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THE ROLE OF HUMAN  
REPLICATIVE DNA  
POLYMERASES IN DNA  
REPAIR AND REPLICATION

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**ANNA RYTKÖNEN**

**THE ROLE OF HUMAN REPLICATIVE  
DNA POLYMERASES IN DNA REPAIR  
AND REPLICATION**

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## **Rytkönen, Anna, The role of human replicative DNA polymerases in DNA repair and replication**

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### ***Abstract***

The maintenance of integrity of the genome is essential for a cell. DNA repair and faithful DNA replication ensure the stability of the genome. DNA polymerases (pols) are the enzymes that synthesise DNA, a process important both in DNA replication and repair. In DNA replication DNA polymerases duplicate the genome during S phase prior to cell division. Pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  are implicated in chromosomal DNA replication, but their exact function in replication is not yet completely clear. The mechanisms of different repair pathways and proteins involved are not yet completely characterised either. The deeper understanding of DNA repair and replication mechanisms is crucial for our understanding on the function of the cell.

The mechanism of repair of DNA double strand breaks (DSBs) by non-homologous end joining (NHEJ) was studied with an *in vitro* assay. DNA polymerase activity was found to be involved in NHEJ and important in stabilising DNA ends. Antibodies against pol  $\alpha$ , but not pol  $\beta$  or  $\epsilon$ , decreased NHEJ significantly, which indicates the involvement of pol  $\alpha$  in NHEJ. In addition, the removal of proliferating cell nuclear antigen (PCNA) slightly decreased NHEJ activity.

The division of labour between pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  during DNA replication was studied. Results from UV-crosslinking, chromatin association, replication in isolated nuclei, and immunoelectron microscopy (IEM) studies showed that there are temporal differences between the activities and localisations of the pols during S phase. Pol  $\alpha$  was active throughout S phase, pol  $\epsilon$  was more active at early S phase, whereas the activity of pol  $\delta$  increased as S phase advanced. These results suggest that pols  $\delta$  and  $\epsilon$  function independently during DNA replication.

Pol  $\epsilon$  could be crosslinked to nascent RNA, and this labelling was not linked to DNA replication, but rather to transcription. Immunoprecipitation studies indicated that pol  $\epsilon$ , but not pols  $\alpha$  and  $\delta$ , associated with RNA polymerase II (RNA pol II). Only the hyperphosphorylated, transcriptionally active RNA pol II was found to associate with pol  $\epsilon$ . A large proportion of pol  $\epsilon$  and RNA pol II colocalised in cells as determined with immunoelectron microscopy. The interaction between pol  $\epsilon$  and RNA pol II suggests that they are involved in a global regulation of transcription and DNA replication.

**Keywords:** DNA replication, non-homologous end joining, proliferating cell nuclear antigen, RNA polymerase II



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Oulu, June 2006

Anna Rytönen



## Abbreviations

BER	base excision repair
CIP	calf intestinal phosphatase
CS	Cockney syndrome
CSA	Cockayne syndrome A protein
CSB	Cockayne syndrome B protein
CTD	carboxyterminal domain
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSB	double strand break
DSBR	double strand break repair
ECL	enhanced chemiluminescence
GGR	global genome repair
HNPCC	hereditary nonpolyposis colorectal cancer
HR	homologous recombination
ICL	interstrand crosslinks
IDL	insertion deletion loops
IEM	immunolectron microscopy
IPTG	isopropyl-beta-D-thiogalactoside
IR	ionising radiation
MCM	minichromosome maintenance
MMR	mismatch repair
NBS	Nijmegen breakage syndrome
NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORC	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
Pol	DNA polymerase
RFC	replication factor C
RNA	ribonucleic acid

RNA pol RNA polymerase  
RPA replication protein A  
SDS sodium dodecyl sulphate  
SUMO small ubiquitin-like modifier  
SV40 Simian virus 40  
T antigen SV40 large tumor antigen  
TCR transcription coupled repair  
TdT terminal deoxynucleotidyl transferase  
TFIIH transcription factor IIH  
TLS translesion synthesis  
UV ultraviolet  
V(D)J variable-diversity-joining  
WRN Werner syndrome protein  
XP Xeroderma pigmentosum  
XRCC X-ray repair cross complementing

## List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Pospiech H\*, Rytönen AK\* & Syväoja JE (2001) The role of DNA polymerase activity in human non-homologous end joining. *Nucleic Acids Res* 29: 3277-3288.
- II Rytönen AK, Vaara M, Nethanel T, Kaufmann G, Sormunen R, Läärä E, Nasheuer H-P, Rahmeh A, Lee MYWT, Syväoja JE & Pospiech H (2006) Distinctive activities of DNA polymerases during human DNA replication. *Febs J* 273: 2984-3001.
- III Rytönen AK\*, Hillukkala T\*, Vaara M, Sokka M, Jokela M, Sormunen R, Nasheuer H-P, Nethanel T, Kaufmann G, Pospiech H & Syväoja JE (2006) DNA polymerase  $\epsilon$  associates with the elongating form of RNA polymerase II and nascent transcripts. Submitted.

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# 1 Introduction

DNA is the carrier of the genetic information. The diversity of life on this planet results from different nucleotide sequences encoded in our genes. DNA is constantly challenged because of damaging agents in the environment, e.g. ultraviolet (UV) radiation from the sun, ionising radiation (IR), and chemicals from our environment and diet insult DNA. In addition, DNA is damaged by internal factors, bases in DNA are spontaneously hydrolysed, the oxidative metabolism of the cell causes lesions, and mistakes arise during DNA replication. Mutations arising in important genes may lead to cancer. Yet, mutations are the driving force of evolution.

In the cell, multiple mechanisms ensure the integrity of the genome. DNA replication, the process where DNA is duplicated prior to cell division, is extremely accurate and multiple DNA repair pathways correct DNA damage. Checkpoints ensure that the cell cycle does not proceed in the event of major DNA damage. Despite the fact that e.g. DNA replication has been studied for decades, the picture is not yet completely clear. The deeper understanding of DNA repair and replication mechanisms is crucial for our understanding on the function of the cell.

In this study, various aspects of nucleic acid metabolism in the cell were studied. First, the mechanism of DNA double strand break repair by non-homologous end joining was studied with an *in vitro* assay. DNA synthesis was discovered to play an important role in NHEJ and to stabilise DNA at the break site. In particular, pol  $\alpha$  was implicated in DNA synthesis during NHEJ. In addition, PCNA was found to be involved in NHEJ. Second, the roles of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  in DNA replication were studied. The results from biochemical and cell biological experiments suggest that pol  $\delta$  and pol  $\epsilon$  function independently during DNA replication. Pol  $\epsilon$  was more involved in DNA replication at early S phase whereas pol  $\delta$  activity increased as S phase advanced. Third, pol  $\epsilon$  was found to associate with transcriptionally active RNA polymerase II. Pol  $\epsilon$ , but not pols  $\alpha$  and  $\delta$ , and RNA pol II were found to coimmunoprecipitate, and a large proportion of RNA pol II and pol  $\epsilon$  colocalised in cells as determined with immunoelectron microscopy.

## 2 Review of the literature

### 2.1 Eukaryotic DNA polymerases

#### *2.1.1 Classification and characteristics of DNA polymerases*

DNA polymerases are enzymes that catalyse the addition of deoxyribonucleotides to a growing polynucleotide chain (Kornberg & Baker 1992). In eukaryotes, at least 15 DNA polymerases have been characterised so far. Pols are template-dependent and they require a primer as well as a 3' free hydroxyl group; they cannot start synthesising DNA *de novo*. The pyrophosphate (PP<sub>i</sub>) released in the reaction is subsequently hydrolysed providing energy for DNA replication. All pols seem to have a conserved overall structure, their shape resembles a right hand, and furthermore, the thumb, palm, and finger domains can be distinguished (Steitz 1999). However, the fine structures of pols differ; the palm domain is more conserved whereas the thumb and the finger domains are more diverged among pols. Analyses of structures of pols from different families suggest that a two-metal-ion-catalysed mechanism is utilised. The fidelity of DNA synthesis is secured by the proofreading exonuclease activity associated with some pols and post-replicative repair.

DNA polymerases are essential in various transactions of the cell (Hübscher *et al.* 2000). Pols are required in DNA replication, DNA repair, translesion DNA synthesis, and DNA recombination. In addition, pols are important in regulatory events. The significant functions of pols in the cell and their great number suggest that there exists a safety mechanism so that the same function can be performed with different pols. Eukaryotic pols are classified to families A, B, X, and Y according to sequence analysis. Family A pols are named after their homology to the *Escherichia coli* pol I, and family B pols are named after their homology to *E. coli* pol II (Ito & Braitwhaite 1991). Family Y contains recently identified pols that share significant amino acid sequence identity and similarity amongst themselves (Ohmori *et al.* 2001). Table 1 summarises the essential information about eukaryotic pols.



*Table 1. Eukaryotic DNA polymerases*

Name	Family	Function(s) <sup>1</sup>
Pol $\alpha$	B	Replication, repair (HR), checkpoint, telomere maintenance
Pol $\beta$	X	Repair (BER, NHEJ)
Pol $\gamma$	A	Mitochondrial DNA replication and repair
Pol $\delta$	B	Replication, repair (BER, MMR, NER, HR), telomere maintenance, recombination
Pol $\epsilon$	B	Replication, repair (BER, HR, NER), checkpoint
Pol $\zeta$	B	Translesion synthesis, antibody diversity generation
Pol $\eta$	Y	Translesion synthesis, antibody diversity generation
Pol $\theta$	A	Repair (ICL), antibody diversity generation
Pol $\iota$	Y	Translesion synthesis
Pol $\kappa$	Y	Translesion synthesis
Pol $\lambda$	X	Meiotic repair, repair (BER, NHEJ), antibody diversity generation
Pol $\mu$	X	Repair (NHEJ), antibody diversity generation
Pol $\sigma$	X	Sister chromatid cohesion
Pol $\phi$	B	rRNA synthesis
Rev1	Y	Translesion synthesis

<sup>1</sup> BER = Base excision repair, HR = Double strand break repair by homologous recombination, ICL = Interstrand crosslinks, MMR = Mismatch repair, NER = Nucleotide excision repair, NHEJ = Non-homologous end joining

## ***2.1.2 Replicative DNA polymerases and replication auxiliary factor***

### ***2.1.2.1 DNA polymerase $\alpha$***

Pol  $\alpha$  consists of four polypeptides, 180, 68, 55, and 48 kDa subunits in mammals (reviewed by Hübscher *et al.* 2002, Muzi-Falconi *et al.* 2003). The largest subunit possesses DNA polymerase activity, the next largest has no known enzymatic activities, but it is thought to mediate interactions or to have a regulatory role. The two smallest subunits are required for primase activity. The smallest subunit contains primase activity itself, but the second smallest subunit is required for optimal stability (Schneider *et al.* 1998).

