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Mechanisms for Replicating DNA

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COMMON STEPS IN DNA REPLICATION

In the most fundamental sense, the general mechanism of DNA replication was first suggested by Watson and Crick as an immediate and obvious consequence of the complementarity of the two strands of the DNA structure. Thus, all replication processes simply involve the melting apart of the two strands followed by the polymerization of each complementary strand on the resulting single-stranded templates. However, when one looks a bit closer at the details of the process of genome duplication, one finds that cells, plasmids, and viruses have evolved a bewildering variety of particular solutions to the problem (Kornberg and Baker 1992). In many cases, the level of complexity of the enzymatic machinery for DNA replication is considerably greater than might have been expected, given that the information required to generate two daughter genomes is encoded in the structure of the parental genome in such a simple way. Such complexity presumably evolved to increase the efficiency and fidelity of DNA replication and to ensure that the duplication of the genome is coordinated with other events in the life of a cell. Below we attempt to distill the observed complexity down to the few basic processes that are common to most DNA replication pathways.

Initial Opening of the Duplex at Origins of Replication

DNA replication usually begins at one or more specific sites within the genome, referred to as origins of DNA replication. The first essential event in the initiation of DNA synthesis is the local opening of the duplex to provide access to the template strands. Origins of replication serve to increase the efficiency of initiation of DNA replication by providing loci for the assembly of multiprotein complexes that mediate DNA synthesis. If the individual components required for DNA synthesis were capable of interacting with random sites along the DNA, a suffi-

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cient local concentration of all of the essential factors might be achieved infrequently. Thus, the required components are usually brought together in one place by specific protein-DNA and protein-protein interactions. Origins of replication also provide specific points for the control of cellular DNA replication, ensuring that replication occurs at the right point in the cell cycle and that each segment of DNA is replicated precisely once. The initial opening of the DNA duplex at origins is generally mediated by specific initiator proteins and can be facilitated by certain structural features of the DNA (e.g., negative supercoiling, easily unwound sequences) and by certain accessory proteins (e.g., single-stranded DNA binding proteins [SSBs]).

Duplex Unwinding at Replication Forks

The initial opening of the duplex allows the establishment of a replication fork(s). The essence of this process is the loading of a DNA helicase on one or both of the exposed single strands. DNA helicases are enzymes that utilize the energy of ATP hydrolysis to translocate unidirectionally along a DNA strand, melting the duplex. At some origins, helicases are loaded onto both DNA strands, resulting in the establishment of two active replication forks (bidirectional DNA replication). In other cases, only a single fork is established (unidirectional DNA replication). An important characteristic of a given helicase activity is its polarity of translocation. Some helicases track along in the 3′ to 5′ direction of the so-called "leading strand" template of the replication fork, and others move in the 5′ to 3′ direction of the "lagging strand" template.

Priming of DNA Synthesis

In most DNA replication systems, the process of starting new DNA chains is distinct from the process of elongating established chains. Thus, all of the known DNA polymerases are incapable of starting chains de novo and require a primer to begin DNA synthesis. In eukaryotic cells, DNA synthesis is generally primed by short RNA chains. The separation of initiation from elongation and the use of RNA, rather than DNA, to initiate DNA synthesis are probably consequences of the requirement for extremely high fidelity in DNA replication. One major mechanism for achieving high fidelity involves proofreading the products of a given polymerization step before proceeding to the next polymerization step. A proofreading exonuclease built into most DNA polymerases recognizes and efficiently excises mismatched nucleotides at the primer terminus. It

is likely that this type of proofreading mechanism would be rather inefficient in detecting errors at or near the beginning of a new DNA chain. This problem is apparently solved by "marking" sites of initiation with a chemically distinct RNA chain. The replication machinery can later remove the RNA and fill the resulting gap by extension of an upstream DNA chain by DNA polymerase with proofreading function. This is a relatively costly solution, but one that maintains the accuracy of the genome.

Although both nuclear and mitochondrial DNA replication make use of RNA priming, the enzymatic mechanism of primer synthesis is different in the two cases. The synthesis of RNA primers for nuclear DNA synthesis is accomplished by a primase enzyme that is a component of DNA polymerase-α (pol-α:primase). This primase appears to be the only enzyme that is capable of priming chromosomal DNA synthesis in eukaryotes and is distinct from the RNA polymerases involved in the transcription of nuclear genes. A few eukaryotic viruses, such as SV40, utilize the cellular priming apparatus for the replication of their genomes. There is no evidence for the presence of pol-α:primase in mitochondria, and it is believed that the synthesis of RNA primers for mitochondrial DNA replication is carried out by uniquely mitochondrial enzymes. Interestingly, initiation of DNA synthesis at the primary origin of DNA replication in the mitochondrial genome is mediated by mitochondrial RNA polymerase, the same enzyme that is responsible for mitochondrial transcription.

Some eukaryotic viruses have evolved mechanisms for priming DNA synthesis that do not involve oligoribonucleotide synthesis. Two examples are discussed below. The parvovirus genomes have self-complementary hairpin termini that allow the 3′ termini of the genomic single strands to prime DNA synthesis. This example represents a very simple case, since the primers for DNA replication are incorporated into the viral genome itself. Adenoviruses make use of a protein to prime DNA replication. In this interesting case, the first phosphodiester bond is formed between the terminal nucleotide and a serine residue of a virus-encoded protein.

The distribution and frequency of priming events on the two parental strands determine the general pattern of DNA replication in a given system (Fig. 1). In continuous DNA replication there is only a single priming event per template strand, so each progeny DNA strand is synthesized continuously from one end to the other. Examples include mitochondrial DNA replication and the replication of the parvoviruses and adenoviruses. In semidiscontinuous DNA replication one progeny

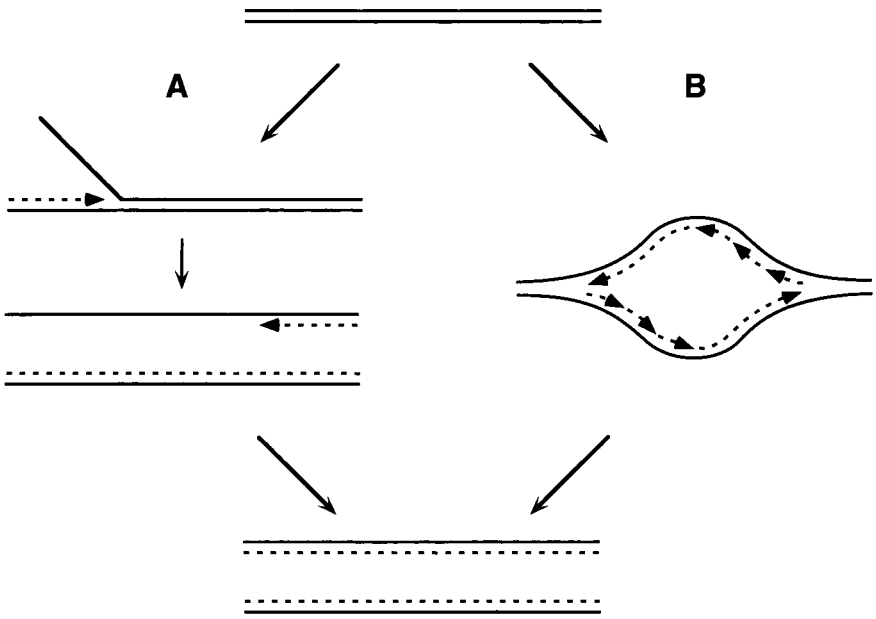

Figure 1 Continuous (*A*) versus semidiscontinuous (*B*) DNA replication. The two basic mechanisms for replicating DNA are shown, with nascent DNA (*broken lines*) synthesized from primers in the direction of the arrows.

strand (leading strand) is elongated continuously from a single primer, whereas the other progeny strand (lagging strand) is constructed from many short DNA chains elongated from multiple primers. Polymerization of the leading strand occurs in the same direction as replication fork movement, and polymerization of the lagging strand occurs in the opposite direction. Nuclear DNA replication and the replication of some viruses (e.g., SV40, herpesviruses) proceed by a semidiscontinuous mechanism. In semidiscontinuous DNA synthesis, completion of the lagging strand requires a repair system to remove the primers, fill in the resulting gaps, and join together the short nascent DNA strands.

Elongation of Nascent DNA Strands

Eukaryotic cells contain several DNA polymerase activities. The nuclear pol-α:primase complex mentioned above appears to be largely concerned with the synthesis of primers during semidiscontinuous DNA synthesis. The DNA polymerase activity of the enzyme is capable of extending the oligoribonucleotides synthesized by the intrinsic primase activity. How-

ever, pol-α:primase is relatively nonprocessive, so that only a short DNA chain is synthesized before the enzyme dissociates from the template. The relatively rapid turnover of the enzyme is consistent with its primary role in the synthesis of multiple primers on the lagging strand as the replication fork advances. The RNA-DNA primers synthesized by pol-α:primase can be extended by the highly processive DNA polymerases δ (pol-δ) and ε (pol-ε). Both of these enzymes can be assembled into complexes with the eukaryotic processivity factor proliferating cell nuclear antigen (PCNA) at primer termini. PCNA serves as a topological clamp, tethering pol-δ or pol-ε to the template, so that many thousands of nucleotides can be polymerized before the enzyme dissociates. It is likely that the bulk of chromosomal DNA synthesis is mediated by either pol-δ or pol-ε or both. In addition to their high processivity, which contributes to the efficiency of DNA replication, both enzymes have active proofreading exonuclease activities that enhance the fidelity of DNA replication.

