Structure of eukaryotic DNA polymerase epsilon and lesion bypass capability

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Till min vackra familj

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ABSTRACT

To transfer the information in the genome from mother cell to daughter cell, the DNA replication must be carried out only once and with very high fidelity prior to every cell division. In yeast there are several different DNA polymerases involved in DNA replication and/or DNA repair. The two replicative DNA polymerases, DNA polymerase δ (Pol δ) and DNA polymerase ε (Pol ε), which both include a proofreading 3′→5′exonuclease activity, can replicate and proofread the genome with a very high degree of accuracy. The aim of this thesis was to gain a better understanding of how the enigmatic DNA polymerase ε participates in DNA transactions.

To investigate whether Pol ε or Pol δ is responsible for the synthesis of DNA on the lagging strand, the processing and assembly of Okazaki fragments was studied. Pol δ was found to have a unique property called "idling" which, together with the flap-endonuclease (FEN1), maintained a ligatable nick for DNA ligase I. In contrast, Pol ε was found to lack the ability to "idle" and interact functionally with FEN-1, indicating that Pol ε is not involved in processing Okazaki fragments. Together with previous genetic studies, it was concluded that Pol δ is the preferred lagging strand polymerase, leaving Pol ε to carry out some other function.

The structure of Pol ε was determined by cryo-electron microscopy, to a resolution of ~20 Å. Pol ε is composed of a globular "head" domain consisting of the large catalytic subunit Pol2p, and a "tail" domain, consisting of the small subunits Dpb2p, Dpb3p, and Dpb4p. The two separable domains were found to be connected by a flexible hinge. Interestingly, the high intrinsic processivity of Pol ε depends on the interaction between the tail domain and double-stranded DNA.

As a replicative DNA polymerase, Pol ε encounters different lesions in DNA. It was shown that Pol ε can perform translesion synthesis (TLS) through a model abasic site in the absence of external processivity clamps under single-hit conditions. The lesion bypass was dependent of the sequence on the template and also on a proper interaction of the "tail"domain with the primer-template.

Yeast cells treated with a DNA damaging agent and devoid of all TLS polymerases showed improved survival rates in the presence of elevated levels of dNTPs. These genetic results suggested that replicative polymerases may be engaged in the bypass of some DNA lesions. *In vitro*, Pol ε was found to bypass 8-OxoG at elevated dNTP levels. Together, the *in vitro* and *in vivo* results suggest that the replicative polymerases may be engaged in bypass of less bulky DNA lesions at elevated dNTP levels.

In conclusion, the low-resolution structure presented represents the first structural characterization of a eukaryotic multi-subunit DNA polymerase. The replicative DNA polymerase Pol ε can perform translesion synthesis due to an interaction between the tail domain and double-stranded DNA. Pol ε may also bypass less bulky DNA lesions when there are elevated dNTP concentrations *in vivo*.

Keywords: DNA polymerase epsilon, DNA replication, Okazaki fragment, Translesion synthesis, DNA lesion, dNTP

List of papers:

This thesis is based on these papers:

I. . Garg P., Stith C. M., Sabouri N., Johansson E., Burgers P. M. Idling by DNA polymerase δ Maintains a Ligatable Nick during Lagging Strand DNA Replication.
Genes Dev. 2004 Nov 15;18(22):2764-73.

II. Asturias F.J., Cheung I. K **., Sabouri N **., Chilkova O., Wepplo. D., and Johansson E. Structure of *S. cerevisiae* DNA polymerase epsilon by cryo-electron microscopy.
Nat Struct Mol Biol. 2006 Jan;13(1):35-43.
**** Equal contributions.**

III. Sabouri N., and Johansson E.
DNA polymerase epsilon bypasses an abasic site in the absence of a processivity clamp.
Manuscript.

IV. Sabouri N**., Viberg J**., Kumar D., Johansson E., and Chabes A.
Evidence for lesion bypass by yeast replicative DNA polymerases during DNA damage.
Submitted manuscript.
**** Equal contributions.**

1. Introduction

"To be or not to be", is a well-known phrase from William Shakespeare's celebrated play Hamlet. It is also a fundamental question regarding all organisms. In order to develop and function, replication of the genetic code, which is stored in the cell as deoxyribonucleic acid (DNA), must be regulated very carefully before each cell division. The genome of the unicellular eukaryote *Saccharomyces cerevisiae* (one species of yeast) consists of 12,000,000 base pairs. Thus, a rapid replication is important in order to complete the synthesis of DNA before every cell division. The synthesis of the genome is only allowed to occur once per cell cycle, and the DNA synthesis is challenged by both endogenous and exogenous agents that continuously damage the DNA.

The replication process is not only required to be fast; it must also be carried out with high fidelity. If not, the eukaryotic cell would prefer the option "not to be". In more biological terms, if the damages to the DNA are beyond repair, the cell would undergo apoptosis, as in the case of multicellular organisms.

In all eukaryotic organisms, there is DNA in both the nucleus and the mitochondria. DNA is built from deoxyribonucleoside triphosphates (dNTPs) that link to form a helix. The proteins in charge of the construction of the DNA helices are called DNA polymerases. DNA polymerases are also involved in repair of damaged DNA.

Quiz: One of these four pictures does not fit with the others and should be removed. Which?




Photos by: Omid Anjam

Correct answer to the quiz:
The car should be removed since it does not contain DNA replication machinery.

2. Background

The replicative DNA polymerases responsible for DNA synthesis in the nucleus are DNA polymerase α (Pol α), DNA polymerase δ (Pol δ), and DNA polymerase ε (Pol ε) (Burgers *et al.*, 2001). DNA polymerase γ (Pol γ) is responsible for mitochondrial DNA synthesis. Eukaryotic DNA polymerases are divided into different classes, A, B, X, and Y, based on their sequence similarities (Table 1).

DNA polymerases have a universal architecture with a right hand-like arrangement: (1) a hand, including fingers that functions to bind the incoming DNA template and dNTPs, (2) a palm, possessing the catalytic activity, and (3) a thumb, holding the exiting replicated DNA (reviewed in Cramer, 2002). Pol α, Pol δ, and Pol ε belong to the B class of DNA polymerases. Unfortunately, the structures of these three polymerases (or of any multi-subunit class B DNA polymerase for that matter) are not known, but it is of great importance to solve them in order to gain a better understanding of their functions.

Class	Name	Subunits
B	**Pol α**[1]	Pri1, Pri2, Pol1, Pol12
	Pol δ[2]	Pol3, Pol31, Pol32
	Pol ε[3]	Pol2, Dpb2, Dpb3, Dpb4
	Pol ζ[4]	Rev3, Rev7
Y	**Pol η**[5]	Rad30
	Rev1[6]	Rev1
X	**Pol IV**[7]	Pol4
A	**Pol γ**[8]	Mip1

Table 1. *S. cerevisiae* DNA polymerases grouped by class.
[1](Plevani *et al.*, 1984), [2](Gerik *et al.*, 1998), [3](Chilkova *et al.*, 2003) [4](Nelson *et al.*, 1996b), [5](Johnson *et al.*, 1999b), [6](Nelson *et al.*, 1996a), [7](Tseng and Tomkinson, 2002), [8](Lucas *et al.*, 2004).

