

## Chapter 2

# Eukaryotic Replicative DNA Polymerases

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**Abstract** DNA replication is a dynamic process that requires the precise coordination of numerous cellular proteins. At the core of replication in eukaryotic cells are three DNA polymerases, Pol  $\alpha$ , Pol  $\delta$ , and Pol  $\epsilon$ , which function cooperatively to ensure efficient and high-fidelity genome replication. These enzymes are members of the B family of DNA polymerases, characterized by conserved amino acid motifs within the polymerase active sites. Pol  $\alpha$  is a DNA polymerase of moderate fidelity that lacks 3'→5' exonuclease activity, while Pols  $\delta$  and  $\epsilon$  are processive, high-fidelity polymerases with functional 3'→5' exonuclease activities. Each polymerase exists as a holoenzyme complex of a large polymerase catalytic subunit and several smaller subunits. The Pol  $\alpha$  holoenzyme possesses primase activity, which is required for de novo synthesis of RNA–DNA primers at replication origins and at each new Okazaki fragment. In one model of eukaryotic DNA replication, Pol  $\epsilon$  functions in leading strand DNA synthesis, while Pol  $\delta$  functions primarily in lagging strand synthesis. This chapter discusses the biochemical properties of eukaryotic replicative polymerases and how biochemical properties shape their functional roles in replication initiation, replication fork elongation, and the check-point responses.

**Keywords** DNA replication fork • S phase checkpoint • DNA polymerase fidelity • primase • proofreading exonuclease • replisome • genome stability

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## 2.1 Biochemical Properties of Replicative Polymerases

### 2.1.1 Polymerase $\alpha$ -Primase

#### 2.1.1.1 Overview

A formal nomenclature for eukaryotic DNA polymerases has been adopted, in which cellular DNA polymerases are given Greek letters in order of their historical discovery (Weissbach et al. 1975; Burgers et al. 1990). Accordingly, Pol  $\alpha$ -primase was the first mammalian polymerase to be purified and characterized (Yoneda and Bollum 1965). Pol  $\alpha$ -primase holoenzyme is a heterotetrameric protein complex in all eukaryotes studied (Table 2.1) (see Muzi-Falconi et al. 2003 for review). The p180 large subunit contains the DNA polymerase active site (Plevani et al. 1985; Wong et al. 1986). The B subunit has no known enzymatic activity, but performs a regulatory function, possibly linking the pol  $\alpha$  holoenzyme to components of the replication fork (Collins et al. 1993). DNA polymerases lack the ability to carry out de novo DNA synthesis and, in eukaryotes, require a 3'-OH provided by an RNA primer in order to initiate DNA synthesis. Primase activity is contained within the tightly associated p49/p58 complex (Plevani et al. 1985; Nasheuer and Grosse 1988). The architecture and subunit arrangement of the *Saccharomyces cerevisiae* (*S. c.*) Pol  $\alpha$ -primase holoenzyme have been examined by X-ray crystallography and electron microscopy. The B subunit is tethered to the p180 subunit through a structured, flexible linker (Klinge et al. 2009). The four-subunit holoenzyme exists as a dumbbell-shaped particle, with the catalytic primase and polymerase active sites present in distinct lobes of the complex, separated by ~100 Å (Nunez-Ramirez et al. 2011).

#### 2.1.1.2 An Essential Polymerase and Primase

Pol  $\alpha$  polymerase activity is essential for chromosomal replication. Genetic analyses of mutants in *S. c.* have demonstrated that both the catalytic and regulatory B subunits are required in vivo for viability, and mutants in either gene exhibit defects in DNA replication and progression through S-phase (Johnson et al. 1985; Foiani et al. 1994; Budd and Campbell 1987). In mammalian cells, Pol  $\alpha$ -primase neutralizing antibodies inhibit DNA synthesis (Miller et al. 1985; Kaczmarek et al. 1986). Thermosensitive yeast *POL1* mutants display elevated genetic instability due to defects in replication (Gutiérrez and Wang 2003; Liu et al. 1999). The primase activity is also essential in eukaryotic cells. Disruption of either the *PR11* or *PR12* genes is lethal in *S. c.* due to replication defects (Foiani et al. 1989; Lucchini et al. 1987). Characterization of conditional *PR11* and *PR12* mutants demonstrates that loss of primase activity causes increased mitotic recombination and spontaneous mutation rates, possibly due to defects in replication and impaired meiosis (Longhese et al. 1993).

**Table 2.1** Composition of the major replicative DNA polymerases in eukaryotes

		Designation (gene/protein)		
Subunit	Function	Human	<i>S. cerevisiae</i>	<i>S. pombe</i>
<i>Pol α holoenzyme</i>				
A	Polymerase	<i>POLA1</i> /p180	<i>POL1</i>	<i>pol1</i>
B	Regulatory	<i>POLA2</i> /p70	<i>POL12</i>	<i>pol12</i>
	Primase regulatory	<i>PRIM2</i> /p58	<i>PRI2</i>	<i>pri2</i>
	Primase catalytic	<i>PRIM1</i> /p49	<i>PRI1</i>	<i>pri1</i>
<i>Pol δ holoenzyme</i>				
A	Polymerase, 3'→5' exonuclease	<i>POLD1</i> /p125	<i>POL3</i>	<i>pol3</i>
B	Regulatory	<i>POLD2</i> /p50	<i>POL31</i>	<i>cdc1</i>
C	Regulatory	<i>POLD3</i> /p68 <sup>a</sup>	<i>POL32</i>	<i>cdc27</i>
D	Regulatory (DNA damage)	<i>POLD4</i> /p12	–	<i>cdm1</i>
<i>Pol ε holoenzyme</i>				
A	Polymerase, 3'→ 5' exo	<i>POLE1</i> /p261	<i>POL2</i>	<i>cdc20</i>
B	Regulatory	<i>POLE2</i> /p59	<i>DPB2</i>	<i>dpb2</i>
C	Double-stranded DNA binding	<i>POLE3</i> /p17	<i>DPB3</i>	<i>dpb3</i>
D	Double-stranded DNA binding	<i>POLE4</i> /p12	<i>DPB4</i>	<i>dpb4</i>