As described in greater detail below, mitochondria possess a DNA polymerase activity distinct from the enzymes involved in nuclear DNA replication. Although some eukaryotic viruses, like SV40, utilize the resident cellular DNA polymerases, others, like the adenoviruses, encode DNA polymerases of their own.

Maturation of Nascent DNA Strands

A consequence of the widespread use of RNA priming mechanisms to initiate DNA synthesis is the requirement for an enzymatic machinery to remove the primers and replace them with DNA. In the case of nuclear DNA replication, this process is rapid and efficient, occurring soon after Okazaki fragment synthesis. Recent work suggests that primers are removed through the combined action of ribonuclease H (RNase H) and a 5′ to 3′ exonuclease. The resulting gaps are filled by DNA polymerase, probably pol-δ or pol-ε, and the final nick is sealed by DNA ligase I. Since this maturation process may result in the nearly complete removal of both RNA and DNA portions of the primers originally generated by the pol-α:primase complex, nearly all of the final DNA product is the result of polymerization by the more processive (and more accurate) DNA polymerases δ and ε. The process of primer replacement in mitochondria is probably mediated by a different set of enzymes, but little work has been done on the problem. A significant fraction of mitochondrial genomes contain residual ribonucleotides at the initiation

sites of DNA synthesis, suggesting that removal of RNA primers may be relatively slow and/or inefficient within the organelle.

The DNA-priming mechanism utilized by the parvoviruses requires a maturation mechanism that is quite different from that needed for RNA priming. Since DNA synthesis is primed by a duplex hairpin at the terminus of the genome (see below), there remains the problem of replicating the hairpin primer sequence itself. This is accomplished by a novel and interesting mechanism in which the hairpin sequence first serves as a primer and then is transferred to the newly synthesized DNA strand where it can serve as a template for DNA synthesis (see below). This maturation scheme regenerates the full-length parvovirus genome. Although a similar scheme could, in principle, be used for the maturation of the ends (telomeres) of linear cellular chromosomes, it is now clear that telomeres are synthesized by a special enzyme, telomerase, which polymerizes short DNA repeats at chromosome termini using an RNA template that is intrinsic to the enzyme. Further discussion of telomerase is beyond the scope of this chapter.

CONTINUOUS DNA REPLICATION

As indicated above, mitochondrial DNA and a number of viruses that replicate in eukaryotic cells have evolved replication mechanisms in which both progeny strands are synthesized continuously from one end to the other. To illustrate this general mode of DNA replication, we summarize the replication pathways of two viral systems, the parvoviruses and the adenoviruses, and then discuss mammalian mitochondrial DNA replication. The examples chosen also illustrate the major modes of priming DNA synthesis: DNA self-priming, protein priming, and RNA priming.

Mechanism of Parvovirus (AAV) DNA Replication

The parvoviruses are the simplest of the viruses that infect animal cells (for reviews, see Berns 1990a; Muzyczka 1992; Berns and Linden 1995). They contain linear single-stranded DNA genomes that are converted to duplex replicating intermediates within infected cells. Progeny single strands are generated from the duplex intermediates by a self-priming, strand-displacement mechanism. Viral DNA replication is largely dependent on host enzymes, probably the factors normally involved in leading-strand synthesis at chromosomal replication forks. Only one viral gene, *rep*, is required for DNA replication, and its product(s) is mainly concerned with processing of the termini of the genome.

Two general classes of parvoviruses have been identified (Berns 1990a). Adeno-associated viruses (AAV) generally require coinfection with a helper virus for efficient replication, although the basis for this requirement is not understood. Other parvoviruses are capable of autonomous replication in the absence of any helper virus (Cotmore and Tattersall 1987). All members of the parvovirus group have a similar genetic organization and follow a roughly similar replication pathway. The replication mechanism of AAV is discussed here as prototypical of the group.

AAV Genome

The AAV genome contains two large open reading frames (ORFs) (Srivastava et al. 1983). The 5′ ORF, referred to as the *rep* gene, encodes four distinct, but overlapping, polypeptide chains generated by alternate promoter utilization and alternate splicing. The two largest gene products, Rep78 and Rep68, have been implicated in AAV DNA replication by genetic studies (Hermonat et al. 1984; Tratschin et al. 1984; Yang et al. 1992). It is not known whether they play different roles in DNA replication in vivo, but either protein appears to be capable of supporting AAV DNA replication in vitro. The Rep proteins have site-specific endonuclease and helicase activities that mediate the novel processing reaction responsible for regenerating AAV termini (Ashktorab and Srivastava 1989; Im and Muzyczka 1990; Snyder et al. 1990a,b; McCarty et al. 1994; Ni et al. 1994).

Replication Pathway

Current evidence indicates that AAV DNA replication is completely continuous (Berns and Hauswirth 1979; Challberg and Kelly 1989; Muzyczka 1992; Berns and Linden 1995). The termini of the viral genome contain 145-bp palindromic sequences, referred to as inverted terminal repeats (ITRs) (Lusby et al. 1980). When folded in such a way as to maximize base-pairing, the ITRs are capable of forming T-shaped hairpin structures containing only seven unpaired bases (Fig. 2). The 3′ end of the genome represents a primer terminus that is used initially to convert the infecting single-stranded genome to a linear duplex form whose strands are covalently linked at one end via the terminal hairpin (Fig. 2) (Hauswirth and Berns 1979). The hairpin terminus is converted to an open duplex through the action of the Rep protein(s) and host enzymes (Tattersall and Ward 1976; Hauswirth and Berns 1979; Snyder et al. 1990b). This processing event, called terminal resolution or hairpin

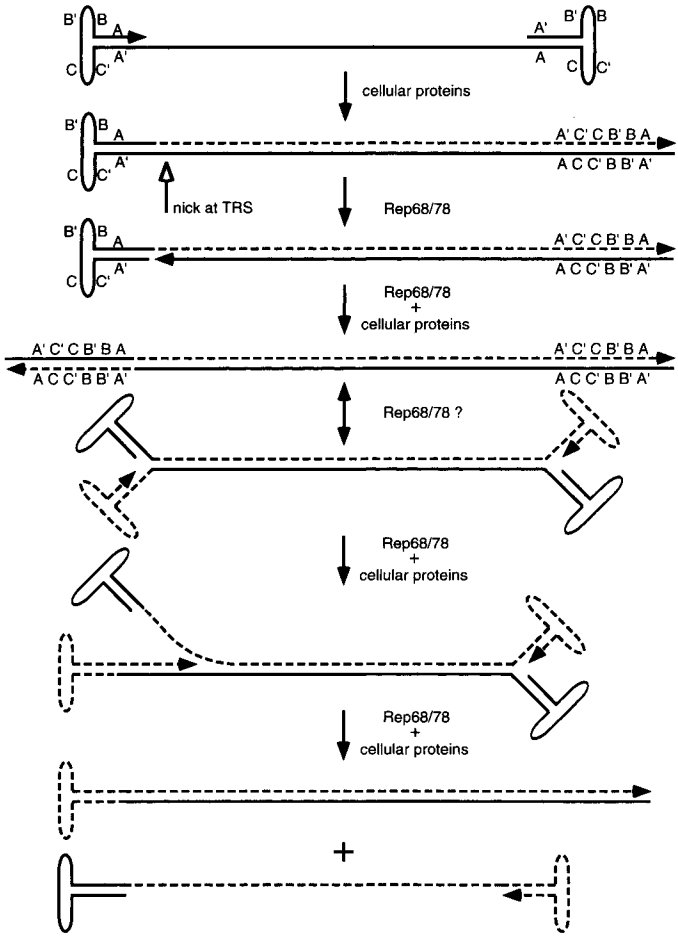

Figure 2 Model for AAV DNA replication. The single-stranded AAV genome contains self-complementary sequences at the termini (indicated by the letters A,A′,B,B′,C,C′) and folds to form the T-shaped hairpin structures shown. The 3′-OH end of one of the two hairpins serves to prime AAV DNA synthesis by host enzymes. Newly synthesized DNA is indicated by broken lines. The terminal resolution site (*trs*) is nicked by the AAV Rep protein to begin the terminal resolution process. DNA synthesis proceeds from the newly created 3′-OH terminus at the *trs* and continues to the end of the genome, resulting in the formation of an open duplex replication intermediate. The two strands of this intermediate are identical to the infecting AAV genomes except for inversion of the terminal palindromic sequence. After rearrangement of the termini to reform the hairpin structures, DNA synthesis proceeds by a displacement mechanism. Each round of DNA replication produces a progeny single strand and a duplex that can undergo terminal resolution to produce the open duplex replication intermediate.

transfer, is initiated by a single endonucleolytic cleavage at a site opposite the original 3′ terminus of the genome. Cleavage at the terminal resolution site (TRS) is carried out by the Rep protein, which becomes covalently linked to the 5′-phosphoryl terminus at the TRS cleavage site (Im and Muzyczka 1990; Snyder et al. 1990a). The 3′-OH terminus is then extended by a cellular polymerase(s) to replicate the terminal hairpin. It is likely that the helicase activity of the covalently bound Rep protein facilitates this process by unwinding the hairpin. The result of the terminal resolution reaction is the generation of a linear duplex replication intermediate whose strands are identical to those of the infecting AAV genome except for inversion of the terminal palindromic sequence (Lusby et al. 1981). The intermediate is then replicated via a self-priming, strand-displacement mechanism (Fig. 2). Priming of this reaction probably involves a rearrangement of the termini reforming the hairpin structures. The resulting structure resembles a replication fork and is presumably a competent substrate for the cellular elongation machinery. It is not clear whether rearrangement of the termini is catalyzed by Rep or a cellular protein or whether it occurs spontaneously. The products of the second stage of the AAV replication reaction are a displaced single strand and a linear duplex with covalently joined termini. The latter can be resolved to an open duplex by Rep and the process can be repeated. Since the two termini of AAV are essentially equivalent, equal numbers of the two complementary strands are synthesized. Both strands are packaged into progeny virus particles (Berns 1990b).

