammalian Cells to the Cell Cycle

Addition of growth factors to G_0-arrested mammalian cells induces transcription of multiple genes, most of which fall into one of two classes—*early-response* or *delayed-response* genes—depending on how soon their encoded mRNAs appear (Figure 21-29a). Transcription of early-response genes is induced within a few minutes after addition of growth factors by signal-transduction cascades that activate preexisting transcription factors in the cytosol or nucleus. Induction of early-response genes is not blocked by inhibitors of protein synthesis (Figure 21-29b) because the required transcription factors are present in G_0 cells and are activated by phosphorylation or removal of an inhibitor in response to stimulation of cells by growth factors (Chapter 14). Many of the early-response genes encode transcription factors, such as c-Fos and c-Jun, that stimulate transcription of the delayed-



▲ FIGURE 21-29 **General time course of expression of early- and delayed-response genes in G_0-arrested mammalian cells after addition of serum.** (a) In the absence of inhibitors of protein synthesis, expression of early-response genes peaks about 1 hour after addition of serum, which contains several mitogens, and then falls as expression of late-response genes begins. (b) Inhibitors of protein synthesis prevent the drop in expression of early-response genes and completely block expression of late-response genes. See text for discussion. [Adapted from A. Murray and T. Hunt, 1993, *The Cell Cycle: An Introduction,* W. H. Freeman and Company.]

response genes. Mutant, unregulated forms of both c-Fos and c-Jun are expressed by oncogenic retroviruses (Chapter 23); the discovery that the viral forms of these proteins (v-Fos and v-Jun) can transform normal cells into cancer cells led to identification of the regulated cellular forms of these transcription factors.

After peaking at about 30 minutes following addition of growth factors, the concentrations of the early-response mRNAs fall to a lower level that is maintained as long as growth factors are present in the medium. Most of the immediate early mRNAs are unstable; consequently, their concentrations fall as their rate of synthesis decreases. This drop in transcription is blocked by inhibitors of protein synthesis (see Figure 21-29b), indicating that it depends on production of one or more of the early-response proteins.

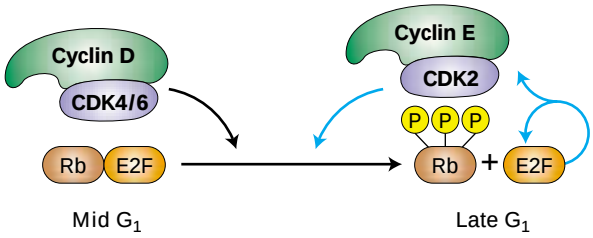
Because expression of delayed-response genes depends on proteins encoded by early-response genes, delayed-response genes are not transcribed when mitogens are added to G_0-arrested cells in the presence of an inhibitor of protein synthesis. Some delayed-response genes encode additional transcription factors (see below); others encode the D-type cyclins, cyclin E, CDK2, CDK4, and CDK6. The D-type cyclins, CDK4, and CDK6, are expressed first, followed by cyclin E and CDK2 (see Figure 21-28). If growth factors are withdrawn before passage through the restriction point, expression of these G_1 cyclins and CDKs ceases. Since these proteins and the mRNAs encoding them are unstable, their concentrations fall precipitously. As a consequence, the cells do not pass the restriction point and do not replicate.

In addition to transcriptional control of the gene encoding cyclin D, the concentration of this mid-G_1 cyclin also is regulated by controlling *translation* of cyclin D mRNA. In this regard, cyclin D is similar to *S. cerevisiae* Cln3. Addition of growth factors to cultured mammalian cells triggers signal transduction via the PI-3 kinase pathway discussed in Chapter 14, leading to activation of the translation-initiation factor eIF4 (Chapter 4). As a result, translation of cyclin D mRNA and other mRNAs is stimulated. Agents that inhibit eIF4 activation, such as TGF-β, inhibit translation of cyclin D mRNA and thus inhibit cell proliferation.

Passage Through the Restriction Point Depends on Phosphorylation of the Tumor-Suppressor Rb Protein

Some members of a small family of related transcription factors, referred to collectively as *E2F factors,* are encoded by delayed-response genes. These transcription factors activate genes encoding many of the proteins involved in DNA and deoxyribonucleotide synthesis. They also stimulate transcription of genes encoding the late-G_1 cyclin (cyclin E), the S-phase cyclin (cyclin A), and the S-phase CDK (CDK2). Thus the E2Fs function in late G_1 similarly to the *S. cerevisiae* transcription factors SBF and MBF. In addition, E2Fs autostimulate transcription of their own genes. E2Fs function as transcriptional repressors when bound to *Rb protein,* which in turn binds histone deacetylase complexes. As discussed in Chapter 11, transcription of a gene is highest when the associated histones are highly acetylated; histone deacetylation causes chromatin to assume a more condensed, transcriptionally inactive form.

Rb protein was initially identified as the product of the prototype **tumor-suppressor gene,** *RB.* The products of tumor-suppressor genes function in various ways to inhibit progression through the cell cycle (Chapter 23). Loss-of-function mutations in *RB* are associated with the disease *hereditary retinoblastoma.* A child with this disease inherits one normal RB^+ allele from one parent and one mutant RB^- allele from the other. If the RB^+ allele in any of the trillions of cells that make up the human body becomes mutated to a RB^- allele, then no functional protein is expressed and the cell or one of its descendants is likely to become cancerous. For reasons that are not understood, this generally happens in a retinal cell leading to the retinal tumors that characterize this disease. Also, in most human cancer cells Rb function is inactivated, either by mutations in both alleles of *RB,* or by abnormal regulation of Rb phosphorylation. ▮



▲ FIGURE 21-30 **Regulation of Rb and E2F activities in mid-late G_1.** Stimulation of G_0 cells with mitogens induces expression of CDK4, CDK6, D-type cyclins, and the E2F transcription factors, all encoded by delayed-response genes. Rb protein initially inhibits E2F activity. When signaling from mitogens is sustained, the resulting cyclin D-CDK4/6 complexes begin phosphorylating Rb, releasing some E2F, which stimulates transcription of the genes encoding cyclin E, CDK2, and E2F (autostimulation). The cyclin E-CDK4 complexes further phosphorylate Rb, resulting in positive feedback loops (blue arrows) that lead to a rapid rise in the expression and activity of both E2F and cyclin E-CDK2 as the cell approaches the $G_1 \rightarrow S$ transition.

Rb protein is one of the most significant substrates of mammalian G_1 cyclin-CDK complexes. Phosphorylation of Rb protein at multiple sites prevents its association with E2Fs, thereby permitting E2Fs to activate transcription of genes required for entry into S phase. As shown in Figure 21-30, phosphorylation of Rb protein is initiated by cyclin D-CDK4 and cyclin D-CDK6 in mid G_1. Once cyclin E and CDK2 are induced by phosphorylation of some Rb, the resulting cyclin E-CDK2 further phosphorylates Rb in late G_1. When cyclin E-CDK2 accumulates to a critical threshold level, further phosphorylation of Rb by cyclin E-CDK2 continues even when cyclin D-CDK4/6 activity is removed. This is one of the principle biochemical events responsible for passage through the restriction point. At this point, further phosphorylation of Rb by cyclin E-CDK2 occurs even when mitogens are withdrawn and cyclin D and CDK4/6 levels fall. Since E2F stimulates its own expression and that of cyclin E and CDK2, positive cross-regulation of E2F and cyclin E-CDK2 produces a rapid rise of both activities in late G_1.

As they accumulate, S-phase cyclin-CDK and mitotic cyclin-CDK complexes maintain Rb protein in the phosphorylated state throughout the S, G_2, and early M phases. After cells complete anaphase and enter early G_1 or G_0, the fall in cyclin-CDK levels leads to dephosphorylation of Rb by unopposed phosphatases. As a consequence, hypophosphorylated Rb is available to inhibit E2F activity during early G_1 of the next cycle and in G_0-arrested cells.

Cyclin A Is Required for DNA Synthesis and CDK1 for Entry into Mitosis

High levels of E2Fs activate transcription of the *cyclin A* gene as mammalian cells approach the $G_1 \rightarrow S$ transition. (Despite its name, cyclin A is a B-type cyclin, not a G_1 cyclin; see Table 21-1.) Disruption of cyclin A function inhibits DNA synthesis in mammalian cells, suggesting that cyclin A-CDK2 complexes may function like *S. cerevisiae* S-phase cyclin-CDK complexes to trigger initiation of DNA synthesis. There is also evidence that cyclin E-CDK2 may contribute to activation of pre-replication complexes.