<sup>a</sup>Also referred to as the p66 subunit

### 2.1.1.3 Coordinated Primase and Polymerase Activities

In eukaryotic cells, *in vivo* studies have demonstrated that RNA primers of Okazaki fragments are attached to short DNA chains (Kitani et al. 1984). The Pol α holoenzyme is a unique replicative protein complex, possessing two coupled catalytic activities (Hu et al. 1984). Primase initiates synthesis of RNA at polypyrimidine tracts within the DNA template, preferentially within a T-rich region, and this occurs at many sites along the DNA template (Yamaguchi et al. 1985; Kitani et al. 1984). The primase catalytic site maps to the p49 subunit (Nasheuer and Grosse 1988), in which a carboxylic triad is essential for its function in generating RNA primers (Copeland and Tan 1995). The *S. c.* Pol α/p49 subunit is sufficient for synthesis of RNA, but highly inefficient without the p58 subunit (Santocanale et al. 1993). Biochemically, p58 is necessary for synthesis of the first dinucleotide of the RNA primer and modulates the rate and stability of subsequent extension steps (Copeland and Wang 1993). GTP is preferentially incorporated into the first dinucleotide, possibly to stabilize the short primer (Sheaff and Kuchta 1993). Although RNA primers generated by purified primase can vary in length from ~2 to 10 nucleotides in the presence of dNTPs (Nasheuer and Grosse 1988), only those ≥7 nucleotides are extended by Pol α, and formation of an RNA primer of this length terminates primase activity (Kuchta et al. 1990). The p58 subunit regulates primase processivity and ensures the correct RNA primer length is synthesized (Zerbe and Kuchta 2002). The DNA polymerase activity of Pol α extends RNA primers with ~20–30 dNTPs to generate an RNA–DNA hybrid molecule (Thompson et al. 1995).

Mechanistically, the primase and polymerase activities of the Pol  $\alpha$  holoenzyme are tightly coupled to ensure that new primers are not synthesized until the previous primer has been extended by polymerase (Sheaff et al. 1994). Functional coordination of the two activities is dependent upon the presence of dNTPs (Hu et al. 1984). An intramolecular transfer of the RNA primer from the primase active site to the polymerase active site occurs rapidly when dNTPs are present (Sheaff et al. 1994). Structurally, flexible tethering of Pol  $\alpha$  holoenzyme lobes containing the primase and polymerase centers increases the efficiency of primer transfer between the two active sites (Nunez-Ramirez et al. 2011). Structural and biochemical studies suggest that the p58 subunit is involved in the switch from primase to polymerase through conformational changes (Arezi et al. 1999; Agarkar et al. 2011). Consistent with its role in synthesizing short RNA–DNA primers, the Pol  $\alpha$  holoenzyme lacks the high processivity possessed by the other major replicative holoenzymes (see below), although it does possess the capacity to carry out robust DNA synthesis *in vitro*. The rate of DNA synthesis by calf thymus Pol  $\alpha$ -primase is similar to that of Pols  $\epsilon$  and  $\delta$  (Weiser et al. 1991), and Pol  $\alpha$ -primase displays an *in vitro* processivity of ~20–100 nucleotides (Hohn and Grosse 1987). Pol  $\alpha$ -primase interacts with the p70 subunit of replication protein A (RPA), which stimulates both the polymerase activity and processivity of the holoenzyme (Braun et al. 1997), possibly by increasing the affinity for primer termini (Maga et al. 2001).

#### 2.1.1.4 A Moderately Accurate Polymerase

The *in vitro* fidelity of the mammalian Pol  $\alpha$  holoenzyme purified from multiple sources has been determined using several genetic assays (Kunkel and Loeb 1981; Kunkel 1985; Eckert et al. 1997). The majority of errors created by Pol  $\alpha$  are single-base substitutions, followed by one-base deletion errors (Kunkel 1985; Eckert et al. 1997). The purified *S. c.* Pol  $\alpha$ /p180 catalytic subunit and the *S. c.* Pol  $\alpha$  holoenzyme have similar overall fidelities but display statistically significant differences in error rates within specific sequence contexts (Kunkel et al. 1989). The *S. c.* Pol  $\alpha$ /p180 subunit exhibits an error rate of 1/9,900 and 1/12,000 per nucleotide synthesized, for single-base substitutions and deletions, respectively (Kunkel et al. 1991). The human Pol  $\alpha$ /p180 subunit base substitution error rate can be as low as 1/42,000 nucleotides in low-pH buffer conditions (Eckert and Kunkel 1993). Direct comparison of purified mammalian Pol  $\alpha$ -primase, Pol  $\delta$ , and Pol  $\epsilon$  preparations demonstrated that Pol  $\alpha$  possesses the lowest fidelity of the three major eukaryotic replicative polymerases (Thomas et al. 1991).

One mechanism by which Pol  $\alpha$  maintains its moderate fidelity is the low rate of extending mismatched DNA primers (Perrino and Loeb 1989). Some calf thymus Pol  $\alpha$  holoenzyme pause sites correspond to sites of increased mismatched 3'-primer termini (Fry and Loeb 1992), consistent with a low rate of mispair extension. Pol  $\alpha$  holoenzyme pausing within microsatellite sequences also is correlated with the rate of misalignment-based errors (Hile and Eckert 2004).

Replication auxiliary factors enhance the DNA fidelity of Pol  $\alpha$ -primase. RPA decreases dNTP misincorporation rate by decreasing the affinity of the Pol  $\alpha$  holoenzyme to mismatches while increasing the affinity to matched DNA primer termini, suggesting that it may function as a “fidelity clamp” that modulates the Pol  $\alpha$  holoenzyme for faithful DNA synthesis (Maga et al. 2001). Terminal misincorporation events at Pol  $\alpha$  pause sites are also avoided in the presence of RPA (Suzuki et al. 1994). Pol  $\alpha$ -primase lacks 3'→5' exonuclease activity due to amino acid changes at catalytic residues within the domain, which limits its intrinsic fidelity (Pavlov et al. 2006b). However, *S. c.* Pol  $\delta$  can perform intermolecular proofreading of errors made by Pol  $\alpha$ -primase (Pavlov et al. 2006a). Based on an estimation that  $\sim 4\text{--}8 \times 10^4$  RNA–DNA primers are synthesized by Pol  $\alpha$ -primase during replication in human cells (Muzi-Falconi et al. 2003), such mechanisms of enhanced fidelity may be essential in maintaining genome stability.

