3

DNA Replication and Chromosome Reproduction

Replication Is Semiconservative

In each DNA synthetic (S) phase, the two strands of the DNA double helix separate by unwinding. Each strand serves as a template for synthesis of a completely new complementary strand from the deoxyribonucleotides dA, dG, dC, and dT, hereafter called A, G, C, and T. Since the new DNA double helix consists of one conserved strand and one newly synthesized strand, replication is called semi-conservative. This was first demonstrated at the chromosomal level by autoradiography, growing cells in the presence of [^{3}H]thymidine during one cell cycle and in the absence of this radioactive DNA precursor during the next cycle. If replication is semi-conservative and each chromatid contains a single DNA molecule, label will be incorporated into the newly replicated strand of DNA in each chromatid at the first cycle but into neither new strand of DNA

O. J. Miller et al., *Human Chromosomes*

at the second cycle. The result will be a radiolabeled strand in only one of the two sister chromatids. This was confirmed autoradiographically by placing a photographic emulsion on metaphase chromosome preparations. Electrons produced by radioactive decay of the tritium (^3H) produced silver grains in the emulsion, mostly within 1 micrometer (μm) of the source, and these were concentrated over one of the two chromatids (Fig. 3.1). Occasionally, however,

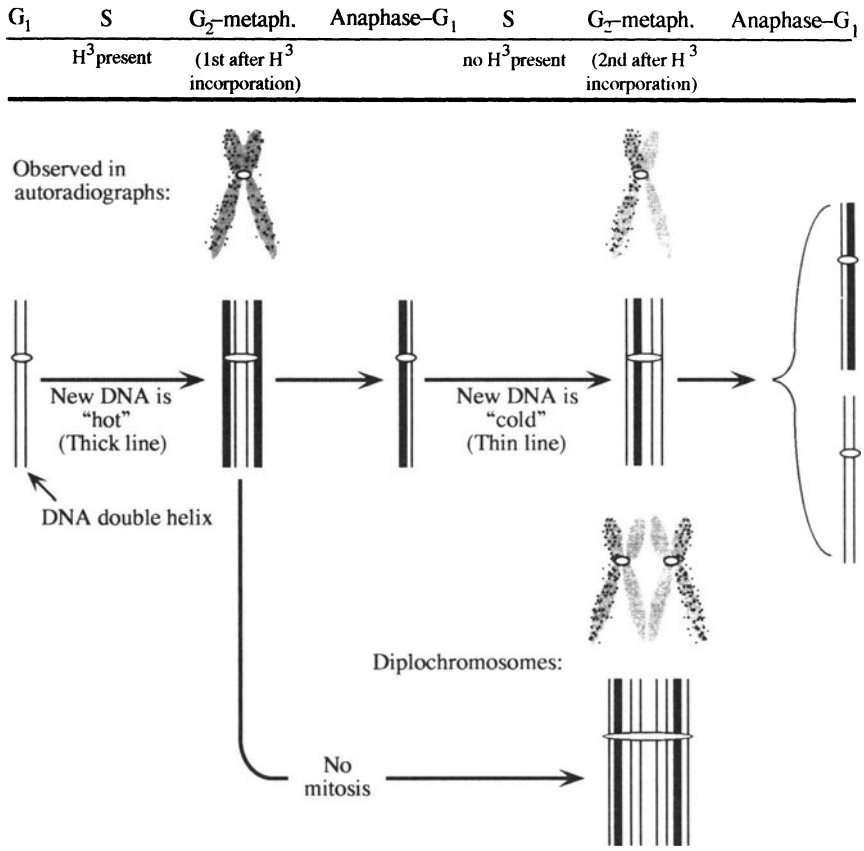

Figure 3.1. Semiconservative replication of chromosomes demonstrated with tritiated thymidine and autoradiography.

a region of the second chromatid would be labeled while the corresponding region of the other chromatid lacked label. This reciprocal labeling pattern is the result of breakage and rejoining of sister chromatids, resulting in an exchange between them (Taylor, 1963).

