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DNA Polymerases that Propagate the Eukaryotic DNA Replication Fork

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ABSTRACT Three DNA polymerases are thought to function at the eukaryotic DNA replication fork. Currently, a coherent model has been derived for the composition and activities of the lagging strand machinery. RNA-DNA primers are initiated by DNA polymerase α-primase. Loading of the proliferating cell nuclear antigen, PCNA, dissociates DNA polymerase α and recruits DNA polymerase δ and the flap endonuclease FEN1 for elongation and in preparation for its requirement during maturation, respectively. Nick translation by the strand displacement action of DNA polymerase δ, coupled with the nuclease action of FEN1, results in processive RNA degradation until a proper DNA nick is reached for closure by DNA ligase I. In the event of excessive strand displacement synthesis, other factors, such as the Dna2 nuclease/helicase, are required to trim excess flaps. Paradoxically, the composition and activity of the much simpler leading strand machinery has not been clearly established. The burden of evidence suggests that DNA polymerase ε normally replicates this strand, but under conditions of dysfunction, DNA polymerase δ may substitute.

KEYWORDS replication fork, DNA polymerase, DNA replication, Okazaki fragment, nuclease, PCNA, FEN1

INTRODUCTION

In eukaryotic cells, replication initiates at many origins, each one of which needs to assemble a replication apparatus that will completely replicate its portion of the chromosome with high fidelity. Fork assembly in eukaryotic cells proceeds along pathways that are basically conserved from yeast to mammalian cells. Insights in the elongation phase of DNA replication and the architecture of the replication fork have mainly derived from *in vitro* replication studies of SV40 viral DNA, from biochemical analysis of replication factors in general, and from genetic analyses in the two yeasts, *Schizosaccharomyces cerevisiae* and *S. pombe*. In this review, we will focus on the role of DNA polymerases α (Pol α), Pol δ, and Pol ε during the elongation of DNA replication. We will briefly describe the assembly of the initiation factors at replication origins, with an emphasis on those factors that may be important in loading of the DNA polymerases. The catalytic activities of each of these enzymes at the leading or at the lagging strand will be described in more detail. Particular emphasis will be given to recent progress in understanding the initiation, elongation, and completion of lagging strand DNA synthesis, one of the most complex DNA metabolic processes during movement of the replication fork.

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BIOGENESIS OF THE DNA REPLICATION FORK

In the yeast *S. cerevisiae*, the origin recognition complex (ORC) is central to the initiation of DNA replication, as it specifically recognizes yeast replication origins (Bell & Stillman, 1992). Beyond this indispensable initial binding event, origin activation is regulated by the binding of many other factors, as well as by posttranslational protein modification events. In recent years, evidence has suggested that these factors are likely present in all eukaryotic organisms, including *S. pombe*, *Drosophila*, *Xenopus*, and humans, attesting to the commonality of replication initiation pathways in eukaryotes. Our current understanding of the initiation of DNA replication and its control derives to a large extent from biochemical and genetic studies in both yeasts. Comparable initiation control mechanisms have emerged from *in vitro* DNA replication studies using *Xenopus* extracts.

The temporal order in which initiation factors are loaded onto chromatin has been delineated in several organisms. In studies using *Xenopus laevis* egg extracts, an effective technique has been to remove a specific factor from extracts by immunodepletion, in order to assay the role of this factor in the association of other factors of interest with chromatin. In *S. cerevisiae* and in *S. pombe*, an anologous strategy involving the use of temperature-sensitive mutants has been informative. For an in-depth discussion of initiation and its control, the reader is referred to recent reviews (Bell & Dutta, 2002; Kearsey & Cotterill, 2003). The tentative hierarchical scheme resulting from these studies is indicated by the folllowing *S. cerevisiae* proteins and complexes:

ORC → Cdc6, Cdt1 → Mcm2-7 → Cdc7/Dbf4 → Mcm10, Dpb11/Sld2, Cdc45/Sld3, GINS, Pol ε → RPA, Pol α-primase → PCNA, RFC → Pol δ

Although it is well established that both Cdc6 and Cdt1 are required for loading the putative helicase Mcm2-7 onto origin chromatin, there is less certainty about the factors following this step. Mcm10 appears to be involved in loading Cdc45 in *S. cerevisiae* (Sawyer *et al.*, 2004). At this point, the loading of a large number of factors appears to be interdependent. The *DPB11* gene (*Cut5* in *S. pombe*) is of particular interest, as it provides the most origin-proximal link to a DNA polymerase, Pol ε (Masumoto *et al.*, 2002). The association of Pol ε with origin chromatin is interdependent on the presence of three other complexes: Sld3/Cdc45, the GINS complex, and Dpb11/Sld2 (Takayama *et al.*, 2003). Protein-protein interaction studies indicate that the Mcm10 protein and the Cdc45 complex are primarily responsible for chromatin loading and retention of Pol α/primase (Mimura *et al.*, 2000; Zou & Stillman, 2000; Uchiyama *et al.*, 2001; Ricke & Bielinsky, 2004). Interestingly, Pol α-primase is not loaded onto chromatin in a temperature-sensitive dpb11-1 mutant at the restrictive temperature, suggesting its entry after Pol ε (Masumoto *et al.*, 2000). An intriguing aspect of this proposed scheme is that Pol ε is loaded onto origin chromatin, even before a primer is available to which it can bind and elongate. It is possible that this initial loading is more relevant for the checkpoint functions of Dpb11-Pol ε than for the actual mechanics of DNA synthesis (Araki *et al.*, 1995; Navas *et al.*, 1995). A double-stranded DNA binding domain in the catalytic polypeptide of Pol ε may be involved in chromation association of this enzyme prior to primer formation (Tsubota *et al.*, 2003). Finally, loading of Pol δ may only occur as one of the last steps in replication fork biogenesis, and then only after loading of PCNA by RFC, presumably at a step after primer synthesis by Pol α-primase.