In contrast to its DNA polymerase fidelity, the priming activity exhibits very low fidelity during RNA synthesis. Primase readily misincorporates NTPs during *in vitro* RNA synthesis, often polymerizing primers containing consecutive mismatches (Sheaff and Kuchta 1994). Importantly, after intramolecular transfer, such mismatched RNA primers are readily extended by the DNA polymerase activity in the presence of dNTP substrates.

### 2.1.1.5 Posttranslational Regulation

The Pol  $\alpha$  holoenzyme exists as an assembled complex throughout the cell cycle (Ferrari et al. 1996), and its activity is regulated by cyclin-dependent kinases in a cell cycle-dependent manner (Voitenleitner et al. 1999). The p180 subunit is a phosphoprotein that becomes hyperphosphorylated in G<sub>2</sub>/M phase, while the B subunit is phosphorylated only in G<sub>2</sub>/M (Nasheuer et al. 1991). Pol  $\alpha$  phosphorylation results in lowered single-stranded DNA binding affinity, lowered DNA synthesis activity, and an inhibition of DNA replication (Nasheuer et al. 1991; Voitenleitner et al. 1999).

## 2.1.2 DNA Polymerase $\delta$

### 2.1.2.1 Overview

Pol  $\delta$  was originally identified as a novel DNA polymerase purified from rabbit bone marrow that possessed a very active 3'→5' exonuclease activity (Byrnes et al. 1976). Subsequently, *S. c.* POL3 was identified as the yeast homolog of Pol  $\delta$  (Boulet et al. 1989). In all eukaryotes, Pol  $\delta$  is a multimeric complex, wherein the largest subunit harbors the DNA polymerase and 3'→5' exonuclease catalytic domains, and is tightly associated with a regulatory B subunit (Table 2.1). Mammalian Pol  $\delta$  holoenzyme is a heterotetrameric protein consisting of two additional

C and D subunits, p68 and p12. The fission yeast *Schizosaccharomyces pombe* (*S. p.*) also encodes a heterotetrameric Pol  $\delta$ , possessing the Cdm1 subunit which shows some sequence similarity to the mammalian p12 subunit. The budding yeast *S. c.* Pol  $\delta$  is a heterotrimer of the A and B subunits together with a C subunit ortholog, Pol32.

The structure of a truncated form of the *S. c.* Pol  $\delta$ /p125 subunit in ternary complex with DNA and dNTP substrates has been solved at 2 Å resolution (Swan et al. 2009). The polymerase domain has the characteristic right-hand structure of palm, fingers, and thumb subdomains. The 3'→5' exonuclease domain is separated from the polymerase domain by 45 Å. Pol  $\delta$  also possesses a novel N-terminal domain that interacts with 10–20 nucleotides of the ssDNA template, upstream of the polymerase active site. The structure of a truncated *S. c.* Pol  $\delta$  holoenzyme as determined by small-angle X-ray scattering analysis consists of a globular catalytic core (A subunit) flexibly linked to an elongated tail comprised of the B and C subunits (Jain et al. 2009). The interaction of Pol3 (A subunit) with the Pol31 and Pol32 (B and C subunits) is stabilized by binding of a 4Fe-4S metal cofactor cluster to four conserved cysteine residues in the CysB motif of the Pol3 C-terminal domain (Netz et al. 2012).

### 2.1.2.2 An Essential Polymerase

Evidence that Pol  $\delta$  is an essential component of the eukaryotic replication machinery has been derived from several models. Disruption of Pol  $\delta$  is lethal in *S. p.*, and thermosensitive mutants of pol3 arrest in S-phase of the cell cycle (Francesconi et al. 1993). Deletion of the *S. p.* *cdc27* gene (Pol  $\delta$ /C subunit) results in cell cycle arrest (MacNeill et al. 1996). Although the orthologous *S. c.* *POL32* gene is not essential, deletion mutants do display abnormal phenotypes (Gerik et al. 1998). *S. c.* *POL3* mutants harboring different substitutions at a catalytically essential residue, Leu<sup>612</sup>, exhibit a wide range of phenotypic deficiencies (Venkatesan et al. 2006). Only 8 of 19 mutants are viable and display varying degrees of genotoxic sensitivity, cell cycle defects, and morphological abnormalities (Venkatesan et al. 2006). Replication studies in *Xenopus* egg extracts demonstrated that immunodepletion of Pol  $\delta$  leads to a significant decrease in DNA synthesis and the accumulation of unreplicated, single-stranded DNA gaps (Fukui et al. 2004). Knockdown of Pol  $\delta$ /p125 in HeLa cells causes an accumulation of cells in early, middle, and late S-phase, and cells do not enter mitosis (Bermudez et al. 2011). Homozygous disruption of *Pold1* in mice was shown to cause embryonic lethality between E4.5 and E7.5, due to significant defects in DNA synthesis (Uchimura et al. 2009). Profoundly, homozygous mutations of highly conserved amino acids within motif A of the Pol  $\delta$  active site (L604G and L604K) are embryonic lethal in mice, and mice heterozygote for the L604K mutant exhibit reduced life spans and accelerated tumorigenesis (Venkatesan et al. 2007). Mouse embryonic fibroblasts heterozygous for these mutant proteins display elevated mutation rates and chromosomal instability relative to wild-type cells (Venkatesan et al. 2007). The severe

replication defects associated with loss of Pol  $\delta$  in each of these models were vital in uncovering the polymerase's role as a major component of the eukaryotic replication fork.