The primase activity of pol  $\alpha$  is crucial for its function in DNA replication (Hübscher *et al.* 2000). First, the primase creates a short stretch of RNA. Subsequently, the RNA primer is further elongated by the pol  $\alpha$  catalytic subunit that synthesises approximately 30-40 nucleotides of DNA. Pol  $\alpha$  functions in DNA replication in priming the leading strand and Okazaki fragments in the lagging strand. Not surprisingly, pol  $\alpha$  is essential for viability in yeast (reviewed by Foiani *et al.* 1997) and evidently in mammals as well (Kaczmarek *et al.* 1986).

In addition to its role in DNA replication, pol  $\alpha$  has other functions in the cell. It is suggested to work in homologous recombination repair of double strand breaks (HR) (Holmes & Haber 1999). Pol  $\alpha$  is also implicated in telomere metabolism (Diede &

Gottschling 1999, Nakamura *et al.* 2005) and in cell cycle control (Michael *et al.* 2000), and in this way it is important in stabilising the genome.

### 2.1.2.2 DNA polymerase $\delta$

Pol  $\delta$  in mammals is arranged from four subunits, 125, 66, 50, and 12 kDa in size (reviewed by Hübscher *et al.* 2000, 2002). In fission yeast, *Schizosaccharomyces pombe*, pol  $\delta$  contains five subunits whereas in budding yeast, *Saccharomyces cerevisiae*, three subunits have been identified so far. The polymerase activity resides in the largest subunit. In addition, the largest subunit has exonuclease activity that removes misincorporated nucleotides. Proofreading by pol  $\delta$  exonuclease guarantees high fidelity of DNA synthesis (Shevelev & Hübscher 2002). Mice with point mutation in the proofreading domain of pol  $\delta$  and thus deficient in pol  $\delta$  proofreading are cancer prone, which indicates the importance of fidelity of DNA replication in the prevention of cancer (Goldsby *et al.* 2001, 2002). The smaller subunits have no known enzymatic activities, but they are important for the architecture and intersubunit interactions of pol  $\delta$ .

Pol  $\delta$  is an essential enzyme in DNA replication where it synthesises the bulk of the DNA (reviewed by Johnson & O'Donnell 2005). However, its specific role in DNA replication, together with pol  $\epsilon$ , in leading and lagging strand synthesis is still unclear. The role of pol  $\delta$  in DNA replication will be described in more detailed in the following chapter. In DNA replication, pol  $\delta$  is associated with the replication auxiliary factors PCNA and replication factor C (RFC), and together they form a moving platform for highly processive DNA synthesis. The association of PCNA and pol  $\delta$  is mediated with three pol  $\delta$  subunits (Brown & Campbell 1993, Reynolds *et al.* 2000, Pohler *et al.* 2005). Earlier, pol  $\delta$  was thought to form a dimer, but recent studies implicate a monomeric pol  $\delta$  complex (Johansson *et al.* 2001, Bermudez *et al.* 2002).

Besides DNA replication, pol  $\delta$  has other functions in the cell. Pol  $\delta$  is implicated in long patch base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and in the repair of double strand breaks by homologous recombination (Wang *et al.* 1993, Blank *et al.* 1994, Longley *et al.* 1997, Holmes & Haber 1999, Araújo *et al.* 2000). In *S. cerevisiae*, pol  $\delta$  is suggested to work in meiotic recombination in addition to simple gap repair (Maloisel *et al.* 2004) and to function in telomere maintenance (Diede & Gottschling 1999). Studies in *S. pombe* hint of an involvement of pol  $\delta$  in cell cycle control (Francesconi *et al.* 1993, 1995).

### 2.1.2.3 DNA polymerase $\epsilon$

Pol  $\epsilon$  in mammals is a complex of four subunits (reviewed by Pospiech & Syväoja 2003). The catalytic A subunit (261 kDa) has the polymerase activity itself and the 3'-5' exonuclease activity for proofreading, thus ensuring the high fidelity of pol  $\epsilon$ . The B subunit (59 kDa) has no known enzymatic activities but together with the catalytic subunit they both are essential in yeast (Morrison *et al.* 1990, Araki *et al.* 1991a). The B subunits are considered to be important in multimerisation, protein-protein interactions,

and regulation. The B subunit in yeast (Dpb2) was found to be phosphorylated in a cell cycle-dependent manner (Kesti *et al.* 2004). The two smallest C and D subunits (12 and 17 kDa) contain histone-fold motifs and they form a stable dimer that interacts with the A and B subunits (Li *et al.* 2000). The two smallest subunits are not essential in *S. cerevisiae* (Araki *et al.* 1991b, Ohya *et al.* 2000), whereas in *S. pombe* the C subunit (Dpb3) is essential but the D subunit (Dpb4) is not (Spiga & D'Urso 2004). Recent evidence suggests that pol  $\epsilon$  forms a heterotetramer with a stoichiometry of 1:1:1:1 (Chilkova *et al.* 2003). The structure of *S. cerevisiae* pol  $\epsilon$  at 20 Å resolution has been solved by using cryo-electron microscopy, and pol  $\epsilon$  seems to contain two separate domains joined by a hinge (Asturias *et al.* 2006).

Pol  $\epsilon$  is considered to play an essential role in chromosomal DNA replication as judged from genetic and biochemical studies (Johnsson & O'Donnel 2005). However, the exact role of pol  $\epsilon$  in DNA replication has remained elusive. The possible roles of pol  $\epsilon$  in DNA replication will be discussed in more detailed in the following chapter. Pol  $\epsilon$  is associated with and stimulated by replication factors PCNA and RFC (Lee *et al.* 1991, Podust *et al.* 1992, Maga & Hübscher 1995), but pol  $\epsilon$  does not absolutely require PCNA for processive DNA synthesis (Syväoja & Linn 1989, Chui & Linn 1995). In addition, pol  $\epsilon$  seems to have greater fidelity than pol  $\delta$  (Chen *et al.* 2000a, Shimizu *et al.* 2002).

Pol  $\epsilon$  catalytic subunit has an interesting feature in its structure; it contains a large C-terminus of unclear function (Hübscher *et al.* 2000). Studies in yeast showed that the carboxyterminal portion of pol  $\epsilon$  is essential and sufficient for viability whereas the polymerase and exonuclease domains of pol  $\epsilon$  are not required for DNA replication, DNA repair, and viability (Dua *et al.* 1999, Kesti *et al.* 1999). However, point mutations in the catalytic residues were lethal (Dua *et al.* 1999) and the polymerase domain of pol  $\epsilon$  was required for efficient DNA replication and telomere length maintenance (Ohya *et al.* 2002). These results suggest that pol  $\epsilon$  is essential for chromosomal replication, but when the catalytic domain is absent, another pol can substitute for pol  $\epsilon$ . If the catalytic domain is inactive, however, it blocks the site and prevents the activity of a substitute pol.

Pol  $\epsilon$  has been linked to many other cellular processes in addition to DNA replication. Pol  $\epsilon$  has been implicated in base excision repair (Wang *et al.* 1993), nucleotide excision repair (Araújo *et al.* 2000), and in the repair of double strand breaks by homologous recombination (Jessberger *et al.* 1996, Holmes & Haber 1999). Pol  $\epsilon$  and the other replication proteins, PCNA, RFC, and Cdc45, were found to have a role in transcriptional silencing in *S. cerevisiae* (Ehrenhofer-Murray *et al.* 1999). In addition, pol  $\epsilon$  has been linked to the epigenetic inheritance of telomeres in *S. cerevisiae* (Iida & Araki 2004). The mouse B subunit was found to interact with proteins involved in chromatin modification (Wada *et al.* 2002). These studies suggest that DNA replication machinery, and especially pol  $\epsilon$  are important in maintaining chromatin structure. Pol  $\epsilon$  has been suggested to play a role in sister chromatid cohesion as well (Edwards *et al.* 2003).

Pol  $\epsilon$  has a role in the S phase checkpoint in budding yeast (Navas *et al.* 1995, 1996) whereas in fission yeast pol  $\epsilon$  has not been implicated in checkpoint control (D'Urso & Nurse 1997). Pol  $\epsilon$  has been shown to interact with various proteins in the cell e.g. with MDM2, which is a regulator of tumor suppressor protein p53 (Vlatkovic *et al.* 2000), and human checkpoint protein Rad17 (Post *et al.* 2003). In addition, pol  $\epsilon$  was found to purify with the RNA pol II complex (Maldonado *et al.* 1996). These findings support the role of pol  $\epsilon$  in various transactions of the cell in addition to its function in DNA replication.

### 2.1.2.4 Proliferating cell nuclear antigen

PCNA is a multifunctional protein in DNA metabolism (reviewed by Maga & Hübscher 2003). PCNA was first discovered from patients with the autoimmune disease *systemic lupus erythematosus* by Miyachi and coworkers in 1978. Later, PCNA has been shown to have an important role in DNA replication, repair, and cell cycle control. Analysis of the structure of PCNA from yeast at 2.3 Å resolution revealed that three PCNA monomers form a ring that can encircle duplex DNA (Krishna *et al.* 1994), and further it was suggested, that two homotrimers may function as a complex (Naryzhny *et al.* 2005).

PCNA has a well-established role in DNA replication where it forms a sliding clamp and enhances the processivity of pols  $\delta$  and  $\epsilon$  (Prelich *et al.* 1987, Prelich & Stillman 1988, Maga & Hübscher 1995). PCNA has been linked to nucleotide excision repair (Shivji *et al.* 1992), mismatch repair (Umar *et al.* 1996), base excision repair (Matsumoto *et al.* 1994), translesion synthesis (TLS) (Haraeska *et al.* 2001), post-replication repair (Hoege *et al.* 2002), and to non-homologous end joining of DNA double strand breaks (Balajee & Geard 2001). In addition, PCNA interacts with many proteins involved in cell cycle control and may link DNA replication to the cell cycle machinery. Many proteins interacting with PCNA contain a consensus binding motif in their structure that mediates the interaction (Warbrick 2000). PCNA is regulated by p53 (Morris *et al.* 1996) and it interacts with p21, an important regulator of cell cycle progression (Waga *et al.* 1994a). Thus, PCNA mediates the response of cells to damage. PCNA modification by the addition of ubiquitin and SUMO (small ubiquitin-like modifier) may direct PCNA to different functional pathways (Hoege *et al.* 2002). PCNA, given its many functions and interacting partners, is well suited for coordinating DNA metabolism.

### 2.1.3 Other DNA polymerases

Pol  $\beta$  was first considered to be a repair enzyme, and indeed, it is an important player in the repair of damaged bases by BER (Beard & Wilson 2000). In addition, in yeast Pol4, a homolog of mammalian pol  $\beta/\lambda$  was implicated in NHEJ of certain end configurations (Wilson & Lieber 1999). Additional novel functions have emerged for pol  $\beta$ , e.g. analysis of pol  $\beta$ -deficient mice revealed abnormal neurogenesis characterised by apoptotic cell death in the developing central and peripheral nervous systems (Sugo *et al.* 2000).