2.1 The cell cycle

A cell divides into two new units during the process of cell division. To create a new cell, the parent cell progresses through a strongly regulated process called the cell cycle. The cell cycle consists of four phases, G_1 (gap 1), S (DNA synthesis), G_2 (gap 2) and M (mitosis) phase (Fig. 1). At the end of the G phase, a checkpoint controls the entry of the cell into the next phase. If everything in the current phase has not proceeded successfully, entry to the following phase is delayed until all the requirements in the current phase have been fulfilled. There are also intra-checkpoints, both in the S phase and the M-phase. The cell grows during the G1 and the G2 phases, preparing for the S-phase and M-phase, respectively. Cells in G1 do not always proceed to the S phase; instead they can enter a resting stage called the G0 phase. The topic of this thesis, DNA replication, occurs during the S phase. In the M phase, the cell separates the duplicated genome and divides into two identical copies.

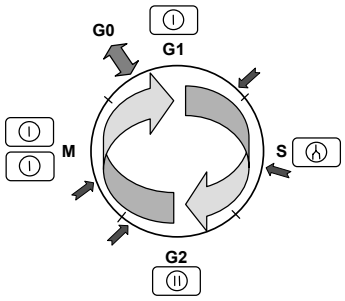

Figure 1. Schematic illustration of the different phases of the cell cycle.
The black notched arrows indicate the checkpoints.

2.2 DNA and its building blocks

The DNA double helix is composed of two anti-parallel complementary strands, which are constructed from four substrates, deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP) (Fig. 2). Ribonucleotide reductase (RNR) is the enzyme responsible for synthesizing the DNA substrates by reducing ribonucleotides to form deoxyribonucleotides (dNTPs) (reviewed in Nordlund and Reichard, 2006). To avoid mutations, the concentration of dNTPs must be kept balanced (Weinberg *et al.*, 1981). However, the amounts of each individual dNTP are not equimolar in the cell and the RNR activity is strictly regulated by allosteric binding sites that regulate both the balance between the four dNTPs and also the total amount of each.

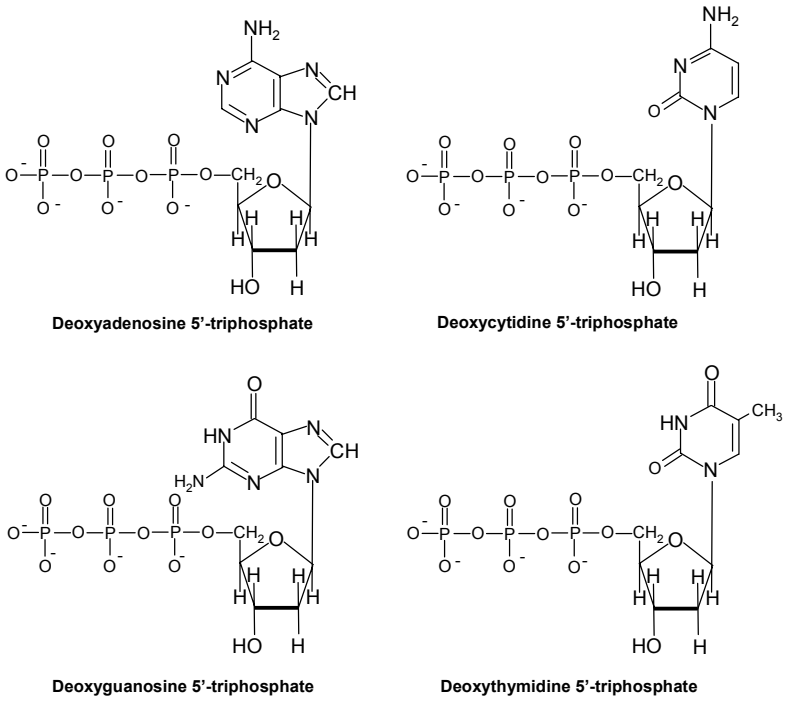

Figure 2. Deoxyribonucleoside triphosphates, the four building blocks of the DNA.

2.3 Initiation of replication

DNA replication is a complicated process which involves a large number of replication factors. The process is initiated at multiple origins and each origin is only initiated once per cell cycle. At each origin, a highly organized pre-replicative complex (pre-RC) is established and forms the start of two bidirectional replication forks. The binding of the origin recognition complex (ORC) to autonomous replicating sequences (ARSs), a specific DNA sequence in *S. cerevisiae*, occurs at the G1 phase, requires ATP, and is the first stage of action in the assembly of the pre-RC (Bell and Stillman, 1992). X-ray crystallographic structures of ORCs from Archaea bound to replication initiator sites have revealed the entry of specific ORC domains into the DNA, which allow distortion of the strands, resulting in an untwisting of the double-stranded DNA (dsDNA) (Gaudier *et al.*, 2007;Dueber *et al.*, 2007). The binding of ORC recruits Cdc6p and Cdt1p. The structure of Cdc6p and ORC bound together (as seen by electron microscopy (EM)) resembles a ring-like structure, such as another AAA+ family member clamp loader RFC (Replication factor C) (Speck *et al.*, 2005). It has been suggested that the ORC-Cdc6 complex forms a ring around the DNA, and together with Cdt1p, loads Mcmc2-7p complex (MCM) on the DNA (Randell *et al.*, 2006). Many origin-associated proteins are believed to belong to the AAA+ ATPase family. A typical function of AAA+ proteins is to remodel other macromolecules by using ATP. MCM is involved in both initiation of replication and in the elongation step, and has been suggested to be the DNA

helicase, which unzips the double stranded DNA helix in the 3'-5' direction by moving ahead of the leading strand (Ishimi, 1997;reviewed in Labib and Diffley, 2001). ORC, Cdc6p, Cdt1p, and MCM together form the pre-RC in an ATP-dependent manner (reviewed in Bell, 2002).

Prior to the start of DNA synthesis, many other factors must be assembled and our knowledge of the exact role of these factors is still quite poor. A summary of how the replisome is activated is given below, although the exact order and function of these factors is still not known with any certainty.

A switch from G phase to S phase occurs through phophorylation by Cdc7/Dbf4 kinase and cyclin-dependent kinase (CDK), which activate MCM, Sld2p, and Sld3p (Zegerman and Diffley, 2007;Tanaka *et al.*, 2007). Cdc45p forms a complex with MCM and is required for DNA unwinding (Walter and Newport, 2000). In *S. cerevisiae*, Mcm10 is needed for loading of Cdc45 onto the origin (Sawyer *et al.*, 2004). Phosphorylated Sld2 and Sld3 bind Dpb11, recruiting the GINS complex and Pol ε to the origin (Zegerman and Diffley, 2007;Tanaka *et al.*, 2007). The loading of these factors promotes the unwinding of the dsDNA. Next, RPA binds to the single-stranded DNA (ssDNA), resulting in its stabilization, followed by the Polα-primase. In the last step, RFC loads PCNA (proliferating cell nuclear antigen), the clamp, onto the newly synthesized primer and Pol δ is recruited.