Enzymology of AAV DNA Replication

Several in vitro systems that carry out various aspects of the AAV DNA replication reaction have been developed (Hong et al. 1992; Ni et al. 1994; Ward et al. 1994). When duplex DNA molecules containing functional AAV origins are used as templates, either Rep68 or Rep78 is required for extensive DNA synthesis (Ni et al. 1994). It has also been reported that DNA synthesis in vitro can be stimulated significantly when the extracts are prepared from adenovirus-infected cells, suggesting that a protein(s) encoded or induced by the helper may play some role in AAV DNA replication (Ni et al. 1994). However, it seems likely that most of the proteins involved in viral DNA replication are derived from the host cell. Recent work indicates involvement of the cellular replication proteins PCNA, replication factor C (RF-C), and replication protein A (RP-A) (N. Muzyczka, pers. comm.). The DNA polymerase responsible for AAV DNA chain elongation has not yet been identified

with certainty, but, given the involvement of the processivity factor PCNA, pol-δ and pol-ε are likely candidates. Thus, AAV is probably replicated mainly by the apparatus responsible for leading-strand synthesis at cellular replication forks (see below). It is not known whether movement of the AAV replication fork is catalyzed by the helicase activity of Rep68/78 or whether a cellular helicase mediates this function. Since the AAV genome is linear and rather short, there is no apparent need for a DNA topoisomerase, consistent with the observation that antibodies against mammalian topoisomerase I and II do not inhibit DNA replication in vitro (N. Muzyczka, pers. comm.).

Mechanism of Adenovirus DNA Replication

Adenovirus Genome

The mechanism of adenovirus DNA replication has been well characterized because of the early development of a cell-free replication system (Challberg and Kelly 1979). The most extensively studied viral serotypes are the closely related Ad2 and Ad5 (for reviews, see Challberg and Kelly 1989; Hay and Russell 1989; Stillman 1989; Salas 1991; Van der Vliet 1991; Kornberg and Baker 1992). The Ad2/5 genome is a linear double-stranded DNA molecule of 36 kb with two novel structural features: (1) The nucleotide sequences at the ends of the genome are identical for the first 103 nucleotides and (2) the 5′ end of each strand is covalently linked to the virus-encoded, 55-kD terminal protein (TP). Viral DNA replication requires three viral proteins: an 80-kD precursor to the terminal protein (pTP), a 140-kD DNA polymerase (Ad pol), and a single-stranded DNA-binding protein (Ad DBP). The efficiency of DNA replication is increased significantly by several cellular proteins, including the transcription factors nuclear factor I (NFI) and octamer-binding protein 1 (Oct-1) and a DNA topoisomerase (NFII) (Nagata et al. 1982; Pruijn et al. 1986; Rosenfeld and Kelly 1986; Rosenfeld et al. 1987). Initiation of adenovirus DNA replication takes place by a protein-priming mechanism at the termini of the genome, and each daughter strand is synthesized continuously from one end to the other (Lechner and Kelly 1977; Rekosh et al. 1977; Challberg et al. 1980; Ikeda et al. 1982).

Initiation of Adenovirus DNA Replication

The adenovirus origin of replication, encompassing roughly the first 50 bp of the viral genome, is the locus of binding of several proteins. NFI and Oct-1 bind to specific recognition sites within the region between

nucleotides 19 and 51 (Nagata et al. 1983b; Rawlins et al. 1984; Pruijn et al. 1986; Rosenfeld and Kelly 1986; Rosenfeld et al. 1987; Wides et al. 1987). A complex of the two viral proteins, pTP and Ad pol (pTP-pol), appears to recognize sequence elements within the first 18 nucleotides that constitute the minimal essential origin (Ikeda et al. 1982; Mul and Van der Vliet 1992; Temperley and Hay 1992). Binding of pTP-pol to the viral origin of replication is the critical first step in the initiation reaction (Fig. 3). This step is greatly facilitated by protein-protein interactions between pTP-pol and the bound cellular proteins, NFI and Oct-1 (Bosher et al. 1990; Chen et al. 1990; Mul et al. 1990). The binding reaction may also be stimulated by the presence of the 55-kD terminal protein at the 5′ end of one of the parental strands (see Fig. 3). Once the pTP-pol is bound at the terminus of the genome, DNA synthesis is initiated by the formation of a phosphodiester bond between the first nucleotide in the new DNA chain, dCMP, and the β-hydroxyl group of a serine residue in the pTP (Challberg et al. 1980). This novel protein-priming reaction is presumably catalyzed by the adenovirus DNA polymerase, which then functions to extend the nascent chain (Challberg et al. 1980; Ikeda et al. 1982). An early intermediate in the elongation reaction of Ad5 is a trinucleotide covalently attached to the pTP (pTP-CAT) (King and Van der Vliet 1994). Interestingly, it has recently been demonstrated that the template for synthesis of pTP-CAT is not the first three nucleotides in the parental strand, but nucleotides 4–6. The sequence at the terminus of the template strand (3′ GTAGTAGTTA...5′) is repetitive, so it is thought that the pTP-CAT, synthesized opposite residues 4–6, translocates to positions 1–3 to start elongation. The function of this rather baroque mechanism is not yet clear, but it has been suggested that it may serve to protect the integrity of the terminal sequences of the viral genome during DNA replication (King and Van der Vliet 1994).

Elongation of Adenovirus DNA Strands

Following initiation at one of the termini, DNA synthesis proceeds by a displacement mechanism, producing a daughter duplex and a free single strand (Fig. 3) (Lechner and Kelly 1977). The latter can cyclize via self-complementary termini to form a panhandle structure. Since the duplex panhandle is identical to the termini of the original genome, initiation of DNA synthesis can presumably occur by the same mechanism, leading to the completion of a second daughter duplex. In vitro studies have demonstrated that the elongation of nascent adenovirus DNA strands re-

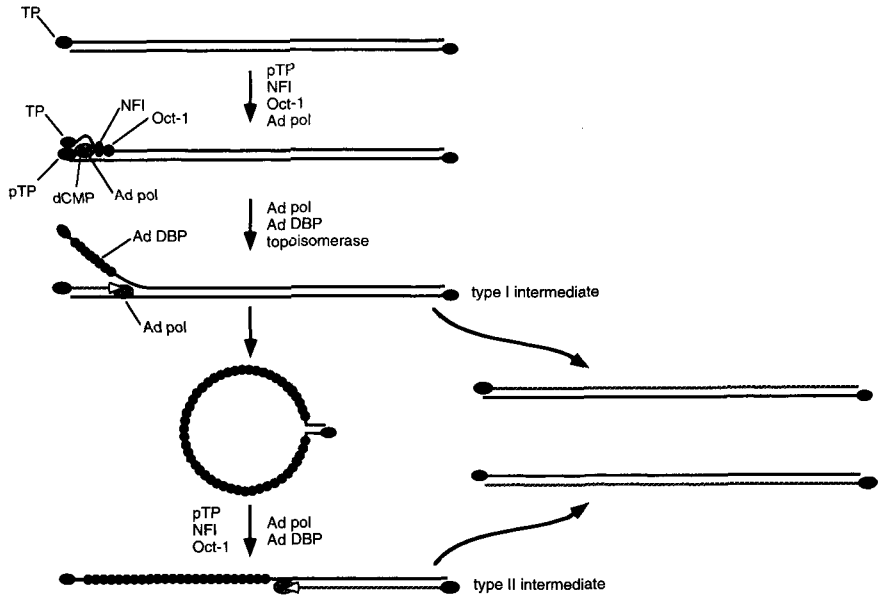

Figure 3 Model for adenovirus DNA replication. The 5′ ends of the adenovirus genome are covalently linked to the 55-kD terminal protein (TP). Initiation of DNA synthesis requires the 80-kD preterminal protein (pTP), the 140-kD adenovirus DNA polymerase (Ad pol), and the cellular proteins nuclear factor I (NFI) and octamer-binding protein 1 (Oct-1). Initiation is facilitated by the adenovirus DNA-binding protein (Ad DBP), which enhances NFI binding. Initiation involves the formation of a phosphodiester bond between the β-OH of a serine residue in pTP and the 5′ phosphoryl group of dCMP (see text for details). Elongation of nascent adenovirus DNA chains (*dotted lines*) requires the Ad pol, Ad DBP, and a cellular topoisomerase (NFII). Displacement synthesis on type I intermediates leads to the generation of a progeny duplex and a displaced single strand. The displaced single strand circularizes via self-complementary termini and serves as a substrate for a second initiation event, identical to the first. Completion of DNA synthesis on type II intermediates results in the formation of a second progeny duplex.

quires only two proteins, Ad pol and Ad DBP (Lindenbaum et al. 1986). The adenovirus DNA polymerase is a highly processive enzyme that extends DNA primers much more efficiently than RNA primers (Field et al. 1984; Lindenbaum et al. 1986). Its activity is specifically stimulated by the Ad DBP; prokaryotic or other eukaryotic DBPs cannot replace Ad DBP in the replication reaction (Lindenbaum et al. 1986). Movement of the displacement fork during adenovirus DNA replication does not appear to require a separate helicase activity. The Ad DNA pol, acting in

concert with the Ad DBP, is sufficient to unwind the parental duplex. The energy required for unidirectional fork movement is derived exclusively from hydrolysis of the deoxyribonucleoside triphosphate precursors for DNA synthesis (Pronk et al. 1994). Thus, adenovirus appears to have evolved a highly efficient two-protein engine for DNA synthesis that combines the functions of helicase and polymerase. Interestingly, in vitro studies have suggested that the replication of the adenovirus genome is facilitated by a cellular DNA topoisomerase (NFII) even though the genome is linear (Nagata et al. 1983a). Replication proceeds efficiently in the presence of Ad DNA pol and Ad DBP until replication is about 25% complete, after which fork movement is slow unless topoisomerase activity is present. The basis for this requirement is not completely clear, but presumably it reflects some hindrance to the free rotation of the unreplicated parental duplex in adenovirus replication intermediates.