Three related *C*DK *i*nhibitory *p*roteins, or *CIPs* ($p27^{KIP1}$, $p57^{KIP2}$, and $p21^{CIP}$), appear to share the function of the *S. cerevisiae* S-phase inhibitor Sic1 (see Figure 21-24). Phosphorylation of $p27^{KIP1}$ by cyclin E-CDK2 targets it for polyubiquitination by the mammalian SCF complex (see Figure 21-2, step 5). The SCF subunit that targets $p27^{KIP1}$ is synthesized as cells approach the $G_1 \rightarrow S$ transition. The mechanism for degrading $p21^{CIP}$ and $p57^{KIP2}$ is less well understood. The activity of mammalian cyclin-CDK2 complexes is also regulated by phosphorylation and dephosphorylation mechanisms similarly to those controlling the *S. pombe* mitosis-promoting factor, MPF (see Figure 21-14). The *Cdc25A phosphatase*, which removes the inhibitory phosphate from CDK2, is a mammalian equivalent of *S. pombe* Cdc25 except that it functions at the $G_1 \rightarrow S$ transition rather than the $G_2 \rightarrow M$ transition. The mammalian phosphatase normally is activated late in G_1, but is degraded in the response of mammalian cells to DNA damage to prevent the cells from entering S phase (see Section 21.7).

Once cyclin A-CDK2 is activated by Cdc25A and the S-phase inhibitors have been degraded, DNA replication is initiated at pre-replication complexes. The general mechanism is thought to parallel that in *S. cerevisiae* (see Figure 21-26), although small differences are found in vertebrates. As in yeast, phosphorylation of certain initiation factors by cyclin A-CDK2 most likely promotes initiation of DNA replication and prevents reassembly of pre-replication complexes until the cell passes through mitosis, thereby assuring that replication from each origin occurs only once during each cell cycle. In metazoans, a second small protein, geminin, contributes to the inhibition of re-initiation at origins until cells complete a full cell cycle.

The principle mammalian CDK in G_2 and mitosis is CDK1 (see Figure 21-28). This CDK, which is highly homologous with *S. pombe* Cdc2, associates with cyclins A and B. The mRNAs encoding either of these mammalian cyclins can promote meiotic maturation when injected into *Xenopus* oocytes arrested in G_2 (see Figure 21-6), demonstrating that they function as mitotic cyclins. Thus mammalian cyclin A-CDK1 and cyclin B-CDK1 are functionally equivalent to the *S. pombe* MPF (mitotic cyclin-CDK). The kinase activity of these mammalian complexes also appears to be regulated by proteins analogous to those that control the activity of the *S. pombe* MPF (see Figure 21-14). The inhibitory phosphate on CDK1 is removed by *Cdc25C phosphatase*, which is analogous to *S. pombe* Cdc25 phosphatase.

In cycling mammalian cells, cyclin B is first synthesized late in the S phase and increases in concentration as cells proceed through G_2, peaking during metaphase and dropping after late anaphase. This parallels the time course of cyclin B expression in *Xenopus* cycling egg extracts (see Figure 21-9).

In human cells, cyclin B first accumulates in the cytosol and then enters the nucleus just before the nuclear envelope breaks down early in mitosis. Thus MPF activity is controlled not only by phosphorylation and dephosphorylation but also by regulation of the nuclear transport of cyclin B. In fact, cyclin B shuttles between the nucleus and cytosol, and the change in its localization during the cell cycle results from a change in the relative rates of import and export. As in *Xenopus* eggs and *S. cerevisiae*, cyclins A and B are polyubiquitinated by the anaphase-promoting complex (APC) during late anaphase and then are degraded by proteasomes (see Figure 21-2, step 9).

Two Types of Cyclin-CDK Inhibitors Contribute to Cell-Cycle Control in Mammals

As noted above, three related CIPs—$p21^{CIP}$, $p27^{KIP2}$, and $p57^{KIP2}$—inhibit cyclin A-CDK2 activity and must be degraded before DNA replication can begin. These same CDK inhibitory proteins also can bind to and inhibit the other mammalian cyclin-CDK complexes involved in cell-cycle control. As we discuss later, $p21^{CIP}$ plays a role in the response of mammalian cells to DNA damage. Experiments with knockout mice lacking $p27^{KIP2}$ have shown that this CIP is particularly important in controlling generalized cell proliferation soon after birth. Although $p27^{KIP2}$ knockouts are larger than normal, most develop normally otherwise. In contrast, $p57^{KIP2}$ knockouts exhibit defects in cell differentiation and most die shortly after birth due to defective development of various organs.

A second class of cyclin-CDK inhibitors called *INK4s* (*in*hibitors of *k*inase *4*) includes several small, closely related proteins that interact only with CDK4 and CDK6 and thus function specifically in controlling the mid-G_1 phase. Binding of INK4s to CDK4/6 blocks their interaction with cyclin D and hence their protein kinase activity. The resulting decreased phosphorylation of Rb protein prevents transcriptional activation by E2Fs and entry into the S phase. One INK4 called p16 is a tumor suppressor, like Rb protein discussed earlier. The presence of two mutant p16 alleles in a large fraction of human cancers is evidence for the important role of p16 in controlling the cell cycle (Chapter 23).

KEY CONCEPTS OF SECTION 21.6

Cell-Cycle Control in Mammalian Cells

- Various polypeptide growth factors called mitogens stimulate cultured mammalian cells to proliferate by inducing expression of early-response genes. Many of these encode transcription factors that stimulate expression of delayed-response genes encoding the G_1 CDKs, G_1 cyclins, and E2F transcription factors.
- Once cells pass the restriction point, they can enter the S phase and complete S, G_2, and mitosis in the absence of growth factors.
- Mammalian cells use several CDKs and cyclins to regulate passage through the cell cycle. Cyclin D-CDK4/6 function in mid to late G_1; cyclin E-CDK2 in late G_1 and early S; cyclin A-CDK2 in S; and cyclin A/B-CDK1 in G_2 and M through anaphase (see Figure 21-28).
- Unphosphorylated Rb protein binds to E2Fs, converting them into transcriptional repressors. Phosphorylation of Rb by cyclin D-CDK4/6 in mid G_1 liberates E2Fs to activate transcription of genes encoding cyclin E, CDK2, and other proteins required for the S phase. E2Fs also autostimulate transcription of their own genes.
- Cyclin E-CDK2 further phosphorylates Rb, further activating E2Fs. Once a critical level of cyclin E-CDK2 has been expressed, a positive feedback loop with E2F results in a rapid rise of both activities that drives passage through the restriction print (see Figure 21-30).
- The activity of cyclin A-CDK2, induced by high E2F activity, initially is held in check by CIPs, which function like an S-phase inhibitor, and by the presence of an inhibitory phosphate on CDK2. Proteasomal degradation of the inhibitors and activation of the Cdc25A phosphatase, as cells approach the $G_1 \rightarrow S$ transition, generate active cyclin A-CDK2. This complex activates pre-replication complexes to initiate DNA synthesis by a mechanism similar to that in *S. cerevisiae* (see Figure 21-26).
- Cyclin A/B-CDK1 induce the events of mitosis through early anaphase. Cyclins A and B are polyubiquitinated by the anaphase-promoting complex (APC) during late anaphase and then are degraded by proteasomes.
- The activity of mammalian mitotic cyclin-CDK complexes also are regulated by phosphorylation and dephosphorylation similar to the mechanism in *S. pombe*, with the Cdc25C phosphatase removing inhibitory phosphates (see Figure 21-14).
- The activities of mammalian cyclin-CDK complexes also are regulated by CDK inhibitors (CIPs), which bind to and inhibit each of the mammalian cyclin-CDK complexes, and INK4 proteins, which block passage through G_1 by specifically inhibiting CDK4 and CDK6.

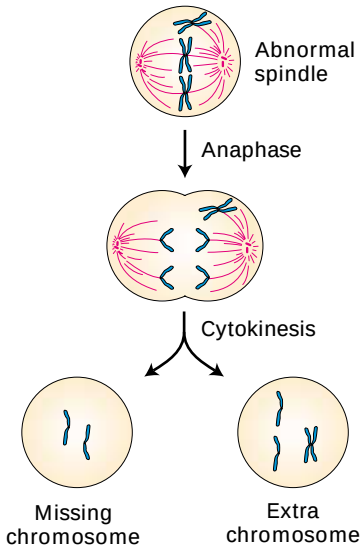
21.7 Checkpoints in Cell-Cycle Regulation

Catastrophic genetic damage can occur if cells progress to the next phase of the cell cycle before the previous phase is properly completed. For example, when S-phase cells are induced to enter mitosis by fusion to a cell in mitosis, the MPF present in the mitotic cell forces the chromosomes of the S-phase cell to condense. However, since the replicating chromosomes are fragmented by the condensation process, such premature entry into mitosis is disastrous for a cell.