### 2.1.2.3 Efficient DNA Synthesis in the Presence of Replication Accessory Factors

Pol  $\delta$  requires replication accessory proteins to achieve its greatest efficiency. Shortly after the discovery of Pol  $\delta$ , an auxiliary protein was identified, which co-eluted with Pol  $\delta$  during purification and significantly enhanced its in vitro DNA synthesis on several DNA templates (Tan et al. 1986). This protein was later identified as proliferating cell nuclear antigen (PCNA) and was shown to greatly enhance calf thymus Pol  $\delta$  processivity in vitro (Prelich et al. 1987; Bravo et al. 1987). PCNA is now known as the eukaryotic sliding clamp protein, a homotrimer ring-shaped protein that encircles DNA and tethers replication proteins, allowing movement along the DNA template (Moldovan et al. 2007). Replication Factor C (RFC), a heteropentameric complex, is responsible for loading PCNA onto DNA through an ATP-dependent mechanism. In the presence of PCNA, RFC, and RPA, *S. c.* Pol  $\delta$  processivity is enhanced from 6 nucleotides to >600 nucleotides (Chilkova et al. 2007). Human Pol  $\delta$  holoenzyme activity increases >50-fold and processivity is stimulated in the presence of PCNA (Xie et al. 2002). However, the human Pol  $\delta$  holoenzyme differs biochemically from *S. c.* Pol  $\delta$  holoenzyme, as human Pol  $\delta$  dissociates more readily from DNA templates, even in the presence of accessory factors (Bermudez et al. 2011).

Recombinant human Pol  $\delta$  can be purified in several subassemblies, all of which retain DNA synthesis activity (Zhou et al. 2012; Podust et al. 2002). The Pol  $\delta$  p125/p50 heterodimer (also referred to as the core dimer) is a tightly associated complex with low specific activity. The three subunit complex, Pol  $\delta$ 3 (core + p68 subunit), displays high DNA synthesis activity but is unstable in vitro. The p68 subunit is essential for synthesis of long DNA products by Pol  $\delta$ . The p12 subunit increases stability of the holoenzyme and increases DNA synthesis activity. All four subunits of human Pol  $\delta$  individually interact with PCNA, which may allow for flexibility during DNA replication, as many proteins functionally interact with PCNA during Okazaki fragment maturation (Zhang et al. 1999; Wang et al. 2011; Li et al. 2006b). PCNA interacting motifs also were identified within the C-termini of all three subunits of *S. c.* Pol  $\delta$ , which are needed for efficient DNA synthesis (Acharya et al. 2011). PCNA stimulation of DNA synthesis activity differs quantitatively among the various human Pol  $\delta$  subassemblies, in the order Pol  $\delta$ 4 > Pol  $\delta$ 3 (core + p68) > Pol  $\delta$ 2 core (Zhou et al. 2012). Kinetically, PCNA reduces the  $K_m$  for DNA template binding and increases the  $V_{max}$  of the calf thymus Pol  $\delta$  catalytic core, suggesting that PCNA might stimulate Pol  $\delta$  processivity by increasing its residence time on the DNA template and the rate of nucleotide incorporation (Ng et al. 1991).

#### 2.1.2.4 An Accurate Polymerase

Replication of the genome requires accurate DNA synthesis in order to avoid the accumulation of deleterious mutations. As demonstrated using in vitro mutation assays, purified Pol  $\delta$  generally possesses a high DNA synthesis fidelity (see Prindle and Loeb 2012 for review). The *S. c.* Pol  $\delta$  holoenzyme incorporates less than one-base substitution error out of 80,000 nucleotides synthesized (Fortune et al. 2005). For human Pol  $\delta$ , less than one-base misinsertion error occurred per 220,000 nucleotides (Schmitt et al. 2009). However, *S. c.* and human Pol  $\delta$  holoenzymes do not exhibit high fidelity during in vitro synthesis of repetitive microsatellite DNA sequences (Hile et al. 2012; Abdulovic et al. 2011). At the heart of Pol  $\delta$ 's high fidelity is its intrinsic 3'→5' exonuclease activity (Simon et al. 1991), which enables proofreading upon incorporation of incorrect nucleotides during DNA synthesis (Kunkel et al. 1987). Both human and *S. c.* exonuclease-deficient Pol  $\delta$  forms exhibit approximately 10-fold higher base substitution error rates than wild-type Pol  $\delta$  in vitro (Fortune et al. 2005; Schmitt et al. 2009). In vivo, yeast strains carrying inactivating mutations within the *POL3* exonuclease domain exhibit a 100-fold increased spontaneous mutation rate (Morrison and Sugino 1994). Furthermore, mice with homozygous substitutions at highly conserved residues within the proofreading domain of Pol  $\delta$  exhibit a high incidence of cancer and decreased survival (Goldsby et al. 2002; Albertson et al. 2009).

Intrinsic kinetic properties of Pol  $\delta$  also are key determinants of its high fidelity. *S. c.* Pol  $\delta$  strongly favors incorporation of correct versus incorrect nucleotides during synthesis (Dieckman et al. 2010). A highly conserved leucine residue within the Pol  $\delta$  active site has been extensively studied in yeast, mice, and humans (Leu-612, 604 and 606, respectively) for its role in ensuring high-fidelity DNA synthesis. Amino acid substitutions at this site cause allele-specific phenotypic effects in *S. c.* and mice (Venkatesan et al. 2006, 2007). In vitro, the human Pol  $\delta$  holoenzyme L606G mutant is highly error prone (Schmitt et al. 2010). Interestingly, the L606K mutant exhibits higher fidelity than wild-type Pol  $\delta$  but decreased bypass of DNA adducts (Schmitt et al. 2010), suggesting that the high fidelity conferred by this active site residue is balanced by the need for the wild-type enzyme to perform other activities, including translesion synthesis at the replication fork.

Efficient proofreading requires partitioning of DNA substrates containing 3' terminal mispairs from the polymerase to the exonuclease active sites (Khare and Eckert 2002). An *S. c.* *POL3* active site mutation that impairs this partitioning results in decreased DNA synthesis fidelity and increased spontaneous mutation rate (McElhinny et al. 2007). Purified *S. c.* Pol  $\delta$  interacts with base pairs distant from the templating base, which may allow the polymerase to “sense” base mismatches (Swan et al. 2009).