Sister chromatid exchanges (SCEs) can be induced by radioactivity, but they also occur spontaneously, as shown by the formation of double-sized dicentric ring chromosomes from simple ring chromosomes (Wolff, 1977; see also Chapter 15). In *diplochromosomes*, which have undergone two rounds of DNA replication without an intervening mitosis, there are four chromatids (see, for example, Fig. 15.2). The outer two chromatids are radioactive (Fig. 3.1). This shows that the diplochromosomes are ordered in some as yet unknown way, with the older, template DNA strands in the two inner chromatids and the newly replicated strands in the two outer chromatids. While semi-conservative replication and SCEs were first visualized autoradiographically, they can be visualized more quickly and the exchange points determined with higher resolution using bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA strands and a non-radioactive detection system (Fig. 24.1). For this approach, one can use enzyme- or fluorescein-labeled antibodies to BrdU. Alternatively, on can use ultraviolet (UV)-enhanced photolysis (degradation) of the BrdU-containing DNA strands, which reduces the amount of DNA and thus the intensity of staining with DNA-binding fluorochromes or Giemsa stain. SCE frequencies are widely used as a test for exogenous or endogenous genotoxic, or chromosome-breaking, agents (*clastogens*).

The Chemistry of Replication

Replication of each new strand of DNA proceeds only in a 5′ to 3′ direction (Fig. 3.2). That is, single nucleotide monophosphates, attached to the 5′ OH of the deoxyribose sugar, are added sequentially to the 3′ end by forming a covalent chemical bond with a 3′ OH. When the 3′ to 5′ strand serves as template, its complementary strand is synthesized from a single short RNA primer in one continuous 5′ to 3′ process. However, when the 5′ to 3′ strand serves as template, a series of thousands of short sequences (*Okazaki fragments*) are synthesized 5′ to 3′, each from a short RNA primer (dotted line in Figs. 3.2 and 3.3), which is then removed. The adjacent fragments are ligated together by an enzyme called *DNA ligase*. The stepwise addition of nucleotides to a growing (nascent)

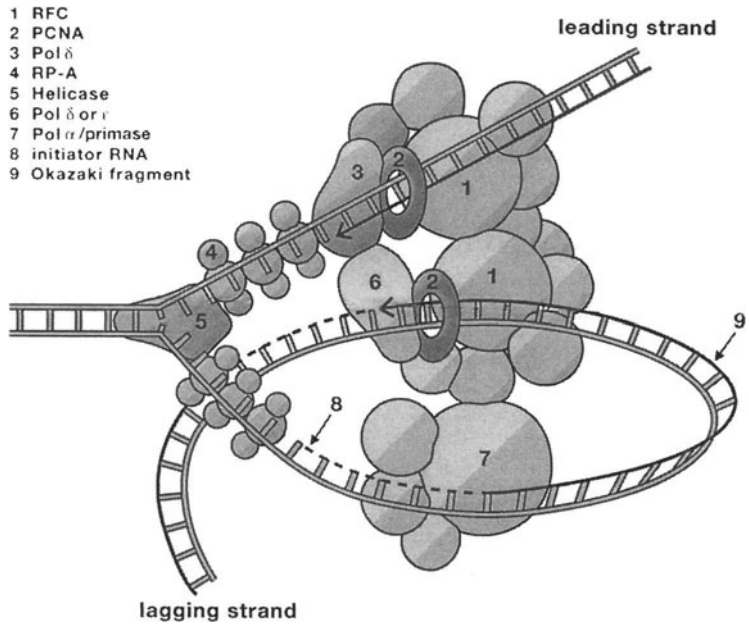

Figure 3.2. A DNA replication fork and components of a replication factory. Note that replication along the leading strand moves continuously towards the fork, whereas replication along the lagging strand proceeds from the fork along a short Okazaki fragment. See text for further explanation (Jónsson and Hübscher, 1997, BioEssays, Vol. 19, p 968; copyright 1997, John Wiley & Sons; reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.).