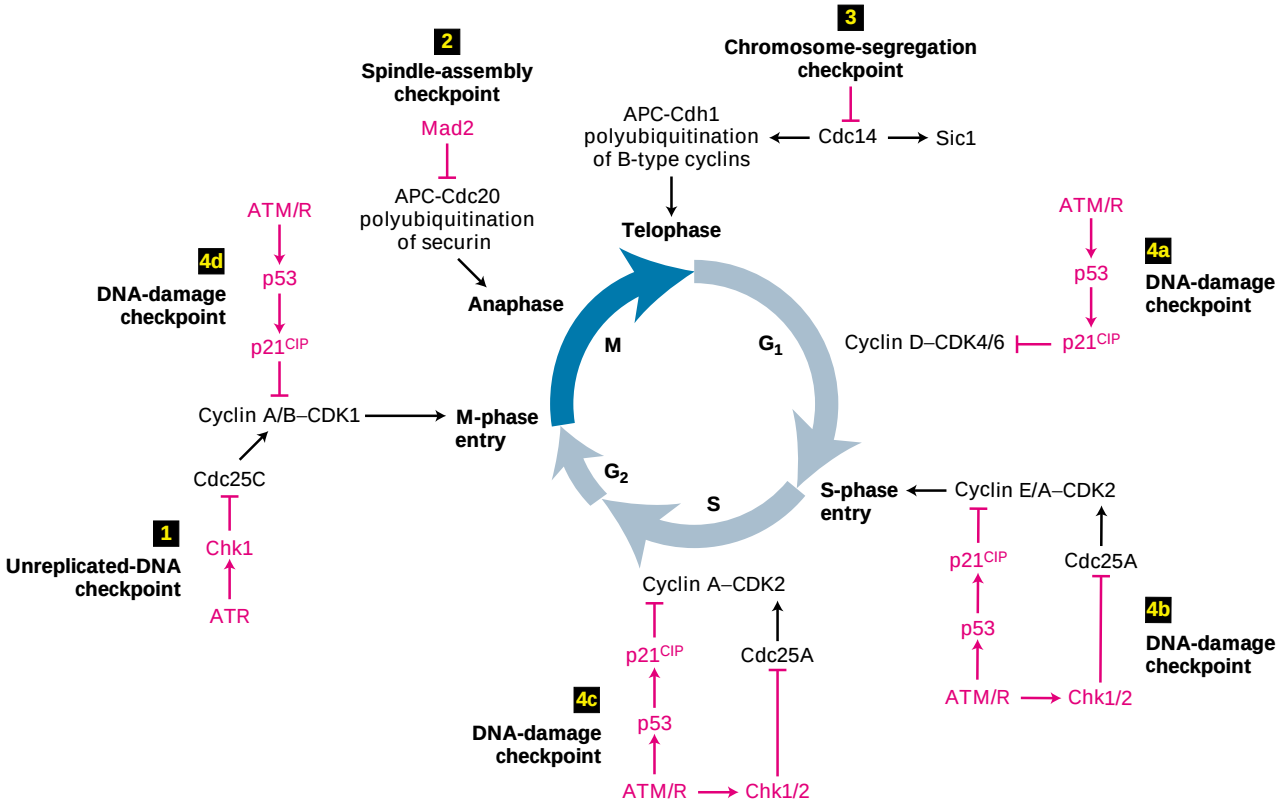
MEDICINE

Another example concerns attachment of kinetochores to microtubules of the mitotic spindle during metaphase. If anaphase is initiated before both kinetochores of a replicated chromosome become attached to microtubules from opposite spindle poles, daughter cells are produced that have a missing or extra chromosome (Figure 21-31). When this process, called *nondisjunction*, occurs during the meiotic division that generates a human egg, Down syndrome can occur from trisomy of chromosome 21, resulting in developmental abnormalities and mental retardation. ▮

To minimize the occurrence of such mistakes in cell-cycle events, a cell's progress through the cycle is monitored at several **checkpoints** (Figure 21-32). Control mechanisms that operate at these checkpoints ensure that chromosomes are intact and that each stage of the cell cycle is completed before the following stage is initiated. Our understanding of these control mechanisms at the molecular level has advanced considerably in recent years.



◀ FIGURE 21-31 **Nondisjunction.** This abnormality occurs when chromosomes segregate in anaphase before the kinetochore of each sister chromatid has attached to microtubules (red lines) from the opposite spindle poles. As a result, one daughter cell contains two copies of one chromosome, while the other daughter cell lacks that chromosome. [Adapted from A. Murray and T. Hunt, 1993, *The Cell Cycle: An Introduction,* W. H. Freeman and Company.]



▲ FIGURE 21-32 **Overview of checkpoint controls in the cell cycle.** The unreplicated-DNA checkpoint (1) prevents activation of cyclin A-CDK1 and cyclin B-CDK1 (i.e., mitosis-promoting factor, MPF) by activation of an ATR-Chk1 protein kinase cascade that phosphorylates and inactivates Cdc25C, thereby inhibiting entry into mitosis. In the spindle-assembly checkpoint (2), Mad2 and other proteins inhibit activation of the APC specificity factor (Cdc20) required for polyubiquitination of securin, thereby preventing entry into anaphase. The chromosome-segregation checkpoint (3) prevents release of the Cdc14 phosphatase from nucleoli, thereby blocking activation of the APC specificity factor (Cdh1) required for polyubiquitination of B-type cyclins as well as induction of Sic1. As a result, the decrease in MPF activity required for the events of telophase does not occur. In the initial phase of the DNA-damage checkpoint (4), the ATM or ATR protein kinase (ATM/R) is activated. The active kinases then trigger two pathways: the Chk-Cdc25A pathway (4b and 4c), blocking entry into or through the S phase, and the p53-p21^{CIP} pathway, leading to arrest in G_1, S, and G_2 (4a–4b). See text for further discussion. Red symbols indicate pathways that inhibit progression through the cell cycle.

The Presence of Unreplicated DNA Prevents Entry into Mitosis

Cells that fail to replicate all their chromosomes do not enter mitosis. Operation of the *unreplicated-DNA checkpoint* control involves the recognition of unreplicated DNA and inhibition of MPF activation (see Figure 21-32, 1). Recent genetic studies in *S. pombe* and biochemical studies with *Xenopus* egg extracts suggest that the ATR and Chk1 protein kinases, which also function in the DNA-damage checkpoint, inhibit entry into mitosis by cells that have not completed DNA synthesis.

The association of ATR with replication forks is thought to activate its protein kinase activity, leading to the phosphorylation and activation of the Chk1 kinase. Active Chk1 then phosphorylates and inactivates the Cdc25 phosphatase (Cdc25C in vertebrates), which normally removes the inhibitory phosphate from CDKs that function during mitosis. As a result, the cyclin A/B-CDK1 complexes remain inhibited and cannot phosphorylate targets required to initiate mitosis. ATR continues to initiate this protein kinase cascade until all replication forks complete DNA replication and disassemble.

Improper Assembly of the Mitotic Spindle Prevents the Initiation of Anaphase

The *spindle-assembly checkpoint* prevents entry into anaphase when just a single kinetochore of one chromatid fails to associate properly with spindle microtubules. Clues about how this checkpoint operates has come from isolation of yeast mutants in the presence of benomyl, a microtubule-depolymerizing drug. Low concentrations of benomyl increase the time required for yeast cells to assemble the mitotic spindle and attach kinetochores to microtubules. Wild-type cells exposed to benomyl do not begin anaphase until these processes are completed and then proceed on through mitosis, producing normal daughter cells. In contrast, mutants defective in the spindle-assembly checkpoint proceed through anaphase before assembly of the spindle and attachment of kinetochores is complete; consequently, they mis-segregate their chromosomes, producing abnormal daughter cells that die.