Mechanism of Mitochondrial DNA Replication

General Features of Mitochondrial DNA Replication

Much of the early work on the mechanism of mitochondrial DNA replication was done in mammalian systems, but studies in other organisms, particularly yeast, have been increasingly important for identifying and characterizing required replication proteins (for reviews, see Clayton 1991, 1992; Kornberg and Baker 1992; Schmitt and Clayton 1993). The available evidence suggests that the basic features of mitochondrial DNA replication have been conserved from yeast to man, so the following description focuses primarily on mammalian cells (Clayton 1982, 1991). The mammalian mitochondrial genome is a closed duplex circle of about 16 kb. Cells contain on the order of 10^3–10^4 mitochondrial genomes with an average of 5–10 per organelle. Unlike the nuclear DNA, mitochondrial genomes are replicated throughout the cell cycle, and templates appear to be drawn at random from the pool of genomes. Thus, in a given cell cycle, some mitochondrial genomes are replicated more than once and some are not replicated at all. The regulatory mechanisms that determine the total number of mitochondrial genomes per cell are not understood.

Studies of the structures of replication intermediates have provided strong evidence that the replication of the mitochondrial genome occurs by a completely continuous mechanism (Fig. 4). In mammalian cells there are two origins of DNA replication, one for each strand, which are located about 11 kb apart (Clayton 1982). At each of these origins, DNA

synthesis is primed by specific RNA molecules (see below). A single priming event apparently suffices for the complete synthesis of each strand. The origin for heavy (H) strand synthesis (O_H) is activated first, resulting in the establishment of a replication fork that moves unidirectionally (Gillum and Clayton 1979; Chang and Clayton 1985). At this fork a new H strand is continuously elongated, and the parental H strand is displaced. The origin for light (L) strand synthesis (O_L) is activated only when it has been rendered single-stranded by the passage of the H-strand replication fork (Wong and Clayton 1985a,b). It is likely that a specific secondary structure that forms in the displaced strand is recognized by the protein(s) responsible for RNA primer synthesis. The L strand is continuously elongated from the primer in what is essentially a large gap-filling reaction. Since the two origins of DNA replication are separated by approximately two-thirds of the genome, the replication of the two strands of mitochondrial DNA is quite asynchronous (see Fig. 4). When the synthesis of the H strand is completed, a process that takes about an hour, two products are generated. One product is a duplex circle with a full-length progeny H strand, and the other is a gapped circle with an incomplete progeny L strand, which is subsequently extended to full length.

The primary event in mitochondrial DNA replication is the activation of the H-strand origin, since the activation of the L-strand origin is secondary to the establishment of the H-strand replication fork. Thus, one likely point of regulation of mitochondrial DNA synthesis is the generation of the initial RNA primer. There may also be a second control point that operates at the level of elongation of the H strand. A majority of mitochondrial genomes in mammalian cells contain a so-called D loop in the vicinity of O_H (Gillum and Clayton 1979). This structure consists of a short nascent H DNA whose 5′ end is located at the origin for H-strand synthesis. The nascent H strands in D loops appear to be metabolically labile and are turned over at a rate that significantly exceeds the rate of replication of mitochondrial genomes. The existence of the short nascent H strands suggests that there may be a barrier to chain elongation just downstream from O_H (Clayton 1982; Madsen et al. 1993). It is possible that mechanisms exist that regulate whether or not the H-strand replication fork can pass this barrier, but at present there is no evidence on this point.

Less work has been done on the replication of the mitochondrial DNA of yeast, but it is likely that DNA synthesis is largely continuous, although there may be more than one priming event per strand (Blanc and Dujon 1980; Baldacci et al. 1984; de Zamaroczy et al. 1984; Schmitt and

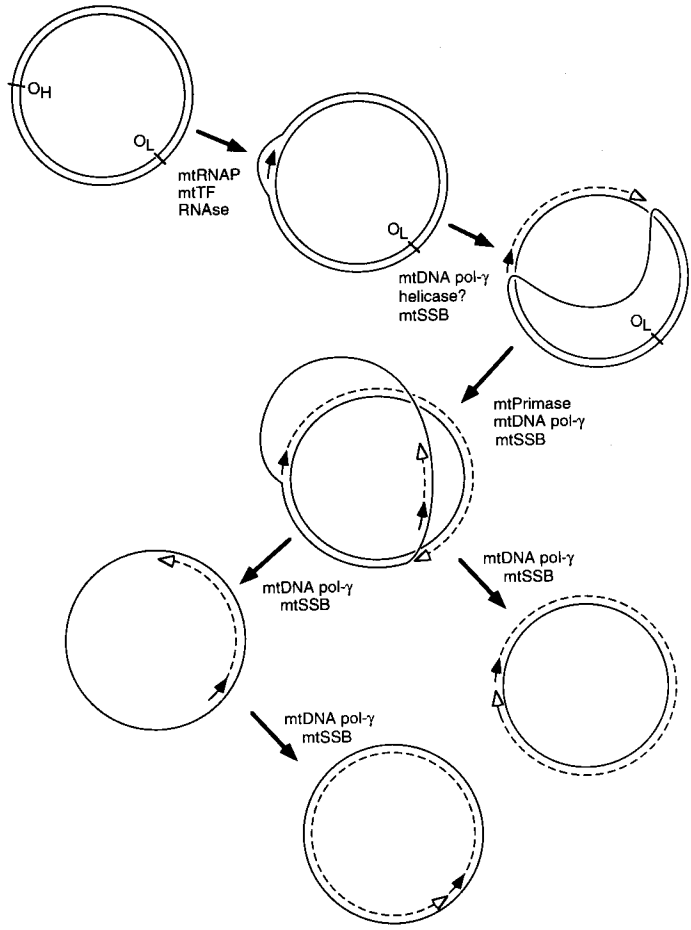

Figure 4 Model for mammalian mitochondrial DNA replication. The two mitochondrial origins O_H and O_L are located about two-thirds of the genome apart. Initiation of DNA replication begins with the synthesis of a transcript by the mitochondrial RNA polymerase (mtRNAP) and an essential transcription factor (mtTF). The transcripts are either processed by an endoribonuclease (RNase) to form a primer for mitochondrial DNA replication or continue to be extended to form mRNA. If cleavage occurs, a nascent H strand (*broken line*) is synthesized by the mitochondrial DNA polymerase (mtDNA pol-γ). Other proteins, including a putative mitochondrial helicase and SSB (mtSSB) probably participate in the chain elongation process. Synthesis of the L strand is initiated by a mitochondrial primase (mtPrimase) after the displacement fork passes O_L. Completion of H-strand synthesis and separation of the two progeny molecules occurs before completion of L-strand synthesis.

Clayton 1993). At least four putative origins of replication have been located in the yeast mitochondrial genome. It is likely that more origins are required because of the large size of the yeast mitochondrial genome. The structural features of yeast origins are similar to those of higher eukaryotes (Schmitt and Clayton 1993). However, unlike mammalian origins, the sites of initiation of synthesis of the two strands are in close proximity rather than being separated by many kilobases.

Priming of Mitochondrial DNA Synthesis

Transcription of the mammalian mitochondrial genome is directed by two divergent promoters located within the D-loop region (Chang and Clayton 1984). Transcripts initiated at one of these promoters (LSP) also serve to prime H-strand synthesis during mitochondrial DNA replication (Chang and Clayton 1985; Chang et al. 1985). A fraction of the LSP transcripts are cleaved by an endoribonuclease at one of several discrete sites at O_H, approximately 100 nucleotides from the promoter. The 3′-OH termini of the transcripts are then elongated by the mitochondrial DNA replication apparatus. Transcription from the LSP requires the mitochondrial RNA polymerase and a specific transcription factor, mtTFA (Fisher and Clayton 1985; Parisi et al. 1993). An RNase capable of processing nascent transcripts at sites near the transition from RNA synthesis to DNA synthesis has been identified in mammalian cells and yeast (Chang and Clayton 1987; Schmitt and Clayton 1992; Stohl and Clayton 1992). The enzyme, called RNase MRP, contains a required RNA moiety that may be involved in recognition of the specific cleavage sites. Recently, it has been suggested that a second mitochondrial nuclease, endonuclease G, may also play a role in processing nascent transcripts to generate primers for DNA replication (Cote and Ruiz-Carrillo 1993).