2.4 The replication fork: leading and lagging strand

Once the DNA polymerases have been loaded onto the unzipped dsDNA, forming two strands, DNA synthesis can begin. Due to the fact that DNA polymerases can only synthesize DNA in the 5'-3' direction, the replication fork is synthesized continuously on the leading strand and discontinuously on the lagging strand (Fig. 3). As a result of discontinuous synthesis, the lagging strand generates short pieces of DNA called Okazaki fragments. Basically, the leading strand only needs to be primed once, but the lagging strand requires continuous priming, as each Okazaki fragment requires one primer. Pol α has both primase and polymerase activity and synthesizes RNA-DNA hybrid primers on both strands. The primer is approximately 30 bps long, 10 bp of RNA synthesized by the primase and 20 bp of DNA synthesized by the polymerase (reviewed in Arezi and Kuchta, 2000). Pol α is not as accurate as Pol δ or Pol ε in synthesizing DNA (Shcherbakova *et al.*, 2003;Fortune *et al.*, 2005;Zhong *et al.*, 2006) and the bulk of DNA synthesis is started by Pol δ and Pol ε, both of which have 3'-5' proofreading exonuclease activity. The 3'-5' exonuclease activity of DNA polymerases is an editing function that contributes to replication of the genome with high fidelity. The error rate for base substitutions measured in gap-filling *lac Z* assays is less than 2×10^{-5} and less than 1.3×10^{-5} for Pol ε and Pol δ, respectively (Shcherbakova *et al.*, 2003;Fortune *et al.*, 2005). However, the exonuclease-deficient Pol ε and Pol δ measured in the same *lac Z* assays have a base substitution error rate that is

greater than 10 times that of the exonuclease-proficient forms, $24x10^{-5}$ and $13x10^{-5}$, respectively (Shcherbakova *et al.*, 2003;Fortune *et al.*, 2005). The role of these two replicative polymerases at the replication fork is unclear and through the years many models concerning which polymerase (Pol δ or Pol ε) is responsible for synthesizing a particular strand have been proposed by different research groups (Johnson and O'Donnell, 2005;Garg and Burgers, 2005a).

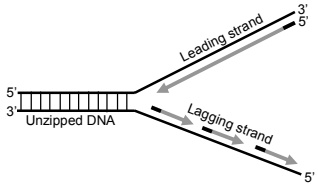

Figure 3. Scheme illustrating a replication fork with the continuous leading strand and the discontinuous lagging strand. The black line on each arrow represents a primer.

2.5 Okazaki maturation

Around 100,000 Okazaki fragments must be processed on the lagging strand in a haploid yeast cell. Each fragment is estimated to be approximately 150 nucleotides (nts) (Ishimi *et al.*, 1988). During the maturation of Okazaki fragment, the RNA-DNA hybrid primer synthesized by Pol α requires to be displaced and degraded, a process called nick translation, and the nick created must be sealed by DNA ligase I in order to avoid double-strand breaks and lethality. The 5'-flap-endonuclease (FEN1) cleaves 5'-unannealed flaps. Long flaps cannot be cleaved by FEN1; thus, and the nuclease/helicase Dna2 was suggested to play an important role in Okazaki fragment maturation (Bae *et al.*, 2001). However the action of Dna2 has subsequently been shown to be limited to long flaps only (Jin *et al.*, 2003;Ayyagari *et al.*, 2003). FEN1 binds to PCNA through a conserved PCNA-binding motif (Warbrick, 1998). Biochemical studies have shown that Pol δ, FEN1, and PCNA are strongly associated with each other in DNA synthesis and DNA degradation (Ayyagari *et al.*, 2003).

2.6 DNA polymerase δ

Pol δ is composed of Pol3p (125 kDa), Pol31p (50 kDa) and Pol32p (40 kDa), and is purified as a monomer in *S. cerevisiae* (Johansson *et al.*, 2001). The *POL3* and *POL31* are essential genes with the catalytic and 3'-5'exonuclease activity located at Pol3p. *POL32* is suggested to be a mutator subunit and to be involved in DNA mutagenesis (Gerik *et al.*, 1998). Fission yeast and mammals have an additional subunit (Zuo *et al.*, 1997;Liu *et al.*, 2000;Podust *et al.*, 2002). Homozygous mice with mutations at the active site

of Pol δ are not viable, and heterozygous mice with the same mutation are genomically unstable and have a shorter life than wild-type mice (Venkatesan *et al.*, 2007). Genetic analysis in *S. cerevisiae* has demonstrated that Pol δ and Pol ε proofread different DNA strands (Shcherbakova and Pavlov, 1996) and replication errors made by Pol α are corrected by Pol δ (Pavlov *et al.*, 2006), thus associating Pol δ with lagging strand DNA synthesis.

PCNA stimulates both Pol δ and Pol ε to a comparable degree of processive DNA synthesis (Chilkova *et al.*, 2007). During processive DNA synthesis, the polymerase synthesizes long stretches of DNA without dissociating itself from the primer-template. There are two plausible PCNA binding domains on Pol δ, one located on Pol3p interacting with PCNA on the DNA and the other located on Pol32p interacting with PCNA in solution (Johansson *et al.*, 2004). Loading of the PCNA-RFC complex has been suggested to be involved in the switching from Pol α to Pol δ, to initiate the bulk replication (reviewed in Garg and Burgers, 2005a).

2.7 DNA polymerase ε

Recombinant Pol ε is purified as a heterotetramer from *S. cerevisiae* and consists of Pol2p (256 kDa), Dpb2p (79 kDa), Dpb3p (23 kDa), and Dpb4p (22 kDa) (Chilkova *et al.*, 2003). *POL2* encodes the catalytic activity of Pol ε and contains both the polymerase activity and the 3'-5'exonuclease activity (Morrison *et al.*, 1991). *POL2* is essential for viability in yeast and is required for chromosomal DNA replication (Araki *et al.*, 1992;Kesti *et al.*, 1999).

Many attempts have been made to reveal the function of Pol ε at the replication fork. Chromatin immunoprecipitation assays have revealed that Pol ε is recruited to the origin (Aparicio *et al.*, 1997) and is loaded both at early and late origins together with Pol α during the S phase (Aparicio *et al.*, 1999). It has also been shown that Pol δ, Pol ε, and Pol α co-localize together at the replication fork (Hiraga *et al.*, 2005).

DNA synthesis in human proliferating cells was found to be reduced by using a neutralizing antibody against Pol ε (Pospiech *et al.*, 1999). This finding supported the function of Pol ε as a replicative DNA polymerase. Also, inactivation of the 3'-5 exonuclease activity of Pol ε in yeast resulted in a 5- to 40-fold increase in the spontaneous mutation rate, also indicating that Pol ε is involved in DNA synthesis (Morrison *et al.*, 1991).

It has been shown that SV40 genome does not require Pol ε for DNA replication (Waga *et al.*, 1994;Zlotkin *et al.*, 1996). Studies in budding yeast showed that deletion of the amino-terminal catalytic portion of *POL2* is not lethal but causes growth defects in yeast (Kesti *et al.*, 1999;Dua *et al.*, 1999) (Fig. 4). In contrast, a point mutation in the catalytic residue of Pol ε was found to be lethal (Dua *et al.*, 1999). Together, these results in yeast suggest

that in the absence of the catalytic domain of Pol2p, the polymerization activity of Pol ε can be substituted by another DNA polymerase, but not in the presence of an inactive Pol2p (Kesti *et al.*, 1999;Dua *et al.*, 1999).

In the same study, Kesti and co-workers also showed that the carboxy-terminal part of Pol2p is indispensable for viability in budding yeast (Kesti *et al.*, 1999), indicating that Pol2p has other essential functions in the cell apart from DNA polymerization. The carboxy-terminal end of Pol2p contains a zinc finger region, which interacts with Dpb2p, the other essential subunit of Pol ε (Dua *et al.*, 1998;Araki *et al.*, 1991a). This domain could possibly play an important role in S/M checkpoint pathways (Dua *et al.*, 1999) and may explain the essential role of the carboxy-terminal of Pol2.

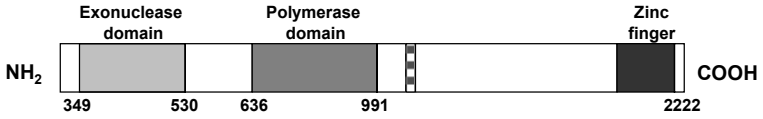

Figure 4. Different domains of the Pol2 subunit.
The squared pattern is the putative PCNA binding motif and is located at amino acid positions 1193-1201.