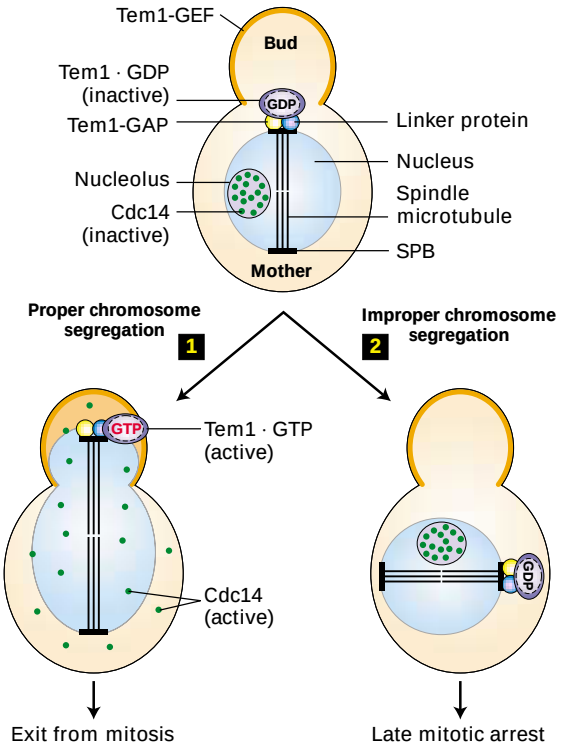
Analysis of these mutants identified a protein called Mad2 and other proteins that regulate Cdc20, the specificity factor required to target the APC to securin (see Figure 21-32, 2). Recall that APC-mediated polyubiquitination of securin and its subsequent degradation is required for entry into anaphase (see Figure 21-19). Mad2 has been shown to associate with kinetochores that are unattached to microtubules. Experiments with Mad2 fused to green fluorescent protein (GFP) indicate that kinetochore-bound Mad2 rapidly exchanges with a soluble form of Mad2. Current models propose that when Mad2 associates with a kinetochore complex that is not bound by a microtubule, it is converted to a short-lived activated form that can interact with and inhibit Cdc20. Microtubule attachment prevents this activation of Mad2. Consequently, once all kinetochore complexes bind a microtubule, generation of the activated form of Mad2 ceases, the inhibition of Cdc20 is relieved, and Cdc20 is free to direct the APC to polyubiquitinate securin, thereby initiating the onset of anaphase.

Proper Segregation of Daughter Chromosomes Is Monitored by the Mitotic Exit Network

Once chromosomes have segregated properly, telophase commences. The various events of telophase and subsequent cytokinesis, collectively referred to as the exit from mitosis, require inactivation of MPF. As discussed earlier, dephosphorylation of the APC specificity factor Cdh1 by the Cdc14 phosphatase leads to degradation of mitotic cyclins and loss of MPF activity late in anaphase (see Figure 21-10). During interphase and early mitosis, Cdc14 is sequestered in the nucleolus and inactivated. The *chromosome-segregation checkpoint*, which monitors the location of the segregating daughter chromosomes at the end of anaphase, determines whether active Cdc14 is available to promote exit from mitosis (see Figure 21-32, 3).

Operation of this checkpoint in *S. cerevisiae* depends on a set of proteins referred to as the *mitotic exit network*. A key component is a small (monomeric) GTPase, called Tem1. This member of the **GTPase superfamily** of switch proteins controls the activity of a protein kinase cascade similarly to the way Ras controls MAP kinase pathways (Chapter 14). During anaphase, Tem1 becomes associated with the spindle pole body (SPB) closest to the daughter cell bud. (The SPB, from which spindle microtubules originate, is equivalent to the centrosome in higher eukaryotes.) At the SPB, Tem1 is maintained in the inactive GDP-bound state by a specific GAP (GTPase-accelerating protein). The GEF (guanosine nucleotide–exchange factor) that activates Tem1 is localized to the cortex of the bud and is absent from the mother cell. When spindle microtubule elongation at the end of anaphase has correctly positioned segregating daughter chromosomes into the bud, Tem1 comes into contact with its GEF and is converted into the active GTP-bound state. The terminal kinase in the cascade triggered by Tem1 · GTP then phosphorylates the nucleolar anchor that binds and inhibits Cdc14, releasing it into the cytoplasm and nucleoplasm in both the bud and mother cell (Figure 21-33, 1). Once active Cdc14 is available, a cell can proceed through telophase and cytokinesis. If daughter chromosomes fail to segregate into the bud, Tem1 remains in its inactive state, Cdc14 is not released from the nucleolus, and mitotic exit is blocked (Figure 21-33, 2).

In the fission yeast *S. pombe*, formation of the septum that divides daughter cells is regulated by proteins homologous to those that constitute the mitotic exit network in *S. cerevisiae*. Genes encoding similar proteins also have been found in higher organisms where the homologs probably



▲ FIGURE 21-33 **Operation of the chromosome-segregation checkpoint.** In *S. cerevisiae,* Cdc14 phosphatase activity is required for the exit from mitosis. (*Top*) During interphase and early mitosis, Cdc14 is sequestered and inactivated in the nucleolus. Inactive Tem1 · GDP (purple) associates with the spindle pole body (SPB) nearest to the bud early in anaphase with the aid of a linker protein (green) and is maintained in the inactive state by a specific GAP (GTPase-accelerating protein). If chromosome segregation occurs properly (**1**), extension of the spindle microtubules inserts the daughter SPB into the bud, causing Tem1 to come in contact with a specific GEF (guanine nucleotide–exchange factor) localized to the cortex of the bud (brown). This GEF converts inactive Tim1 · GDP to active Tem1 · GTP, which triggers a protein kinase cascade leading to release of active Cdc14 and exit from mitosis. If the spindle apparatus fails to place the daughter SPB in the bud (**2**), Tem1 remains in the inactive GDP-bound state and Cdc14 remains associated with nucleoli. Arrest in late mitosis results. [Adapted from G. Pereira and E. Schiebel, 2001, *Curr. Opin. Cell Biol.* **13**:762.]

function in an analogous checkpoint that leads to arrest in late mitosis when daughter chromosomes do not segregate properly.

Cell-Cycle Arrest of Cells with Damaged DNA Depends on Tumor Suppressors

MEDICINE

The *DNA-damage checkpoint* blocks progression through the cell cycle until the damage is repaired. Damage to DNA can result from chemical agents and from irradiation with ultraviolet (UV) light or γ-rays. Arrest in G_1 and S prevents copying of damaged bases, which would fix mutations in the genome. Replication of damaged DNA also promotes chromosomal rearrangements that can contribute to the onset of cancer. Arrest in G_2 allows DNA double-stranded breaks to be repaired before mitosis. If a double-stranded break is not repaired, the broken distal portion of the damaged chromosome is not properly segregated because it is not physically linked to a centromere, which is pulled toward a spindle pole during anaphase.

As we discuss in detail in Chapter 23, inactivation of tumor-suppressor genes contributes to the development of cancer. The proteins encoded by several tumor-suppressor genes, including *ATM* and *Chk2,* normally function in the DNA-damage checkpoint. Patients with mutations in both copies of *ATM* or *Chk2* develop cancers far more frequently than normal. Both of these genes encode protein kinases.

DNA damage due to UV light somehow activates the ATM kinase, which phosphorylates Chk2, thereby activating its kinase activity. Activated Chk2 then phosphorylates the Cdc25A phosphatase, marking it for polyubiquitination by an as-yet undetermined ubiquitin ligase and subsequent proteasomal degradation. Recall that removal of the inhibitory phosphate from mammalian CDK2 by Cdc25A is required for onset of and passage through the S phase mediated by cyclin E-CDK2 and cyclin A-CDK2. Degradation of Cdc25A resulting from activation of the ATM-Chk2 pathway in G_1 or S-phase cells thus leads to G_1 or S arrest (see Figure 21-32, 4b and 4c). A similar pathway consisting of the protein kinases ATR and Chk1 leads to phosphorylation and polyubiquitination of Cdc25A in response to γ-radiation. As discussed earlier for the unreplicated-DNA checkpoint, Chk1 also inactivates Cdc25C, preventing the activation of CDK1 and entry into mitosis.

Another tumor-suppressor protein, **p53,** contributes to arrest of cells with damaged DNA. Cells with functional p53 arrest in G_1 and G_2 when exposed to γ-irradiation, whereas cells lacking functional p53 do not arrest in G_1. Although the p53 protein is a transcription factor, under normal conditions it is extremely unstable and generally does not accumulate to high enough levels to stimulate transcription. The instability of p53 results from its polyubiquitination by a ubiquitin ligase called Mdm2 and subsequent proteasomal degradation. The rapid degradation of p53 is inhibited by ATM and probably ATR, which phosphorylate p53 at a site that interferes with binding by Mdm2. This and other modifications of p53 in response to DNA damage greatly increase its ability to activate transcription of specific genes that help the cell cope with DNA damage. One of these genes encodes $p21^{CIP}$, a generalized CIP that binds and inhibits all mammalian cyclin-CDK complexes. As a result, cells are arrested in G_1 and G_2 until the DNA damage is repaired and p53 and subsequently $p21^{CIP}$ levels fall (see Figure 21-32, 4a–4d).