Pol  $\gamma$  is a mitochondrial pol responsible for DNA replication in mitochondria, and it is most likely involved in repair and recombination in mitochondria as well (Kaguni 2004, Graziewicz *et al.* 2006). Pol  $\gamma$  consists of two subunits and it possesses proofreading activity. Interestingly, mice with defective pol  $\gamma$  proofreading activity were found to age prematurely suggesting a link between mitochondrial replication fidelity and ageing (Trifunovic *et al.* 2004).

For long time just five DNA polymerases (pols  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ) were characterised and thought to fulfil the needs for DNA synthesis in cell. Progress in biochemical methods and genomics contributed to the discovery of at least 10 novel pols in the last few years. Many of the novel pols are required for translesion synthesis (reviewed by Lehmann 2005, Prakash *et al.* 2005). Despite efficient repair machinery, not all lesions in

DNA are instantly repaired. These lesions will block transcriptional elongation by RNA pol II and DNA replication by DNA polymerases. Specific pols, namely pols  $\zeta$ ,  $\eta$ ,  $\iota$ ,  $\kappa$ , and Rev1 can replicate damaged DNA in a process called translesion synthesis (Nelson *et al.* 1996a,b, Masutani *et al.* 1999a, Johnson *et al.* 2000a, Ohashi *et al.* 2000). These pols have lower stringency than replicative pols, and their active site is more open to fit damaged bases. These pols may have specificities for different kinds of damage and/or they may play different roles during TLS. One of these pols may insert a nucleotide opposite to a damage site, whereas another pol maybe required for extension after damage.

Pol  $\theta$  was first proposed to repair interstrand crosslinks according to extensive homology to *Drosophila mus308*, which encodes a protein that functions in this repair pathway (Sharief *et al.* 1999, Johnson *et al.* 2000b). Subsequently, pol  $\theta$  was shown to play an important role in somatic hypermutation, a process necessary for generation of high-affinity antibodies (Masuda *et al.* 2005, Zan *et al.* 2005). Pol  $\lambda$  shares homology with pol  $\beta$ , and it has been linked to DNA repair associated with meiosis, base excision repair, and repair of DNA double strand breaks by non-homologous end joining (Garcia-Diaz *et al.* 2000, 2001, Lee *et al.* 2004b). Pol  $\mu$  has been linked to NHEJ as well (Mahajan *et al.* 2002). In addition, pol  $\mu$  is preferentially expressed in peripheral lymphoid tissues, and it takes part in Ig gene rearrangement (Dominguez *et al.* 2000, Bertocci *et al.* 2003). In addition, pol  $\eta$ , pol  $\zeta$ , and pol  $\lambda$  may have roles in the generation of antibody diversity (reviewed by Seki *et al.* 2005).

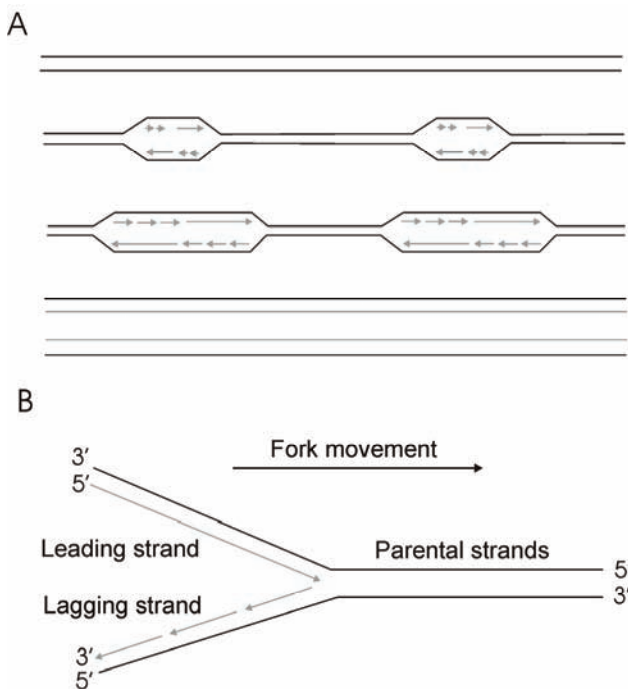
Pol  $\sigma$ , formerly named pol  $\kappa$ , is required for sister chromatid cohesion (Wang *et al.* 2000). Pol  $\phi$  localises exclusively to the nucleolus, and is involved in regulating the synthesis of ribosomal RNA (Shimizu *et al.* 2002). Given the different properties of the new pols and the multiple tasks where DNA synthesis is required, it is likely that further novel functions will be identified for them.

## 2.2 Eukaryotic DNA replication

DNA replication is a coordinated process where DNA is duplicated once, and only once, prior to cell division (reviewed by Waga & Stillman 1998, Bell & Dutta 2002, Alberts 2003). DNA replication requires the concerted action of numerous proteins. Common sets of proteins are involved in DNA replication in different organisms, but the number of polypeptides that generate these protein activities tend to increase as the organisms get more complex.

During DNA replication, duplex DNA is opened and DNA strands serve as a template for DNA polymerisation where adenine is added opposite to thymine and guanine opposite to cytosine. The overall replication process is as follows. The prereplication complex forms during the G1 phase of the cell cycle at certain places called origins. At the initiation of replication these origins are fired, and two replication forks start the synthesis of DNA in opposite directions (Fig. 1A). In the elongation phase, the replication forks progress and synthesise the bulk of DNA. Finally, the replication forks meet. Because of the features of DNA synthesis by pols, overall chain growth of DNA is in the 5'→ 3' direction (Fig. 1B). One strand, designated the leading strand, is synthesised

continuously, whereas the synthesis of the other strand, designated the lagging strand, is discontinuous. This results in synthesis of short fragments called Okazaki fragments that are subsequently ligated together.



**Fig. 1. Outline of eukaryotic DNA replication A: Schematic diagram of the DNA replication of a chromosome segment. B: Architecture of one replication fork.**

The following core components are required for DNA replication in bacteria, eukaryotes, and most viruses: 1) DNA helicase for unwinding DNA, 2) single-strand DNA binding proteins for coating the template DNA, 3) DNA primase for producing RNA primers for leading strand and each Okazaki fragment in the lagging strand, 4) DNA polymerases for leading and lagging strand synthesis and DNA polymerase accessory factors for efficient function of the pols, 5) nuclease to remove the RNA primers, and 6) DNA ligase for joining Okazaki fragments. Eukaryotic DNA replication is more challenging compared to more simple organisms since eukaryotic chromosomes are large and contain multiple replication origins. Furthermore, the eukaryotic chromosome is a highly packed complex of DNA and proteins, which have to be unwrapped and duplicated as well. The nuclear matrix is believed to be involved in DNA replication both by providing a structural support and key protein factors (Anachkova *et al.* 2005). In DNA replication, replication machinery is considered to remain stationary, attached to nuclear matrix, while DNA is moving.

Much what is known about eukaryotic DNA replication comes from studies with the Simian Virus 40 (SV40) system and yeast genetics. The measurement of replication *in vitro* by the SV40 system is based on a plasmid DNA with an SV40 origin of replication that is efficiently replicated by mammalian cell extracts or purified proteins (Li & Kelly 1984). The SV40 large tumour antigen (T antigen) is the only viral component required for replication. However, the viral SV40 system measuring DNA replication of a small plasmid may give an oversimplified picture of the more complex mammalian DNA replication. Novel methods have been developed for studying mammalian DNA replication, and they have challenged part of the results gained with the SV40 system. Cell free systems for measuring DNA replication *in vitro* from *Xenopus laevis* egg extracts and from permeable human nuclei have been developed, and the results gained with the new methods have provided new insight into mammalian replication (Blow & Laskey 1986, Krude *et al.* 1997).

### ***2.2.1 Initiation of DNA replication***

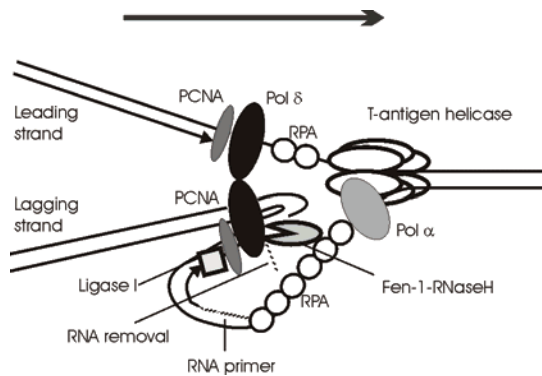
DNA replication is initiated from specific chromosomal positions called replication origins (reviewed by Weinreich *et al.* 2004). In eukaryotic cells, many replication origins exist, which enables fast and efficient duplication of the large genome (Kearsey & Cotterill 2003). The most studied model for initiation of DNA replication is *S. cerevisiae*, which has approximately 400 replication origins. Metazoan systems are more complex. There are more origins than necessary for efficient DNA replication, and the origins are not identical allowing regulation. There is temporal regulation in origin activation, some origins fire early during S phase whereas other origins fire later during S phase. For example, transcriptionally active regions in mammalian cells tend to replicate in early S phase (Woodfine *et al.* 2004). The state of differentiation may affect the origin usage as well. Posttranslational chromatin modifications, heterochromatin-binding proteins, and nucleosome positioning can regulate the origin usage. Transcription factors may be involved in the origin selection process (Kohzaki & Murakami 2005).

The initiation of DNA replication starts when a six-subunit origin recognition complex (ORC) binds to the origin (reviewed in Bell & Dutta 2002, Machida *et al.* 2005). Subsequently, initiation factors Cdc6 and Cdt1 associate with ORC. Cdc6 and Cdt1 are required for loading the MCM2-7 complex (minichromosome maintenance) onto chromatin (Coleman *et al.* 1996, Nishitani *et al.* 2000). MCMs form the presumptive replicative helicase that unwinds DNA (Forsburg 2004). In the transition to replication at least Cdc45, MCM10, the GINS complex, and the action of two kinases, CDK and Cdc7, are required. Finally, replication fork components are recruited to the origin, and the replisome is ready to start DNA synthesis.