Biochemical studies have shown that Pol ε can interact with primed DNA, ssDNA, and dsDNA (Maki *et al.*, 1998;Tsubota *et al.*, 2003;Chilkova *et al.*, 2007). Dpb3 and Dpb4 both contain a conserved histone-fold motif and form a heterodimer (Ohya *et al.*, 2000;Li *et al.*, 2000;Tsubota *et al.*, 2003). Gel mobility shift assays have shown that the Dpb3-Dpb4 heterodimer has affinity for dsDNA (Tsubota *et al.*, 2003). Yeast strains lacking *DPB3* have a higher rate of spontaneous mutation rate, indicating that Dpb3p has a role in maintaining the fidelity of DNA synthesis (Araki *et al.*, 1991b).

Pol ε has also been suggested to be involved in other functions, such as DNA repair and sister chromatid cohesion (Edwards *et al.*, 2003). In summary, Pol ε has been shown to be involved in many different functions. Many have questioned the function of Pol ε as a replicative DNA polymerase; however, recently it was demonstrated that Pol ε participates in the replication of the leading strand (Pursell *et al.*, 2007).

2.8 PCNA in DNA repair

PCNA is a DNA sliding clamp, which is loaded onto the dsDNA by RFC in an ATP dependent manner (Gomes and Burgers, 2001). PCNA forms a ring-shaped homotrimer (Krishna *et al.*, 1994) and one of its functions is to tether DNA polymerases tightly to the DNA to enhance their processivity. *POL30*,

which codes for PCNA, is an essential gene and numerous cellular processes involve interaction with PCNA.
Post-translational modifications of PCNA by monoubiquitylation or polyubiquitylation recruit translesion synthesis (TLS) polymerases to stalled replication forks (Fig. 5). TLS is a mechanism that involves one or several specialized DNA polymerases (mostly Y-family polymerases) that tolerate bypass of DNA adducts. The recruitment of TLS polymerases is dependent on ubiquitin-binding domains, which have been found in several TLS polymerases (Bienko *et al.*, 2005). The ubiquitylation of PCNA is activated by Rad18p, a DNA binding protein, which binds the ubiquitin conjugating enzyme Rad6p to PCNA on DNA (Hoege *et al.*, 2002). The monoubiquitylation is suggested to promote error-prone TLS and the polyubiquitylation is suggested to promote error-free post-replication repair (PRR) (Hoege *et al.*, 2002;Kannouche *et al.*, 2004). The error-free PRR pathway includes Rad5p, Mms2p and Ubc13p. Rad5p has been reported to comprise a helicase activity that may promote a template switch and error-free lesion bypass (Blastyak *et al.*, 2007).

Deubiquitination of PCNA is promoted by Usp1p, an enzyme belonging to a family of cysteine proteases termed DUBSs (deubiquitinating enzymes) (Huang *et al.*, 2006). Usp1p is degraded during UV damage, thereby activating monoubiquitination of PCNA (Huang *et al.*, 2006).

PCNA can also be modified by a small ubiquitin-like modifier (SUMO). Genetic analysis in yeast has shown that SUMOylated PCNA functionally cooperates with the helicase Srs2 (Pfander *et al.*, 2005). Immunofluorescence images of yeast chromatin demonstrated partial co-localization of PCNA and Srs2 in S phase (Ulrich *et al.*, 2005). It has also been shown *in vitro* that the helicase Srs2 disrupts Rad51 filaments (Veaute *et al.*, 2003). It was therefore suggested that SUMOylated PCNA recruits Srs2 to the replication fork, which prevents association of Rad51 filaments, leading to reduced levels of homologous recombination. It is proposed that this action of Srs2 facilitates PCNA ubiquitylation pathways when the fork encounters a lesion.

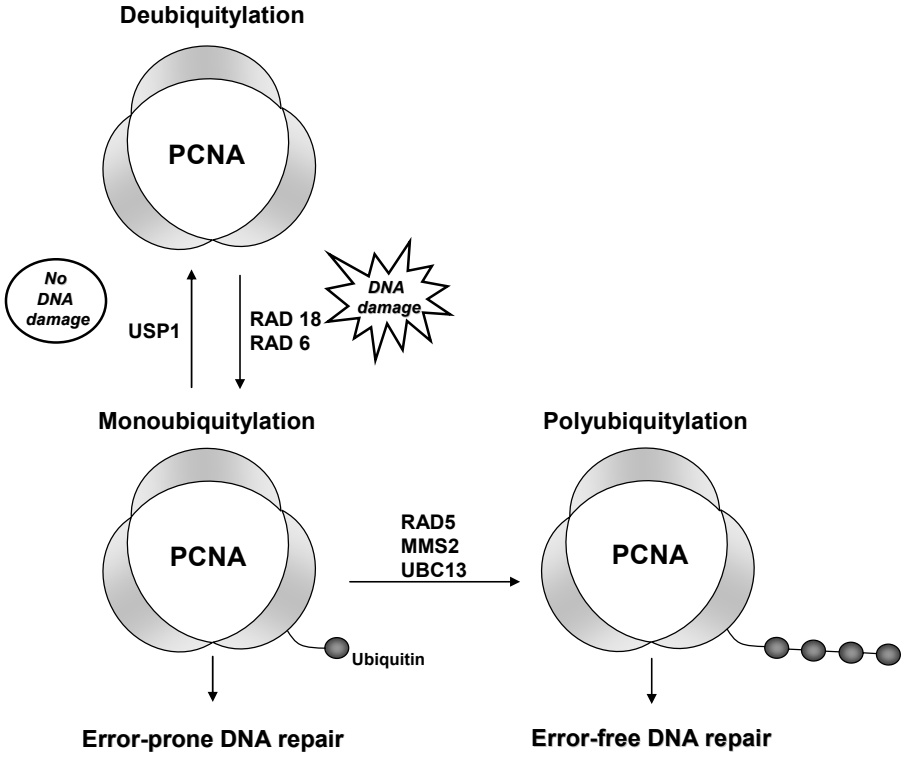

Figure 5. Ubiquitylation and deubiquitylation of PCNA.
The picture is simplified, with ubiquitin on only one of the subunits of the homotrimer PCNA. The ubiquitylation occurs only when PCNA is loaded on DNA.

2.9 Lesions and DNA repair pathways

DNA can be damaged in several different ways. Thus, numerous different base modifications can occur, single- or double-strand breaks can occur in the DNA. However, there are many different DNA repair pathways—before, during, and after DNA replication, which help the cell to keep the genome intact during the life cycle of the cell. These repair pathways are nucleotide excision repair (NER), base excision repair (BER), mismatch repair, homologous recombination (HR), non-homologous end joining, and PRR.

During BER, specific DNA glycosylases remove the modified, altered base and thereafter the two endonucleases, Apn1p and Apn2, cleave the sugar-phosphate backbone. Next, a DNA polymerase inserts the correct nucleotide in the gap and the nick is sealed by a DNA ligase (reviewed in Memisoglu and Samson, 2000).

Examples of DNA modifications are 7,8-dihydro-8-oxoguanine (8-OxoG) from oxidative damage, thymine dimers from UV exposures, or apurinic (abasic) sites (Fig. 6).