Under some circumstances, such as when DNA damage is extensive, p53 also activates expression of genes that lead to apoptosis, the process of programmed cell death that

normally occurs in specific cells during the development of multicellular animals. In vertebrates, the p53 response evolved to induce apoptosis in the face of extensive DNA damage, presumably to prevent the accumulation of multiple mutations that might convert a normal cell into a cancer cell. The dual role of p53 in both cell-cycle arrest and the induction of apoptosis may account for the observation that nearly all cancer cells have mutations in both alleles of the *p53* gene or in the pathways that stabilize p53 in response to DNA damage (Chapter 23). The consequences of mutations in *p53*, *ATM*, and *Chk2* provide dramatic examples of the significance of cell-cycle checkpoints to the health of a multicellular organism. ▮

KEY CONCEPTS OF SECTION 21.7

Checkpoints in Cell-Cycle Regulation

- Checkpoint controls function to ensure that chromosomes are intact and that critical stages of the cell cycle are completed before the following stage is initiated.
- The unreplicated-DNA checkpoint operates during S and G_2 to prevent the activation of MPF before DNA synthesis is complete by inhibiting the activation of CDK1 by Cdc25C (see Figure 21-32, 1).
- The spindle-assembly checkpoint, which prevents premature initiation of anaphase, utilizes Mad2 and other proteins to regulate the APC specificity factor Cdc20 that targets securin for polyubiquitination (see Figures 21-32, 2, and 21-19).
- The chromosome-segregation checkpoint prevents telophase and cytokinesis until daughter chromosomes have been properly segregated, so that the daughter cell has a full set of chromosomes (see Figure 21-32, 3).
- In the chromosome-segregation checkpoint, the small GTPase Tem1 controls the availability of Cdc14 phosphatase, which in turn activates the APC specificity factor Cdh1 that targets B-type cyclins for degradation, causing inactivation of MPF (see Figure 21-10).
- The DNA-damage checkpoint arrests the cell cycle in response to DNA damage until the damage is repaired. Three types of tumor-suppressor proteins (ATM/ATR, Chk1/2, and p53) are critical to this checkpoint.
- Activation of the ATM or ATR protein kinases in response to DNA damage due to UV light or γ-irradiation leads to arrest in G_1 and the S phase via a pathway that leads to loss of Cdc25A phosphatase activity. A second pathway from activated ATM/R stabilizes p53, which stimulates expression of $p21^{CIP}$. Subsequent inhibition of multiple CDK-cyclin complexes by $p21^{CIP}$ causes prolonged arrest in G_1 and G_2 (see Figure 21-32, 4a–4d).
- In response to extensive DNA damage, p53 also activates genes that induce apoptosis.

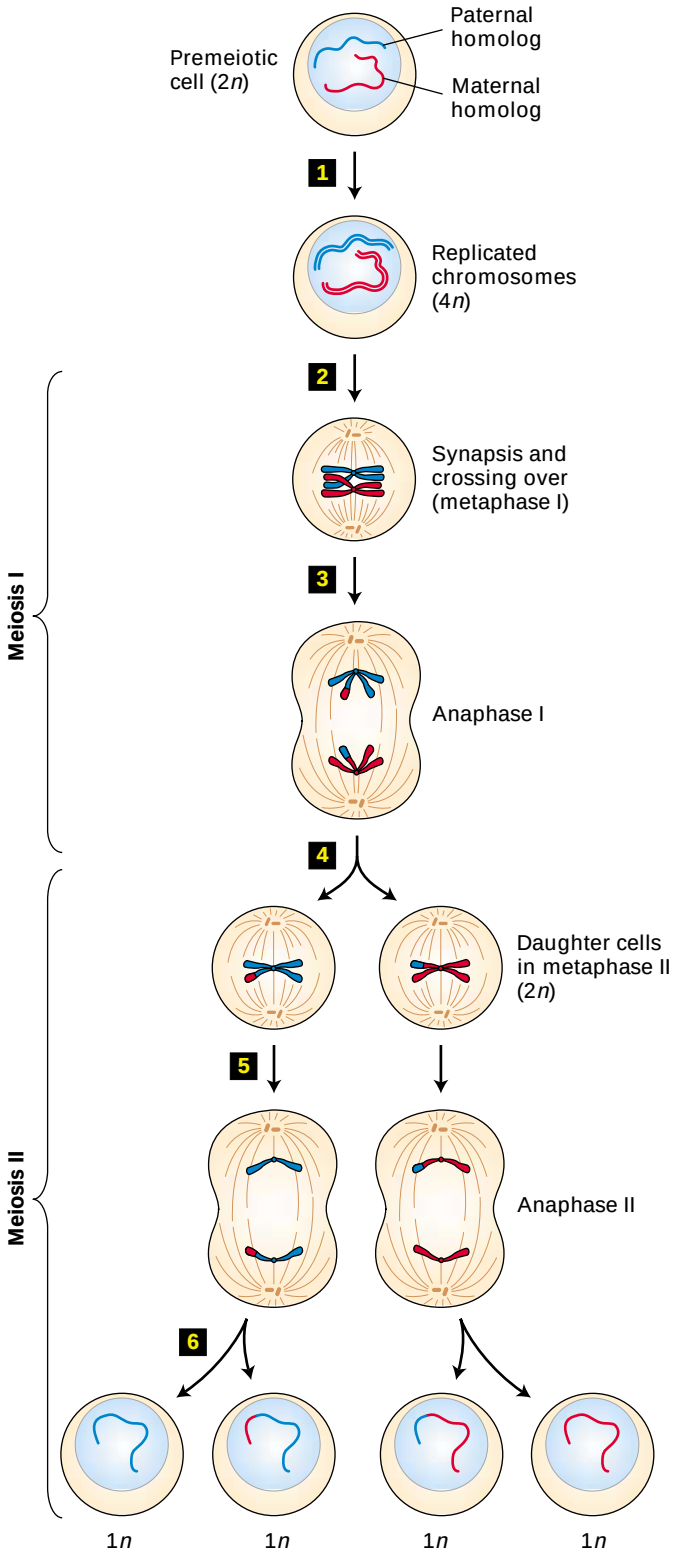
21.8 Meiosis: A Special Type of Cell Division

In nearly all eukaryotes, **meiosis** generates haploid germ cells (eggs and sperm), which can then fuse to generate a diploid zygote (Figure 21-34). During meiosis, a single round of DNA replication is followed by two cycles of cell division, termed *meiosis I* and *meiosis II*. **Crossing over** of chromatids, visible in the first meiotic metaphase, produces **recombination** between parental chromosomes. This increases the genetic diversity among the individuals of a species. During meiosis I, both chromatids of each homologous chromosome segregate together to opposite spindle poles, so that each of the resulting daughter cells contains one homologous chromosome consisting of two chromatids. During meiosis II, which resembles mitosis, the chromatids of one chromosome segregate to opposite spindle poles, generating haploid germ cells. Meiosis generates four haploid germ cells from one diploid premeiotic cell.

Repression of G_1 Cyclins and Meiosis-Specific Ime2 Prevents DNA Replication in Meiosis II

In *S. cerevisiae* and *S. pombe*, depletion of nitrogen and carbon sources induces diploid cells to undergo meiosis, yielding haploid spores (see Figure 1-5). This process is analogous to

▶ FIGURE 21-34 **Meiosis.** Premeiotic cells have two copies of each chromosome (2*n*), one derived from the paternal parent and one from the maternal parent. For simplicity, the paternal and maternal homologs of only one chromosome are diagrammed. Step 1: All chromosomes are replicated during the S phase before the first meiotic division, giving a 4*n* chromosomal complement. Cohesin complexes (not shown) link the sister chromatids composing each replicated chromosome along their full lengths. Step 2: As chromosomes condense during the first meiotic prophase, replicated homologs become paired as the result of at least one crossover event between a paternal and a maternal chromatid. This pairing of replicated homologous chromosomes is called synapsis. At metaphase, shown here, both chromatids of one chromosome associate with microtubules emanating from one spindle pole, but each member of a homologous chromosome pair associates with microtubules emanating from opposite poles. Step 3: During anaphase of meiosis I, the homologous chromosomes, each consisting of two chromatids, are pulled to opposite spindle poles. Step 4: Cytokinesis yields the two daughter cells (now 2*n*), which enter meiosis II without undergoing DNA replication. At metaphase of meiosis II, shown here, the chromatids composing each replicated chromosome associate with spindle microtubules from opposite spindle poles, as they do in mitosis. Steps 5 and 6: Segregation of chromatids to opposite spindle poles during the second meiotic anaphase followed by cytokinesis generates haploid germ cells (1*n*) containing one copy of each chromosome (referred to as chromatids earlier).



the formation of germ cells in higher eukaryotes. Multiple yeast mutants that cannot form spores have been isolated, and the wild-type proteins encoded by these genes have been analyzed. These studies have identified specialized cell-cycle proteins that are required for meiosis.