The proposed structure of the replication fork in Figure 1 can only be considered the most common form. As we will discuss later, alterations to this proposed fork structure can be tolerated in certain mutants. The existence of altered fork structures in mutants poses the question of whether these structures may also

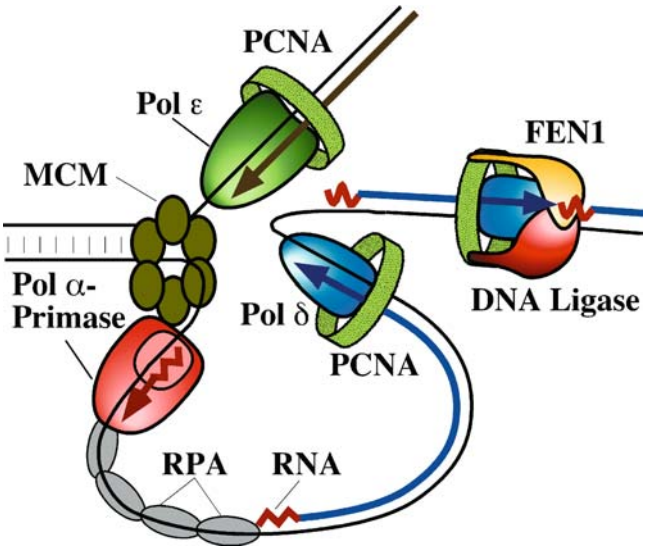

FIGURE 1 **Eukaryotic DNA replication fork. The minimal set of proteins for fork propagation are indicated.**

occur in wild-type cells under specialized conditions, *e.g.*, when structural blocks or DNA damage impede progression of the regular replication fork.

DNA POLYMERASES

The three DNA polymerases responsible for fork propagation all belong to the B class of DNA polymerases (Burgers *et al.*, 2001). Structural information about this class of enzymes derives from distantly related cousins, *i.e.*, from bacteriophage RB69 and from thermophiles (Hopfner *et al.*, 1999; Zhao *et al.*, 1999; Rodriguez *et al.*, 2000; Franklin *et al.*, 2001; Hashimoto *et al.*, 2001). The crystal structures of these enzymes show a remarkable difference from class A DNA polymerases, for which *E. coli* DNA polymerase I, Klenow fragment, forms the prototype (Derbyshire *et al.*, 1988). The highest degree of structural conservation between these two classes of enzymes localizes to the palm subdomain of the polymerase domain, which contains the residues important for polymerase catalysis (Figure 2). There is much less structural and sequence conservation in the thumb and fingers subdomains. Despite this divergence at the structural level, however, the fingers domains show a high degree of functional conservation. The incoming dNTP binds to the opened fingers domain through interactions with a conserved group of positively charged interactions on a fingers helix. Binding of this nucleotide is followed by a conformational change that is associated with a large rotation of the fingers domain to form a closed complex competent for catalysis (Doublie *et al.*, 1998; Li *et al.*, 1998; Franklin *et al.*, 2001). A comparison of the closed ternary complexes of bacteriophage T7 DNA polymerase (A class) and bacteriophage RB69 DNA polymerase (B class) clearly shows the conservation of the palm domain and the active site arrangement but, beyond this, also highlights large differences between these two classes (Doublie *et al.*, 1998; Franklin *et al.*, 2001). Particularly, the arrangement of the exonuclease domain is radically different. Whereas in the A class enzymes, the exonuclease domain projects from the bottom of the palm subdomain, this domain projects from the top of the fingers domain in the B-class enzymes.

This arrangment has distinct consequences for proofreading and for binding of the single-stranded template DNA. Upon nucleotide misincorporation, the path that the mismatched primer terminus must traverse to reach the exonuclease domain is across and down the surface of the palm domain for an A-type enzyme, but up along the tip of the fingers domain for a B-type enzyme (Figure 2). In both types of enzymes, the single-stranded template nucleotide adjacent to the template nucleotide positioned in the active site, makes a 90° turn. This sharp turn positions solely the template base in the active site for base-pairing interaction with the incoming dNTP. However, the environment of the rest of the single-stranded DNA template is distinctly different. The template strand enters the active site of an A-type enzyme from the fingers domain, but in the

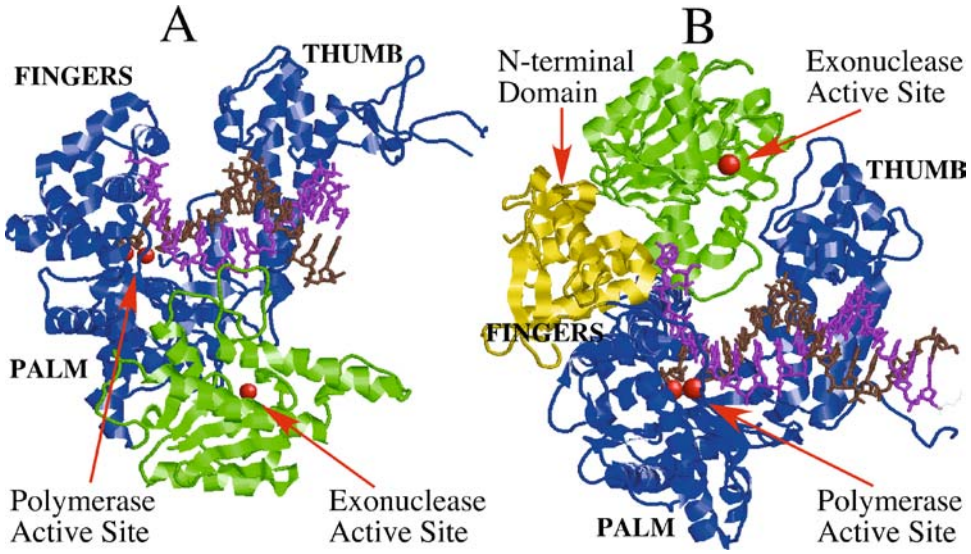

FIGURE 2 **Structural comparison of class A and B DNA polymerases. Bacteriophage T7 DNA polymerase, lacking thioredoxin, is compared to bacteriophage RB69 DNA polymerase. Both enzymes are in a closed complex with a dideoxy-terminated primer-template DNA and an incoming base-paired dNTP. The polymerase active sites are in the same relative orientation. Coordinates are from (Doublie *et al.*, 1998; Franklin *et al.*, 2001).**

B-class enzyme, the template strand projects into a positively charged cleft between the N-terminal domain and the exonuclease domain. These distinct structural differences may have emerged to allow an optimal interaction of these enzymes with specialized processivity factors and other cofactors. Most B-type enzymes function with a circular clamp as processivity factor, whereas A-class enzymes normally function without a processivity factor, processivity through binding of thioredoxin being an exception (Bedford *et al.*, 1997).