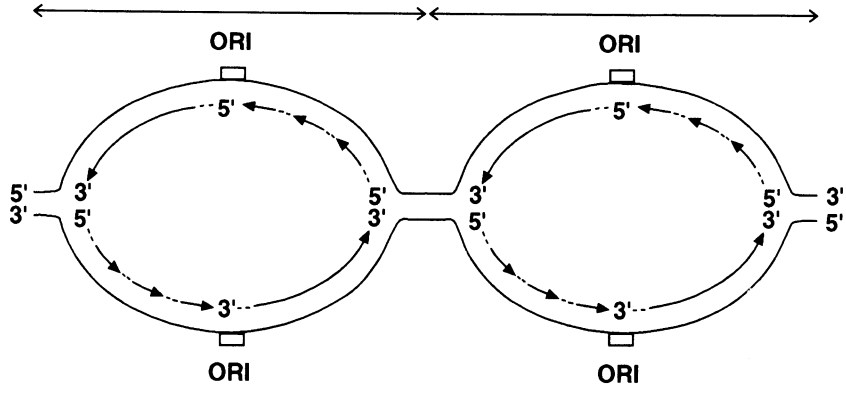

Figure 3.3. Diagram showing synchronous replication in two adjacent units of replication (replicons). DNA synthesis begins at an origin (ORI) and proceeds in both directions, always 5′ to 3′. Synthesis is continuous along the leading strand and discontinuous (occurring in Okazaki fragments) along the trailing strand. Adjacent replication bubbles coalesce to yield two identical double helices of DNA.

DNA strand is an error-prone process. Several error-correcting mechanisms have evolved and been incorporated into the replication machinery, resulting in remarkably, though not completely, accurate duplication of the DNA molecule in each chromosome of the genome.

DNA replication takes place in nuclear foci called replication factories (Fig. 3.2). These are anchored to the nuclear matrix (Chapter 4) and are enormous multiprotein complexes. They contain DNA polymerases α and δ, DNA ligase, several nucleotide mismatch repair proteins, a DNA methylase, and a fascinating protein called proliferating cell nuclear antigen (PCNA), which plays a key role in DNA replication and its control (Jónsson and Hübscher, 1997). The initiation of DNA replication requires the activation of cyclin-dependent kinases (CDKs) by binding to cyclins. When CDK2 is bound to cyclin E or A, it can phosphorylate CDC6 (cell division cycle protein 6), which is essential for the initiation of replication (Chapter 2). RCC1, a DNA-binding protein that regulates chromosome condensation, is also essential. It forms a complex with the Ran/TC4 protein and targets it to the nucleus, where the complex somehow monitors the progress of replication and couples its completion to the onset of mitosis (Ren et al., 1993).

Figure 3.2 presents a diagrammatic model of the key workers in a replication factory at a replication fork. Two molecules of PCNA join to form a doughnut-shaped homodimer through which DNA is threaded during replication. This requires the interaction of PCNA with a protein complex called replication factor C (RF-C). The two sides of the PCNA doughnut have different features, which may provide the basis for distinguishing the newly replicated strand from the conserved strand. This is essential in order that nucleotide mismatches produced by replication errors in the new strand can be correctly repaired by the mismatch repair proteins, MSH2 and MLH1. PCNA is also important for cell cycle regulation. The *WAF1* gene product, p21, is a cell cycle inhibitor that acts by binding to PCNA and blocking DNA replication, thus arresting the cell cycle (Chuang et al., 1997).