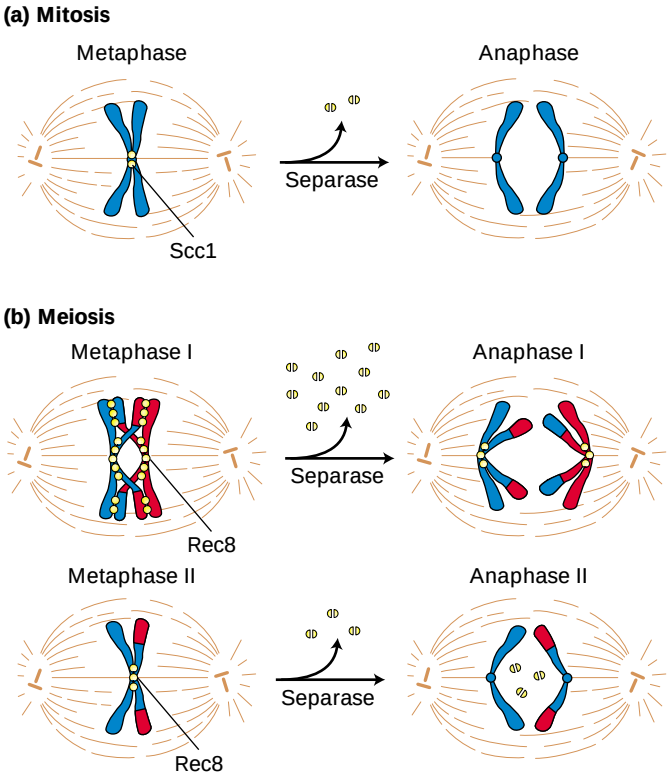
Under starvation conditions, expression of G_1 cyclins (Cln1/2/3) in *S. cerevisiae* is repressed, blocking the normal progression of G_1 in cells as they complete mitosis. Instead, a set of early meiotic proteins are induced. Among these is *Ime2*, a protein kinase that performs the essential G_1 cyclin-CDK function of phosphorylating the S-phase inhibitor Sic1, leading to release of active S-phase cyclin-CDK complexes and the onset of DNA replication in meiosis I (see Figure 21-34, step 1). The absence of Ime2 expression during meiosis II and the continued repression of Cln expression account for the failure to replicate DNA during the second meiotic division (steps 5 and 6).

Crossing Over and Meiosis-Specific Rec8 Are Necessary for Specialized Chromosome Segregation in Meiosis I

Recall that in mitosis, sister chromatids replicated during the S phase are initially linked by cohesin complexes at multiple positions along their full length (see Figure 21-19, *left*). As chromosomes condense, cohesin complexes become restricted to the region of the centromere, and at metaphase, the sister chromatids composing each (replicated) chromosome associate with microtubules emanating from opposite spindle poles. Although motor proteins pull sister chromatids toward opposite spindle poles, their movement initially is resisted by the cohesin complexes linking them at the centromere. The subsequent separase-catalyzed cleavage of the Scc1 cohesin subunit permits movement of the chromatids toward the spindle poles to begin, heralding the onset of anaphase (Figure 21-35a; see also Figure 21-19, *right*).

In metaphase of meiosis I, both sister chromatids in one (replicated) chromosome associate with microtubules emanating from the *same* spindle pole, rather than from opposite poles as they do in mitosis. Two physical links between homologous chromosomes are thought to resist the pulling force of the spindle until anaphase: (a) crossing over between chromatids, one from each pair of homologous chromosomes, and (b) cohesin cross-links between chromatids distal to the crossover point.

Evidence for the role of crossing over in meiosis in *S. cerevisiae* comes from the observation that when recombination is blocked by mutations in proteins essential for the process, chromosomes segregate randomly during meiosis I; that is, homologous chromosomes do not necessarily segregate to opposite spindle poles. Such segregation to opposite spindle poles normally occurs because both chromatids of homologous chromosome pairs associate with spindle fibers emanating from opposite spindle



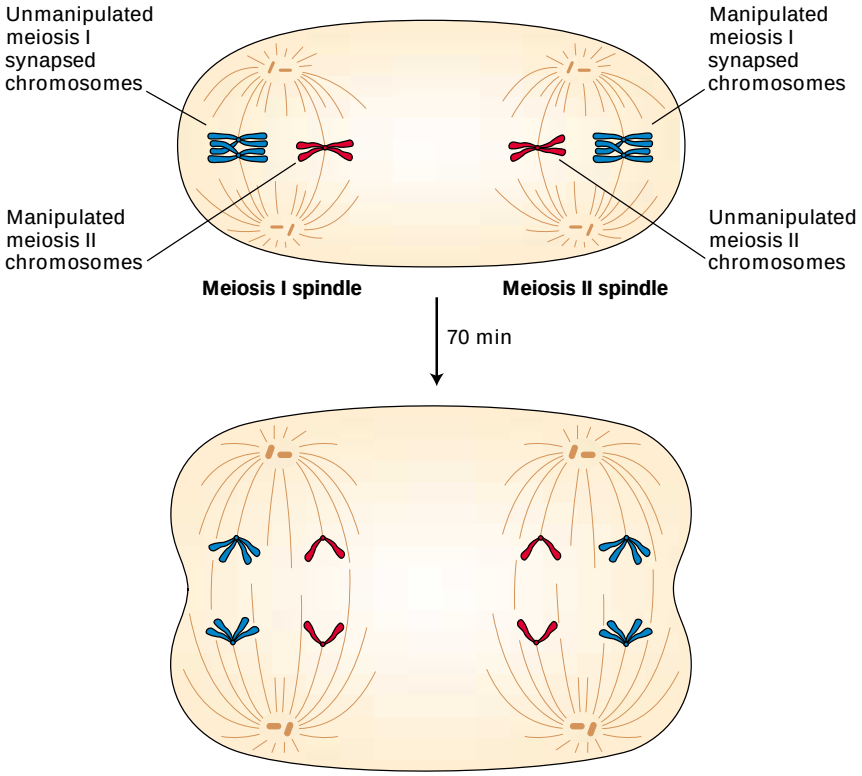
▲ FIGURE 21-35 **Cohesin function during mitosis and meiosis.** (a) During mitosis, sister chromatids generated by DNA replication in the S phase are initially associated by cohesin complexes along the full length of the chromatids. During chromosome condensation, cohesin complexes (yellow) become restricted to the region of the centromere at metaphase, as depicted here. Once separase cleaves the Scc1 cohesin subunit, sister chromatids can separate, marking the onset of anaphase (see Figure 21-19). (b) In metaphase of meiosis I, crossing over between maternal and paternal chromatids produces synapsis of homologous parental chromosomes. The chromatids of each replicated chromosome are cross-linked by cohesin complexes along their full length. Rec8, a meiosis-specific homolog of Scc1, is cleaved in chromosome arms but not in the centromere, allowing homologous chromosome pairs to segregate to daughter cells. Centromeric Rec8 is cleaved during meiosis II, allowing individual chromatids to segregate to daughter cells. [Modified from F. Uhlmann, 2001, *Curr. Opin. Cell Biol.* **13**:754.]

poles (see Figure 21-34, step 3). This in turn requires that homologous chromosomes pair during meiosis I, a process called *synapsis* that can be visualized microscopically in eukaryotes with large chromosomes. Consequently, the finding that mutations that block recombination also block proper segregation in meiosis I implies that recombination is required for synapsis in *S. cerevisiae*. In higher eukaryotes, processes in addition to recombination and chromatid linking through cohesin complexes contribute to synapsis in meiosis I.

During meiosis I, the cohesin cross-links between chromosome arms are cleaved by separase, allowing the homologous chromosomes to separate, but cohesin complexes at the centromere remain linked (Figure 21-35b, *top*). The maintenance of centromeric cohesion during meiosis I is necessary for the proper segregation of chromatids during meiosis II. Recent studies with a *S. pombe* mutant have shown that a specialized cohesin subunit, *Rec8*, maintains centromeric cohesion between sister chromatids during meiosis I. Expressed only during meiosis, Rec8 is homologous to Scc1, the cohesin subunit that forms the actual bridge between sister chromatids in mitosis (see Figure 21-19). Immunolocalization experiments in *S. pombe* have revealed that during early anaphase of meiosis I, Rec8 is lost from chromosome arms but is retained at centromeres. However, during early anaphase of meiosis II, centromeric Rec8 is degraded by separase, so the chromatids can segregate, as they do in mitosis (Figure 21-35b, *bottom*). A specific protein expressed during meiosis I, but not during meiosis II, protects centromeric Rec8 from separase cleavage in meiosis I.