DNA Polymerase α-Primase

This DNA polymerase has the unique ability to initiate DNA replication in eukaryotic cells because it couples the primase and DNA polymerase activities in the same four-subunit complex. The subunit structure of this heterotetrameric enzyme is conserved in all organisms and has been firmly established for many years (Figure 3) (reviewed in Hubscher *et al.*, 2002; Muzi-Falconi *et al.*, 2003). The largest subunit (Pol1) contains the DNA polymerase activity, but lacks exonuclease activity, despite the presence of an exonuclease domain, which is likely maintained for structural purposes. The Pri1 subunit (p48) catalyzes formation of the short RNA primers utilized for elongation by Pol α. The remaining two subunits, the B subunit (Pol12, p79) and Pri2 (p58) play a role in stabilizing and regulating the catalytic subunits, and are found tightly associated with the polymerase and primase subunit, respectively.

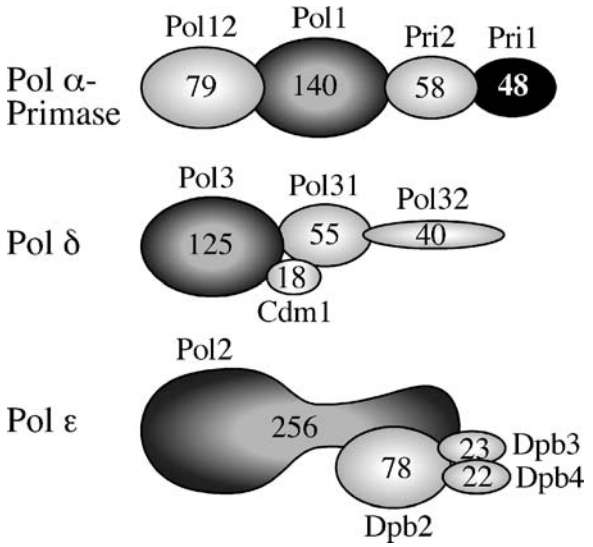

FIGURE 3 **Subunit interactions in DNA polymerases. Subunit interactions are summarized as reviewed in detail in (MacNeill *et al.*, 2001; Muzi-Falconi *et al.*, 2003; Pospiech and Syvaoja, 2003). Sizes and names of the subunits are from *S. cerevisiae*, except for Cdm1 (*S. pombe*), which subunit is not found in *S. cerevisiae*. The polymerase subunits are shaded in dark and the primase subunit Pri1) of Pol α in black. The third subunit Pol32) of Pol δ is extremely elongated in shape, and the catalytic subunit of Pol ε Pol 2) is a two-domain polypeptide, interactions with the other subunits being localized to the C-terminal domain. See text for details and references.**

The DNA primase activity in Pol α/primase is the only activity known to prime DNA replication in eukaryotes (reviewed in Arezi & Kuchta, 2000; Frick & Richardson, 2001). The primase binds the single-stranded DNA template and catalyzes primer formation. The final size of the RNA primer is determined by the length of the oligoribonucleotide that fits in the primase initiation groove. For the eukaryotic primases, this size varies from 8 to 12 nucleotides. Although the primase accessory subunit does not contain catalytic activity, its presence is important for primase stability, for the efficiency of initiation, and for primer length determination (Santocanale *et al.*, 1993; Zerbe & Kuchta, 2002). This subunit also may mediate transfer of the nascent RNA primer terminus to the polymerase subunit (Arezi *et al.*, 1999). Following the synthesis of the RNA primer, the Pol1 subunit of Pol α extends the primer by approximately 20 nucleotides, from which lagging strand DNA replication continues.

As Pol α is the true initiator of DNA replication, it is not surprising that its activity is tightly regulated by post-translational modification and by interactions with many other proteins, from proteins involved in chromatin remodeling to replication initiation and elongation. Interactions of Pol α have been mapped to many initiation proteins, including Mcm10 and Cdc45, both of which have been shown to play critical roles in the initation of DNA replication (Bell & Dutta, 2002; Fien *et al.*, 2004; Ricke & Bielinsky, 2004). Further cell-cycle–dependent regulation of Pol α function is accomplished through phosphorylation of the B subunit. The C-terminus of this subunit is phosphorylated by Cdk2/cyclin A (Cdc28/Clb in yeast) during the S and G2 phases, and it has been speculated that this phosphorylated form is involved in ongoing lagging strand replication, whereas the hypophosphorylated form may play a role in accurate initiation of DNA replication (Nasheuer *et al.*, 1991; Desdouets *et al.*, 1998; Muzi-Falconi *et al.*, 2003).

DNA Polymerase δ

Pol δ is the lagging strand DNA polymerase and, as discussed below, has evolved to deal efficiently with the

recurring problem of Okazaki fragment maturation. Pol δ from *S. cerevisiae* has three subunits of 125 (Pol3), 55 (Pol31/Hys2), and 40 kDa (Pol32) (Gerik *et al.*, 1998). The enzymes from *S. pombe* and humans have an additional small fourth subunit that functions to stabilize the complex (Figure 3) (Zuo *et al.*, 1997; Liu *et al.*, 2000; Podust *et al.*, 2002). The enzymes from the three different sources show roughly similar structure-function characteristics (reviewed in MacNeill *et al.*, 2001). The catalytic and the second subunit form a stable complex, to which the third subunit is tethered solely via interactions with the second subunit (MacNeill *et al.*, 1996; Gerik *et al.*, 1998). The third subunit of Pol δ is extremely elongated in shape, which prompted early speculations that forms of Pol δ containing this subunit might form higher-ordered structures (Burgers & Gerik, 1998; Mo *et al.*, 2000; Zuo *et al.*, 2000). However, further biophysical studies showed that the complex contains one of each of the subunits, *i.e.*, it is a monomeric catalytic complex (Johansson *et al.*, 2001; Bermudez *et al.*, 2002).

The *S. cerevisiae POL32* gene for the third subunit is dispensible for growth, although deletion mutants show poor growth, are sensitive to replication inhibitors and DNA damage, are defective for mutagenesis, and show synthetic lethality with a host of other genes that function in DNA metabolism (Gerik *et al.*, 1998; Huang *et al.*, 2002; Tong *et al.*, 2004). The orthologous *S. pombe Cdc27* gene is essential for growth (Hughes *et al.*, 1992; Bermudez *et al.*, 2002).