Priming of L-strand synthesis at O_L appears to be mediated by a mitochondrial primase that recognizes a specific stem-loop structure which forms in the H-strand template (Wong and Clayton 1985a,b; Hixson et al. 1986). The enzyme initiates the synthesis of a short oligoribonucleotide at a run of T residues in this loop. The transition from RNA synthesis to DNA synthesis occurs immediately adjacent to the base of the stem. The mitochondrial primase appears to require an RNA component for activity, and the most likely candidate is cellular 5.8S RNA (Wong and Clayton 1986). Thus, the priming mechanisms for the synthesis of both strands of the mitochondrial genome require ribonucleoprotein enzymes.

Mechanism of Chain Elongation

Although some of the required enzymes have been identified, the detailed mechanism of continuous DNA synthesis in mitochondria is not well understood. This is mainly due to the absence of an efficient cell-free replication system. All of the components involved in mitochondrial DNA replication, including both the priming and elongation stages, are encoded by nuclear genes and imported into the organelle. A highly processive mitochondrial DNA polymerase, referred to as DNA polymerase-γ, has been purified from several sources (Wernette and Kaguni 1986; Wernette et al. 1988; Insdorf and Bogenhagen 1989a,b; Gray and Wong 1992). The human enzyme consists of subunits of 140 kD and 54 kD. The larger subunit contains the polymerase active site as well as a potent 3′ to 5′ exonuclease activity. The latter shows strong preference for unpaired primer termini and presumably plays a proofreading role. The yeast gene encoding the catalytic subunit of DNA polymerase-γ (*MIP1*) has been cloned (Foury 1989). The amino acid sequence of the Mip1 protein is similar to both eukaryotic nuclear DNA polymerases and reverse transcriptases, but has no discernible resemblance to prokaryotic DNA polymerases.

Mitochondrial DBPs have also been identifed from several organisms (Pavco and Van Tuyle 1985; Mignotte et al. 1988; Van Dyck et al. 1992; Tiranti et al. 1993; Stroumbakis et al. 1994). Interestingly, these proteins are homologous to *Escherichia coli* SSB, and their physicochemical properties are quite similar to the prokaryotic enzyme as well. Yeast mutants lacking the *RIM1* gene, which encodes the mitochondrial SSB, are completely devoid of mitochondrial DNA, consistent with an essential role for the protein in mitochondrial DNA replication (Van Dyck et al. 1992). Presumably, the mitochondrial SSB facilitates the elongation of nascent DNA chains by the mitochondrial DNA polymerase. However, the functional interactions of the two proteins have not been extensively analyzed to date.

Another likely accessory protein for mitochondrial DNA synthesis is a DNA helicase, since H-strand synthesis requires melting of the parental strands at the replication fork. A helicase activity has been identified in highly purified mitochondria from bovine brain (Hehman and Hauswirth 1992). This activity, which translocates in the 3′ to 5′ direction on the single-stranded portion of partially duplex substrates, is a likely candidate for a replicative helicase in mitochondria, but this role has not been verified directly. The Pif1 helicase of budding yeast has been implicated in the repair, recombination, and replication of the mitochondrial genome (Lahaye et al. 1991). Genetic interactions have been observed between

the *PIF1* gene and the *RIM1* gene, encoding the mitochondrial SSB (Van Dyck et al. 1992). Moreover, a yeast strain deficient in the Pif1 enzyme loses mitochondrial DNA at elevated temperatures.

Mitochondrial DNA replication remains the best example of a naturally occurring, completely continuous DNA replication system in eukaryotic cells. Although a number of the components required for DNA replication have been identified, and we have a general picture of the overall replication pathway, much remains to be learned about the biochemical mechanisms involved. Future in vitro studies of mitochondrial DNA synthesis with purified components will likely fill in many of the gaps in our knowledge.

SEMIDISCONTINUOUS DNA SYNTHESIS

Replication of Chromosomal DNA

In the continuous DNA replication systems that we have discussed so far, the two complementary DNA strands are synthesized relatively independently and asynchronously. In contrast, the replication of eukaryotic chromosomal DNA occurs by a semidiscontinuous mechanism in which the synthesis of the two strands is strongly coupled in space and time. One advantage of semidiscontinuous DNA replication over completely continuous DNA replication is that the generation of single-stranded DNA is much more localized and transient. This may help preserve the integrity of the genome, since breaks and other lesions in single-stranded DNA are difficult to repair.

Semidiscontinuous DNA replication is mediated by a complex protein machine assembled at each replication fork. The proteins comprising this replication machine act in concert to unwind the parental strands and carry out the simultaneous synthesis of the two progeny strands. Both progeny strands are synthesized in the 5′ to 3′ direction, but since the parental DNA strands are antiparallel, two distinct mechanisms of DNA synthesis are required. One of the two progeny strands (leading strand) is synthesized continuously in the direction of fork movement. The other strand (lagging strand) is synthesized discontinuously in the direction opposite to fork movement. Discontinuous DNA synthesis on the lagging-strand template involves the repeated synthesis of oligoribonucleotide primers, which are then elongated into short DNA chains (Okazaki fragments). Following their synthesis, Okazaki fragments are processed to remove the RNA primers and joined together to form an uninterrupted progeny strand.

The semidiscontinuous DNA replication mechanism appears to be quite ancient in origin, as its essential features have been conserved from bacteria to man. Although the basic biochemical processes that occur at eukaryotic and prokaryotic replication forks are similar, there are many differences in detail. For example, in *E. coli*, a single DNA polymerase is thought to form a dimeric complex that mediates DNA synthesis on both the leading and lagging strands (Maki et al. 1988; McHenry 1988), whereas eukaryotes contain at least three distinct DNA polymerase activities, all of which participate in DNA replication. The critical protein-protein interactions that are required for formation of an efficient replica tion machine also appear to differ between prokaryotes and eukaryotes. In *E. coli*, the primase activity responsible for initiating new DNA chains forms a specific complex with the helicase activity responsible for unwinding the parental strands as the fork moves (Kornberg and Baker 1992) whereas in eukaryotes, the primase is a subunit of one of the DNA polymerase molecules (Lehman and Kaguni 1989; Wang 1991). Some of the accessory proteins involved in DNA synthesis have more complex structures in eukaryotes than in prokaryotes. One example is the single-stranded DNA-binding protein, which is a single polypeptide chain in *E. coli* (Meyer and Laine 1990) but a large heterotrimeric protein in eukaryotes (Fairman and Stillman 1988; Wold and Kelly 1988). The greater complexity may be related to the more complex mechanisms required to regulate DNA replication in eukaryotes. Despite these apparent differences, there are many similarities between prokaryotic and eukaryotic DNA replication that reflect a common evolutionary origin (see Stillman, this volume). One particularly striking example is the remarkable structural similarity of the β subunit of *E. coli* DNA polymerase III and the eukaryotic protein PCNA, both of which function as "sliding clamps" to increase the processivity of DNA synthesis (Kong et al. 1992; Krishna et al. 1994).

A novel feature of eukaryotic chromosomes is the packaging of DNA into chromatin. This fact may account for the surprising disparity in the rate of fork movement in prokaryotes and eukaryotes. Whereas the *E. coli* replication machinery moves at the prodigious rate of 100 kb/minute, unwinding DNA at some 10,000 rpm, eukaryotic fork movement is much slower (0.5–5 kb/min) (Kornberg and Baker 1992). The presence of histones may limit the maximal rate at which DNA polymerization can occur. Such a constraint might have contributed to the evolution of intrinsically slower polymerases in eukaryotes. In addition, special mechanisms may have evolved to allow fork movement without disruption of either the replication complex or the nucleosomes. In this

manner, the DNA remains packaged even while DNA synthesis is taking place. However, the ability of large protein assemblies to negotiate one another is not unique to eukaryotes, as in vitro experiments have shown that the phage T4 replication apparatus can bypass an RNA polymerase complex moving in either direction without complete dissociation of either set of proteins (Liu et al. 1993; Liu and Alberts 1995).

Viral Model Systems

Direct biochemical investigation into the mechanisms underlying cellular DNA replication has proven difficult. With the exception of special cases such as *Xenopus* eggs, it has not yet been possible to develop an in vitro cellular DNA replication system. In the absence of such a system, investigators have turned to viral models to study the mechanism of cellular DNA replication. Viruses present several advantages, including small genomes and well-defined origins of replication. The Papovaviridae, which include the SV40, polyoma, and papilloma viruses, have been particularly important to the study of chromosomal replication. Upon infection of suitable host cells, the genomes of these viruses are transported to the nucleus, where the double-stranded viral minichromosomes are replicated by mechanisms that closely resemble the cellular process. Initiation occurs at a single origin within the viral genome and proceeds bidirectionally in a semidiscontinuous manner. The great advantage of SV40 and other papovaviruses is that many of the important steps in DNA replication are performed by host proteins.

A significant advance in the study of eukaryotic DNA replication came with the development of a cell-free SV40 system in 1984 (Li and Kelly 1984). Primate cytoplasmic extract and a single viral protein, the large T antigen, carry out all the functions required for complete replication of SV40-origin-containing plasmid DNA. T antigen recognizes the origin and unwinds the duplex, providing access for the numerous host replication proteins that function coordinately to synthesize the daughter DNA strands (for review, see Stillman 1989; Borowiec et al. 1990; Kelly 1991). Fractionation of human extract and reconstitution of replication activity using an in vitro complementation assay have led to the identification of those cellular proteins necessary and sufficient for viral DNA replication (Ishimi et al. 1988; Wold et al. 1989; Tsurimoto et al. 1990; Weinberg et al. 1990; Eki et al. 1992; Waga et al. 1994). Given that the mechanisms of SV40 and cellular DNA replication appear to be very similar, there is considerable confidence that the same proteins have identical functions in the replication of the chromosomes. It should be

noted that analysis of the viral system has not identified all of the proteins involved in eukaryotic DNA replication. For example, genetic studies in yeast strongly suggest that pol-ε is necessary for DNA replication in vivo (Araki et al. 1992; Budd and Campbell 1993), but this enzyme was not originally identified as necessary for SV40 DNA replication.