Initiation at Many Sites: Origins of Replication

When cells are incubated with [^{3}H]thymidine for very short intervals during the S phase, many sites of DNA synthesis are seen by autoradiography, both in inter-

phase nuclei and along the length of metaphase chromosomes. That is, the single giant DNA molecule in each chromosome is not replicated continuously from one end to the other, which would take months, but from a large number of initiation sites, called origins of replication (Fig. 3.3). Fiber autoradiography, the analysis of DNA fibers isolated on microscope slides from cells grown for varying periods in [^{3}H]thymidine, has shown that replication proceeds in both directions from almost every origin. The replication fork progresses at a rate of about 0.6 μm, or 2 *kilobases* (kb), per minute. Fiber autoradiography has shown that units of replication (*replicons*) range in size from about 15 to 100 μm, or 50 to 330 kb, with an average of about 100 kb (Edenberg and Huberman, 1975). More recent technological developments enabled Tomilin et al. (1995) to visualize the elementary units of DNA replication and show that they correspond in size to the DNA loop domains described in Chapter 5. Since replication of the roughly 3.4 billion base pairs of DNA in the haploid genome proceeds at about 2 kb per minute, or 120 kb per hour, and takes approximately eight hours (S phase), there must be about 34,000 replicons. Replicons that initiate synthesis at the same time are clustered in linear tandem arrays of four or more, as shown by the apparent continuity of adjacent silver grain tracks in fiber autoradiographs. In fact, these clusters can contain 10–25 or more replicons, or 1–3 megabases (Mb) of DNA, each perhaps representing an entire chromosome band (Chapter 6).

The nature of human origins of replication is still unclear. Some origins appear to be very short DNA sequences, while in other regions of the genome replication may begin anywhere within a long stretch of DNA. Methods to define origins are still limited. Vassilev and Johnson (1990) used PCR (polymerase chain reaction) amplification of very short, newly replicated (*nascent*) strands of DNA to localize the origin of replication of the cellular oncogene c-*MYC*. This fell within a 2-kb region centered about 1.5 kb upstream of the first coding region, exon 1, and replication from this origin was bidirectional. Using the same technique, Kumar et al. (1996) localized an origin on chromosome 19 within a 500-bp segment at the 5′ end of the gene for the nuclear envelope protein, lamin B2. They showed that the same origin was used in six different cell types, of myeloid, neural, epithelial, and connective tissue origin. Aladjem et al. (1995) similarly localized the β-globin origin. An alternative approach using DNA fibers from yeast artificial chromosomes (YACs) and fluorescence in situ hybridization (fiber-FISH; see Chapter 8) allowed identification of two origins in a 400-kb region of the huge 2.4-Mb dystrophin (Duchenne muscular dystrophy, or *DMD*) gene on the X chromosome (Rosenberg et al., 1995).

Replication Is Precisely Ordered: Replication Banding

DNA replication follows a precise order, with a corresponding progression and arrangement of replication foci and a consistent pattern of replication along each chromosome. Some origins consistently initiate replication early in S and others late in S; the mechanism underlying this is not clear. The initial autoradiographic studies of human DNA replication by Morishima, Grumbach, and Taylor showed that one X chromosome in XX cells and two in XXX cells terminate replication much later than do the autosomes and the other X chromosome. This indicates that the heteropycnotic, Barr body–forming X chromosomes are late replicating. With the development of a simple method (C-banding) to recognize constitutive heterochromatin, it became clear that constitutive as well as facultative heterochromatin is consistently late replicating. Even with the limited resolution of tritium autoradiography, the distinctive patterns of replication of a few autosomes permitted a distinction to be made between morphologically similar chromosomes, such as numbers 4 and 5, as demonstrated in patients with 5p- and 4p- deletion syndromes by German and Wolf, respectively.

BrdU incorporation makes DNA sensitive to photolysis in the presence of DNA-binding fluorochromes, enabling sites of replication to be visualized at high resolution. Latt et al. (1976) grew cells for 40 to 44 hours in a medium containing BrdU and then substituted thymidine for BrdU during the last 6 to 7 hours before fixation. After this treatment, the late-replicating X was more intensely stained with the fluorochromes Hoechst 33258 or coriphosphine O than was the other X (Fig. 3.4). More important, this procedure produced highly consistent and detailed replication banding patterns along metaphase and prometaphase chromosomes (Dutrillaux et al., 1976). These *replication banding* patterns closely resemble Q-, G-, and R-banding patterns (Figs. 3.4 and 6.4). Limiting the incorporation of BrdU to narrow windows (fractions) of the S phase has allowed detailed analyses of the replication timing of individual bands to be carried out. These indicate that the DNA in each band is replicated independently of the DNA in the adjacent bands and that chromosomes are replicated in 8–10 or more successive waves of replication, with each band always replicated in the same wave (Drouin et al., 1991). Dutrillaux et al. (1976) recognized as many as 18 successive replication times.