Although PCNA stimulates Pol  $\delta$  activity, it may decrease its fidelity. In vitro, PCNA increases the rate of nucleotide misincorporation by *S. c.* Pol  $\delta$ , resulting in a

significant reduction in fidelity (Hashimoto et al. 2003). Similarly, nucleotide misincorporations by calf thymus Pol  $\delta$  increase ~27-fold in the presence of PCNA, and PCNA may enable Pol  $\delta$  to extend mismatched base pairs by stabilizing the Pol  $\delta$ -template-primer complex (Mozzherin et al. 1996). However, addition of both PCNA and RPA had no effect on *S. c.* Pol  $\delta$  base substitution error rates, and the addition of PCNA and RPA to Pol  $\delta$  DNA synthesis reactions contributed to a decreased rate of large deletion errors within directly repeated sequences (Fortune et al. 2006). Further studies are needed to fully understand the effect of accessory factors on Pol  $\delta$  fidelity. Interestingly, the Werner syndrome protein (WRN), a RecQ family helicase and 3'→5' exonuclease, can enable high-fidelity DNA synthesis by Pol  $\delta$  through excision of primer-template mismatches prior to polymerase extension (Kamath-Loeb et al. 2012).

### 2.1.2.5 Posttranslational Regulation

Phosphorylation may represent an important mechanism of Pol  $\delta$  regulation (see Lee et al. 2012 for review). Mammalian Pol  $\delta$  protein levels peak at the G1/S border, and the p125 subunit is most actively phosphorylated during S-phase (Zeng et al. 1994). The B subunit (p50) is also phosphorylated in vivo and is an in vitro substrate of the cyclin A-Cdk2 cell cycle-dependent kinase (Li et al. 2006a). The C subunit (p68) can be phosphorylated by cyclin-dependent kinases (CycE-Cdk2, CycACdk1, or CycA-Cdk2) in vitro, and PCNA interferes with this phosphorylation (Ducoux et al. 2001). In addition, mammalian Pol  $\delta$ /p125, p68, and p12 subunits can be phosphorylated by casein kinase 2 in vitro and subsequently dephosphorylated by protein phosphatase-1 (Gao et al. 2008), suggesting an additional regulatory circuit for regulation. Phosphorylation of Pol  $\delta$ /p68 coincides with Pol  $\delta$  association with chromatin at the start of S-phase (Lemmens et al. 2008). The Pol  $\delta$ /p68 subunit also contains a phosphorylation site for protein kinase A, and phosphomimetic mutation of this residue decreases Pol  $\delta$  affinity for PCNA and processivity (Rahmeh et al. 2011). Thus, phosphorylation may serve to regulate Pol  $\delta$  activity by controlling its interaction with DNA and/or auxiliary proteins during replication.

## 2.1.3 DNA Polymerase $\epsilon$

### 2.1.3.1 Overview

Purified Pol  $\epsilon$  was first characterized as a larger form of Pol  $\delta$  that was highly processive, but not stimulated by PCNA in vitro (Syvaola and Linn 1989). The Pol  $\epsilon$  holoenzyme exists as a heterotetrameric protein in all eukaryotes studied (Table 2.1) (Pursell et al. 2008). The large catalytic subunit contains the polymerase and 3'→5' exonuclease active sites within the N-terminus, and binding domains for smaller

subunits and PCNA within the C-terminus. The C and D subunits form a subcomplex that is important for double-stranded DNA binding (Tsubota et al. 2003). The *S. c.* Pol  $\epsilon$  holoenzyme structure has been solved at 20 Å resolution using cryo-electron microscopy and single-particle analyses (Asturias et al. 2006). The three small subunits (B–D) form a discrete extended tail structure, separated from the large catalytic subunit by a flexible hinge. Similar to the *S. c.* Pol  $\delta$ , a 4Fe-4S cluster is bound to four conserved cysteine residues (the CysB motif) within the C-terminal domain of Pol2 and may be essential for stabilizing the *S. c.* Pol  $\epsilon$  holoenzyme (Netz et al. 2012).

### 2.1.3.2 An Essential Component of the Eukaryotic Replication Machinery

The Pol  $\epsilon$  holoenzyme is essential for chromosomal replication. In *S. c.*, *POL2* reading frame disruptions are nonviable due to arrest of DNA replication; however, truncation mutations that maintain catalytic activity are viable with a slow growth phenotype (Morrison et al. 1990). Deletion of the *POL2* N-terminus, which encodes the polymerase catalytic domain, also is viable, although mutants display severe replication defects, including slow fork movement, prolonged S-phase, and shortened telomeres (Kesti et al. 1999; Feng and D’Urso 2001; Dua et al. 1999). Thermosensitive yeast mutants also demonstrate that nonfunctional Pol  $\epsilon$  leads to defective chromosomal replication and the accumulation of short DNA fragments (Araki et al. 1992; Budd and Campbell 1993). Immunodepletion of Pol  $\epsilon$  from *Xenopus* egg extracts significantly impedes elongation of nascent DNA strands and causes the accumulation of short replication intermediates (Waga et al. 2001). In HeLa cells, transient knockdown of Pol  $\epsilon$  causes an accumulation of cells in early S-phase and a decreased rate of replication fork movement (Bermudez et al. 2011). Disruption of Pol  $\epsilon$  in mice results in embryonic lethality (Menezes and Sweasy 2012).

### 2.1.3.3 A Highly Efficient and Processive Polymerase

Pol  $\epsilon$  and Pol  $\delta$  differ in their biochemical interactions with PCNA. PCNA binds the Pol  $\epsilon$  p261 (N-terminus), p59, and p12 subunits and increases the rate of nucleotide incorporation by the holoenzyme (Bermudez et al. 2011). In the presence of PCNA, RFC, and RPA, *S. c.* Pol  $\epsilon$  processivity is stimulated ~6-fold, less than the ~100-fold stimulation observed for Pol  $\delta$  (Chilkova et al. 2007). *S. c.* Pol  $\epsilon$  has a high affinity for DNA and low affinity for PCNA, while *S. c.* Pol  $\delta$  displays the opposing affinities for each, suggesting that Pol  $\epsilon$  might load onto DNA independently of PCNA, while Pol  $\delta$  requires preloading of PCNA (Chilkova et al. 2007). However, *S. c.* and human Pol  $\epsilon$  are more processive than Pol  $\delta$ , even in the absence of accessory factors (Dua et al. 2002) (Bermudez et al. 2011). High *S. c.* Pol  $\epsilon$  processivity requires a minimal primer duplex stem length of 40 base pairs, and

structural studies suggest that the tail domain formed by the B–D subunits contributes directly to Pol  $\epsilon$  processivity by binding double-stranded DNA, precluding the need for PCNA to enhance DNA affinity (Asturias et al. 2006). The processivities of both the *S. c.* Pol  $\epsilon$  polymerase and exonuclease activities are reduced in subassemblies lacking the C and D subunits, relative to the holoenzyme form (Aksenova et al. 2010).