### ***2.2.2 Elongation of DNA replication***

The reconstitution of SV40 DNA replication *in vitro* with purified proteins was the hallmark in the replication field (Waga & Stillman 1994, 1998, Waga *et al.* 1994b). The

model for DNA replication according to SV40 studies is as follows (Fig. 2). First, T-antigen binds to an origin of replication and unwinds the DNA strands together with replication protein A (RPA). Pol  $\alpha$  is recruited and it primes DNA synthesis by creating RNA-DNA primers. RFC binds to nascent DNA and loads PCNA and pol  $\delta$  onto the template and subsequently pol  $\alpha$  is released. The process where pol  $\delta$  replaces pol  $\alpha$  is called the polymerase switch (Tsurimoto *et al.* 1990). Pol  $\delta$  then synthesises bulk DNA, continuously on the leading strand and discontinuously together with pol  $\alpha$  on the lagging strand. The synchrony of leading and lagging strand synthesis requires a system that prevents the leading strand synthesis from outpacing the slower lagging strand synthesis. DNA primase was found to act as a molecular brake in DNA replication and keep the leading and lagging strand synthesis in synchrony (Lee *et al.* 2006). The torsional stress caused by the progressing replication fork is released by topoisomerase I and II. Trimming of the lagging strand is required, 5'-3' exonuclease Fen1, RNase H, pol  $\delta$ , and DNA ligase I work together to remove primers and seal the Okazaki fragments. Despite the power of the reconstituted SV40 system, it still is an oversimplified version of eukaryotic DNA replication where numerous additional proteins and factors are involved.



**Fig. 2. A model of an SV40 replication fork. Adapted from Waga and Stillman (1994), Tuusa (2001).**

The DNA polymerases utilised in DNA replication deserve a more careful description. In the SV40 system, pol  $\alpha$  and pol  $\delta$  are responsible for DNA synthesis. In addition, the requirement for pols  $\alpha$  and  $\delta$  in chromosomal DNA replication has been well documented with genetic and biochemical methods and is not described here. However, the role of pol  $\epsilon$  in DNA replication has been more enigmatic. Pol  $\epsilon$  has been linked to SV40 DNA replication *in vitro* in a few studies (Lee *et al.* 1991), but *in vivo* SV40 crosslinking experiments showed that pol  $\epsilon$  is not essential for SV40 replication (Zlotkin *et al.* 1996). However, numerous studies implicate pol  $\epsilon$  in chromosomal DNA replication both in yeast and higher eukaryotes. The *S. cerevisiae* and *S. pombe* pol  $\epsilon$  genes (*pol2* and *cdc20<sup>+</sup>*) have an essential function in cellular DNA replication (Araki *et al.* 1992, D'Urso & Nurse 1997). Pol  $\epsilon$  did crosslink to replicating cell chromosomal DNA (Zlotkin *et al.* 1996). A neutralising antibody against pol  $\epsilon$  inhibited DNA synthesis both in nuclei of growing fibroblasts and in isolated HeLa cell nuclei (Pospiech *et al.* 1999).



Immunodepletion of pol  $\epsilon$  from *Xenopus* egg extracts decreased DNA synthesis significantly (Waga *et al.* 2001).

The core question in DNA replication is the division of labour between pol  $\delta$  and pol  $\epsilon$ . Even though both pols are required in the synthesis of the bulk of DNA during nuclear replication, their exact function in DNA replication has remained elusive. Several models have been described. Many models place pol  $\delta$  and pol  $\epsilon$  on opposite arms of the replication fork, one pol on the leading strand and the other pol on the lagging strand. This model is supported by findings from yeast when mutation spectra of exonuclease deficient pol  $\delta$  and pol  $\epsilon$  were studied (Shcherbakova & Pavlov 1996, Karthikeyan *et al.* 2000). However, the pols have not been firmly assigned to certain strands. Nevertheless, pol  $\delta$  is often regarded as the lagging strand enzyme and pol  $\epsilon$  as the leading strand enzyme (e.g. Fukui *et al.* 2004, Garg & Burgers 2005). It is as well possible that one pol replicates most of both strands while the other pol plays a more specific role, for example during initiation or termination of DNA replication (Kunkel 1992). Indeed, pol  $\epsilon$  has been linked to initiation of DNA replication with chromatin immunoprecipitation studies (Aparicio *et al.* 1997). Still, pol  $\epsilon$  did travel with replication forks that were formed at early replication origins suggesting that pol  $\epsilon$  functions in DNA replication in addition to its role in initiation. Furthermore, the second largest subunit of pol  $\epsilon$  in fission yeast was found to bind to origin DNA early in S phase (Feng *et al.* 2003). In contrast, immunofluorescence studies hinted at the involvement of pol  $\epsilon$  in late S phase (Fuss & Linn 2002). In addition, the two pols may contribute equally to replication, but they may be specialised for different templates that are temporally or spatially separate. For example, one pol may replicate heterochromatin whereas the other pol replicates euchromatin. After all, the exact architecture of the DNA replication fork has remained enigmatic.

### 2.3 DNA repair

Cells are challenged continuously with agents that damage the genome, and they use several strategies to cope with the harmful effects of damaged DNA (reviewed by Christmann *et al.* 2003, Friedberg 2003). Repair machineries are dedicated to repair damaged DNA. Checkpoints cause cell cycle arrest to give the repair machinery time to fix the damage. However, apoptosis may take place, if damage to DNA is very severe. Despite multiple and efficient repair pathways in cells, gene mutations will arise. Some mutations interfere with the proper function of the cell causing diseases and cancer, and DNA repair has a crucial role in preventing cancer (Hoogervorst *et al.* 2005). Despite the deleterious consequences that mutations have, they nevertheless are necessary for evolution.

DNA damage has three main causes (Hoeijmakers 2001). First, environmental agents such as ionising radiation, UV radiation from the sun, and genotoxic chemicals, e.g. in tobacco smoke, damage DNA. Second, normal cellular metabolism, especially oxidative respiration, creates reactive oxygen species injuring DNA. Finally, some bonds in DNA spontaneously decompose to some extent even under normal physiological conditions.

There are plenty of proteins involved in protecting the genome. Over 150 human DNA repair associated genes have been found (Ronen & Glickman 2001, Wood *et al.* 2005). Repair pathways can be broadly divided to direct repair, excision repair, and repair of strand breaks (Eisen & Hanawalt 1999, Friedberg 2003). These pathways split to subpathways. In direct repair, abnormalities are chemically reversed such as in enzymatic photoreactivation of thymidine dimers. In excision repair, the damaged strand is removed and the complementary strand is used as a template for repair resulting in faithful repair of DNA. Different excision repair pathways, base excision repair, nucleotide excision repair, and mismatch repair, are utilised depending on the type of damage. Double strand breaks can be repaired either by homologous recombination or non-homologous end joining. Repair pathways are overlapping indicating the importance of the repair in cells. For example, oxidative damage to DNA can be repaired by BER, NER or MMR (Fortini *et al.* 2003). Table 2 summarises the essential information about major mammalian repair pathways.

*Table 2. DNA repair pathways in mammals*

	Base excision repair	Mismatch repair	Nucleotide excision repair	Non-homologous end joining	Homologous recombination
Type of the damage	Damaged or lost bases in DNA	Mismatched bases in DNA, small deletions and insertions	Bulky DNA lesions e.g. cyclobutane pyrimidine dimers	DNA double strand break	DNA double strand break
Main cause of damage	Reactive oxygen species, spontaneous deamination and methylation of DNA bases	Replication errors	Ultraviolet radiation, chemicals	Ionising radiation, mechanical stress, replication of nicked DNA	Ionising radiation, mechanical stress, replication of nicked DNA
Major components	Glycosylase, endonuclease, DNA ligase I/III, Fen1, PCNA, RFC	MSH2-MSH6, MSH2-MSH3, MLH1-PMS2, PCNA, RPA, EXO1, Fen1	XPA-XPG, ERCC1, PCNA, RFC, RPA	DNA-PK, XRCC4-DNA ligase IV	Rad52 epistasis group
Pol implicated	Pol $\beta$ , $\delta$ , $\epsilon$ , $\lambda$	Pol $\delta$	Pol $\delta$ , $\epsilon$	Pol $\alpha$ , $\beta$ , $\mu$ , $\lambda$	Pol $\alpha$ , $\delta$ , $\epsilon$

### ***2.3.1 Excision repair***

#### ***2.3.1.1 Nucleotide excision repair***

Nucleotide excision repair is a critical pathway for removing damage from DNA (reviewed by Hoeijmakers 2001, Christmann *et al.* 2003). The most significant source of

the damage repaired by NER is UV radiation from the sun. UV radiation causes cyclobutane pyrimidine dimers and 6-4 photoproducts. In addition, NER recognises e.g. bulky chemical adducts and intrastrand and interstrand crosslinks being the most versatile in terms of lesion recognition. Overall, damage resulting from various agents that cause significant distortion of the DNA helix is eliminated by NER.

NER can be divided to two pathways (Hoogervorst *et al.* 2005). Mellon and coworkers found in 1987 that after UV radiation, the transcribed strand is repaired much faster than the nontranscribed strand. Global genome NER (GGR) scans the entire genome for distortions in DNA, whereas transcription coupled NER (TCR) eliminates DNA damage after transcriptive RNA pol II has encountered a block that cannot be bypassed (Gong *et al.* 2005). The mechanism of NER, particularly GGR, has been extensively examined with reconstitution studies (de Laat *et al.* 1999). NER is a complicated process requiring the concerted action of roughly 30 proteins. The following steps are required both in GGR and TCR: DNA damage recognition, DNA unwinding, excision of the DNA lesion, repair synthesis, and finally ligation. Recognition of damage in GGR and TCR differs, but later stages of repair are likely to be identical (Hoeijmakers 2001).

In GGR different protein complexes have been implicated in the damage recognition (Thoma & Vasquez 2003). Protein complex XPC-HR23B senses lesions and recruits other factors to the site of damage (Sugasawa *et al.* 1998, 2001). In addition, XPA is a DNA-binding protein capable of recognising lesions in DNA *in vitro* (Robins *et al.* 1991). The XPA-RPA complex is involved in early phases of NER and it is speculated to have a role in verifying damage before incision of DNA (Thoma & Vasquez 2003). Furthermore, DNA damage binding factor DDB, a heterodimer of DDB1 and DDB2, which is mutated in a subset of Xeroderma pigmentosum (XP) complementation group E patients, has been implicated in the early stages of GGR (Tang & Chu 2002, Wagasugi *et al.* 2002). The specific roles and sequence of actions of different protein complexes in early phases of NER are still controversial. In TCR, RNA pol II stalls at damage and Cockayne syndrome A (CSA) and B (CSB) proteins are recruited to the site of the damage (van Gool *et al.* 1997, van Hoffen *et al.* 2003).

DNA unwinding is necessary before subsequent steps in NER can proceed. Transcription factor IIIH (TFIIH) is a protein complex involved in the initiation and elongation of transcription (reviewed by Zurita & Merino 2003). In addition, TFIIH has an important role in cell-cycle control and NER (Drapkin *et al.* 1994). XPB and XPD are part of TFIIH. They contain DNA helicase activities with opposite polarity and they unwind the DNA duplex around damage (Schaefferr *et al.* 1993, 1994, Roy *et al.* 1994).