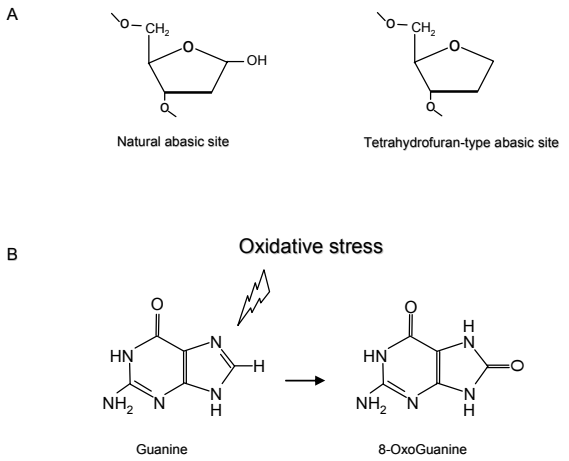

Figure 6. DNA lesions relevant to this thesis.
A. Structures of natural and tetrahydrofuran-type abasic sites. Tetrahydrofuran is widely used as a model abasic site in *in vitro* assays.
B. Guanine damaged by oxidative stress, forming an 8-OxoG.

Abasic sites are formed spontaneously, through exposure to toxic agents such as ionizing radiation, or by removal of uracil or a damaged base by DNA glycosylases during BER (Guillet and Boiteux, 2003;Boiteux and Guillet, 2004). The number of bases lost in a mammalian cell per day has been calculated to be between 10,000 and 200,000 bases (Lindahl and Nyberg, 1972;Nakamura and Swenberg, 1999). The large variation in these numbers is due to the use of different measurement assays and on the tissues in different experiment. However, one can certainly say that abasic sites are one of the most frequent lesions in the cell. Although BER is responsible for removal of base modifications and NER is responsible for repair of helix-distorting lesions, some lesions still escape the repair machineries.

2.10 Replication of damaged DNA

Lesions that have escaped the repair machinery before the S phase and are encountered by the replication fork may arrest the fork, leading to double-strand breaks and cell death. However, there are several mechanisms that may process these lesions. Apart from the error-free PRR, two models or ideas are currently suggested (Fig. 7). The most recent model is the TLS. The second, older model suggests that replicative polymerases leave a gap opposite the damage and that repriming occurs on the other side of the lesion

such that the replication is reinitiated. These two models do not need to be contradictory; both can be true depending on the lesion that the fork meets. The TLS model may assist smaller lesions such as 8-OxoG and the gap model may assist in the case of more severe obstacles.

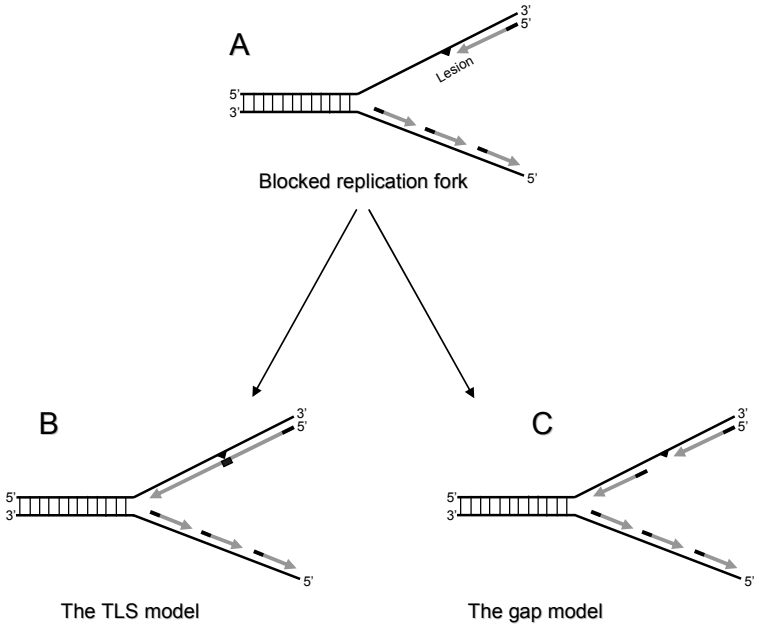

Figure 7. Replication of damaged DNA.
A. The leading strand is blocked by a lesion.
B. The lesion is directly bypassed by the TLS pathway.
C. A new primer is synthesized and replication is continued downstream of the lesion, forming a single-strand gap opposite the lesion.

2.10.1 The gap model

The gap model was proposed as early as 1968. Rupp and Howard-Flanders observed that NER-deficient bacteria exposed to UV-light were able to continue to synthesize DNA almost as fast as unirradiated cells (Rupp and Howard-Flanders, 1968). They also discovered that the replicated DNA in the UV-irradiated cells was discontinuous, but still 10 times longer than Okazaki fragments. These fragments could be sealed, and after the sealing the DNA in the UV-irradiated cells was as long as the DNA in the control cells. These results suggest that a gap is formed opposite the DNA damage, followed by gap-filling of the ssDNA—which seals the gap and repairs the damage.

These long stretches of ssDNA were also detected in UV-irradiated NER-deficient yeast cells by electron microscopy (Lopes *et al.*, 2006). In another study, Heller and Marians demonstrated restart of replication downstream of

the UV-irradiated leading-strand DNA (Heller and Marians, 2006). Together, these studies suggest a model in which a gap is left opposite the lesion (both for the leading and the lagging strand), a repriming event occurs after the lesion and the fork can continue the replication. The gap is thought to be repaired by HR and/or by TLS behind the replication fork.

2.10.2 The TLS model

The Y-family polymerases were discovered as late as in the end of 1990s (Ohmori *et al.*, 2001). Y-family polymerases are specialized polymerases that, in contrast to replicative polymerases, replicate undamaged DNA with lower processivity and fidelity due to a more open active site, which can accommodate bulky lesions in the catalytic active site (Wang *et al.*, 1997;Ling *et al.*, 2001;Trincao *et al.*, 2001;Franklin *et al.*, 2001) (Table 1). This open conformation of the palm domain is caused by smaller thumb and finger domains than those possessed by replicative polymerases (Ling *et al.*, 2001;Trincao *et al.*, 2001;Franklin *et al.*, 2001). Y-family polymerases also have an additional domain called the little finger. The little finger is the most mobile part of Y polymerases, and is believed to control the catalytic efficiency of the polymerase. This domain also influences what kind of damage a particular Y polymerase "prefers" and specializes in (Boudsocq *et al.*, 2004;Yang, 2005). Translesion synthesis is a mechanism involving one or several of these specialized polymerases; it tolerates bypass of different DNA lesions but mostly generates mutations for the cost of survival. Some of these polymerases specialize in inserting a base opposite the damage, others are better at extending synthesis from a base that has already been inserted opposite the damage, and some may do both. Switching between a replicative polymerase and a TLS polymerase may vary depending on the nature of the damage and also the genome sequence. Our knowledge of which of the different polymerases that is responsible for what is still quite poor (reviewed in Lehmann *et al.*, 2007). Ubiquitination of the processivity clamp PCNA has been suggested to attract TLS polymerases to a blocked replication fork and to act as a connecting link for the polymerase switch (Kannouche *et al.*, 2004). After the first switch and bypass of the lesion by the TLS polymerase, a second switch occurs and the replicative polymerase re-engages to PCNA and continues to synthesize the DNA. TLS polymerases have also been suggested recently to be involved in HR of double-strand breaks (McIlwraith *et al.*, 2005;Kawamoto *et al.*, 2005;Hirano and Sugimoto, 2006).

2.11 DNA Polymerases involved in TLS

In *S. cerevisiae*, Rad30p, Rev1p, Rev3p, Rev7p, and Pol32p are involved in TLS. TLS polymerases have different abilities regarding insertion of or extension of nucleotides opposite different lesions, and contribute to either error-free or error-prone bypasses.