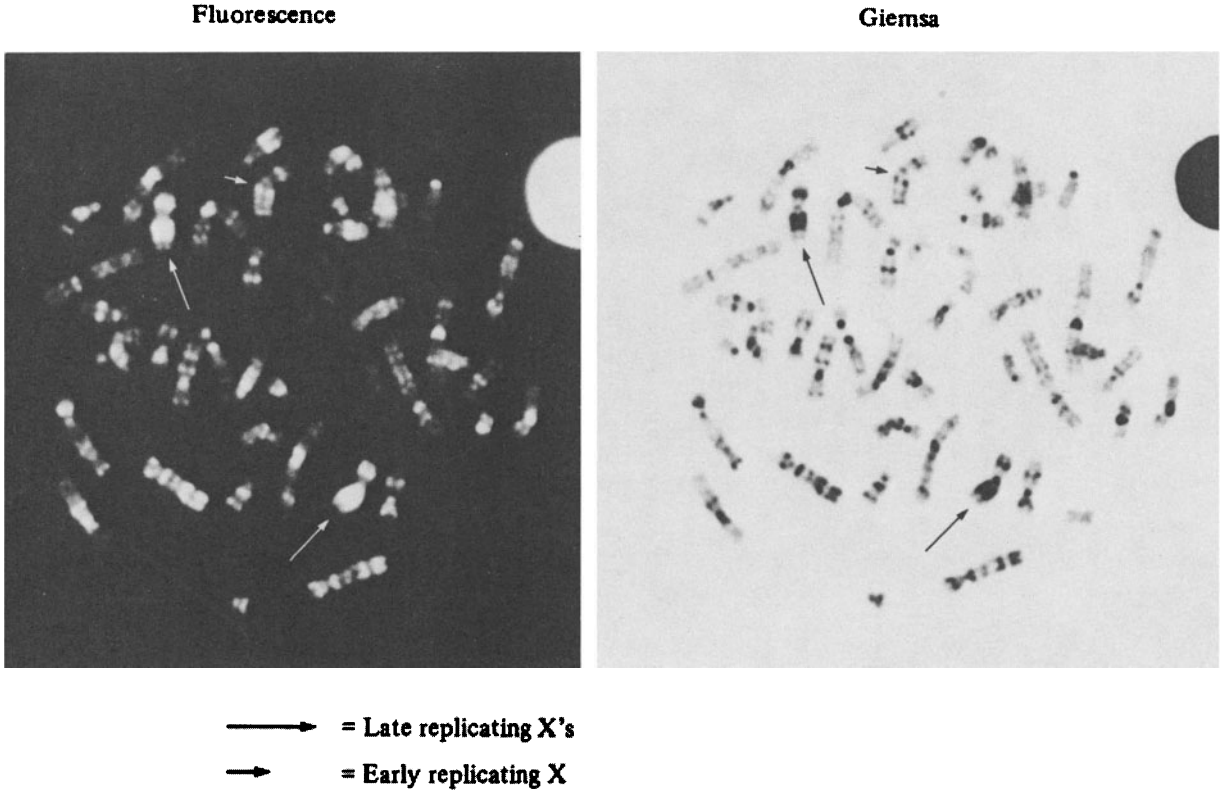

Figure 3.4. Two late-replicating X chromosomes (long arrows) and one early-replicating X (short arrow) in a 47,XXX cell, demonstrated using BrdU (left, stained with Hoechst 33258; right, with Giemsa) (Latt et al., 1976).