After damage recognition and helix unwinding, the DNA strand containing the damage is excised by endonucleases (Evans *et al.* 1997a). NER endonucleases are suggested to work on an opened “bubble” intermediate (Evans *et al.* 1997b, Wood 1997). Two endonucleases have been implicated in NER. XPG creates the 3' incision (Habracken *et al.* 1994, O'Donovan *et al.* 1994) and a complex of XPF-ERCC1 makes the 5' incision (Sijbers *et al.* 1996). The endonucleases cleave only the damaged strand generating a 24-32 base oligonucleotide containing the lesion, but leaving the intact strand untouched to serve as a template in the subsequent repair synthesis (Hoeijmakers 2001).

Finally, the gap in DNA is filled by DNA polymerase and the nick is sealed by DNA ligase. Normal cellular DNA replication machinery, namely pol  $\delta$  and/or pol  $\epsilon$ , RFC, RPA, and PCNA, are responsible for this synthesis (Shivji *et al.* 1992, Aboussekhra *et al.*

1995). Numerous *in vitro* studies with chemical inhibitors, inhibitory antibodies, and *in vitro* reconstitution of NER with purified proteins indicate that both pol  $\delta$  and pol  $\epsilon$ , but neither pol  $\alpha$  nor pol  $\beta$ , function in NER DNA synthesis (Dresler & Frattini 1986, Nishida *et al.* 1988, Hunting *et al.* 1991, Coverley *et al.* 1992, Aboussekhra *et al.* 1995, Shivji *et al.* 1995, Araújo *et al.* 2000). However, the role of recently characterised pols in NER has not been studied. DNA ligase I is probably the enzyme in the final step of NER (Aboussekhra *et al.* 1995).

Several disorders are linked to NER (reviewed by Lehmann 1995). Defective repair of UV damaged DNA was found to cause Xeroderma pigmentosum, which is a rare autosomal recessive disorder (Cleaver 1968). XP patients are sensitive to UV radiation and exhibit a more than 1000 fold increased risk for light-induced skin cancer. Furthermore, the immune system of XP patients seems to be impaired due to UV exposure suggesting that in addition to impaired DNA repair, a defective immune system may contribute to skin cancer susceptibility in XP patients (Morison *et al.* 1985, Gaspari *et al.* 1993). Some of the XP patients also have neurological abnormalities (Rapin *et al.* 2000). Eight complementation groups for XP have been characterised, from XPA to XPG and a variant XPV (Hoogervorst *et al.* 2005). XPA to XPG have direct defect in NER. XPV cells carry out normal NER but are deficient in the replication of UV damaged DNA. XPV was identified to encode pol  $\eta$ , a pol capable of bypassing thymidine dimers in translesion repair synthesis (Masutani *et al.* 1999a,b).

In addition, Cockayne syndrome (CS) patients have mutations in proteins involved in NER (reviewed by Anderssoo & Hoeijmakers 2005). CS is a rare disorder causing variety of symptoms. Developmental defects including mental and physical retardation, neurological dysfunction, premature aging, and acute sun sensitivity are linked to CS (Nance & Berry 1992). Interestingly, despite the fact that cells from CS patients are hypersensitive to UV radiation, their cells display normal repair efficiency and the patients are not particularly prone to cancer (van Hoffen *et al.* 2003). Transcription coupled repair, but not global genome NER, malfunctions in CS patients (Venema *et al.* 1990). Mutations in CSA, CSB, XPB, XPD, and XPG have been found to cause CS (Troelstra *et al.* 1992, Henning *et al.* 1995, Berenburg & Lehmann 2001). CSA and CSB function in early phases of TCR and are recruited to the site of damage (van den Boom *et al.* 2002). However, the exact roles of CSA and CSB in TCR have remained elusive. At least purified CSB (Selby & Sancar 1997) and yeast RAD26 (Lee *et al.* 2001), a homolog of the human CSB gene, have been shown to stimulate RNA pol II elongation suggesting that CSB has a role in the progression of transcription as well. The underlying cause of CS is suggested to be combination of repair and transcriptional defects (van Gool *et al.* 1997, Lee *et al.* 2001, 2002).

### 2.3.1.2 Mismatch repair

Mismatch repair is important in maintaining genetic stability (reviewed by Modrich & Lahue 1996, Stojic *et al.* 2004). MMR repairs errors arising from DNA replication that escape the 3'  $\rightarrow$  5' exonucleolytic proofreading activity of DNA polymerases. Mismatched bases do not form Watson-Crick basepairs, namely AT and GC. Also small

insertion and deletion mutations arising during DNA replication are repaired by MMR. MMR is not limited to the repair of DNA replication errors, but MMR proteins recognise certain DNA lesions arising from normal intracellular metabolism and from physical and chemical agents from the environment (Colussi *et al.* 2002, Young *et al.* 2003, Russo *et al.* 2004). In addition, MMR has other roles in cells (reviewed by Kunkel & Erie 2005). MMR proteins prevent recombination between similar, but not identical (homeologous) sequences (Selva *et al.* 1995). MMR repairs mismatched bases resulting from recombination. MMR proteins may be involved in the TCR pathway of NER (Lee *et al.* 2004a). Furthermore, MMR proteins act as lesion sensors, and they have been implicated in the activation of cell cycle checkpoints that signal apoptosis (Brown *et al.* 2003, Li 2003, Stojic *et al.* 2004).

The MMR process can be divided into three steps (reviewed by Modrich 1991). First a mismatch is recognised. Second, the DNA strand containing the mismatch is excised. Here, the discrimination of the newly synthesised, mutated strand is essential. Finally, resynthesis creates a new DNA strand. In human cells, the mismatch recognition is mediated mainly by the heterodimer of hMSH2 and hMSH6, which recognises single base-base mismatches and small insertion deletion loops (IDL) (Kunkel & Erie 2005). In addition, a heterodimer of hMSH2 and hMSH3 is responsible for recognition of longer IDL (Harfe & Jinks-Robertson 2000). Following binding to a mismatch, the hMSH2-hMSH6-complex undergoes an ATP-dependent conformational change (Blackwell *et al.* 1998). A second heterodimer, hMLH1 and hPMS2, is bound to the hMSH2-hMSH6-ATP complex, again in an ATP-dependent manner (Li & Modrich 1995). This complex can translocate along DNA to search for strand discontinuities, which most likely in eukaryotes serve as markers for strand discrimination (Iams *et al.* 2002, Pavlov *et al.* 2003). PCNA has been implicated both in the early and late phases of MMR (Johnson *et al.* 1996, Umar *et al.* 1996, Gu *et al.* 1998). Exonuclease EXO1 degrades the newly synthesised strand (Genschel & Modrich 2003). EXO1 is stimulated by hMSH2-hMSH6, and multiple rounds of mismatch recognition, translocation, and degradation are required for removal of a strand containing the mismatch (Stojic *et al.* 2004). RPA stabilises the ssDNA (Ramilo *et al.* 2002). The gap is filled most likely by pol  $\delta$  (Longley *et al.* 1997), and the nick is sealed by DNA ligase.

Defective MMR is common in several cancers, especially in hereditary nonpolyposis colon cancer (HNPCC), but also in sporadic cancers in various organs (reviewed by Peltomäki 2003). Defective MMR was linked to HNPCC on the basis of different findings (Kolodner 1995). First, HNPCC tumours were shown to have microsatellite instability. Second, germline mutations in MMR genes were found to predispose to HNPCC (Fishel *et al.* 1993, Leach *et al.* 1993, Peltomäki *et al.* 1993, Bronner *et al.* 1994, Nicolaides *et al.* 1994, Papadopoulos *et al.* 1994, Wu *et al.* 1999a). So far, more than 400 different predisposing MMR gene mutations have been identified (Peltomäki 2003). MLH1 and MSH2 are the most often mutated protein components (90% of mutations) (Mitchell *et al.* 2002). Defective MMR may cause cancer in many ways. First, defective MMR decreases genomic stability and increases the mutation rate. Second, loss of suppression of recombination between homeologous sequences may lead to gene conversion and translocations.

### 2.3.1.3 Base excision repair

Base excision repair removes damaged bases from DNA (reviewed by Fortini *et al.* 2003). Bases in DNA are constantly being damaged both by endogenous and exogenous factors. Most of the damage is endogenous and is caused by oxidation of DNA by reactive oxygen species or by spontaneous deamination or methylation of DNA bases. Modifications in bases do not generate large disturbances in the backbone of DNA.

BER is divided into two different pathways called short patch repair and long patch repair (Matsumoto *et al.* 1994, Frosina *et al.* 1996). BER has been reconstituted with purified proteins. Kubota and coworkers (1996) reconstituted short patch BER and showed that uracil-DNA glycosylase, apurinic/apyrimidinic endonuclease, pol  $\beta$ , and DNA ligase III were sufficient in BER. Long patch BER of apurinic/apyrimidinic sites was reconstituted with just six proteins, namely with AP endonuclease, RFC, PCNA, pol  $\delta$  or  $\epsilon$ , Fen1, and DNA ligase I (Matsumoto *et al.* 1999, Pascucci *et al.* 1999).

The mechanism of BER has been characterised rather well. BER consists of five steps (Fan & Wilson 2005): 1) the damaged base is recognised and removed by DNA glycosylase; 2) the DNA backbone is incised resulting in an abasic site; 3) the termini generated are processed; 4) DNA is synthesised and; 5) the nick is sealed by ligase. DNA glycosylases are needed to hydrolyse the N-glycosylic bond between the base and deoxyribose (reviewed by Krokan *et al.* 1997, Dizdaroglu 2003). Many DNA glycosylases have been characterised and they show various substrate specificities.

Pol  $\beta$  has been implicated in BER in many studies, and it evidently is the major gap-filling enzyme in BER (Sobol *et al.* 1996, Fan & Wilson 2005). However, pol  $\beta$ -deficient cells were able to perform both BER pathways, albeit with slower kinetics in the case of short patch BER (Fortini *et al.* 1998, Bennet *et al.* 2001). Several *in vitro* and *in vivo* studies implicate the involvement of pols  $\delta$  and  $\epsilon$  in BER (Wang *et al.* 1993, Blank *et al.* 1994, Matsumoto *et al.* 1994, 1999, Pascucci *et al.* 1999). Initially, pol  $\beta$  was thought to be required only for short patch BER, but now an increasing body of evidence supports a role for pol  $\beta$  also in long patch BER (Klungland & Lindahl 1997, Dianov *et al.* 1999, Prasad *et al.* 2000, Podlutzky *et al.* 2001). Evidently, there is redundancy in pol usage in BER and cells have backup systems for BER. In addition to pols  $\beta$ ,  $\delta$ , and  $\epsilon$ , pol  $\lambda$  was shown to contribute to BER and protect cells against oxidative DNA damage (Braithwaite *et al.* 2005a,b).