RAD30 encodes for DNA polymerase η (Pol η). Pol η has the ability to incorporate adenine opposite thymine dimers with comparable efficiency to

that seen for undamaged thymine (McCulloch *et al.*, 2004). Bypass of thymine dimers is an error-free pathway and inactivation of Pol η causes Xeroderma pigmentosum variant in humans (Masutani *et al.*, 1999;Johnson *et al.*, 1999a). In conditions in which multiple cycles of polymerization of the DNA substrate were allowed in primer extension assays, Pol η was found to be capable of bypassing 8-OxoG (Haracska *et al.*, 2000) but not abasic sites (Northam *et al.*, 2006). Under the same condition, bypass of abasic sites for yeast DNA polymerase ζ (Pol ζ) was only 3% (Northam *et al.*, 2006). PCNA stimulates this bypass for both Pol η and Pol ζ (Northam *et al.*, 2006). Ub-PCNA stimulates the bypass of abasic sites by Rev1 and Pol η to a much greater extent than unmodified PCNA (Garg and Burgers, 2005b).

Rev1 is a more accurate Y-family polymerase and has a deoxycytidyl transferase activity (Nelson *et al.*, 1996a). It inserts a dCTP opposite the abasic site (Kow *et al.*, 2005) but cannot bypass it (Haracska *et al.*, 2002), and the bypass is not stimulated by PCNA (Haracska *et al.*, 2006). Rev1 has been suggested to participate in TLS in association with Pol ζ (Lawrence, 2002).

Pol ζ is a B-family DNA polymerase involved in mutagenesis, and consists of two subunits (Rev3p and Rev7p) (Zhong *et al.*, 2006;Nelson *et al.*, 1996b). Pol ζ can bypass thymine dimers and is involved in many insertion and extension steps at different lesions. Pol ζ is an efficient extender of nucleotides inserted by Pol δ opposite 8-OxoG (Haracska *et al.*, 2003).

Pol32—the smallest subunit of Pol δ—together with Pol ζ and Rev1 has been suggested to be involved in bypass of abasic sites (Haracska *et al.*, 2001;Gibbs *et al.*, 2005). Pol δ inserts a dAMP opposite an abasic site (Haracska *et al.*, 2001) and *in vitro* assays have shown that Pol δ can bypass abasic sites in the presence of PCNA (Garg *et al.*, 2005;Northam *et al.*, 2006).

2.12 DNA damage increases the dNTP levels

The regulation of dNTPs is controlled through several mechanisms. The transcription of RNR genes is increased during S phase, and thereby the levels of dNTPs. In budding yeast, Sml1p regulates RNR by inactivating the enzyme (Zhao *et al.*, 1998;Chabes *et al.*, 1999) and Dun1p inactivates Sml1p and upregulates the RNR genes (Zhou and Elledge, 1993).

RNR has two allosteric sites, the specificity site and the activity site. The allosteric activity site consists of a controlling mechanism that regulates the amount of ATP reduction to dATP. When dATP levels reach a certain concentration, the reduction is decreased—and thereby also the concentration of dNTPs in the cell. The allosteric activity site in *S. cerevisiae* has been found to be more insensitive to dATP levels than that in mammals (Domkin

et al., 2002). The specificity allosteric site is responsible for controlling the levels of the different dNTPs required in the cell.

After DNA damage, checkpoint proteins slow down or arrest the progress of the cell cycle to give the repair pathways the possibility to repair the damage before S phase. The checkpoint proteins also induce the transcription of RNR genes. For example, DNA damage induces expression of p53R2, which is a small RNR subunit in mammals (Tanaka *et al.*, 2000). It has been demonstrated that *Saccharomyces cerevisiae* increases its dNTP levels by 6- to 8-fold during DNA damage (Chabes *et al.*, 2003). This increase leads to improved survival, but also to an increased mutation rate—which has been suggested to be due to increased translesion synthesis by the TLS polymerases (Chabes *et al.*, 2003). This high increase in dNTP concentration has not been found in other organisms; however, it has been speculated that high dNTP levels exist in these organisms, but that these are compartmentalized close to the site of the DNA damage (Hakansson *et al.*, 2006b;Hakansson *et al.*, 2006a).

3. Aim of thesis

The aim of this thesis was to gain a better knowledge of the function of the replication fork by investigating the biochemical and/or genetic properties of yeast DNA polymerases in DNA replication and repair, the main focus being on the enigmatic Pol ε.

Specific aims:

- To investigate the role of Pol ε and Pol δ in processing Okazaki fragments.
- To obtain a structure of Pol ε for a better functional understanding of the enzyme.
- To understand the role of Pol ε when it encounters the most common DNA lesion in the cell.
- To identify the role of TLS polymerases and replicative polymerases in TLS during DNA damage at elevated dNTP concentration.

4. Results and discussion

4.1 Paper I - Okazaki fragment maturation

The aim of this particular study was to investigate the role of Pol δ and Pol ε in Okazaki fragment maturation, in order to determine which of these enzymes may function on the lagging strand.

A ^{32}P- end-labeled (30-mer) DNA primer and a downstream blocking oligonucleotide were together annealed to a 113-nt long DNA template to test the ability of Pol δ and of Pol ε in strand-displacement synthesis. To achieve a processive DNA synthesis, the assays were run with RPA bound to the single-stranded DNA and PCNA loaded on the DNA. We analyzed several forms of Pol δ and two forms of Pol ε, and found that all of them could replicate to the exact position of the nick or one to four nucleotides into the dsDNA (the blocking primer). We called this strand displacement ability "strand opening", since it only involved a few nucleotides and not the entire blocking primer.

The exonuclease-deficient forms of Pol δ produced a larger amount of strand-opening products compared to wild-type Pol δ, wild-type Pol ε, and surprisingly also than exonuclease-deficient Pol ε. Two other forms of Pol δ with *in vivo* defects in Okazaki fragment maturation also showed much more strand opening products than the wild-type Pol δ.

Turnover of dNTPs to dNMPs during DNA synthesis can occur during a process called idling. Idling is a process in which the polymerase incorporates and degrades the primer terminus without synthesizing or degrading any further. By measuring the first two nucleotides (dGMPs) displaced on the blocking primer, we could compare the ability of the DNA polymerases in idling. We observed that Pol δ could idle very well when encountering the blocking primer, but this was not the case for Pol ε. The idling of Pol δ decreased if we added DNA ligase I to the reactions, which sealed the nicks. Addition of FEN1 to the reactions eliminated the idling activity, due to efficient cutting by FEN1 of the flap that was produced.

By separating nicked or closed plasmid on an agarose gel, we also showed that Pol δ (but not Pol ε) was very efficient in performing nick closure together with FEN1 and DNA ligase I on a plasmid. In contrast, FEN1 and Pol ε did not collaborate efficiently together at the nick.

We demonstrated that the main products liberated from the downstream blocking primer by FEN1 and Pol δ were mononucleotides, indicating that repeated cycles of strand opening and degradation of the blocking primer occur.

We proposed a model whereby in the presence of FEN1, Pol δ displaces the downstream RNA primer, creating a flap. In this model, FEN1 cuts the created flap through multiple cycles by degrading mono- and dinucleotides. In the absence of FEN1, Pol δ idles at the nick. Both ways, Pol δ can maintain a ligatable nick, which is a crucial property for a lagging-strand DNA polymerase in processing Okazaki fragments. In conclusion, we proposed that Pol δ synthesizes DNA on the lagging strand and that Pol ε does not. In addition, we suggested that Pol ε replicates the leading strand, which was confirmed very recently (Pursell *et al.*, 2007).