PCNA as Accessory Factor for Pol δ

Two-subunit forms of Pol δ, lacking the Pol32 subunit, and here designated Pol3/Pol31, have been isolated and studied in some detail. In fact, the first form of Pol δ isolated was the two-subunit form from calf thymus, and until a few years ago, all studies of mammalian Pol δ were carried out with this two-subunit form (Lee *et al.*, 1984; Sun *et al.*, 1997; Zhou *et al.*, 1997). Furthermore, the processivity clamp PCNA was first discovered as an auxiliary factor for the two-subunit Pol δ (Tan *et al.*, 1986; Prelich *et al.*, 1987).

The various subassemblies of Pol δ have been employed to identify both physical and functional interaction with PCNA. Although it has been firmly established that multiple interactions exist between PCNA and the subunits of Pol δ, both their identity and function have largely remained elusive, in part because some interactions with PCNA are only manifested when PCNA encircles the DNA (Figure 4). In the absence of DNA, direct interactions between PCNA and Pol3/Pol31 are negligible, whereas they are very strong when PCNA encircles the DNA. Interactions, if detected have been very weak, and considerable disagreement about their relevance exists among the investigators who have worked on this problem (Eissenberg *et al.*, 1997; Tratner *et al.*, 1997; Gerik *et al.*, 1998; Hughes *et al.*, 1999; Zhang *et al.*, 1999; Shikata *et al.*, 2001; Lu *et al.*, 2002). On the other hand, the observation that DNA replication by this two-subunit Pol δ is stimulated by PCNA is indicative of a functional interaction on the DNA (Tan *et al.*, 1986; Zhang *et al.*, 1995; Burgers and Gerik, 1998). Indeed, stable mammalian DNA · PCNA · Pol3/Pol31 complexes have been isolated (McConnell *et al.*, 1996; Mozzherin *et al.*, 1999). Therefore, loading of PCNA onto DNA appears to reveal a binding domain for Pol3/Pol31 that previously had been inaccessible (Figure 4).

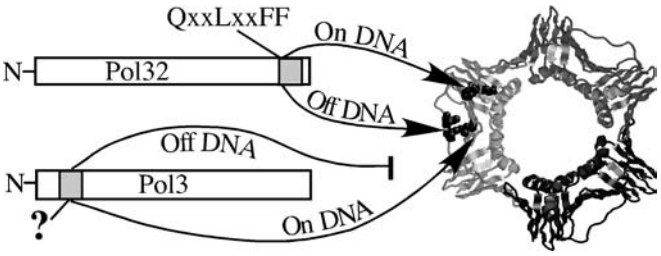

FIGURE 4 **DNA-dependent interactions between Pol δ and PCNA. Interactions between Pol32 and PCNA reposition from the interdomain connector loop region of PCNA in the absence of DNA (Off DNA) to the C-terminus when PCNA encircles the DNA (On DNA). Interactions of PCNA with Pol3 require that PCNA encircles the DNA. Pol31 (not shown) may also contribute to PCNA binding.**

In contrast, the Pol32 subunit has at its carboxy-terminus a consensus PCNA-binding domain **QxxLxxFF**, like in the Cdk inhibitor p21 and FEN1 (reviewed in Warbrick, 2000; Majka & Burgers, 2004). Similarly to FEN1, binding of Pol32 off the DNA is directed to the interdomain connector loop of PCNA, and binding on the DNA to the carboxy-terminus of PCNA (Figure 4) (Gomes & Burgers, 2000; Johansson *et al.*, 2004). Consequently, binding of the complete Pol δ assembly to PCNA off the DNA is largely determined by the C-terminus of Pol32 (Bermudez *et al.*, 2002; Johansson *et al.*, 2004). Finally, the importance of the PCNA-binding domain in Pol32 is unclear. *In vitro*, the contribution of this domain to processivity by Pol δ is minor, and only uncovered under unfavorable replication conditions. *In vivo*, deletion of this domain

in *S. pombe* leads to growth defects while in *S. cerevisiae* a similar deletion merely affects the efficiency of DNA damage-induced mutagenesis (Bermudez *et al.*, 2002; Johansson *et al.*, 2004).

DNA Polymerase ε

Of all three DNA polymerases proposed to act at the replication fork, Pol ε is the most enigmatic, and the most reluctant to release pertinent and clear information about its role in replication fork propagation. Identified many years ago as a proofreading DNA polymerase in yeast, it was first isolated as a multipolypeptide complex by Sugino and coworkers in 1990 (Wintersberger & Wintersberger, 1970; Morrison *et al.*, 1990). Most progress has been made with the enzyme from *S. cerevisiae.* The four-subunit enzyme has been overproduced in baculovirus and in yeast (Dua *et al.*, 2002; Chilkova *et al.*, 2003). Biophysical studies show it to be a heterotetramer of the Pol2 (256 kDa), Dpb2 (78 kDa), Dpb3 (23 kDa), and Dpb4 (22 kDa) subunits (Figure 3) (Chilkova *et al.*, 2003). Because the small subunits have also been identified in other organisms, and both biochemical and genetic interactions have been identified between these small subunits and the catalytic subunit, it is likely that Pol ε is also at least a four-subunit enzyme in other organisms (reviewed in Pospiech & Syvaoja, 2003).

Genetic analyses of the four-subunit genes provide a complex picture of the role of Pol ε in DNA replication. While the *DPB2* gene is essential in both yeasts, the *S. cerevisiae DPB3* and *DPB4* genes are both non-essential for growth, but their phenotypes indicate that they provide a stabilizing function to the Pol ε core (Araki *et al.*, 1991a; Araki *et al.*, 1991b; Ohya *et al.*, 2000; Feng *et al.*, 2003). The *S. pombe dpb3* gene is essential for growth while the *dpb4* gene is dispensible. *S. pombe dpb3* depletion studies and synthetic lethality studies with *dpb4* indicate functions for these genes in replication initiation, S phase progression, and during late stages of replication and cell separation (Spiga & D'Urso, 2004).