As discussed previously, the natural template for eukaryotic DNA replication is chromatin. Whereas the cell-free system was originally developed with naked DNA templates, a number of studies have attempted to more accurately reflect the in vivo replicative process by employing chromatin templates. Although in vitro replication of such minichromosomes is very inefficient relative to that of naked DNA, the repression of initiation due to tightly bound histones can be relieved by the presence of transcription factors bound near the origin (Cheng and Kelly 1989; Cheng et al. 1992). A similar stimulatory effect of transcription factors on replication efficiency has been observed in vivo. Several possible mechanisms could account for this derepression, including increased accessibility of the origin to the initiator protein or activation of the replication apparatus by contact with factors bound near the origin (see DePamphilis, this volume). Further studies have focused on the fate of parental nucleosomes during DNA replication and indicate that nucleosomes remain associated with the replicating DNA during DNA synthesis (Randall and Kelly 1992). In addition, the assembly of new chromatin appears to be coupled to SV40 DNA replication in vitro (Stillman 1986). Experiments such as these have added to our understanding of the mechanism by which the replication machinery negotiates other DNA-bound structures, a process that must occur continuously in the nucleus.

Enzymology of the Replication Fork

Replication Proteins

Studies employing the SV40 model system have resulted in the identification of many cellular replication proteins. Recent work employing both genetic and biochemical techniques has added to this list. A summary of known replication proteins is presented in Table 1 and in some detail below.

Helicases

The focal point of all replication forks is the helicase, which catalyzes the transition from double- to single-stranded DNA. In *E. coli,* the DnaB

Table 1 Cellular replication proteins

Protein	Subunit (kD)[a]	Replicative function
DNA polymerases		
pol-α:primase	180, 70, 58, 48	DNA polymerase, primase
pol-δ	125, 48	DNA polymerase, 3′ to 5′ exonuclease
pol-ε	258, 55	DNA polymerase, 3′ to 5′ exonuclease
Accessory proteins		
RP-A	70, 32, 14	single-stranded DNA binding
PCNA	36	pol-δ/ε processivity factor
RF-C	145, 40, 38, 37, 36.5	loads PCNA onto template
Nucleases		
ribonuclease H1	89	Okazaki fragment maturation
FEN-1 (MF-1)	44	Okazaki fragment maturation
Others		
DNA ligase I	102	joins Okazaki fragments
topoisomerase I	100	unlinks parental strands
topoisomerase II	172	unlinks parental strands and progeny duplexes

References include those cited within the text as well as Kesti et al. (1993), Syvaoja and Linn (1989), Eder and Walder (1991), and Miller et al. (1981).

[a]Approximiate molecular weights of human polypeptides.

helicase translocates in a 5′ to 3′ direction while unwinding DNA (LeBowitz and McMacken 1986) and therefore is bound to the lagging-strand template during DNA replication. The same mechanism is employed by the phages T4 and T7 (Matson et al. 1983; Richardson and Nossal 1989), which induce their own helicases to engage in semi-discontinuous replication. In direct contrast, SV40 T antigen is bound to the leading-strand template and moves in the 3′ to 5′ direction advancing the fork (Goetz et al. 1988; Wiekowski et al. 1988). Although the polarity of translocation is not conserved, the replicative helicases of *E. coli*, T4, T7, and SV40 are all hexamers, suggesting a common quaternary structure for enzymes with this function. To date, several eukaryotic helicases have been identified, but conclusive identification of the enzyme that acts at chromosomal replication forks must await further biochemical and genetic investigation.

Single-stranded DNA-binding Protein

As the replication fork advances, a helix-destabilizing protein is required to maintain the single-stranded DNA structure that serves as a template for RNA priming and DNA synthesis. In eukaryotic cells, replication protein A (RP-A; RF-A; HSSB) performs this function (Wobbe et al. 1987; Fairman and Stillman 1988; Wold and Kelly 1988). This phosphoprotein has three subunits with molecular weights of 70,000, 32,000, and 14,000. The large subunit contains the DNA-binding activity but cannot support SV40 DNA replication in vitro by itself (Kenny et al. 1990; Erdile et al. 1991); therefore, at least one of the two smaller subunits is likely to be required for replication. In support of this hypothesis, all three yeast genes encoding RP-A are essential for viability (Heyer et al. 1990; Brill and Stillman 1991), and antibodies directed against any of the three subunits inhibit SV40 DNA replication in vitro (Erdile et al. 1990, 1991; Kenny et al. 1990; Umbricht et al. 1993). Although the roles of the two smaller RP-A subunits remain unknown, the middle subunit is phosphorylated during S phase of the cell cycle (Din et al. 1990), suggesting that this subunit may play some role in regulating DNA replication.

The DNA-binding properties of RP-A have been investigated extensively, but there remains some disagreement about fundamental aspects of the protein-DNA interaction. RP-A binds relatively nonspecifically to single-stranded DNA but exhibits a modest preference for DNA sequences rich in pyrimidines (Kim et al. 1992). Several different binding site sizes have been reported, ranging from 8 to 30 nucleotides for human RP-A and up to 100 nucleotides for the yeast protein (Alani et al. 1992; Kim et al. 1992; Blackwell and Borowiec 1994). Some evidence has been presented that RP-A forms two different types of complexes with DNA, which may account for the variability in site size estimates. Further disagreement centers on the degree of cooperativity of the DNA-binding reaction. Most prokaryotic single-stranded DNA-binding proteins display a high level of cooperativity, allowing the rapid and complete binding of any exposed single-stranded regions in the genome. Steady-state fluorescence experiments indicate that this is also the case with yeast RP-A (Alani et al. 1992). However, a series of studies employing electrophoretic mobility shift of oligonucleotides in the presence of human RP-A have demonstrated little cooperativity (Kim et al. 1992; Kim and Wold 1995). Other studies employing a similar technique suggest that only one of two proposed binding modes is highly cooperative (Blackwell and Borowiec 1994). The lack of consensus with regard to the DNA-binding properties may result from differences in experimental

technique, or possibly differences in the state of the protein itself, such as the extent of phosphorylation.

DNA Pol-α:Primase Complex

DNA synthesis is initiated by the bifunctional pol-α:primase complex, a heterotetrameric phosphoprotein (Wang 1991). The primase activity resides in the 48-kD D subunit and is tightly associated with the 58-kD C subunit, which is thought to tether the primase to the 180-kD polymerase A subunit (Copeland and Wang 1993; Santocanale et al. 1993; Bakkenist and Cotterill 1994; Stadlbauer et al. 1994). With the exception of the *Drosophila* enzyme (Cotterill et al. 1987), there is no proofreading exonuclease activity associated with the A subunit. The remaining 70-kD B subunit has no known catalytic function, but it may contribute to recruitment of pol-α:primase to the replication fork (Collins et al. 1993) (see below).

The main function of pol-α:primase is to serve as a priming enzyme. The primase catalyzes the synthesis of complementary oligoribonucleotides, which are then extended a short distance by the A subunit DNA polymerase activity. Although high concentrations of pol-α:primase can support the complete replication of SV40 origin-containing plasmid DNA in vitro (Ishimi et al. 1988), it is unlikely that cellular replication relies heavily on this polymerase during elongation. The low processivity of the enzyme and the lack of an associated proofreading exonuclease suggest that pol-α:primase serves exclusively to initiate DNA synthesis on the lagging strand. Dissociation of pol-α:primase from the DNA provides a primer terminus for the assembly of the PCNA/pol-δ or /pol-ε complexes, highly processive polymerases that can efficiently extend the RNA/DNA primers originally synthesized by pol-α:primase.

DNA Polymerases δ and ε

The heterodimeric DNA polymerases δ and ε are involved in the elongation stage of DNA replication. Unlike pol-α:primase, polymerases δ and ε do not act alone but require the action of two auxiliary factors. The multisubunit replication factor C (RF-C) (Tsurimoto and Stillman 1989; Lee et al. 1991a) binds to the primer terminus immediately after RNA/DNA primer synthesis has been completed by pol-α:primase, allowing the subsequent assembly of a functional pol-δ or pol-ε complex. Once bound to the primer-template junction, RF-C loads PCNA onto the DNA in an energy-dependent reaction. PCNA then functions as a proces-

sivity factor, binding to pol-δ or pol-ε and maintaining a stable interaction between polymerase and template (Tan et al. 1986; Prelich et al. 1987; Burgers 1991; Lee et al. 1991a; Tsurimoto and Stillman 1991; Podust and Hubscher 1993). Ultimately, one or both of these processive polymerases, with their intrinsic proofreading activities, probably synthesize all of the cellular DNA, thereby ensuring faithful duplication of the genome (see below).

DNA polymerases δ and ε have some similarities in catalytic function and are both essential for viability in yeast (for reviews, see Wang 1991; So and Downey 1992). However, it is not yet clear whether they have specialized roles in replication. pol-ε does not substitute well for pol-δ in the cell-free SV40 DNA replication system with purified proteins (Lee et al. 1991b), so it may contribute to DNA synthesis only when certain additional factors are present. It is also possible that these enzymes have important roles that are not limited to DNA replication. For example, recent experiments indicate that pol-ε is one member of the S-phase checkpoint pathway (Navas et al. 1995), a biochemical feedback mechanism that delays cell-cycle progression upon damage to the DNA or inhibition of DNA synthesis during the replicative phase. Thus, in addition to synthesizing DNA, pol-ε may be involved in monitoring the status of DNA replication.