S. cerevisiae Rec8 has been shown to localize and function similarly to *S. pombe* Rec8. Homologs of Rec8 also have been identified in higher organisms, and **RNA interference (RNAi)** experiments in *C. elegans* (Chapter 9) indicate that the Rec8 homolog in that organism has a similar function. Recent micromanipulation experiments during grasshopper spermatogenesis support the hypothesis that kinetochore-bound proteins protect centromeric Rec8 from cleavage during meiosis I but not during meiosis II and also direct kinetochore attachment to microtubules emanating from the correct spindle pole (Figure 21-36). Thus crossing over, Rec8, and special kinetochore-associated proteins appear to function in meiosis in all eukaryotes.

Recent **DNA microarray** analyses in *S. cerevisiae* have revealed other proteins that are required for meiosis. As discussed in other chapters, researchers can monitor transcription of thousands of genes, indeed entire genomes, with DNA microarrays (see Figure 9-35). One of the multiple genes found to be expressed in *S. cerevisiae* cells during meiosis but not during mitosis is *MAM1*, which encodes a protein called *monopolin*. Subsequent gene-specific mutagenesis studies revealed that deletion of *MAM1* causes sister chromatids in metaphase of meiosis I to associate with the first meiotic spindle as though they were mitotic chromatids or chromatids in meiosis II. That is, kinetochores of the sister chromatids composing a single (replicated) chromosome attached to microtubules emanating from opposite spindle poles rather than from the same spindle pole. This result indicates that monopolin is required for formation of a specialized kinetochore in meiosis I responsible for the unique co-orientation of sister chromatids of synapsed homologous chromosomes in the first meiotic division.



▲ EXPERIMENTAL FIGURE 21-36 **Anaphase movements and cohesion of meiotic chromosomes are determined by proteins associated with the chromosomes.** Grasshopper spermatocytes in meiosis I and II were fused so that both types of spindles with their associated chromosomes were present in a single fused cell. Then a micromanipulation needle was used to move some meiosis I chromosomes (blue) and meiosis II chromosomes (red) from one spindle to another; other chromosomes were left attached to their normal spindles. After 70 minutes, both spindles with their attached chromosomes had completed anaphase movements. The synapsed meiosis I chromosomes (blue) separated normally (i.e., one homologous pair toward one spindle pole and the other homologous pair toward the other) whether attached to the meiosis I spindle (*left*) or meiosis II spindle (*right*). Similarly, the meiosis II chromosomes (red) separated normally (i.e., one chromatid toward one spindle pole and the other chromatid toward the opposite pole) independent of which spindle they were attached to. These results indicate that the association of kinetochores with spindle microtubules and the stability of cohesins linking chromosomes are determined by factors that are associated with the chromosomes and not by the spindles or soluble components of cells in meiosis I and II. [See L. V. Paliulis and R. B. Nicklas, 2000, *J. Cell Biol.* **150**:1223.]

KEY CONCEPTS OF SECTION 21.8

Meiosis: A Special Type of Cell Division

■ Meiosis involves one cycle of chromosome replication followed by two cycles of cell division to produce haploid germ cells from a diploid premeiotic cell (see Figure 21-34).

■ During meiosis I, replicated homologous chromosomes pair along their lengths in a process called synapsis. At least one recombination between chromatids of homologous chromosomes almost invariably occurs.

■ Most of the cell-cycle proteins that function in mitotically dividing cells also function in cells undergoing meiosis, but some proteins are unique to meiosis.

■ In *S. cerevisiae*, expression of G_1 cyclins (Clns) is repressed throughout meiosis. Meiosis-specific Ime2 performs the function of G_1 cyclin-CDK complexes in promoting initiation of DNA replication during meiosis I. DNA replication does not occur during meiosis II because neither Ime2 nor G_1 cyclins are expressed.

■ In *S. cerevisiae*, recombination (crossing over) between chromatids of homologous parental chromatids and cohesin cross-links between chromatids distal to the crossover are responsible for synapsis of homologous chromosomes during prophase and metaphase of meiosis I. A specialized cohesin subunit, Rec8, replaces the Scc1 cohesin subunit during meiosis.

■ During early anaphase of meiosis I, Rec8 in the chromosome arms is cleaved, but a meiosis-specific protein associated with the kinetochore protects Rec8 in the region of the centromere from cleavage. As a result, the chromatids of homologous chromosomes remain associated

during segregation in meiosis I. Cleavage of centromeric Rec8 during anaphase of meiosis II allows individual chromatids to segregate into germ cells (see Figure 21-35b).

■ Monopolin, another meiosis-specific protein, is required for both chromatids of homologous chromosomes to associate with microtubules emanating from the same spindle poles during meiosis I.

PERSPECTIVES FOR THE FUTURE

The remarkable pace of cell-cycle research over the last 25 years has led to the model of eukaryotic cell-cycle control outlined in Figure 21-2. A beautiful logic underlies these molecular controls. Each regulatory event has two important functions: to activate a step of the cell cycle and to prepare the cell for the next event of the cycle. This strategy ensures that the phases of the cycle occur in the proper order.

Although the general logic of cell-cycle regulation now seems well established, many critical details remain to be discovered. For instance, although researchers have identified some components of the pre-replication complex that must be phosphorylated by S-phase CDK-cyclin complexes to initiate DNA replication, other components remain to be determined. Also still largely unknown are the substrates phosphorylated by mitotic cyclin-CDK complexes, leading to chromosome condensation and the remarkable reorganization of microtubules that results in assembly of the beautiful mitotic spindle. Much remains to be learned about how the activities of Wee1 kinase and Cdc25 phosphatase are controlled; these proteins in turn regulate the kinase activity of the CDK subunit in most cyclin-CDK complexes.

Much has been discovered recently about operation of the cell-cycle checkpoints, but the mechanisms that activate ATM and ATR in the DNA-damage checkpoint are poorly understood. Likewise, much remains to be learned about the control and mechanism of Mad2 in the spindle-assembly checkpoint and of Cdc14 in the chromosome-segregation checkpoint in higher cells. As we learn in the next chapter, asymmetric cell division plays a critical role in the normal development of multicellular organisms. Many questions remain about how the plane of cytokinesis and the localization of daughter chromosomes are determined in cells that divide asymmetrically. Similarly, the mechanisms that underlie the unique segregation of chromatids during meiosis I have not been elucidated yet.

Understanding these detailed aspects of cell-cycle control will have significant consequences, particularly for the treatment of cancers. Radiation and many forms of chemotherapy cause DNA damage and cell-cycle arrest in the target cells, leading to their apoptosis. But this induction of apoptosis depends on p53 function. For this reason, human cancers associated with mutations of both *p53* alleles, which is fairly common, are particularly resistant to these therapies. If more were understood about cell-cycle controls and checkpoints, new strategies for treating p53-minus cancers might be possible. For instance, some chemotherapeutic agents inhibit microtubule function, interfering with mitosis. Resistant cells selected during the course of treatment may be defective in the spindle-assembly checkpoint as the result of mutations in the genes encoding the proteins involved. Perhaps the loss of this checkpoint could be turned to therapeutic advantage. Only better understanding of the molecular processes involved will tell. ■

KEY TERMS

anaphase-promoting complex (APC) *863*
APC specificity factors *863*
Cdc25 phosphatase *866*
checkpoints *887*
CIPs *885*
cohesins *872*
condensin *870*
crossing over *890*
cyclin-dependent kinases (CDKs) *855*
destruction box *863*
DNA-damage checkpoint *889*
E2F proteins *834*
G_1 cyclins *876*
interphase *854*
meiosis *890*
mitogens *856*
mitosis-promoting factor (MPF) *860*
mitotic cyclin *861*
p53 protein *889*
quiescent cells *883*
Rb protein *884*
restriction point *883*
S-phase inhibitor *868*
S phase–phase promoting factor (SPF) *876*
securin *872*
synapsis *892*
Wee1 protein-tyrosine kinase *866*

REVIEW THE CONCEPTS

1. What strategy ensures that passage through the cell cycle is unidirectional and irreversible? What is the molecular machinery that underlies this strategy?

2. When fused with an S-phase cell, cells in which of the following phases of the cell cycle will initiate DNA replication prematurely—G1? G2? M? Predict the effect of fusing a cell in G1 and a cell in G2 with respect to the timing of S phase in each cell.

3. In 2001, the Nobel Prize in Physiology or Medicine was awarded to three cell cycle scientists. Sir Paul Nurse was recognized for his studies with the fission yeast *S. pombe,* in particular for the discovery and characterization of the *wee1* gene. What is the wee phenotype? What did the characterization of the *wee1* gene tell us about cell cycle control?