Genetic studies of the catalytic subunit are much more confounding. Although *POL2* is an essential gene and mutations in the active site of the polymerase domain confer lethality, the entire catalytic polymerase domain of Pol2 is dispensable in both yeasts (see below for a discussion) (Dua *et al.*, 1999; Kesti *et al.*, 1999; Feng and D'Urso, 2001; Pavlov *et al.*, 2001). However, these mutants have severe phenotypic defects in several aspects of the cell cycle including the progression of DNA replication (Ohya *et al.*, 2002). In contrast, the C-terminal domain of *POL2* is essential for growth. It does not contain polymerase motifs, but it does contain a zinc finger region that both is essential for growth and required for the S-phase checkpoint in *S. cerevisiae* (Navas *et al.*, 1995; Dua *et al.*, 1999, 2000). An attractive hypothesis is that the C-terminus of Pol ε participates as an essential component in the assembly of the replication complex at origins. This hypothesis is in agreement with the observation that Pol ε loads onto origin complexes prior to primer synthesis, *i.e.*, that a non-polymerase function of Pol ε is involved in assembly. In further support of this hypothesis is the identification of a double-stranded DNA binding domain in Pol ε (Tsubota *et al.*, 2003). Although this interpretation may seem logical just from the viewpoint of DNA replication, one caveat is that additional interactions of Pol ε, *e.g.*, with *TRF4* during the establishment of sister chromatid cohesion, may color the overall genetic portrait in unexpected ways (Edwards *et al.*, 2003).

PCNA Interaction with Pol ε

PCNA stimulates DNA synthesis by Pol ε (Hamatake *et al.*, 1990; Burgers, 1991; Podust *et al.*, 1992; Dua *et al.*, 2002). Because Pol ε is a highly processive enzyme by itself, the observed stimulation by PCNA was generally not very large. Interestingly, stimulation of processivity by PCNA was observed both for the four-subunit form of Pol ε and for a commonly encountered ~140 kDa monomeric form of Pol ε generated by proteolysis during purification (Burgers, 1991; Dua *et al.*, 2002). A putative PCNA-binding site localizes to aa 1193 to 1200 of Pol2, which is retained in the 140 kDa proteolytic fragment (Maki *et al.*, 1998). Deletion of the consensus PCNA-binding motif in the catalytic subunit conferred essentially no growth defects, but strong damage sensitivity (Dua *et al.*, 2002). This result indicates that the phenotype of a PCNA-interaction deletion mutant in *POL2* is much less severe than that of the polymerase domain deletion mutant. However, whether additional PCNA-binding domains exist in Pol ε, like in Pol δ, still requires investigation.

ROLES OF POL δ AND POL ε IN CHROMOSOMAL DNA REPLICATION

At first glance, it would seem that the participation of Pol ε at the replication fork is infrequent at best. In

human fibroblast cells, Pol ε colocalizes with PCNA in replication foci only in late S phase, whereas in early S phase it localizes adjacent to PCNA foci, suggesting that in early S phase, Pol ε is not present in PCNA containing forks (Fuss & Linn, 2002). One explanation for this observation is that Pol ε-containing forks only assemble in late S phase in order to replicate heterochromatic DNA. However, an alternative explanation would retain Pol ε as the leading strand polymerase at all times, although the leading strands may not always contain PCNA. Pol ε is highly processive by itself, and its interaction with PCNA may only be required during DNA repair, or during late stages of DNA replication (Dua *et al.*, 2002). Chromatin immunoprecipitation studies in yeast have shown that Pol ε does travel with replication forks that are formed at early replicating origins (Aparicio *et al.*, 1997).

Further uncertainty regarding a regular role for Pol ε at the fork follows from the observed viability of yeast mutants containing deletions of the Pol ε polymerase domain. However, the observed lethality of *POL2* mutants with point mutants in the polymerase active site seems to suggest an alternative explanation (Dua *et al.*, 1999; Pavlov *et al.*, 2001). Possibly, the replisome is more flexible to changes than previously anticipated, and the polymerase domain of Pol ε may normally participate in DNA replication, but another polymerase can substitute in the absence of this domain. That replication forks with only Pol α and Pol δ can assemble and function under specialized conditions follows from the mechanism of replication of the SV40 genome, which does not require Pol ε *in vitro* nor *in vivo* (Zlotkin *et al.*, 1996; Waga and Stillman, 1998).

Several lines of evidence strongly indicate an involvement of Pol ε in DNA replication, in addition to Pol α and Pol δ. DNA replication in *Xenopus* extracts depleted for Pol δ or Pol ε resulted in a marked decrease in DNA synthesis (Fukui *et al.*, 2004). The products formed in the absence of Pol δ were most consistent with a defect in lagging strand DNA synthesis, suggesting that Pol ε may be the leading strand enzyme.

Further support comes from elegant genetic studies by Shcherbakova and Pavlov of 6-N-hydroxylaminopurine (HAP) induced mutagenesis in yeast cells deficient either for the exonuclease activity of Pol ε (*pol2exo*$^-$) or Pol δ (*pol3exo*$^-$) (Shcherbakova & Pavlov, 1996). HAP base-pairs with T and with C, leading to GC-AT and AT-GC transitions, depending on whether HAP is present as the incoming nucleotide or as a template nucleotide, respectively. As HAP mutagenesis is unaffected by mismatch repair, recombination, or postreplication repair, the mutations induced by HAP are a direct consequence of the misinsertion rate by the polymerase and its ability to proofread these misinsertions (Shcherbakova *et al.*, 1996). In a *pol2exo*$^-$ mutant, the frequencies of HAP-induced reversion of specific missense mutations in the *URA3* gene dramatically changed in magnitude when the orientation of this target was reversed with regard to the ARS306 replication origin on chromosome III. In contrast, in a *pol3exo*$^-$ mutant, a similar change in magnitude of reversion frequencies was observed upon target reversal, but exactly opposite to those in the *pol2exo*$^-$ mutant. Therefore, the exonuclease activities of Pol δ and Pol ε proofread opposite strands of the replication fork, and by extension Pol δ and Pol ε are proposed to replicate opposite strands of the fork. Analogous results were obtained when the mutator specificity of a tRNA gene was examined in *pol2exo*$^-$ and *pol3exo*$^-$ mutants with regard to its origin orientation (Karthikeyan *et al.*, 2000). These studies did not specifically address which strand is replicated by Pol δ and which by Pol ε.