Nucleases

Synthesis of the lagging strand results in the generation of DNA fragments with RNA primers at the 5′ ends. For lagging-strand synthesis to be completed, these primers must be removed and the resulting gap must be filled. In *E. coli,* DNA polymerase I, with its intrinsic 5′ to 3′ exonuclease activity, mediates both functions (Kornberg and Baker 1992). None of the replicative eukaryotic polymerases contains a 5′ to 3′ exonuclease, indicating that other factors are involved in the initial processing of Okazaki fragments. A 44-kD 5′ to 3′ exonuclease (FEN-1; MF-1) that is required for the formation of covalently closed circular DNA during in vitro SV40 DNA replication has been identified in human cells (Ishimi et al. 1988; Harrington and Lieber 1994; Waga et al. 1994). Recently, studies employing purified calf proteins and a model lagging-strand template have shown that FEN-1 and RNase H1 act together to process Okazaki fragments. RNase H1 nicks the primer on the 5′ side of the 3′ ribonucleotide, providing a suitable substrate for FEN-1, which removes the 3′-terminal ribonucleotide of the RNA primer. The oligoribonucleotide is displaced and the gap is filled by DNA polymerase,

resulting in nicked double-stranded DNA (Turchi and Bambara 1993; Huang et al. 1994; Murante et al. 1994; Turchi et al. 1994).

DNA Ligase

After removal of the RNA primers and extension of the DNA chains through the resulting gaps, the nascent DNA fragments must be joined to complete the synthesis of the lagging strand. Of the three DNA ligases that have been identified in mammalian cells (Lindahl and Barnes 1992), DNA ligase I is the most likely candidate to carry out this function in vivo. In a cell-free SV40 system containing highly purified proteins, DNA ligase I, but not DNA ligase III, catalyzes the formation of covalently closed daughter molecules (Waga et al. 1994). Although DNA ligase II has not been tested, this enzyme is present at low abundance and is likely to be involved in DNA repair. Genetic studies support the conclusion that DNA ligase I is involved in cellular replication in vivo. It has been shown that the human DNA ligase I gene can complement a *Saccharomyces cerevisiae* cdc9 mutant, which is defective in DNA replication due to DNA ligase deficiency (Barnes et al. 1990).

Topoisomerases

As replication proceeds and the parental strands are unwound, positive supercoils are potentially introduced ahead of the replication fork. The resulting accumulation of torsional strain could lead to inhibition of fork movement if not relieved by a DNA topoisomerase. In eukaryotic cells, two types of topoisomerases have been discovered (Wang 1985). The type I enzyme introduces a transient single-strand break in the DNA, thereby relaxing either negatively or positively supercoiled DNA. Type II topoisomerase introduces a transient double-strand DNA break through which duplex DNA is passed. In addition to relaxing DNA, this enzyme can decatenate intertwined molecules. Studies with the SV40 model system employing naked DNA have revealed that either type of topoisomerase is capable of removing the positive supercoils ahead of the fork, allowing rapid and efficient DNA synthesis (Yang et al. 1987). However, the progeny DNA molecules that are formed remain multiply intertwined because of failure to remove all of the links between the parental strands during DNA synthesis. Topoisomerase II is required to resolve this tangled structure into two separate progeny genomes, allowing subsequent segregation (Yang et al. 1987).

The topological problems accompanying SV40 DNA replication in vitro are likely to arise during cellular replication as well. The length of

chromosomal DNA and its association with nuclear proteins to form chromatin probably precludes free rotation of the DNA during replication. Therefore, the two eukaryotic topoisomerases are likely to function in vivo as they do in the SV40 system. It has been suggested that topoisomerase II may have a special role in the completion of DNA synthesis where two adjacent forks converge (Ishimi et al. 1992). However, this conclusion is based on studies of a cell-free SV40 system containing only one DNA polymerase (pol-α:primase) and may not accurately reflect the normal cellular process.

Organization of the Replication Fork

Most of the studies aimed at defining the organization of the cellular replication fork have revolved around the SV40 system. Although the identity and mechanism of the cellular helicase remain unknown, it is likely that the known cellular proteins play similar roles in chromosomal replication as in the viral model. The current picture of the eukaryotic replication fork is summarized in Figure 5. Leading-strand synthesis is performed by a processive polymerase complex (PCNA/pol-δ or PCNA/pol-ε) and a single auxiliary protein (RP-A). Lagging-strand synthesis requires additional proteins. pol-α:primase synthesizes RNA/DNA primers that are extended by complexes similar to those operating on the leading strand. Primer removal and completion of DNA synthesis require FEN-1 nuclease, RNase H1, and DNA ligase I as described above.

Studies with both crude and purified systems have demonstrated that assembly of replication proteins at the replication fork is mediated by specific protein-protein contacts. These interactions are critical for the coordinated synthesis of both leading and lagging strands, and are likely to be important at all stages of the replicative process. Specific mechanisms involving multiple proteins at the replication fork are discussed below.

Protein-protein Interactions

In the SV40 system, the viral T antigen and the cellular proteins pol-α:primase and RP-A are required for initiation of DNA replication, and a variety of studies have provided evidence for significant molecular interactions among the three proteins. For example, biochemical analysis of model reactions has uncovered a number of instances in which one protein affects the activity of another (Kenny et al. 1989; Collins and Kelly 1991; Collins et al. 1993; Melendy and Stillman 1993; Murakami

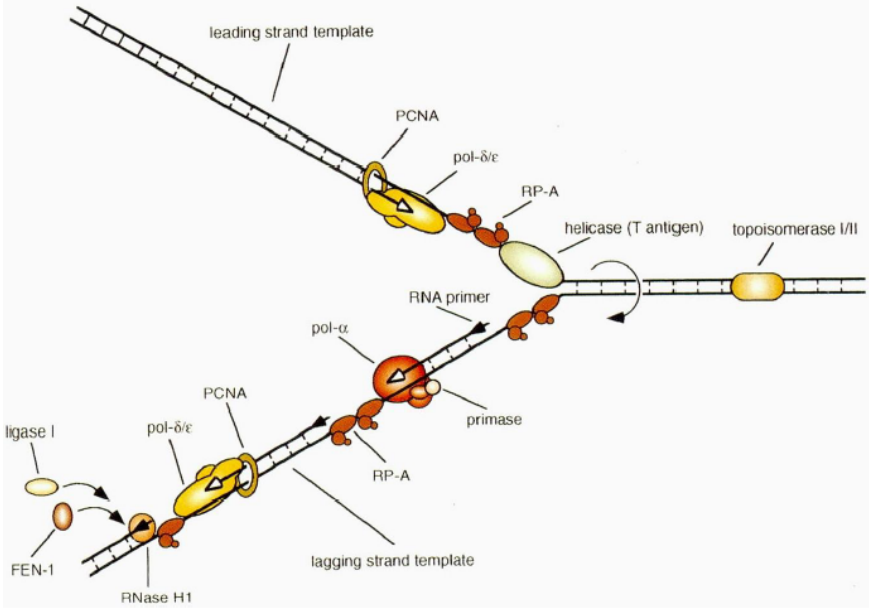

Figure 5 Semidiscontinuous DNA synthesis. This figure shows a diagrammatic view of the organization of the eukaryotic replication fork. The pol-δ/ε auxiliary factor RF-C is not included in this diagram because it is not known whether this protein stays associated with the replication apparatus after loading PCNA. See text for further details.

and Hurwitz 1993a). These biochemical data are supported by physical studies demonstrating direct association of each combination of two proteins. In particular, much information has been gathered on the pol-α/T-antigen interaction. This association appears complex, possibly involving three of the four pol-α subunits (A, B, and D) (Dornreiter et al. 1990, 1992; Gannon and Lane 1990; Collins et al. 1993; Bruckner et al. 1995).

It is very likely that the molecular interactions among initiation proteins are important determinants of efficiency and specificity in the initiation reaction. Unwinding of the SV40 origin requires T antigen and RP-A, and contact between these two proteins is likely to be important at this early stage of replication. Although the isolated large subunit of RP-A does not interact with T antigen, intact RP-A does (Dornreiter et al. 1992), suggesting that one or both of the smaller subunits could play a role in this interaction. Once the duplex is unwound, interaction between T antigen and pol-α may contribute to efficient priming on the RP-A-coated, single-stranded DNA template. It is possible that the different in-

teraction domains on pol-α have different functional roles during replication. For example, the D subunit may help to coordinate initiation at the origin (Schneider et al. 1994), and the A and B subunits may be important for tethering pol-α:primase to the advancing helicase (see below). Priming is also likely to be influenced by an RP-A/primase interaction, which has been demonstrated to occur in vitro through the large subunit of RP-A (Dornreiter et al. 1992). The close association of the three proteins involved in initiation of SV40 DNA replication is quite striking and suggests that similar interactions may occur at cellular origins of replication. Confirmation of this possibility awaits identification of the cellular counterpart(s) of T antigen.

Protein-protein interactions are also critically important during elongation of the nascent DNA chains. As discussed above, efficient elongation requires a highly processive polymerase, and it is likely that pol-δ (or pol-ε), with its auxiliary factors RF-C and PCNA, carries out this function on both leading and lagging strands. The processivity of pol-δ is absolutely dependent on interaction with PCNA, which is topologically linked to the template DNA. There is no evidence for interaction between the processive polymerases and other replication proteins, although RP-A does stimulate pol-δ activity to some extent (Kenny et al. 1989).