4. Tim Hunt shared the 2001 Nobel Prize for his work in the discovery and characterization of cyclin proteins in eggs and embryos. What experimental evidence indicates that cyclin B is required for a cell to enter mitosis? What evidence indicates that cyclin B must be destroyed for a cell to exit mitosis?

5. Leeland Hartwell, the third recipient of the 2001 Nobel Prize, was acknowledged for his characterization of cell cycle checkpoints in the budding yeast *S. cerevisiae*. What is a cell cycle checkpoint? Where do checkpoints occur in the cell cycle? How do cell cycle checkpoints help to preserve the fidelity of the genome?

6. In *Xenopus*, one of the substrates of MPF is the Cdc25 phosphatase. When phosphorylated by MPF, Cdc25 is activated. What is the substrate of Cdc25? How does this information explain the autocatalytic nature of MPF as described in Figure 21-6?

7. Explain how CDK activity is modulated by the following proteins: (a) cyclin, (b) CAK, (c) Wee1, (d) p21.

8. Three known substrates of MPF or kinases regulated by MPF are the nuclear lamins, subunits of condensin, and myosin light chain. Describe how the phosphorylation of each of these proteins affects its function and progression through mitosis.

9. Describe the series of events by which the APC promotes the separation of sister chromatids at anaphase.

10. A common feature of cell cycle regulation is that the events of one phase ensure progression into a subsequent phase. In *S. cerevisiae*, Cdc28-Clns catalyze progression through G1, and Cdc28-Clbs catalyze progression through S/G2/M. Name three ways in which the activity of Cdc28-Clns promotes the activation of Cdc28-Clbs.

11. For S phase to be completed in a timely manner, DNA replication initiates from multiple origins in eukaryotes. In *S. cerevisiae*, what role do S-phase CDK-cyclin complexes play to ensure that the entire genome is replicated once and only once per cell cycle?

12. What is the functional definition of the restriction point? Cancer cells typically lose restriction point controls. Explain how the following mutations, which are found in some cancer cells, lead to a bypass of restriction point controls: (a) overexpression of cyclin D, (b) loss of Rb function, (c) infection by retroviruses encoding v-Fos and v-Jun.

13. Individuals with the hereditary disorder ataxia telangiectasia suffer from neurodegeneration, immunodeficiency, and increased incidence of cancer. The genetic basis for ataxia telangiectasia is a loss-of-function mutation in the ATM gene (ATM = ataxia telangiectasia-mutated). Name two substrates of ATM. How does the phosphorylation of these substrates lead to inactivation of CDKs to enforce cell cycle arrest at a checkpoint?

14. Meiosis and mitosis are overall analogous processes involving many of the same proteins. However, some proteins function uniquely in each of these cell division events. Explain the meiosis-specific function of the following: (a) Ime2, (b) Rec8, (c) monopolin.

ANALYZE THE DATA

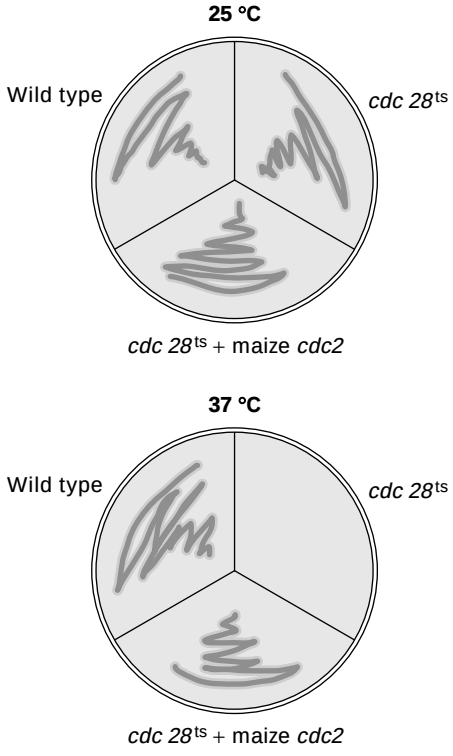
As genes encoding cyclin-dependent kinases were cloned from fission yeast, budding yeast, and animal cells, investigators working in plant systems designed experiments to determine whether plants also possessed CDKs that functioned as the catalysts for cell cycle progression. To isolate a cDNA encoding a CDK in maize, investigators aligned the predicted amino acid sequences of human *cdc2*, *S. pombe cdc2*, and *S. cerevisiae cdc28* genes (see the figure below). Degenerate

```
S. pombe cdc2          ----MENYQKVEKIGEGTYGVVYKARHKLSG---RIVAMKKIRLEDESEGVPSTAIREIS
human cdc2             ----MEDYTKIEKIGEGTYGVVYKGRHKTTG---QVVAMKKIRLESEEEGVPSTAIREIS
S. cerevisiae cdc28     MSGELANYKRLEKVGEGTYGVVYKALDLRPGQGQRVVALKKIRLESEDEGVPSTAIREIS
                            : :* ::**:********** . .  .*   ::**:******.*.**********

S. pombe cdc2          LLKEVNDENNRSNCVRLLDILHAES-KLYLVFEFLDMDLKKYMDRISETGATSLDPRLVQ
human cdc2             LLKELR----HPNIVSLQDVLMQDS-RLYLIFEFLSMDLKKYLDSIPPG--QYMDSSLVK
S. cerevisiae cdc28     LLKELK----DDNIVRLYDIVHSDAHKLYLVFEFLDLDLKRYMEGIPKD--QPLGADIVK
                        ****:.        * * * *::  :: :***:****.:***:*:: *.       :.. :*:

S. pombe cdc2          KFTYQLVNGVNFCHSRRIIHRDLKPQNLLIDKEGNLKLADFGLARSFGVPLRNYTHEIVT
human cdc2             SYLYQILQGIVFCHSRRVLHRDLKPQNLLIDDKGTIKLADFGLARAFGIPIRVYTHEVVT
S. cerevisiae cdc28     KFMMQLCKGIAYCHSHRILHRDLKPQNLLINKDGNLKLGDFGLARAFGVPLRAYTHEIVT
                        .:   *: :*: :***:*::********** ..*.:**.******:**:*:* ****:**

S. pombe cdc2          LWYRAPEVLLGSRHYSTGVDIWSVGCIFAEMIRRSPLFPGDSEIDEIFKIFQVLGTPNEE
human cdc2             LWYRSPEVLLGSARYSTPVDIWSIGTIFAELATKKPLFHGDSEIDQLFRIFRALGTPNNE
S. cerevisiae cdc28     LWYRAPEVLLGGKQYSTGVDTWSIGCIFAEMCNRKPIFSGDSEIDQIFKIFRVLGTPNEA
                        ****:******. :*** ** **:* ****:  :.*:* ******::*:**:.*****:

S. pombe cdc2          VWPGVTLLQDYKSTFPRWKRMDLHKVVPNGEEDAIELLSAMLVYDPAHRISAKRALQQNY
human cdc2             VWPEVESLQDYKNTFPKWKPGSLASHVKNLDENGLDLLSKMLIYDPAKRISGKMALNHPY
S. cerevisiae cdc28     IWPDIVYLPDFKPSFPQWRRKDLSQVVPSLDPRGIDLLDKLLAYDPINRISARRAAIHPY
                        :** :  * *:* :**:*:  .* . * . :  .::**. :* *** :***.: *  : *

S. pombe cdc2          LRDFH------
human cdc2             FNDLDNQIKKM
S. cerevisiae cdc28     FQES-------
```

oligonucleotide PCR primers corresponding to the boxed amino acids were generated and a DNA fragment of the expected size was synthesized by PCR using these primers and maize cDNA library as a template. Why were the boxed regions selected as the basis for primer design?

The PCR product was then used as a probe to screen the maize cDNA library, and a full-length cDNA clone was isolated. The clone was sequenced, and the predicted amino acid sequence was 64% identical to human *cdc2* and 63% identical to *S. pombe cdc2* and to *S. cerevisiae cdc28*. What does the level of sequence similarity suggest about the evolution of *cdc2* genes?

A complementation experiment was performed with *S. cerevisiae* cells possessing a temperature-sensitive *cdc28* mutation. Wild-type cells, $cdc28^{ts}$ cells, and $cdc28^{ts}$ cells transformed with the maize *cdc2* cDNA under the influence of a strong promoter were grown at the permissive (25 °C) or restrictive (37 °C) temperatures. Cell proliferation was monitored by the growth of colonies on the culture plates (shown in the figure below).



Why do the $cdc28^{ts}$ cells form colonies at 25 °C but not 37 °C? What is the significance of colony formation of the $cdc28^{ts}$ + maize *cdc2* cells at 37 °C? What does this experiment tell us about the functional homology of cyclin-dependent kinase genes among eukaryotic species?

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