These results fit well with the earlier demonstration, using fiber autoradiography, of the synchronous replication of long tandem arrays of replicons (Edenberg and Huberman, 1975). They also fit with more recent findings using confocal laser-scanning microscopy to examine sites of replication in interphase nuclei throughout the S phase. These sites, called replication factories, are anchored to the nuclear matrix, whose structure and functions are reviewed by Berezny et al. (1995). The sites can be identified by the incorporation of BrdU during a limited fraction of S and by staining of the metaphase spreads with fluorescein isothiocyanate (FITC)–labeled antibodies to BrdU. At the beginning of S, about 150 sites, or factories, are visible. Replication proceeds for 45–60 minutes in these sites, and then secondary replication sites appear, to be replaced in another 45–60 minutes by tertiary sites, and so on throughout the roughly 8-hour S phase (Jackson and Pombo, 1998). Fluorescence laser-scanning confo-

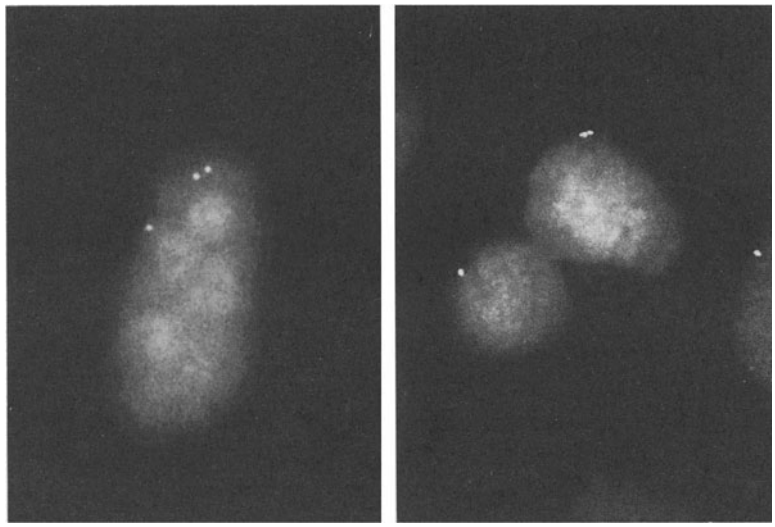

Figure 3.5. Fluorescence in situ hybridization (FISH) with a plasmid *XIST* probe (left) or a cosmid *FMRI* probe (right). Each nucleus shows a doublet signal (fully replicated) and a singlet signal (not fully replicated). This indicates the asynchronous replication of the two *XIST* alleles (left) and the two *FMR1* alleles (right) in XX cells (reproduced from the Am J Hum Genet, Torchia et al., copyright 1994, the American Society of Human Genetics, with permission of the University of Chicago Press).

cal microscopy has revealed that individual sites of replication appear to be grouped into some 22 higher-order domains, each containing many of the individual sites (Wei et al., 1998).

Methods have been developed to determine the time of replication of specific genes. The standard method for this is filter hybridization. DNA is isolated from cells that had incorporated BrdU at different intervals of S using flow cytophotometry to separate cells that completed a different fraction of S in the presence of BrdU. Antibodies to BrdU can be used to isolate the BrdU-containing (newly replicated) strand of DNA and determine its genic content. Alternatively, photolysis can destroy the BrdU-containing strand and single-strand-specific nuclease used to digest the exposed regions of the other strand. The remaining DNA, which was not replicating during the BrdU exposure interval, is digested with a restriction endonuclease; the fragments are separated by size by gel electrophoresis and transferred to a nitrocellulose filter. Gene probes are hybridized to these Southern blots. These studies have shown that most *housekeeping genes* (those that are expressed in all cell types) are replicated early, while genes whose expression is tissue specific are replicated early in the tissues in which they can be expressed and replicated late in other tissues (Chapter 7).