Recent data suggest the possibility that protein-protein interactions may even be important during the final stage of DNA replication, when the Okazaki fragments are joined to form a continuous DNA chain. Studies with purified proteins have shown that DNA ligase I, but not DNA ligase III, can catalyze the formation of covalently closed daughter DNA molecules in the SV40 model system (Waga et al. 1994). Since both ligases are capable of sealing nicks in double-stranded DNA, the strict requirement for DNA ligase I may reflect specific contacts between the enzyme and other proteins involved in the maturation of nascent strands.

A number of interesting interactions between replication proteins and other cellular factors have been uncovered. Although the functions of these interactions are not entirely clear, some of these may be involved in regulating replication. RP-A interacts with several proteins, including the transcription factors p53, GAL4, and VP16 (He et al. 1993; Li and Botchan 1993) and the repair proteins XPA and XPG (He et al. 1995; Matsuda et al. 1995). Another example, discovered with the SV40 model system, is the coupling of chromatin assembly to DNA replication (Stillman 1986). This coupling is strongly suggestive of a direct interaction between the chromatin assembly apparatus and the replication ma-

chinery and probably evolved to ensure rapid and efficient packaging of the progeny DNA following synthesis.

Synthesis of Okazaki Fragments and Cycling of Pol-α:Primase

Because synthesis of the lagging strand proceeds in the direction opposite to fork movement, repeated priming events by the pol-α:primase are required. There are two general mechanisms by which this priming could occur. One possibility is that pol-α:primase completely dissociates from the template following completion of each RNA/DNA primer (distributive mechanism). In this scenario, synthesis of each RNA/DNA primer would involve association of a different molecule of pol-α:primase with the template. Alternatively, pol-α:primase might be tethered to the replication complex at the fork via specific protein-protein interactions. In this case, the enzyme would not leave the domain of the template following the completion of an RNA/DNA primer but would immediately reassociate with the template at a new site to initiate primer synthesis (processive mechanism).

At this point, the available data are not sufficient to choose between the two mechanisms. Evidence supporting a distributive model has come from dilution experiments in the cell-free SV40 DNA replication system (Murakami and Hurwitz 1993b). When the pol-α:primase concentration is decreased by dilution, the rate of DNA replication decreases proportionately. Although these data clearly indicate that pol-α:primase dissociates from the template at a measurable rate during DNA synthesis, it has not yet been possible to directly measure the number of priming events mediated by a given pol-α:primase prior to dissociation. Evidence for processive priming has been provided by the strong physical interaction between pol-α and T antigen (Collins et al. 1993). It is known that T antigen is capable of unwinding long segments of DNA without dissociation. Binding of pol-α:primase to T antigen could significantly increase the lifetime of pol-α:primase at the fork. Thus, a single pol-α:primase molecule could proceed through several priming cycles, allowing efficient synthesis of the lagging strand. In agreement with this model, the presence of T antigen increases the apparent processivity of pol-α:primase on model templates (Collins and Kelly 1991).

In addition to possibly increasing processivity of priming, interaction between pol-α:primase and T antigen may facilitate access of pol-α:primase to the template during replication. Experiments with model templates have shown that RP-A-coated single-stranded DNA is resistant to priming. In the presence of T antigen, this inhibition is relieved (Col-

lins et al. 1993; Melendy and Stillman 1993). Therefore, interaction of pol-α:primase with T antigen may be required for primase to productively interact with the template DNA. During cellular DNA replication, a similar mechanism must be employed to allow efficient priming of the RP-A-coated DNA. It is possible that the cellular process is also mediated by a direct physical interaction between pol-α:primase and the replicative helicase.

Proofreading Mechanisms

The catalytic subunits of polymerases δ and ε contain 3′ to 5′ exonuclease activities, allowing high-fidelity DNA synthesis. Therefore, the majority of nuclear DNA synthesis employs the conventional proofreading mechanism first outlined in prokaryotic systems. However, pol-α:primase contains no obvious proofreading activity. It has been reported that a cryptic 3′ to 5′ exonuclease is uncovered when the *Drosophila* pol-α:primase complex is dissociated (Cotterill et al. 1987), but this phenomenon has not been observed with pol-α:primase complexes from other species. It is not clear how errors are corrected in DNA synthesized by pol-α:primase. One possibility is that a separate exonuclease proofreading activity exists. However, it is more likely that maturation of lagging-strand DNA fragments leads to removal of mismatches downstream from the RNA primer in pol-α-catalyzed regions. Prior to ligation of the nicked strand, FEN-1 nuclease and a proofreading polymerase could act in conjunction to accurately replace the DNA originally polymerized by pol-α. Since FEN-1 does not function on a gapped template but requires a nick, removal of DNA and resynthesis would have to occur concurrently. Alternating nucleolytic and synthetic steps by these two enzymes would closely resemble the "nick translation" reaction of *E. coli* DNA polymerase I (Murante et al. 1994).

Replication Centers

An interesting recent development has been the discovery that DNA replication may occur in relatively discrete foci in the nuclei of mammalian cells. When nascent DNA is labeled by exposure of cells to a short pulse of BrdU, the label is localized to a relatively small number of intranuclear sites. Enumeration of these sites or "replication centers" suggests that each may contain as many as 100 replication forks. Thus, DNA replication may be highly compartmentalized in eukaryotic cells (for review, see Cook 1991). Both PCNA and RP-A have been detected at these replication centers by immunofluorescence (Bravo and Macdonald-

Bravo 1987; Cardoso et al. 1993; Brenot-Bosc et al. 1995). Interestingly, the large subunit of RP-A has been detected at foci prior to the onset of replication and is a possible component of a prereplication complex at the origin (Adachi and Laemmli 1992).

The organization of replication centers is not well understood. One hypothesis invokes the nuclear matrix as an insoluble support that serves as a foundation for DNA replication and organizes the replication centers. However, a clear understanding of the structure and function of foci will have to await further biochemical investigation.

Control of Eukaryotic DNA Replication—Unanswered Questions

The basic enzymology of the eukaryotic replication fork has been uncovered through the identification and characterization of the replication proteins required for DNA synthesis in vitro. However, many fundamental questions remain regarding the control of replication in vivo. These problems focus on the coordination of DNA replication with other events in the cell cycle, including the timing of initiation, the prevention of multiple rounds of replication during a single replicative phase of the cell cycle, and the response to insults that may compromise the faithful duplication of the genetic material. Much of the current research on eukaryotic DNA replication is directed at characterizing these mechanisms.

Initiator proteins, which recognize origins of replication and unwind the double-stranded DNA, are likely to be central to the regulation of cellular DNA replication. The presence or the activation of these proteins may be responsible for controlling the timing of initiation during the cell cycle, and their subsequent removal or inactivation may be necessary to prevent re-replication during a single S phase. Identification of eukaryotic initiator proteins has proven to be quite difficult. However, a complex of six polypeptides that specifically recognizes yeast origin sequences has been purified recently (Bell and Stillman 1992). This origin recognition complex (ORC) is likely to function as an initiator, and thorough characterization of the protein should provide insight into the mechanisms that operate to regulate initiation. Studies have already shown that ORC protein level is constant through the cell cycle (Diffley and Cocker 1992). This observation suggests that the initiator protein is activated at the G_1/S transition, possibly by protein phosphorylation or by specific interactions with other cellular proteins.

Studies with the cell-free SV40 system have provided some evidence that protein phosphorylation may be involved in replication control.

SV40 DNA replication does not occur until the G_1/S transition has been reached in the host cell. Interestingly, extracts prepared from cells in G_1 are incompetent for in vitro SV40 DNA replication unless supplemented with protein phosphatase 2Ac (PP2Ac) or cdc2 kinase (Virshup et al. 1989; D'Urso et al. 1990). Although the key target of these G_1-activating enzymes is not yet clear, T antigen is one possible candidate. Both PP2Ac and cdc2 kinase can directly modify the phosphorylation state of T antigen in vitro, thereby regulating its ability to unwind the SV40 origin. It is possible that cellular initiator proteins may be activated at the G_1/S boundary by similar phosphorylation and/or dephosphorylation events.

Two cellular proteins involved in the initiation of SV40 DNA replication in vitro, pol-α:primase and RP-A, are phosphorylated in cell-cycle-dependent manners (Din et al. 1990; Nasheuer et al. 1991). There has been no clear demonstration that phosphorylation affects the replicative function of either protein, but this remains a reasonable possibility. It has also been suggested that RP-A phosphorylation might be involved in a signaling pathway that coordinates DNA replication with the cell cycle (Brush et al. 1994). In this case, RP-A would have both a replicative and a regulatory role. Such bifunctionality has recently been demonstrated with pol-ε, which appears to act as a replicative polymerase and as a member of the S-phase checkpoint pathway (Navas et al. 1995). This finding may help to explain the need for two rather redundant DNA polymerase activities in eukaryotic cells.

Observations such as those described above have provided some clues into the mechanism of DNA replication control, but it is clear that the majority of the process is not well understood. The characterization of the pathways and their components that allow communication between the replication machine and other cellular apparati will rely on both the biochemistry of the viral model systems and the genetics afforded by yeast. Since the basic mechanism of DNA replication has been conserved from yeast to humans, many of the regulatory mechanisms are likely to be conserved as well. As a result, these systems will continue to be used interchangeably to provide us with an understanding of how the cell efficiently and faithfully replicates its DNA in preparation for cell division.

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