Defective BER has been associated with diseases, especially cancer. Mutations in 8-oxoguanine DNA-glycosylase have been found in lung and kidney tumours (Chevallard *et al.* 1998). Mutations in MYH, a human homolog for the *E. coli* DNA glycosylases mutY, were found to predispose to colorectal tumours (Al-Tassan *et al.* 2002, Jones *et al.* 2002). Mice with deficient uracil-DNA glycosylase have been studied. B-cell differentiation was disturbed and mice were found to develop spontaneous malignancies (Rada *et al.* 2002, Nilsen *et al.* 2003). In addition, pol  $\beta$  mutations are linked to cancer (Starcevic *et al.* 2004).

### 2.3.2 Double strand break repair

DNA double strand breaks are extremely dangerous for a cell (reviewed by Lees-Miller & Meek 2003, Hefferin & Tomkinson 2005). Even one DSB can lead to cell death (Bennett *et al.* 1993). Inaccurate repair of DSBs can lead to chromosomal aberrations such as chromosomal fragmentation, translocations, and deletions (Varga & Aplan 2005). These changes may result in genomic instability and to cancer predisposition through activation of oncogenes, inactivation of tumour-suppressor genes or loss of heterozygosity (Kanaar *et al.* 1998). DSBs are caused both by external and internal agents. Ionising radiation, chemicals e.g. anticancer drugs, mechanical stress, and the oxidative metabolism of the cell create breaks in DNA. DNA replication of single strand breaks causes DSBs. Site specific DSBs arise also during natural processes of the cell, for example in variable-diversity-joining [V(D)J] recombination, a process necessary for the diversity of the immune system (Lieber *et al.* 2004). NHEJ and V(D)J recombination utilise a similar set of components for processing breaks.

DSBs are repaired efficiently in the cell. The repair pathways can be broadly divided into two classes, namely homologous recombination and non-homologous end joining (Hoeijmakers 2001). In HR the intact sister chromatid is used as a template for repair and the repair is thus very accurate. In contrast, in NHEJ no template is used and the repair is often inaccurate resulting in loss of a few nucleotides. Both repair pathways are found from mammals and yeast. However, there seems to be differences in the contribution of these different repair pathways in different organisms. In *S. cerevisiae*, HR predominates, whereas in mammals, NHEJ is thought to be preferred (Lieber *et al.* 2003). The developmental phase and cell cycle state and thus the availability of the close template for HR probably plays a role in selection of a repair mode (Pastwa & Blasiak 2003).

#### 2.3.2.1 Non-homologous end joining

Characterisation of radiosensitive mutants from mammalian cells has been important for our understanding of double strand break repair (DSBR)(reviewed by Thacker & Zdzienicka 2003, 2004). So far 10 complementation groups for rodent mutants sensitive to IR have been identified. The genes defined by these groups are named *XRCC* for X-ray-repair cross-complementing gene (Thompson & Jeggo 1995). Naturally not all of the proteins encoded by *XRCC* genes are involved in DSBR since IR causes other forms of damage to DNA as well.

In addition to genetic approaches, numerous biochemical *in vivo* and *in vitro* studies have been crucial for deepening our understanding of NHEJ (reviewed by Labhart 1999). In the traditional *in vitro* assay, a linearised plasmid is incubated with extract proteins or partially purified fractions. The repair products are detected with agarose gel electrophoresis, Southern hybridisation, PCR or transformation. The experimental setup can be modified and the requirement for various factors can be tested, for example, by using inhibitors or antibodies. *Xenopus* egg extracts were first used to study cell-free NHEJ (Pfeiffer & Vielmetter 1988). *Xenopus* eggs are well suited for studies on DNA replication and repair, since they contain high amounts of stored proteins involved in

DNA metabolism. A mammalian cell-free system for NHEJ was first described by North and colleagues (1990) from nuclei of a transformed human cell line. Numerous studies have followed using either cytoplasmic (e.g. Farnet & Haseltine 1991) or whole cell extracts (e.g. Daza *et al.* 1996). In most of the *in vitro* studies, plasmids linearised with restriction enzymes are utilised. However, these substrates do not present an ideal situation since radiation causes more complicated damage (Labhart 1999). Some studies have been conducted with modified DNA ends, and they suggest that there are limitations to the conclusion based on these simplified substrates (Beyert *et al.* 1994, Gu *et al.* 1998). An apparent paradox in the *in vitro* end-joining assays was found (Blanco *et al.* 2004). The DNA/protein ratio in the reaction seems to determine whether DNA ends are degraded or protected and joined together.

NHEJ can be divided to separate stages (Hefferin & Tomkinson 2005). First, DNA-dependent protein kinase (DNA-PK) is bound to the broken ends where it protects DNA from degradation and brings the ends into close proximity. Since IR causes diverse damage in addition to breaks in the backbone of DNA, the DSB site often requires trimming by nucleases and polymerases. Finally, the break is sealed by a ligase. The involvement of DNA-PK and XRCC4-DNA ligase IV in NHEJ have been firmly established both with genetic and biochemical studies (Lees-Miller & Meek 2003). In addition, numerous other repair factors are implicated in NHEJ, but their exact role has in most cases remained elusive.

DNA-PK is a holoenzyme consisting of a Ku70-Ku80 heterodimer and a 469 kDa catalytic subunit, DNA-PK<sub>cs</sub> (Collis *et al.* 2005). These proteins are coded by *XRCC6*, *XRCC5* and *XRCC7*, respectively (Thacker & Zdzienicka 2004). The Ku70-Ku80 heterodimer binds DNA ends with high affinity (Dyran & Yoo 1998). Ku binds to various end configurations of linear DNA, but not to closed circular DNA. Ku protects DNA ends from degradation (Liang & Jasin 1996). Structural studies of Ku revealed that it forms an open ring-shaped structure, negatively charged DNA being threaded into the positively charged interior of the molecule (Walker *et al.* 2001). After binding to DNA ends, Ku can translocate along DNA (de Vries *et al.* 1989, Paillard & Strauss 1991). Ku also has helicase and ATPase activities (Tuteja *et al.* 1994). Ku recruits DNA-PK<sub>cs</sub> to the site of damage (Dvir *et al.* 1992). DNA-PK<sub>cs</sub> has at least two important functions in NHEJ, it functions in end bridging (DeFazio *et al.* 2002) and it functions as a kinase. DNA-PK<sub>cs</sub> has a serine/threonine protein kinase activity that is stimulated by association with Ku70-80 and DNA (Dvir *et al.* 1992, Gottlieb & Jackson 1993). The kinase activity is considered to be essential in NHEJ as supported by inhibitor and complementation studies (Baumann & West 1998, Kurimasa *et al.* 1999). DNA-PK<sub>cs</sub> can phosphorylate many substrates *in vitro*, such as RNA pol II, p53, Ku70, Ku80, Artemis, XRCC4, and itself (Lees-Miller *et al.* 1990, Dvir *et al.* 1992, Chan & Lees-Miller 1996, Leber *et al.* 1998, Ma *et al.* 2002). However, it is not clear which of the substrates are relevant in NHEJ *in vivo*. From analysis of the DNA-PK<sub>cs</sub> structure at 20 Å resolution it was found that the molecule contains cavities large enough for double stranded DNA (Chiu *et al.* 1998), and it was found to form a complex with XRCC4-DNA ligase IV and stimulate its activity (Chen *et al.* 2000b).

Numerous additional factors are implicated in NHEJ. Most of them are suggested to have a role in trimming the break site. The Werner syndrome protein (WRN) has helicase and exonuclease activities. WRN exonuclease was shown to interact with DNA-PK and



its exonuclease activity was stimulated by Ku (Li & Comai 2001, Orren *et al.* 2001). In addition, cells deficient in WRN were slightly sensitive to IR supporting the involvement of WRN in DSB (Yannone *et al.* 2001). In *S. cerevisiae* RAD27 nuclease, Fen1 of yeast, was implicated in NHEJ (Wu *et al.* 1999b). Phosphorylation of the 5' hydroxyl group by polynucleotide kinase was required for end joining in an *in vitro* assay (Chappell *et al.* 2002). This activity was dependent on DNA-PK<sub>cs</sub> and XRCC4. Tyrosyl-DNA phosphodiesterase is suggested to work in the processing of NHEJ by removing blocks from DNA 3' ends (Inamdar *et al.* 2002). Interestingly, a carbohydrate inositol phosphate can interact with Ku70-Ku80 and stimulate NHEJ *in vitro* (Hanakahi *et al.* 2000, Hanakahi & West 2002). Artemis is a novel nuclease implicated in NHEJ (Moshous *et al.* 2001, Noordzij *et al.* 2003). Artemis interacts with DNA-PK<sub>cs</sub> and functions in the processing of 5' and 3' overhang ends (Ma *et al.* 2002). The nuclease complex Mre11-Rad50-Nbs1 is required for NHEJ *in vitro* prior to ligation (Paull & Gellert 1998, Chen *et al.* 2001, Huang & Dynan 2002). In addition, it is involved in DNA damage signalling (D'Amours & Jackson 2002).

DNA repair synthesis in NHEJ has remained controversial. First *in vitro* studies were conducted in the absence of nucleotides (North *et al.* 1990, Fairman *et al.* 1992) but later the involvement of pol activity (Mason *et al.* 1996) and furthermore, various pols have been suggested in NHEJ. Islas and colleagues (1998) studied DNA synthesis on discontinuous oligonucleotide templates, a process resembling NHEJ. Pol  $\alpha$  showed the most discontinuous DNA synthesis activity. In yeast, pol4 (pol  $\beta/\lambda$ ) has been implicated in NHEJ for certain end configurations both with transformation of linearised plasmid into yeast and with the chromosomal DSB assay (Wilson & Lieber 1999). Studies of mammalian NHEJ suggest the involvement of various pols in NHEJ. Pol  $\mu$  was found to localise to discrete nuclear foci after IR, and pol  $\mu$  and terminal deoxynucleotidyl transferase (TdT) were found to associate with Ku and XRCC4-DNA ligase IV (Mahajan *et al.* 1999, 2002). In contrast, immunodepletion studies suggested pol  $\lambda$  but not pol  $\mu$ , to function in NHEJ (Lee *et al.* 2004b). What is more, pol  $\lambda$  can perform gap-filling synthesis on DNA substrates mimicking NHEJ, and it interacts with the XRCC4-DNA ligase IV complex (Fan & Wu 2004). In an *in vitro* reconstitution of NHEJ, family X members pol  $\mu$ , pol  $\lambda$ , and TdT, but not pol  $\beta$ , contributed to end joining (Ma *et al.* 2004). Further studies on the role of family X pols, pol  $\lambda$ , pol  $\mu$ , and TdT, in NHEJ suggested that the template makes a difference, and may influence pol usage (Nick McElhinny *et al.* 2005). Studies from yeast with *pol4* mutation complemented with mammalian pol X family pols supported the hypothesis that the structure of the DSB end influences the pol usage in NHEJ (Daley *et al.* 2005). After the ends are trimmed and the gaps filled, the break is sealed with XRCC4-DNA ligase IV (Lee *et al.* 2003). Prior to ligation, the XRCC4-DNA ligase IV complex may be involved in alignment and gap-filling as well.