Several lines of genetic evidence suggest that Pol α and Pol δ function in the initiation and elongation of Okazaki fragment synthesis, respectively. Strong evidence for the hypothesis that Pol δ is the lagging strand enzyme follows from a genetic analysis of telomere replication. The action of telomerase results in the formation of a single-stranded T-G rich strand. Its conversion back to double stranded DNA, a process which by nature represents lagging strand DNA replication, requires both functional Pol α and Pol δ (Diede and Gottschling, 1999).

Additional genetic support for Pol δ as the lagging strand enzyme comes from studies of *POL3 RAD27* double mutants. The *RAD27* gene encodes the FEN1 flap endonuclease that functions in initiator RNA degradation during Okazaki fragment maturation (Figure 5). Most *pol3-exo*$^-$*rad27* double mutants confer lethality [Jin, 2001 #1753; Jin, 2005 #2341]. However, a few double mutants with mild mutations in both genes are viable. The double mutants, but neither one of the single mutants, accumulate small duplications, consistent with a defect in Okazaki fragment maturation [Jin, 2001 #1753; Jin, 2005 #2341]. Therefore, Pol δ functions in the maturation of Okazaki fragments *in vivo*, and likely also during the elongation phase. In further support of this hypothesis, Pol32, the small subunit of

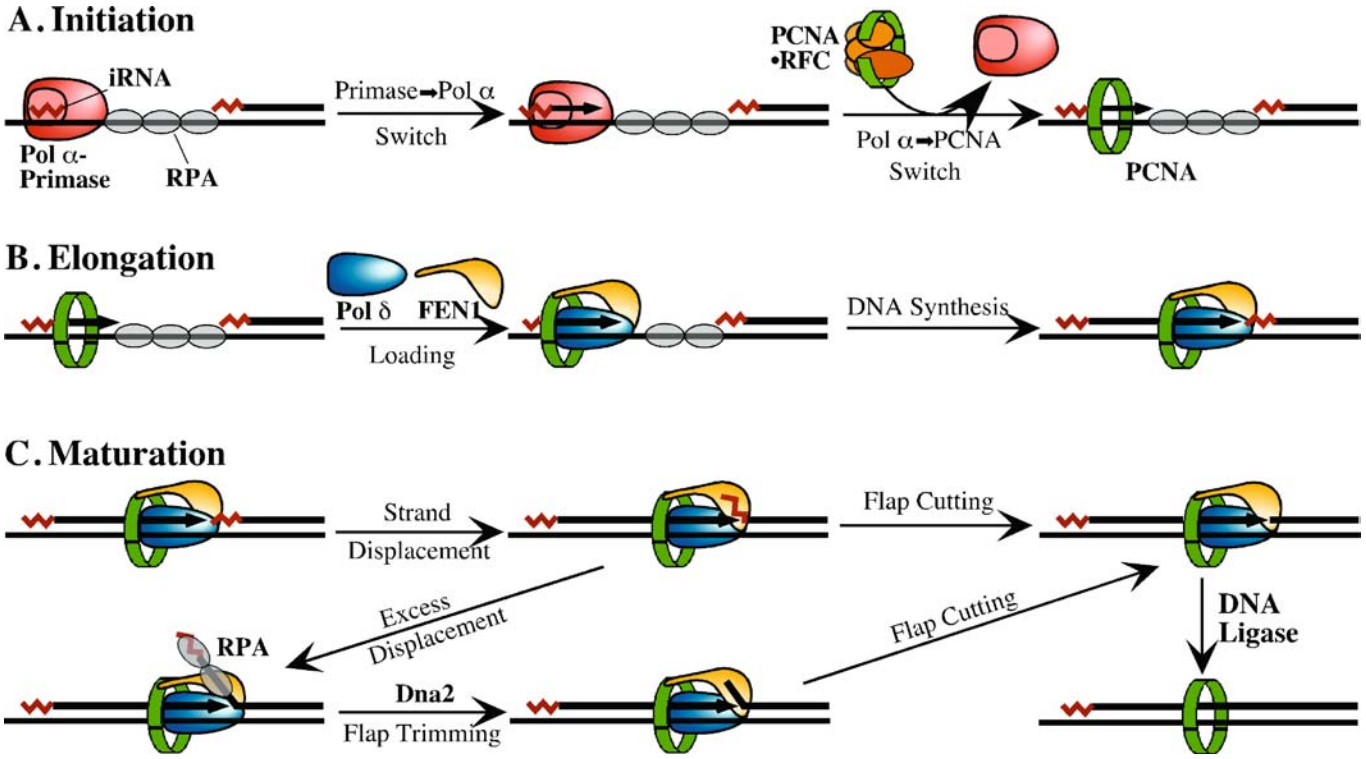

FIGURE 5 **Replication stages of the lagging strand. The Pol $\alpha \rightarrow$ PCNA switch promotes loading of Pol ε on the leading strand not shown), and Pol δ on the lagging strand. During elongation, FEN1 is proposed to be loaded together with Pol δ, but it is only activated upon encountering downstream DNA or RNA. In the model shown in Figure C, RPA binds to long flaps only, thus preventing cleavage by FEN1 and stimulating cleavage by Dna2. The trimmed flap then becomes a substrate for FEN1.**

Pol δ, has been shown to interact with Pol α, possibly linking the two enzymes together in the process of lagging strand DNA replication (Huang *et al.*, 1999; Johansson *et al.*, 2004). In conclusion, although definite proof that Pol ε is the leading strand DNA polymerase is still lacking, the burden of circumstantial evidence, mainly based on studies with Pol δ, supports this assertion.

LAGGING STRAND DNA REPLICATION MACHINERY

Lagging strand DNA replication can be thought to proceed in several discrete stages, *i.e.*, initiation by DNA primase, limited elongation of the RNA primer by Pol α, a switch of the primer terminus from Pol α to Pol δ, elongation by Pol δ, and maturation of the completed Okazaki fragment. Each transition is believed to be mediated by a specific protein or protein complex and has to occur with very high efficiency. In a mammalian cell, this process occurs 20 to 50 million times during every cell cycle, and even in a yeast cell with its very compact genome, about 100,000 Okazaki fragments need to be initiated, elongated, and matured in a single S phase.