It is still unclear just how large a segment of DNA is involved in such developmental switches in replication timing, although it may be considerably less than a standardsized band. In the case of the β-globin gene cluster, more than 200 kb of DNA, encompassing the entire gene cluster, is early replicating in erythroid cells and late replicating in other cells. A more recent technique takes advantage of the fact that after a gene has been replicated, a copy will be present on each chromatid. In later S or G2 nuclei, these may appear by FISH (Chapter 8) as paired dots instead of the single dot seen in earlier S or G1 nuclei (Fig. 3.5; Selig et al., 1992).

The Control of DNA Replication

There are various levels of control of DNA replication, most of them not well understood. For example, what mediates the synchronous initiation of replication of all the replicons in a band? How are all the bands organized into 8–10 or more cohorts that initiate sequentially? What determines which cohort will initiate first? How does the completion of replication by each synchronous group of replicons trigger the initiation of replication in the next

group? How is cell cycle regulation mediated, so that every replicon is replicated once, and only once, in each cycle? What happens when these controls break down?

Cell fusion studies by Johnson and Rao in 1970 provided the first major step in understanding how DNA replication is controlled. Fusion of a G1-phase cell with an S-phase cell leads to rapid initiation of replication in the G1 nucleus. Fusion of a G2-phase cell with an S-phase cell has no such effect. Thus, cells in G1, but not cells in G2, are competent to initiate replication. This competence is gained late in mitosis, when a replication licensing complex containing seven MCM (minichromosome maintenance) proteins and the CDC6 protein binds to the six-protein origin of replication complex (ORC) that is permanently associated with each origin to form an even larger pre-replication complex, or pre-RC. CDC6 expression is turned on by the transcription factor E2F when this is released from its inactive complex with the RB protein (Chapter 2). Phosphorylation of the CDC6 protein by cyclin E/CDK2 or cyclin A/CDK2 and of the MCM proteins by the CDC7 protein kinase leads to activation of an origin. After an origin is activated (fires), the CDC6 and MCM proteins dissociate from the origin, leaving it unable to fire again until a new pre-RC is assembled late in mitosis. This may be the mechanism ensuring that each origin fires once, and only once, in each cycle (Stillman, 1996).

Povirk (1977) provided one of the earliest clues to the mechanism of synchronous replication of a group of adjacent replicons. Taking advantage of the fact that ultraviolet (UV) irradiation damages DNA that contains BrdU and suppresses the initiation of replication, Povirk demonstrated that one UV-induced lesion per 100–500 μm of DNA could suppress initiation in a region 500–750 μm (1.6–2.4 Mb) long, or about 10–15 replicons. This suggests that a single critical site may control the initiation of replication of all the replicons in a synchronized cluster, or *band*. Using more precise methods, Aladjem et al. (1995) showed that, while replication in the β-globin gene cluster in erythroid cells is initiated in the δ–β region 50 kb downstream of the locus control region (LCR), deletion of the LCR abolished initiation within this cluster. This indicates that the LCR controls replication in this region, which is replicated early in erythroid cells in which the genes are transcriptionally competent and late in cells in which the globin genes cannot be expressed. The LCR also controls the transcription and chromatin structure of the entire gene cluster (Chapter 5).

Previous studies support the view that sites of early initiation of replication tend to be close to transcribed sequences and that the earliest sites of replica-

tion in interphase nuclei are adjacent to sites of transcription (Jackson and Pombo, 1998). It is possible that the more open chromatin conformation in these regions facilitates access by the massive replication factory. If so, sequential alterations in the chromatin structure of the various replication cohorts might play a role in the temporal regulation of replication. It is not clear how such alterations might be triggered.

Replication of Chromosome Ends: Telomerase and Cell Aging

DNA replication proceeds 5′ to 3′ from a short RNA primer. When this primer is removed after replication is completed, it leaves an unreplicated stretch about 50–200 nucleotides long at the 5′ end of each new strand. Therefore, one might expect that DNA synthesis at the very end of the chromosome would be incomplete, and it is. In fact, chromosome duplication in normal somatic cells leads to progressive shortening of the chromosome ends (*telomeres*; Chapter 4), until the chromosomes begin to fuse after a number of cell divisions and the cell dies. However, in early embryonic cells and in the stem cells that provide a constant supply of bone marrow, gut and skin epithelium, spermatogonia, and so on, telomeres compensate for this limitation of DNA end-replication.