Few diseases are linked to defective NHEJ (reviewed by O'Driscoll *et al.* 2004). Ligase IV syndrome is a rare disorder where DNA ligase IV, a key player in NHEJ, is mutated (Riballo *et al.* 1999, O'Driscoll *et al.* 2001). Patients show immunodeficiency, developmental and growth delay and characteristic facial features. Inactivation of Ku has been connected to cancer predisposition (Li *et al.* 1998), and a polymorphism in Ku70 and XRCC4 was linked to an increased risk of breast cancer (Fu *et al.* 2003). Artemis was found to be mutated in patients suffering from radiosensitive severe combined

immunodeficiency and to be important in lymphoid differentiation in bone marrow (Moshous *et al.* 2001).

### 2.3.2.2 Homologous recombination

Most of the genes engaged in HR were first identified in *S. cerevisiae* as mutants sensitive to IR and they were named “rad” for radiation sensitive (reviewed by Aylon & Kupiec 2004a,b). The Rad52 epistasis group in yeast includes *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *XRS2*, and *MRE11*. These genes are conserved among eukaryotes in evolution, and human and mouse genes with sequence similarity to most of them are found (Kanaar *et al.* 1998).

HR begins with nucleolytic degradation of DSB most likely by the Mre11-Rad50-Nbs1 complex (Tsuchi *et al.* 2002). Subsequently, the Rad52 heptameric complex binds single stranded DNA (Shinohara *et al.* 1998, Stasiak *et al.* 2000). Rad52 interacts with Rad51 and stimulates the DNA strand exchange activity of Rad51 (New *et al.* 1998). Rad51 forms nucleofilaments, facilitates a search for the homologous DNA and interaction with a homologous region of an undamaged DNA molecule, and also catalyses strand exchange (Benson *et al.* 1994, Baumann *et al.* 1996, Sigurdsson *et al.* 2001). After the identical template is found, DNA is synthesised by replicative pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  (Holmes & Haber 1999). Finally, resolvases resolve the Holliday junction (Constantinou *et al.* 2001).

*BRCA1* and *BRCA2* mutations predispose to breast and ovarian cancer (Nathanson *et al.* 2001). A strong body of evidence suggests that they participate in various aspects of cellular surveillance after DNA damage, such as in cell cycle checkpoint control, repair of DSBs, and transcription regulation (Thompson & Schild 2002). *BRCA1* and *BRCA2* have been found to interact with Rad51, which provided the first evidence for their involvement in HR (Scully *et al.* 1997a, Sharan *et al.* 1997, Chen *et al.* 1998). In addition, *BRCA1* was found to associate with the Mre11-Rad50-Nbs1 complex (Zhong *et al.* 1999). Furthermore, *BRCA1* is suggested to function in NHEJ (Zhong *et al.* 2002). The exact role of *BRCA1* and *BRCA2* in DSBR has remained enigmatic, but they may act as damage sensors, mediate response from damage sensors to repair proteins or perform a scaffolding function by bringing key players in DSBR into close proximity (Scully *et al.* 2004, Zhang & Powell 2005).

Ataxia telangiectasia (AT) is a rare disorder characterized by neuronal degeneration, cancer predisposition and ataxia (Thompson & Schild 2002). The gene defective in AT is ATM (Savitsky *et al.* 1995). ATM is a kinase that regulates signalling events upon DNA damage and it is implicated in HR (Morrison *et al.* 2000). The Nijmegen breakage syndrome (NBS) results from defective DSBR (Matsuura *et al.* 2004). NBS patients suffer from growth retardation, immunodeficiency, and increased risk of malignancies. Nbs1 functions in HR together with Mre11 and Rad50 (Carney *et al.* 1998).

## 2.4 RNA polymerase II

RNA polymerases transcribe genes, and are essential for protein synthesis (reviewed by Paule & White 2000). In eukaryotes three RNA pols, numbered RNA pol I, II, and III, are involved in nuclear transcription. However, there is a strict division of labour between RNA pols with respect to the genes they transcribe. RNA pol I synthesises the large ribosomal RNA (rRNA), RNA pol II creates messenger RNA, transcribing protein encoding genes, and RNA pol III produces transfer RNA, 5S rRNA, and U6 snRNA. Though RNA pol I and III transcribe only a limited set of genes, the activities by RNA pol I and III dominate cellular transcription.

RNA pol II is composed of 12 distinct subunits and it has a mass of about 500 kDa (Shilatfard *et al.* 2003). RNA pol II can also be purified as a larger complex called the holoenzyme (Koleske & Young 1994). The holoenzyme contains transcription factors, transcriptional mediators and accessory proteins (Boeger *et al.* 2005). The holoenzyme is considered to be functionally significant, being responsible for transactivator-stimulated transcription *in vivo*. The composition of the RNA pol II holoenzyme depends on the purification protocol suggesting that there exist multiple forms of the complex *in vivo*. The RNA pol II holoenzyme containing DNA replication and repair proteins like pol  $\epsilon$ , Ku, Rad51, XPC, XPG, RPA, and RFC was purified by Maldonado and coworkers (1996). Subsequently, RNA pol II has been shown to interact with other DNA replication and repair proteins, e.g. with BRCA1 and MCM proteins (Scully *et al.* 1997b, Yankulov *et al.* 1999). These findings suggest that the RNA pol II holoenzyme may integrate transcription with DNA replication and repair.

Regulation of RNA pol II is crucial for its proper function. The largest subunit of RNA pol II contains the conserved C-terminal repeat domain (CTD) that has been implicated to be important for the function, regulation, and interactions of RNA pol II (Palancade & Bensaude 2003). The CTD is comprised of multiple repeats of a seven amino acid motif and five of the amino acids are phosphate acceptors. Phosphorylation of CTD is indeed the major post-translational modification of CTD *in vivo* and crucial for the regulation of RNA pol II. During the transcription cycle, the phosphorylation status of RNA pol II CTD changes. In the pre-initiation phase, RNA pol II is hypophosphorylated, at the initiation phase serine 5 of the heptapeptide is phosphorylated, and during elongation, phosphorylation of serine 5 is lost whereas serine 2 becomes phosphorylated (Svejstrup 2004). Finally, CTD has to be dephosphorylated for a new round of transcription.

RNA pol II has other roles in cells besides transcription. Transcription factors are suggested to regulate DNA replication (Kohzaki & Murakami 2005). The recruitment of the RNA pol II transcription complex was shown to activate DNA replication in yeast, and the CTD was sufficient for the activation (Stagljar *et al.* 1999, Gauthier *et al.* 2002). The CTD of RNA pol II has been proposed to be involved in coordination of transcription and DNA replication.

During transcription, RNA pol II machinery may encounter damage at the template that blocks transcription (Tornaletti 2005). TCR, a form of NER, is activated and the damage is repaired. Stalled RNA pol II at a lesion is considered to be a prerequisite for the initiation of TCR. In addition, components of the RNA pol II holoenzyme, like TFIIH, have been implicated in NER (de Laat *et al.* 1999).

### **3 Aims of the present work**

DNA is the carrier of genetic information, and faithful DNA replication and repair is essential for the proper function of the cell. Identification of proteins involved in these pathways is crucial for the elucidation of the underlying mechanisms. DNA synthesis by DNA polymerases is an essential step in DNA replication and repair. Even though DNA replication has been extensively studied, the exact roles of pol  $\delta$  and pol  $\epsilon$  in DNA replication have remained enigmatic. The mechanism and proteins involved in non-homologous end joining are still only partially characterised. Furthermore, besides its role in DNA replication and repair, pol  $\epsilon$  has additional functions in cells.

The specific aims of the present work were:

1. To elucidate the mechanism and the role of DNA synthesis in non-homologous end joining.
2. To further elucidate the roles of pol  $\alpha$ , pol  $\delta$ , and pol  $\epsilon$  during DNA replication.
3. To characterise the interaction between pol  $\epsilon$  and RNA pol II.

## 4 Materials and methods

The materials and methods are described in more detail in the original articles referred to by their Roman numerals.

### 4.1 Measurement of non-homologous end joining *in vitro*

#### 4.1.1 *Non-homologous end joining assay (I)*

The substrates for NHEJ measurements were constructed from circular double stranded DNA of M13mp18. Novel recognition sites for *Mlu*I and *Aat*II were inserted into M13mp18 to create different substrate ends. Originally, a 23 bp fragment containing a novel site for *Mlu*I was introduced into M13mp18 to create M13dsb1. Alternatively, a substrate was employed that contained a 599 bp  $\lambda$  fragment inserted in M13dsb1, creating M13dsb2. M13dsb4 contained a 19 bp fragment that created a novel *Aat*II site. M13mp18, M13dsb2, and M13dsb4 were digested with *Sma*I, *Xma*I or its schizoisomer *Cfr*9I and *Mlu*I or with *Kpn*I and *Aat*II to create substrates S, XM, and AK, respectively. The S substrate contained blunt ends, the XM substrate contained a 5' protruding end, and the AK substrate contained a 3' protruding end. The digested DNA was separated from the insert and undigested DNA by agarose gel electrophoresis and purified with a GeneClean II kit.

The standard repair reaction contained 30 mM Hepes-KOH, pH 7.8, 7 mM MgCl<sub>2</sub>, 50  $\mu$ g/ml BSA, 4 mM ATP, 100  $\mu$ M dNTPs, 40 mM phosphocreatine, 1  $\mu$ g of creatine phosphokinase, 50-100  $\mu$ g of cell extract protein prepared as described (Krude *et al.* 1997), and 100 ng substrate DNA in a final volume of 25  $\mu$ l. A reaction mixture containing heat-inactivated cell extract was included as a negative control. Reactions were premixed on ice, started by transfer to 25°C, and incubated for two to four hours. Reactions were terminated by the addition of an equal volume of stop buffer (40 mM EDTA, 1 % SDS) and 10  $\mu$ g of proteinase K followed by incubation at 37°C for 30 minutes. After the repair reaction, DNA was purified by extensive phenol/chloroform extraction, precipitated with ethanol, and dissolved in TE.

### **4.1.2 Transformation and sequence analysis of repair products (I)**

Recovered substrate DNA was transformed into *Escherichia coli* XL1blue by using standard procedures (Inoue *et al.* 1990) and plated with X-Gal and isopropyl-beta-D-thiogalactoside (IPTG) on tetracycline plates. Transformations were repeated at least three times. Repair efficiency was measured as the number of plaques formed after transformation, and a complete reaction was regarded as 100 % repair activity. The sequences of the repair joints were determined from transformed repair products by isolation of M13 single stranded or replicative form DNA and subsequent sequencing on an ABI PRISM 377 automated DNA sequencer.