If for yeast, one assumes a mean Okazaki fragment length of 150 nucleotides (nt) and an average rate of fork movement of 50 nt/sec, it follows that an Okazaki fragment needs to be initiated, elongated, and matured in a period of 3 sec (Raghuraman *et al.*, 2001). However, there are two reservations with this simple calculation. First, although Okazaki fragments in eukaryotes are generally assumed to be 100 to 200 nt in length, these estimates derive mainly from *in vitro* SV40 replication studies, and their exact size or range in yeast remain to be determined (Ishimi *et al.*, 1988; Tsurimoto *et al.*, 1990). Second, this calculation is only valid if one Okazaki fragment at the time is being synthesized on the lagging lagging strand, with the start of a new Okazaki fragment coupled to the maturation of the previous one. If, on the other hand, a more distributive mechanism is allowed with many Okazaki fragments being synthesized simultaneously, the rate per fragment could obviously be much slower. Such a distributive mode has been proposed to occur during SV40 DNA replication *in vivo* (Nethanel *et al.*, 1992). The abundance of Okazaki fragments isolated from several archaeabacterial organisms also suggests a distributive mode of Okazaki fragment synthesis in this kingdom

(Matsunaga *et al.*, 2003). However, an electronmicroscopic mapping study of yeast DNA replication forks revealed not only that the mean single-stranded DNA region on the lagging strand is only ~220 nt, but also that nucleosomes are assembled very close to the single-stranded region (Sogo *et al.*, 2002). The latter indicates that the DNA close to the single-stranded region is already fully replicated and ligated as chromatin assembly has occurred. Therefore, the number of Okazaki fragments being synthesized at a fork at any given time appears to be very limited, and if these fragments are of the size we presume them to be, it may just be one.

Given this limitation, *in vitro* measured rates of each of the steps in lagging strand DNA synthesis fall woefully short of the assumed *in vivo* rapidity of this process. Although polymerase extension rates are fast, 50 to 100 nt/sec for Pol α and Pol δ, average times required for primer synthesis by DNA primase are in the range of hundreds of seconds, and maturation of an Okazaki fragments require ten seconds or more (Frick & Richardson, 2001; Ayyagari *et al.*, 2003). Therefore, our discussion of lagging strand DNA replication is with the understanding that some factors that promote rapidity of this process are still lacking.

Initiation and the Pol α-Pol δ Switch

During the initiation of DNA replication, Pol α-primase alone is unable to initiate primer synthesis on RPA-coated single-stranded DNA, rather its recruitment by the MCM complex, Cdc45, and Mcm10 facilitates loading and the initiation of primer synthesis (Collins & Kelly, 1991; Melendy & Stillman, 1993). During the progression of replication on the lagging strand, these same factors may continue to interact with Pol α-primase and enable iterative primer synthesis (Aparicio *et al.*, 1997, 1999; Labib *et al.*, 2000; Ricke & Bielinsky, 2004; Sawyer *et al.*, 2004). Mcm10 may also stimulate the switch from primase to DNA synthesis by Pol α (Fien *et al.*, 2004).

The switch from Pol α to Pol δ has been proposed to be mediated by binding of replication factor C (RFC). In this model, binding of RFC to a replicating Pol α complex serves to abrogate primer synthesis at a length of approximately 30 nt (10 nt of RNA and 20 nt of DNA) and to dissociate Pol α-primase from the DNA (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991; Maga *et al.*, 2000; Mossi *et al.*, 2000). However, there are several reasons why it is more likely that in the cellular environment this switch is accomplished by a PCNA-RFC complex. First, in the cell, PCNA is present in large excess over RFC, and it is likely that all RFC is complexed in a stable ATP-driven PCNA-RFC complex (Gerik *et al.*, 1997; Gomes and Burgers, 2001). Second, DNA binding by RFC is not only transient, but the DNA-bound form of RFC is also unable to recruit PCNA and load it (Gomes *et al.*, 2001). Only a RFC–PCNA complex is capable of productively binding DNA and loading PCNA. Therefore, we envisage the inhibition and dissociation of Pol α-primase to be coupled to loading of PCNA as shown in Figure 5A.

Once the switch from Pol α to the PCNA-Pol δ machinery has been made, further elongation of an Okazaki fragment is very rapid. It is reasonable to assume that the PCNA-stabilized elongation complex not only contains Pol δ, but also FEN1, as shown in Figure 5B. Kinetic studies of Okazaki fragment maturation indicate the presence of a pre-existing PCNA-FEN1-Pol δ complex prior to the polymerase encountering a downstream Okazaki fragment (Ayyagari *et al.*, 2003; Garg *et al.*, 2004). Biochemical studies have shown the PCNA-Pol δ complex to be very processive, replicating at least 7 kb of DNA without dissociating (Burgers, 1991). It was this highly processive character of Pol δ, in fact much higher than that of the PCNA-Pol ε complex, that promoted initial suggestions that Pol δ would be better suited as a leading strand polymerase. Currently, we think that this may only occur under conditions of Pol ε dysfunction.

Okazaki Fragment Maturation

Maturation of Okazaki fragments needs to be carried out with extraordinary efficiency and fidelity. Any unligated nick or gap results in the formation of a double-stranded break during the next cell cycle. Considering that a yeast cell has the capacity to repair only about 30 double-stranded breaks, it follows that a 0.03% failure of ligation would result in lethality in a wild-type strain (Resnick & Martin, 1976). An even higher efficiency must be imposed for mammalian genomes, where Okazaki fragments are expected to be 100 to 1000-fold more numerous, but the number of double-stranded breaks tolerated is comparable (Resnick, 1978). The successful completion of Okazaki fragment maturation hinges on the exquisite coordination between Pol δ and FEN1 action in order to produce and maintain nicks that can be ligated by DNA ligase I. It is in

this process that critical biochemical differences are expressed between Pol δ and Pol ε that make Pol δ the ideal lagging strand enzyme. Pol δ shows a strong coordination with FEN1 for producing a ligatable nick, whereas Pol ε appears to lack this (Garg *et al.*, 2004). As a leading strand enzyme, it certainly would not need it.