Telomeres consist of a tandemly repetitive six-base-pair unit, TTAGGG in the G-rich strand and CCCTAA in the complementary C-rich strand. The G-rich strand is oriented 5′ to 3′ toward the end of the chromosome. The C-rich strand ends somewhat short of the G-rich strand, so that the latter forms a single-stranded tail at the end of the chromosome. Telomeres are synthesized not by the usual replication mechanism but by a special ribonucleoprotein complex called *telomerase*, which can add new repeat units to the 3′-end of the G-rich strand. The enzyme is a *reverse transcriptase*, synthesizing DNA from an RNA rather than a DNA template, in this case the RNA oligonucleotide, CUAACCCUAAC, which is an intrinsic part of this multisubunit enzyme (Harrington et al., 1997). The enzyme uses the end of the G-rich strand as its primer, thus replacing the DNA repeat units that are lost during chromosome replication.

Telomerase is abundant in embryonic and cancer cells but is absent from non-embryonic cells other than stem cells. Consequently, telomeric shortening occurs in diploid fibroblasts, which lose 50–200 bp of telomeric DNA per cell doubling (Levy et al., 1992). This may account for the well-known phenome-

non of cell senescence, with diploid cells losing their ability to divide and ultimately dying after a certain number of cell doublings, as first noted in 1961 by Hayflick and Moorhead. This number, about 50, is sufficient for the cell to lose all its telomeric repeats from a few chromosomes and for telomeric fusions to lead to chromosome loss or cell death. Evidence that telomere shortening does trigger cell senescence has come from studies in which a telomerase gene was introduced into telomerase-negative normal human cells and the transfected cells were able to grow in culture for many more cell doublings than usual (Bodnar et al., 1998).

Certain viruses or chemical mutagens can transform diploid, telomerase-deficient cells with a limited life span into aneuploid cells that have reactivated their telomerase, have stable telomeres, and are immortal, being able to grow indefinitely. Cancer cells are also immortal, and more than 90% of them show a high level of telomerase activity compared to adjacent normal cells (Kim et al., 1994). The product of the *MYC* oncogene induces expression of the catalytic subunit of telomerase in cultured human epithelial cells and fibroblasts and extends their proliferative life span (Wang et al., 1998; see also Chapter 27).

Postreplication Steps: DNA Methylation and Chromatin Assembly

DNA methylation occurs almost immediately after replication of each DNA segment. DNA (cytosine 5) methyltransferase (MCMT, or DNA methylase) is part of the PCNA replication complex referred to earlier in this chapter. MCMT methylates newly replicated DNA only at CpG sites where the CpG in the complementary (template) strand is methylated. In this way, the pattern of DNA methylation can be maintained without change through successive mitotic cell divisions, and even throughout life. When the cell cycle is arrested by p21, the product of the *WAF1* gene, the MCMT-PCNA complex is also disrupted and DNA methylation is inhibited (Chuang et al., 1997).

Chromosomal proteins are added to the newly replicated DNA so as to reproduce the chromatin conformation present before mitosis began. Chromatin assembly factor 1 (CAF-1) assembles nucleosomes in a replication-dependent manner. CAF-1 has three protein subunits: p150, p60, and p48; the last is a histone acetylase. This protein complex acts on histones H3 and H4, which are

added first (Verreault et al., 1996). Histones H2A and H2B are then added, with histone H1 and the nonhistone proteins following. Histones can be acetylated or phosphorylated. This maintains the tissue-specific chromatin structure (and thus the state of differentiation) unchanged throughout successive mitotic divisions, except during the differentiation process itself. DNA methylation and chromosomal protein acetylation and phosphorylation are called *epigenetic* processes because they can be maintained through a series of mitotic divisions but do not involve an alteration of the basic nucleotide sequence.

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