#### 2.1.3.4 A Highly Accurate Polymerase

Pol  $\epsilon$  is perhaps the most accurate eukaryotic DNA polymerase. Purified calf thymus Pol  $\epsilon$  base substitution error rates determined in vitro ranged from 1/30,000 to 1/400,000 mutations per nucleotide synthesized, lower than either Pol  $\delta$  or Pol  $\alpha$  (Thomas et al. 1991). The *S. c.* Pol  $\epsilon$  holoenzyme exhibits very low base substitution and single-base deletion error rates,  $\leq 2 \times 10^{-5}$  and  $\leq 5 \times 10^{-7}$  (Shcherbakova et al. 2003), respectively. Error rates of human Pol  $\epsilon$  have not yet been determined because they are close to background rates for in vitro mutation assays (Korona et al. 2011). Although the in vitro fidelity of purified *S. c.* Pol  $\epsilon$  lacking the C and D subunits is the same as the Pol  $\epsilon$  holoenzyme form, deletion of *DPB3* and *DPB4* results in a slightly increased spontaneous mutation rate in vivo (Aksenova et al. 2010).

The high fidelity of Pol  $\epsilon$  is due, in part, to its intrinsic 3'→5' exonuclease activity. Amino acid substitutions of a conserved motif within the exonuclease active site result in a ~20-fold increase in the *S. c.* spontaneous mutation rate (Morrison et al. 1991). Purified exonuclease-deficient *S. c.* Pol  $\epsilon$  exhibits single-base substitution and deletion errors rates that are ~10- and 100-fold higher, respectively, than wild-type Pol  $\epsilon$  (Shcherbakova et al. 2003). Kinetically, *S. c.* Pol  $\epsilon$  mutants harboring a C1089Y substitution within the polymerase active site exhibit an increased rate of base misincorporation that may result from the inability to perform proper DNA shuffling between the polymerase and exonuclease domains (Shimizu et al. 2002). Human exonuclease-deficient Pol  $\epsilon$  is ~5-fold more accurate than wild-type *S. c.* Pol  $\epsilon$  for both base substitutions and single-base deletions (Korona et al. 2011). Homozygous loss of Pol  $\epsilon$  exonuclease activity in mice results in an elevated spontaneous mutation rate and tumor incidence (Albertson et al. 2009). Specific polymerase active site residues also play a role in determining Pol  $\epsilon$  fidelity. For instance, an M644F substitution within the *S. c.* Pol  $\epsilon$  active site reduces fidelity due to an increased base misincorporation rate (Pursell et al. 2007a). The Pol  $\epsilon$  M644 active site residue may modulate fidelity by maintaining proper geometry of the substrate binding pocket (Pursell et al. 2007a).

#### 2.1.3.5 Posttranslational Modification

The posttranslational regulation of Pol  $\epsilon$  has not been extensively studied. However, the *S. c.* B subunit, required for chromosomal replication (Araki et al. 1991), is

phosphorylated during late G1 phase in a cell cycle-dependent manner (Kesti et al. 2004).

## 2.2 Functions at the Eukaryotic Replication Fork

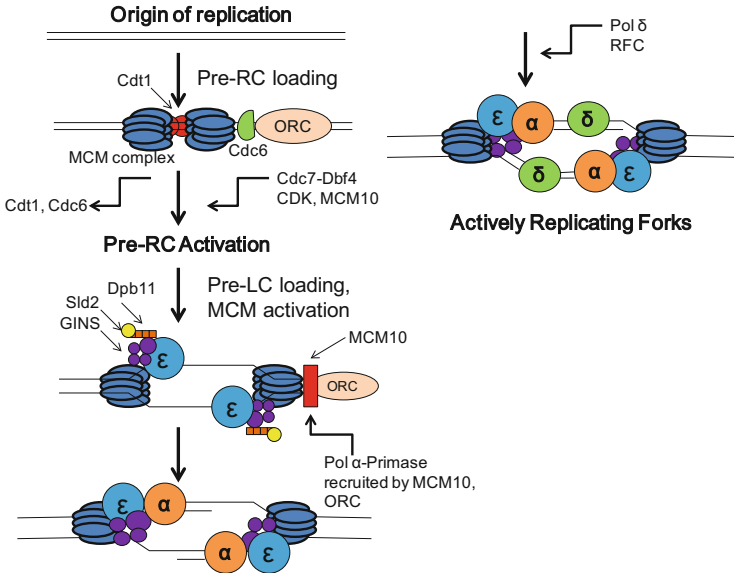
### 2.2.1 Replication Initiation

The process of replication initiation takes place as two steps (see Masai et al. 2010 for review). The pre-RC complex (ORC; Cdc6; Cdt1; MCM10 and MCM2-6 helicase complex) is first loaded onto chromosomes at origins of replication. In the second step, pre-RCs are activated to generate replication fork through the kinase activities of Cdc7-Dbf4 and CDK, which allows loading of other proteins required for replication. Loading of the GINS complex, a heterotetrameric complex essential for replication (Takayama et al. 2003), leads to activation of the MCM helicase complex, which enables unwinding of duplex DNA at the replication fork. All three *S. c.* replicative polymerases ( $\alpha$ ,  $\delta$ ,  $\epsilon$ ) bind to origins in early S-phase (Hiraga et al. 2005). Pol  $\epsilon$  is a component of the preloading complex (Pre-LC), which also contains GINS, Sld2, and Dpb11 (Muramatsu et al. 2010), suggesting that Pol  $\epsilon$  may play an active role in replication initiation (Fig. 2.1). Indeed, loss of the Pol  $\epsilon$  catalytic subunit in *S. p.* prevents loading of GINS and several other initiation proteins to origins (Handa et al. 2012). GINS function is required for recruitment of Pol  $\epsilon$  to chromatin (Pai et al. 2009) and can stimulate Pol  $\epsilon$  activity (Bermudez et al. 2011).