When a replicating Pol δ complex runs into a double-stranded region, it displaces 2 to 3 nt of the downstream RNA or DNA (Figure 5C). Limited displacement by Pol δ is a reversible process. In the absence of FEN1, Pol δ degrades the newly replicated DNA using its 3′ to 5′ exonuclease activity, in a process referred to as idling. This reiterative process of extension, followed by degradation, limits strand displacement to only a few nucleotides and allows the polymerase to effectively maintain a ligatable nick (Figure 6) (Garg *et al.*, 2004). The reversible form of limited strand opening by Pol δ contrasts with its capacity to also carry out extended strand displacement synthesis. Although idling at a nick can maintain Pol δ at a nick for some time, eventually the enzyme will shift to an irreversible strand displacement synthesis mode during which extended regions of DNA are unwound (Maga *et al.*, 2001; Ayyagari *et al.*, 2003).

When FEN1 is present in the replicating complex that runs into the double-stranded region, efficient nick translation ensues, and idling is inhibited (Figure 6). Indicative of the extremely tight coupling between Pol δ and FEN1, mostly mononucleotides are released during nick translation (Garg *et al.*, 2004). Finally, with DNA ligase I also present, the nick translation process can be terminated by ligase action, as rapidly as a few nucleotides past the RNA-DNA junction of an Okazaki fragment (Ayyagari *et al.*, 2003). In this scheme, the participation of DNA ligase I deserves further attention, as in yeast maturation studies ligase did not appear to be a integral component of the maturation complex (Ayyagari *et al.*, 2003). Having a similar PCNA-binding domain as FEN1 and as the Pol32 subunit of Pol δ, one might expect that an appropriate domain on one PCNA monomer of the trimer might still be available for binding DNA ligase. Simultaneous binding of the polymerase, FEN1, and DNA ligase to individual monomers of a heterotrimeric PCNA has been proposed to function in lagging strand DNA replication in some archaea (Dionne *et al.*, 2003). Nevertheless, it appears that DNA ligase only transiently associates with the maturation complex (Ayyagari *et al.*, 2003).

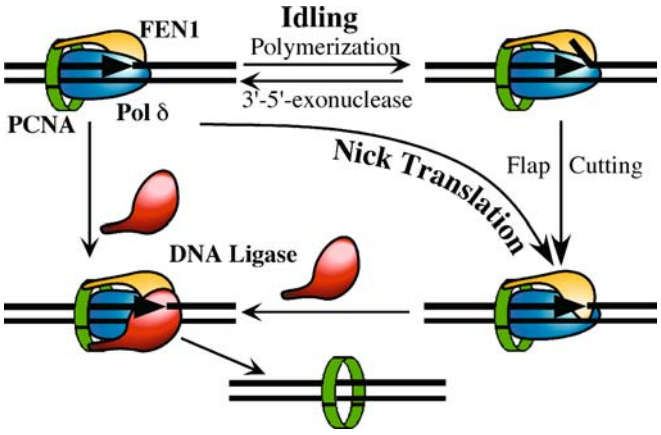

FIGURE 6 **Nick maintenance by polymerase idling or by nick translation. During Okazaki fragment maturation, Pol δ and FEN1 go through multiple cycles of displacement synthesis and FLAP cutting (nick translation) until all RNA has been degraded. In the absence of FEN1, idling predominates.**

Although this simple machinery appears to be highly efficient *in vitro*, it apparently is not sufficient *in vivo*. This follows from studies with the Dna2 nuclease/helicase. Genetic studies show that *DNA2* is an essential gene, and its essentiality likely derives from its function during lagging strand DNA replication (Budd *et al.*, 1995; Budd & Campbell, 1997). The nuclease provides the essential function of Dna2, consistent with a degradative role in Okazaki fragment maturation (Budd *et al.*, 2000; Lee *et al.*, 2000). In the model shown in Figure 5C, Dna2 is only proposed to act when extensive strand displacement synthesis by Pol δ causes binding by proteins, which inhibits access by FEN1. In biochemical studies, flaps of ~30 nt in length bind RPA, inhibit FEN1 action, and activate Dna2 action (Murante *et al.*, 1995; Bae *et al.*, 2001; Ayyagari *et al.*, 2003; Kao *et al.*, 2004). In addition, flaps that show secondary structure are poor substrates for FEN1, necessitating the action of Dna2 (Kao *et al.*, 2004). Genetic studies support the proposed back-up mechanism for Dna2. When either the exonuclease activity of Pol δ or FEN1, activity is compromised, the tight control of the machinery to maintain a nick position is lost, and *pol3-exo⁻ rad27* double mutants are inviable [Jin, 2001 #1753; Jin, 2005 #2341]. However, overexpression of *DNA2* rescues the double mutant, again suggesting that increased formation of long flaps can be counteracted by increased Dna2 function (Jin *et al.*, 2003). Conversely, the temperature sensitivity of a *dna2-1* mutant is suppressed by overexpression of *RAD27*, the gene for FEN1 (Budd & Campbell, 1997).

Recycling of the Lagging Strand Machinery

Once maturation is completed, what happens to the elongation machinery? Simple models would predict that the entire machinery is recycled to the position of a new primer synthesized by Pol α-primase. Currently, this question has only been adressed for the replication clamp PCNA. Recycling of PCNA at the replication fork is suggested from photobleaching studies in mammalian cells, which show a lack of PCNA turnover during multiple rounds of Okazaki fragment synthesis (Sporbert *et al.*, 2002). However, these studies could not exclude the possible existence of a stably localized PCNA pool in a replication factory, from which a new PCNA is recruited to each Okazaki fragment being made. There are a few studies that indicate that recycling may not be an obligatory process. In phage T4, clamps left on the DNA after replication have been shown to serve another purpose in transcriptional activation of late genes (Kolesky *et al.*, 2002). In a study of SV40 DNA replication in human cell extracts, chromatin assembly of replicated DNA was regulated by interaction of chromatin assembly factor CAF-1 with the PCNA left on the replicated DNA (Shibahara & Stillman, 1999). However, it is not certain whether these are PCNA clamps left behind habitually at the lagging strand, or perhaps clamps that stem from leading strand DNA replication. Therefore, although from a viewpoint of economy and speed, it is reasonable to propose that the entire machinery is translocated, by the action of RFC, to a new primer, the possibility exists for more complex regulation at this step in lagging strand DNA replication as well.

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