4.2 Paper II - Structure of Pol ε

In this study, we used cryo-electron microscopy to determine the structure of Pol ε in order to gain a better understanding of its function.

By collecting 1,159 stained individual Pol ε particles and 19,240 unstained single-particle images, we were able to reconstruct the structure of Pol ε to a resolution of ~20 Å, the first structure of a eukaryotic multisubunit DNA polymerase to be published. The study revealed that Pol ε is composed of a globular head domain connected to an extended tail domain.

To be able to localize the subunits of Pol ε in the structure, we collected images of stained Pol2 subunit and stained Pol2/Dpb2 complex. From these images, we could reconstruct the structure of these molecules and concluded that Pol2 is the globular head domain and that Dpb2, Dpb3, and Dpb4 are located in the tail domain. By solving the structure of Pol2/Dpb2 complex, we expected to be able to detect where the smallest subunits of Pol ε, Dpb3 and Dpb4, are located. However, the reconstruction only showed a small increase in density compared to the Pol2 structure alone, although the size of Dpb2 is as large as 79 kDa. We believe that this result was due to a very flexible connection between Pol2 and Dpb2, and that this mobility is increased in the absence of the smallest subunits. In fact, by clustering the images of the largest group of stained Pol ε particles, we showed a flexibility of 25° to 70° between the head and the tail domain. Actually, by comparing the X-ray crystallographic structures of RB69 DNA polymerase in its polymerase or exonuclease mode, it can be seen that there is a large difference in orientation between these structures (Shamoo and Steitz, 1999;Franklin *et al.*, 2001). Thus, we suggest that the flexibility that we observed in Pol ε is not only important for its interaction with the mobile DNA helix, but also for the switching between Pol ε's polymerase and exonuclease active site.

The structure of Pol ε contains only one cleft in the Pol2 subunit that would be spacious enough to accommodate dsDNA, and this cleft faces the tail domain. From these observations, we proposed that the dsDNA may interact with the tail domain. To examine the position of dsDNA in our Pol ε

structure, we measured processivity by using primer extension assays with both Pol ε and Pol2 subunit under single-hit conditions and with primer-templates of various lengths. Accurate binding of the dsDNA with the tail domain would provide a processive DNA synthesis, while an inaccurate binding would provide a distributive DNA synthesis. We determined that Pol ε required 40 bp or longer of dsDNA to perform processive DNA synthesis and that our negative control, the Pol2 subunit, lacking the tail domain was only able to perform distributive DNA synthesis. In fact, the length from the end of the tail domain to the proposed active site-containing cleft is 40 bp long, thus supporting our model for the location of double-stranded DNA helix positioned on the tail domain. From these observations, we proposed that the intrinsic processivity of Pol ε is due to its tail domain.

4.3 Paper III - Pol ε and abasic sites

To understand how the leading strand operates when it encounters a DNA lesion, we wanted to determine the action of the leading-strand Pol ε when meeting the most frequent lesion in eukaryotes, an abasic site. We used primer extension assays and used three different template sequences containing a tetrahydrofuran moiety mimicking an abasic site. To our surprise, the replicative exonuclease-proficient Pol ε was able to bypass the abasic site under single-hit condition. Next, we tested exonuclease-deficient Pol ε (Pol ε exo$^-$) and expected an even higher increase in bypass—due to the inactivation of the proofreading ability—but, surprisingly under single-hit conditions bypass of the lesion was not dramatically increased compared to the result with wild-type Pol ε. These results contrast with previous work on T4 DNA polymerase, were bypass of abasic sites was found to be allowed only with an inactivated exonuclease activity; the later results were, however, not obtained under single-hit conditions (Tanguy Le *et al.*, 2004). The insertion efficiency calculated for Pol ε was lower than for Pol ε exo$^-$; however, the extension efficiency of Pol ε was significantly higher than for Pol ε exo$^-$. We also found that the bypass efficiency was dependent on the sequence of the template.

To test whether the tail domain of Pol ε was involved in the TLS bypass of abasic sites, we used two strategies. The first strategy was to purify different variants of Pol ε that lacked the entire tail domain or parts of it. The second strategy was to use a shorter dsDNA, so that the short DNA duplex would not establish a completely functional contact with the tail domain of Pol ε. We purified Pol2/Dpb2 (Pol2/Dpb2 exo$^+$), Pol2 (Pol2 exo$^+$), and exonuclease deficient Pol2/Dpb2 (Pol2/Dpb2 exo$^-$) and tested them with the same long templates as earlier in this study. We found that overall the exonuclease-proficient variants of Pol ε (Pol2/Dpb2 exo$^+$ and Pol2 exo$^+$) and the exonuclease-deficient variant of Pol ε (Pol2/Dpb2 exo$^-$) had less lesion bypass efficiency than Pol ε exo$^+$ and Pol ε exo$^-$, respectively. The

importance of proper binding of the DNA duplex to the tail domain was even more obvious when we performed the experiments with the short dsDNA. Under these conditions, TLS by Pol ε exo$^+$ was prohibited and Pol ε exo$^-$ was only capable of adding one nucleotide upstream of the abasic site. These observations show the importance of proper binding of the primer-template to the tail domain and processive DNA synthesis during TLS of an abasic site by Pol ε.

We allowed all variants of Pol ε to reinitiate DNA synthesis by adding excess polymerase to the primer-template and by running the reactions for 10 min. The lesion bypass capability of exonuclease-proficient variants of Pol ε did not increase, however, the exonuclease-deficient variants, Pol ε exo$^-$ and Pol2/Dpb2 exo$^-$, were capable of increasing their bypass of the lesion considerably. Once again, the tail domain of Pol ε played an important role in the bypass of the lesion, even with multiple reinitiation opportunities. This also shows that with an inactivated exonuclease activity, Pol ε can extend from the nucleotide inserted opposite the abasic site; however, wild-type Pol ε with the exonuclease activity excises the nucleotide opposite the abasic site rather than extend it. These results may suggest that exonuclease-proficient Pol ε, only has one opportunity to bypass the abasic sites before dissociating from the template.

In this study, we also developed an assay to examine which nucleotide Pol ε prefers to add opposite the abasic site and showed that Pol ε follows the "A-rule" by inserting a dAMP opposite a model abasic site.

4.4 Paper IV - Lesion bypass and dNTP pools

S. cerevisiae increases its dNTP levels in response to DNA damage, resulting in better survival (Chabes *et al.*, 2003). This improved survival has been suggested to be due to more efficient bypass of DNA lesions by TLS polymerases (Chabes *et al.*, 2003).

To investigate whether this proposition was correct, we deleted all yeast nuclear DNA polymerases including *REV1*, *RAD30* (Pol η), *REV3* (the catalytic subunit of Pol ζ), and *POL4,* but excluding the three essential replicative polymerases Pol α, Pol ε, and Pol δ. Apart from constructing single- and multiple-deletion strains with each polymerase alone or all of the polymerases deleted together, we also established one other kind of strain. These other strains not only contained the deletions mentioned above but also had an extra *RNR1* gene introduced into the genome. This extra *RNR1* gene was under control of the *GAL1*-promotor. In these strains we were able to increase the dNTP concentration by a factor of 9-13 by inducing the RNR1 gene with galactose. To determine whether a certain TLS polymerase was able to perform a more efficient TLS at high dNTP concentrations in presence of DNA damage, we tested all the different yeast strains in the

presence or absence of the overexpressed *RNR1* and treated the cells with the DNA damaging agent 4-NQO. 4-NQO is a stable agent that produces 80% quinoline adducts and 20% 7,8-dihydro-8-oxoguanine (8-OxoG) (Kohda *et al.*, 1986). To our surprise, increase in the dNTP concentration improved the cell survival of all single- and multiple polymerase-deleted strains. The improved survival is not due to activation of checkpoint proteins (Chabes and Stillman, 2007). The results suggest that in the presence of high dNTP concentrations, some adducts produced by 4-NQO can be bypassed by replicative polymerases.