Pol  $\alpha$ -primase interacts with several protein players in DNA replication initiation (Fig. 2.1). The B subunit of Pol  $\alpha$ -primase interacts directly with ORC for its recruitment to initiation sites (Uchiyama and Wang 2004). MCM10 binds to the Pol  $\alpha$ /p180 subunit and physically interacts with the Pol  $\alpha$  holoenzyme in vitro and in vivo (Fien et al. 2004; Ricke and Bielinsky 2004; Warren et al. 2009). MCM10 maintains steady-state levels of the Pol  $\alpha$ /p180 subunit (Ricke and Bielinsky 2004), loads Pol  $\alpha$  holoenzyme onto chromatin (Zhu et al. 2007), and facilitates Pol  $\alpha$  holoenzyme binding to primed DNA templates (Fien et al. 2004). Additionally, the GINS complex interacts with the primase subunits and stimulates DNA synthesis activity (De Falco et al. 2007).

### 2.2.2 Leading and Lagging Strand Replication

Upon activation of replication origins, Pol  $\alpha$ -primase is responsible for generating RNA–DNA primers that initiate DNA synthesis on the leading and lagging strands of the replication fork. Temporal studies using *Xenopus* extracts demonstrated that Pol  $\alpha$ -primase is loaded onto DNA after helicase-mediated unwinding has been



**Fig. 2.1** Simplified schematic of replication initiation in eukaryotes. Initiation occurs at origins of replication, at which a pre-RC complex, consisting of the MCM complex, Cdc6, and Cdt1, is first loaded and then activated by the Cdc7-Dbf4 and CDK kinases. This leads to the recruitment of the pre-LC complex, consisting of GINS, Sld2, Dpb11, and Pol  $\epsilon$ . Pol  $\alpha$ -primase is recruited through interactions with MCM10 and also directly interacts with GINS. Mechanisms leading to Pol  $\delta$  recruitment are currently unknown. Upon recruitment of all three replicative Pols, an active, bidirectional replication fork is generated, in which Pol  $\epsilon$  is modeled as the leading strand Pol, and Pol  $\delta$  is modeled as the lagging strand Pol. For simplicity, some replication-initiating factors and protein–protein interactions are not displayed. The reader is referred to Masai et al. 2010 and Araki 2010 for further details

initiated (Walter and Newport 2000). Switching from Pol  $\alpha$  to Pol  $\delta$  or Pol  $\epsilon$  involves RFC. In vitro, RFC inhibits Pol  $\alpha$  activity once a sufficient RNA–DNA primer is generated, by decreasing its affinity for the DNA template, thus coordinating a switch from Pol  $\alpha$  to Pol  $\delta$  (Mossi et al. 2000; Maga et al. 2000).

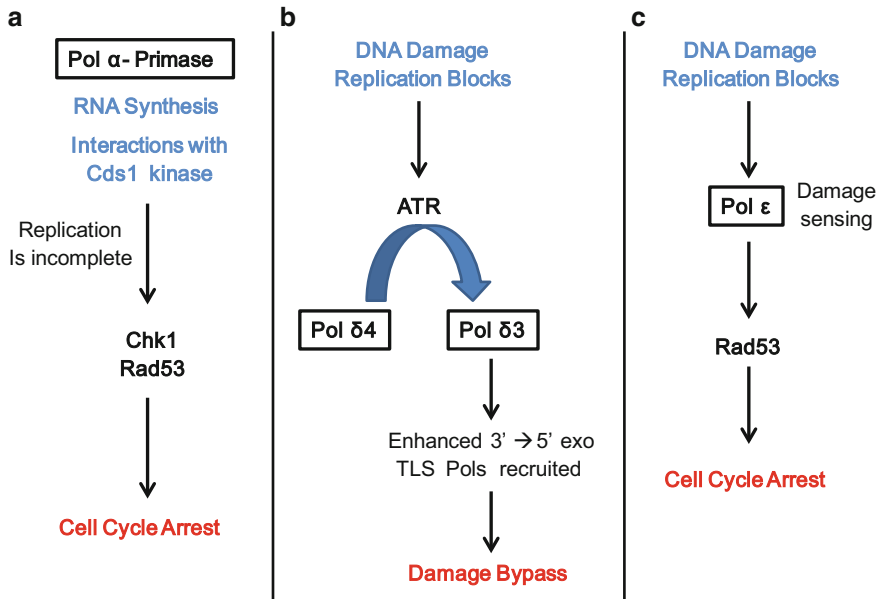
Several studies have suggested that Pols  $\delta$  and  $\epsilon$  have distinct responsibilities at the replication fork (Kunkel and Burgers 2008). In HeLa cells, Pol  $\epsilon$  is more active in early S-phase while Pol  $\delta$  activity increases in late S-phase (Rytönen et al. 2006). Early biochemical evidence that Pol  $\delta$  functions as the lagging strand Pol came from studies demonstrating its role in Okazaki fragment maturation, in which Pol  $\delta$  functions together with FEN1, a 5' flap endonuclease; DNA ligase 1; and PCNA (Burgers 2009). As replication takes place on the lagging strand, Pol  $\delta$  encounters RNA primers within downstream Okazaki fragments every ~100–200 nucleotides, which must be removed in order to prevent deleterious duplication mutations (Burgers 2009). Yeast genetic analyses and in vitro studies demonstrated that Pol  $\delta$  carries out strand displacement of one to two nucleotides within the RNA primer, followed by idling through its 3'→5' exonuclease activity, which allows for FEN1 to remove the resulting ribonucleotide flap. This process occurs in several

cycles until all ribonucleotides are removed and a DNA–DNA nick is generated, which is sealed by DNA ligase 1 (Garg et al. 2004; Jin et al. 2003; Burgers 2009). In contrast, Pol  $\epsilon$  does not undergo idling at downstream primers or interact with FEN1 to generate ligatable nicks (Garg et al. 2004), evidence that is not consistent with a role in lagging strand replication.