To test whether the replicative polymerases are capable of bypassing DNA adducts in the presence of dNTPs at high concentrations, we performed primer extension assays with Pol ε and undamaged and damaged template. The damaged template contained an 8-OxoG four positions downstream of the 3'end of the annealed primer. The experiments were performed with low, normal (S phase dNTP concentrations) and high (damage state dNTP concentration) dNTP levels, with an excess of Pol ε relative to primer-template or under single-hit conditions. When Pol ε was allowed to reinitiate DNA synthesis multiple times, the 8-OxoG lesion bypass with S phase dNTP levels and damage-state dNTP levels was 19% and 93%, respectively. These observations suggested that with damage-state dNTP levels, Pol ε is almost as efficient at bypassing the 8-OxoG as in bypassing an undamaged G (100% bypass). Under single-hit conditions, Pol ε was not able to bypass the 8-OxoG at S phase dNTP levels. However, at damage-state dNTP levels, Pol ε had an approximate bypass probability of 20%. These results suggest that on every fifth occasion that the leading-strand polymerase Pol ε encounters an 8-OxoG at damage-state dNTP levels, it may bypass it without dissociating from the template. From our *in vitro* and *in vivo* data, we proposed that an increased dNTP concentration during DNA damage may allow replicative DNA polymerases to bypass certain less bulky DNA lesions.

5. Concluding Remarks

In *Escherichia coli*, DNA polymerase III replicates both the leading and the lagging strand. Thus, over the years many models concerning one or two replicative polymerases on the eukaryotic replication fork have been suggested. It has been proposed that Pol δ is synthesizes both strands, or Pol ε replicates the lagging strand and Pol δ the leading strand—or vice versa (Burgers, 1998). We have shown that together with FEN1, Pol δ maintains a ligatable nick through idling during replication of the lagging strand. We were also able to show that Pol ε is not involved in the processing of Okazaki fragments, which suggest Pol δ is the lagging strand polymerase and not Pol ε. Based on these results, we proposed that Pol ε may participate in leading strand replication and this proposal was very recently shown to be correct (Pursell *et al.*, 2007). However, it is important to keep in mind that these data

do not exclude the possibility that Pol δ/Pol ε would participate in synthesis of the other strand on some occasions.

By using cryo-electron microscopy, it was possible to determine the structure of Pol ε at a resolution of ~20 Å. This was the first structure of a eukaryotic multi-subunit DNA polymerase to be published. We determined that Pol ε is composed of a globular "head" domain consisting of Pol2p linked to a flexible extended "tail" domain, consisting of Dpb2p, Dpb3p, and Dpb4p. We also observed that the intrinsic processivity of Pol ε is dependent on the interaction between the tail domain and the primer-template. The processivity of Pol ε is enhanced by the interaction of PCNA and is comparable to the processivity of Pol δ together with PCNA (Chilkova *et al.*, 2007). Nevertheless, DNA synthesis by Pol ε is not as efficient as that by Pol δ (Chilkova *et al.*, 2007). The less efficient DNA synthesis of Pol ε, has been suggested to be due to easier binding of PCNA to Pol δ than to Pol ε (Chilkova *et al.*, 2007). Unfortunately, the structure of Pol δ is not available yet, but it is tempting to speculate that the less efficient binding of PCNA to Pol ε may be due to the tail domain, if PCNA binds in-between the head domain and the tail domain.

Abasic sites are the most common DNA lesions in a eukaryotic cell. We showed that Pol ε can perform translesion synthesis through an abasic site in the absence of external processivity clamps under single-hit conditions. It has been shown that the lagging-strand Pol δ is also capable in bypassing abasic sites, however only in the presence of PCNA (Garg *et al.*, 2005;Northam *et al.*, 2006). We have demonstrated that the lesion bypass by Pol ε is dependent on the sequence of the template, and also on proper interaction of the tail domain with the primer-template, showing the importance of the tail domain of Pol ε in TLS. When comparing our data to published data, we observed that the extension efficiencies for Pol ε are more comparable to data for TLS polymerases such as *Sulfolobus solfataricus* Dpo4 and human Pol η (Kokoska *et al.*, 2003), than for replicative polymerases such as T7 DNA polymerase and T4 DNA polymerase (McCulloch and Kunkel, 2006;Tanguy Le *et al.*, 2004). These differences may be due to a more different active site of Pol ε than T4 DNA polymerase or T7 DNA polymerase. In fact, inactivation of the exonuclease activities of Pol ε, T4 DNA polymerase and T7 DNA polymerase in *lac Z* fidelity assays show a 4−6-fold higher base substitution rate for Pol ε exo$^-$ than for T7 DNA polymerase exo$^-$ and T4 DNA polymerase exo$^-$ (Shcherbakova *et al.*, 2003). For a deeper understanding, an X-ray crystallographic structure of Pol ε with a primer-template containing an abasic site bound to the active site of Pol ε would be very helpful. We also showed that Pol ε follows the A-rule (similar to results shown for Pol δ) by inserting a dATP opposite a model abasic site (Haracska *et al.*, 2001). It is important to point out that a dAMP might not be inserted opposite a natural abasic site in the cell, due to the OH residue on the natural

abasic site (Fig. 6). Thus, *in vivo* data regarding what is inserted opposite the abasic site may not reflect the *in vitro* data. To avoid stalling of the replication fork, the bypass properties of the different polymerases in the cell may have evolved differently at different parts of the chromatin, and also based on the origin of the abasic site. For example, if the source of the abasic sites in the cell is excision of dUMP (Guillet *et al.*, 2006), an insertion of dAMP by Pol ε opposite the abasic sites would be a non-mutagenic bypass.

The increase in dNTP levels in *S. cerevisiae* in response to DNA damage results in improved survival (Chabes *et al.*, 2003). This improved survival has been suggested to be due to more efficient bypass of DNA lesions by TLS polymerases. By applying genetics, we showed that yeast strains exposed to the DNA damaging agent 4-NQO (which induces quinoline adducts and 8-OxoG) had improved survival at elevated dNTP levels, even though they lacked all TLS polymerases, suggesting that damage is bypassed by the replicative polymerases. By running primer extension assays, we observed that Pol ε has the ability to bypass 8-OxoG at high dNTP levels. It has been shown that Pol δ can also bypass 8-OxoG at high dNTP concentrations (Haracska *et al.*, 2003). Thus, we suggest a new bypass pathway during elevated dNTP levels in *S. cerevisiae*, in which the replicative polymerases can bypass less bulky DNA lesions. This pathway may perhaps also exist in other organisms, in response to a local increase in dNTP levels, since a similar increase in dNTP levels has not been found. An important and exciting experiment would be to investigate which nucleotide is inserted opposite the 8-OxoG by Pol ε, since insertion of a dCMP would be a non-mutagenic bypass on the leading strand. However, an insertion of dAMP opposite the 8-OxoG, such as seen for Pol δ (Haracska *et al.*, 2003), would be a mutagenic bypass.

In conclusion, one should not forget that synthesis of the DNA strands occurs differently on the leading and the lagging strand. These differences may also reflect the lesion bypass properties of the different polymerases, since the discontinuous DNA synthesis of the lagging strand can leave lesions behind without arresting the progression of the replication fork.

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