Early studies of exonuclease-deficient *S. c.* Pol  $\delta$  and Pol  $\epsilon$  strains provided genetic evidence that the two polymerases act on opposite DNA strands during replication (Morrison and Sugino 1994; Shcherbakova and Pavlov 1996; Karthikeyan et al. 2000). More recently, yeast DNA polymerases  $\delta$  and  $\epsilon$  variants that have distinctive error signatures in vitro have been used to infer the strand preference for DNA synthesis (e.g., leading versus lagging) during in vivo DNA replication. Such genetic studies of Pol  $\epsilon$  and Pol  $\delta$  mutants are consistent with roles as the leading and lagging strand polymerase, respectively. The asymmetric *S. c.* Pol  $\epsilon$  mutant (M644G) exhibits a high rate of T-dTMP mismatches relative to complementary A-dAMP mismatches in vitro and an elevated rate of T-dTMP mismatches in an origin orientation-dependent manner in vivo, consistent with its role in replication on the leading strand (Pursell et al. 2007b). Conversely, the *pol3-L612M* is an asymmetric Pol  $\delta$  mutator that increases the rate of dT-dGMP mismatches but has a low rate for generating the complementary dA-dCMP mismatch in vitro (McElhinny et al. 2007), and genetic analysis of the *pol3-L612M* mutant showed that its signature errors occurred in vivo primarily on the lagging strand (Nick McElhinny et al. 2008). Deep DNA sequencing and analyses of L612M mutant genomes revealed the Pol error signature genome wide, suggesting that Pol  $\delta$  is responsible for lagging strand replication across the *S. c.* genome (Larrea et al. 2010). Although such evidence supports the model in which Pol  $\delta$  functions as the lagging strand Pol, alternative models have been proposed in which Pol  $\delta$  may also contribute to replication on the leading strand (as reviewed in Kunkel and Burgers 2008). Importantly, it remains to be determined whether this model applies to replication in mammalian cells and to the fork after replication restart.

### 2.2.3 Replicative Polymerase Functions in Checkpoint Responses

Replicative polymerases are integral components of pathways monitoring progression of the mitotic cell cycle (Fig. 2.2). Pol  $\alpha$ -primase is a central player in checkpoint signaling that ensures DNA replication is complete prior to progression into mitosis. Checkpoint activation of the Chk1 kinase in *Xenopus* extracts requires RNA synthesis by primase, but not DNA synthesis (Michael et al. 2000), and yeast mutants defective for primase activity are unable to activate the Chk1 and Rad53 kinases (Griffiths et al. 2001; Marini et al. 1997). The Cds1 protein kinase interacts directly with Pol  $\alpha$ -primase, possibly to signal downstream cell cycle checkpoint



**Fig. 2.2** Eukaryotic DNA polymerases in cellular checkpoint responses. (a) During S-phase of the cell cycle, Pol α-primase activity results in RNA synthesis, which serves as a signal that replication is incomplete and prevents cell cycle progression through activation of Chk1 and Rad53. Interactions between Pol α-primase and the Cds1 kinase also act as an upstream signal for Chk1 and Rad53 activation. (b) DNA damage and replication blocks act as a signal for ATR activation in cells, which leads to degradation of the p12 subunit of Pol δ4 and the formation of Pol δ3. The three-subunit form of Pol δ is proposed to play a role in damage bypass. (c) DNA damage and replication blocks act as an upstream signal for Pol ε, which then triggers signaling for activation of Rad53 and cell cycle arrest under these conditions

responses (Murakami and Okayama 1995). Thus, the presence of RNA primers synthesized by primase during S-phase acts as a signal that replication is not yet complete in order to prevent cell cycle progression.

Over the past several years, a three subunit form of Pol δ, devoid of the p12 subunit, has been extensively studied for its role in regulating the DNA damage response in human cells (reviewed in Lee et al. 2012). Upon treatment of cells with various DNA damage-inducing agents, including UV, methyl methanesulfonate, hydroxyurea, and aphidicolin, the p12 subunit undergoes ubiquitylation-dependent degradation to form Pol δ3 (core + p68 subunit). Under conditions of low UV doses, this is dependent on activation of ATR, a major checkpoint response pathway in eukaryotic cells (Zhang et al. 2007). Interestingly, Pol δ3 displays increased exonuclease partitioning and decreased potential for bypass of various DNA lesions (Lee et al. 2012). These findings led to a model in which Pol δ3 is responsible for slowing replication progression at sites of DNA damage, which might allow for switching to a translesion synthesis polymerase (Lee et al. 2012).

Pol  $\epsilon$  also is an essential component of cell cycle checkpoint responses. Pol  $\epsilon$  functions upstream of Rad53 in the DNA damage response pathway (Navas et al. 1996). The C-terminus of *S. c.* POL2 has an essential role in initiating the S-phase checkpoint response, but is not involved in the G1 or G2/M checkpoints (Navas et al. 1995; Dua et al. 1998). In response to replication stress, Pol  $\epsilon$  is required for one of two independent pathways leading to checkpoint activation (Puddu et al. 2011).

## 2.3 Perspective

Intensive basic research over the past 4 decades, using biochemical and genetic approaches and model systems from yeast to humans, has demonstrated that DNA polymerases cooperate to ensure efficient and accurate eukaryote genome replication. This chapter has provided a glimpse into how the biochemical properties of Pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  shape their functional roles at the replication fork. The multisubunit holoenzyme structure of each polymerase provides a mechanism for regulating polymerase activities, allowing proper cell cycle progression and cellular responses to replication roadblocks, such as DNA damage. Studies of budding yeast have provided strong evidence that a fourth polymerase holoenzyme complex is present at the eukaryotic replication fork, namely, Pol  $\zeta$ , whose biochemical activities are critical for translesion DNA replication (see Pavlov and Shcherbakova 2010 for review). Intriguingly, very recent papers have shown that the B and C subunits of Pol  $\delta$  are shared with Pol  $\zeta$ , suggesting that the catalytic domains of replicative polymerases can be exchanged within a holoenzyme complex (Baranovskiy et al. 2012; Johnson et al. 2012). This new evidence highlights the very dynamic nature of DNA polymerases at the replication fork to efficiently process all aspects of genomic maintenance as they arise during replication. Clearly, elucidating exactly how polymerases are regulated during DNA replication in eukaryotic cells is an emerging area of research but one that is already providing new and unexpected twists to the current paradigm.

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