### **4.1.3 Southern analysis (I)**

For direct analysis of repair products by Southern hybridisation, recovered substrate DNA was resolved by electrophoresis in 0.8% agarose and transferred to a Hybond-N neutral Nylon membrane according to standard protocols (Ausubel *et al.* 1989). DNA was detected either using the Rad-free system or DIG chemiluminescent detection and DIG high prime DNA labelling with M13mp18 single stranded DNA as a probe.

## **4.2 Measurement of non-homologous end joining *in vivo* (I)**

Exponentially growing HeLa S3 spinner cells were collected by centrifugation (200 g for 5 minutes) followed by two washes with fresh medium containing 2.5 % fetal calf serum. The cells were resuspended at  $5 \times 10^7$  cells/ml in the same medium, and 400  $\mu$ l aliquots were mixed with 1  $\mu$ g DSB substrate and 50-100 ng pBluescript II SK[+] and transferred to electroporation cuvettes (4 mm electrode spacing). After 5 minutes, the cells were electroporated at 150 V with a 70 msec pulse using an ECM830 square wave electroporator. After 5 minutes recovery, cells were transferred to 100 mm tissue culture dishes and cultured for 30 minutes, 1, 2 or 4 hours in 20 ml conditioned medium supplemented with 5 % fetal calf serum. The NHEJ substrate was re-isolated from the cultured cells by the method of Sadiev and Taylor (1996). NHEJ activity was measured by transforming recovered DNA into *E. coli* cells.

## **4.3 Fractionation of cell extract and purification of PCNA and pol $\epsilon$ (I, III)**

To separate PCNA from most other NHEJ proteins HeLa cell extract was fractionated as described (Prelich *et al.* 1987, Shivji *et al.* 1992) with minor modifications. Briefly, cell extract was adjusted to 0.1 M NaCl and proteins were first separated through phosphocellulose (Whatmann P11) in TDEG (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1

mM EDTA, 10 % glycerol) with 0.1 M NaCl. Bound proteins were eluted with TDEG with 1 M NaCl. Peak fractions were combined resulting in fractions I (flow through) and II (bound), respectively. Fraction I was adjusted to 0.18 M NaCl and separated through DEAE-Sephacel with TDEG with 0.18 M NaCl resulting in fractions IA (flow through) and IB (bound, eluted as above). When necessary, fractions were concentrated by dialysis against polyethylene glycol 22 000 and all samples were dialysed against TDEG.

PCNA was purified as described by Downey and So (1995). Briefly, HeLa cell extract was purified through DEAE-Sephacel, P11 phosphocellulose, phenyl-Sepharose, P11 phosphocellulose, DEAE-Sephacel, and hydroxylapatite. The purity of PCNA was more than 60 % after the hydroxylapatite chromatography.

Pol  $\epsilon$  was purified as previously described (Syväoja & Linn 1989). Steps included  $(\text{NH}_4)_2\text{SO}_4$  fractionation, ion exchange chromatography on DEAE-Sephacel and phosphocellulose, and absorption chromatography on hydroxylapatite.

#### 4.4 PCNA depletion (I)

PCNA was removed from cell extract using a twenty amino acid peptide (KRRQTSMTDEFYHSKTTLIFS) from the PCNA interaction site of p21 (amino acids 141-160) containing biotin in its amino terminus. This peptide strongly interacts with PCNA (Warbrick *et al.* 1995). The peptide was synthesised with a 433A peptide synthesiser (Applied Biosystems). A peptide in which the critical residues Q, M, F, and Y (underlined) were mutated to alanine was unable to precipitate PCNA and served as a control.

For PCNA removal, 1  $\mu\text{g}$  of peptide was linked to 80  $\mu\text{l}$  streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin) in IP-buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) at 4°C for 40 minutes and unbound peptide was washed with IP-buffer. Coupled magnetic beads were then used for depletion of PCNA from 350  $\mu\text{g}$  of cell extract protein in IP buffer in a total volume of 100  $\mu\text{l}$  for 90 minutes on a rocker at 4°C. Multiple rounds of depletion were required to achieve efficient removal of PCNA. Removal of PCNA was monitored by Western analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membrane, incubated with appropriate antibodies, and detected with enhanced chemiluminescence (ECL) reagents.

#### 4.5 Cell synchronisation (II, III)

HeLa cells and IMR-90 fibroblasts were synchronised by blocking the progress of the cell cycle at the G1/S border with 0.5 mM mimosine for 16-18 h (Krude 1999). The cells were released from the block by changing to fresh medium. T98G glioblastoma and IMR-90 cells had been brought to quiescence by incubation in medium containing 0.25 % serum for more than 6 days and cells were stimulated to proliferate by elevating the serum concentration. The efficacy of the synchronisation was demonstrated by

fluorescence activated cell sorter analysis of propidium iodide stained cells (Vindelöv *et al.* 1983) or by the incorporation of [ $^3\text{H}$ ] thymidine in parallel cell cultures.

#### **4.6 UV-crosslinking of proteins to nascent DNA or RNA in a monolayer of isolated nuclei (II, III)**

UV-crosslinking of proteins to nascent DNA or RNA in a monolayer of isolated nuclei was performed as described previously (Zlotkin *et al.* 1996) with minor modifications. HeLa cells were washed with buffer KM (10 mM MOPS-NaOH, pH 7.0, 10 mM NaCl, 1 mM  $\text{MgCl}_2$ , 2 mM DTT, 1x complete protease inhibitor) and lysed by incubation on ice with buffer KM containing 0.5 % Nonidet P-40 resulting in a monolayer of isolated nuclei. The nuclear monolayer was then washed twice with the buffer KAc (30 mM Hepes-KOH, pH 7.5, 5 mM K-acetate, 0.5 mM  $\text{MgCl}_2$ , 2 mM DTT, 1 x complete) and the replication reaction was carried out for 2.5 min at 30°C. After labelling, the nuclei were washed with buffer KAc and UV-irradiated. The irradiated nuclei were treated with DNase to remove bulk DNA. Protein-DNA complexes were extensively extracted with phenol, precipitated with acetone, collected, and washed extensively. The dried protein-DNA pellet was resuspended by boiling in denaturation buffer and renatured.

Pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were immunoprecipitated and immunoprecipitates were washed three times. Proteins were eluted with SDS loading buffer and separated through 6 % SDS-PAGE, transferred to PVDF membranes, and autoradiographed. After autoradiography, proteins were detected using appropriate antibodies and ECL reagents.

#### **4.7 Chromatin association (II)**

HeLa cells were lysed and proteins fractionated to give a detergent-soluble, high salt (bound), and remaining (high salt resistant) fractions as follows. The cells were washed with TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and twice with buffer ME (10 mM MOPS-NaOH, pH 7.0, 10 mM NaCl, 1 mM EDTA, 2 mM DTT). The cells were lysed for 30 minutes with buffer ME containing 0.5 % NP-40. The resulting supernatant was the detergent soluble fraction. The permeabilised nuclei were washed twice with buffer KAcE (30 mM Hepes-KOH, pH 7.5, 5 mM K-acetate, 1 mM EDTA, 2 mM DTT, 1x complete), and proteins were extracted with high salt buffer containing 500 mM NaCl yielding the high salt fraction. The plates were washed twice with high salt buffer ME, and the remaining material was solubilised in buffer ME containing 100 mM NaCl and 0.5 % SDS using a cell scraper. This was considered the high salt resistant fraction. In parallel, cells from a second plate were solubilised directly in buffer ME containing 100 mM NaCl and 0.5 % SDS followed by sonication to give total cell extract. Proteins were separated by 6 % SDS-PAGE and equality of loading was monitored by SYPRO Orange staining and proteins were detected using appropriate antibodies and ECL reagents.



## **4.8 Evaluation of the crosslinking intensity and protein levels from Western analysis (II)**

The radioactive signals of the crosslinked derivatives of the pols and the immunoreactive signal from Western blots were analysed after scanning with an Image Scanner (Amersham Pharmacia) and quantified using the ImageQuant (Molecular Dynamics) software.

## **4.9 DNA replication in isolated nuclei (II)**

Preparation of HeLa and T98G monolayer cell nuclei, and HeLa monolayer or spinner cell cytoplasmic extract as well as subsequent permeabilisation of the nuclei with lysolecithin were performed as described (Krude *et al.* 1997, Stoeber *et al.* 1998). Nuclei were permeabilised immediately before use, washed, and suspended in a Dounce homogeniser by 10 strokes with a loose fitting pestle. DNA replication reactions in isolated nuclei were performed at least in triplicate per experiment as described (Pospiech *et al.* 1999). Nuclei, cytoplasmic extract, and neutralising antibodies against the indicated pols were incubated for 90 min on ice in the reaction mixture prior to the reaction at 37°C for 60 minutes.

## **4.10 Immunoelectron microscopy (II, III)**

Synchronised IMR-90 and T98G cells were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, containing 2.5 % sucrose for 30 min, detached, and centrifuged to form a tight pellet. The pellet was mixed with a small volume of 4 % gelatine or 2 % NuSieve agarose in PBS at 37°C, cooled, and immersed to 2.3 M sucrose in PBS. The specimens were frozen in liquid nitrogen and thin cryosections were cut with a Leica Ultracut UCT microtome. The sections were first incubated in 5 % BSA, 0.1 % gelatine in PBS. Antibodies and gold conjugates were diluted in 0.1 % BSA-C in PBS. All washing steps were performed in 0.1% BSA-C in PBS.

For the double labelling experiments, after blocking as described above, sections were first exposed to the first primary antibody for 60 min. Subsequently, samples were incubated with rabbit anti-mouse IgG at 1.9 µg/ml or anti-rat IgG at 1.6 µg/ml for 30 min. Then samples were treated with 5 nm protein A-gold complex for 30 min (Slot & Geuze 1985). After washings, 1 % glutaraldehyde in 0.1 M phosphate buffer was used to block free binding sites on protein A. Finally, the sections were incubated with a second primary antibody for 60 min followed by anti-mouse or rat IgG for 30 min and protein A-gold complex (size 10 nm) for 30 min. The controls were prepared by carrying out the labelling procedure without primary antibody. The efficiency of blocking was controlled by performing the labelling procedure in the absence of the second primary antibody. The sections were embedded in methylcellulose and examined in a Philips CM100

transmission electron microscope. Images were captured by a CCD camera equipped with the TCL-EM-Menu version 3 from Tietz Video and Image Processing Systems GmbH.

#### 4.11 Statistical methods (II)

The data from UV-crosslinking, chromatin association, and DNA replication in isolated nuclei experiments were analysed by normal linear regression models appropriately specified for each of them. The time trends in the measured outcomes were described by including linear as well as quadratic terms of the time factor in the model. The outcome variable was transformed onto the natural logarithmic scale when modelling the time trends both in crosslinking intensity and in chromatin association, because relative values of the outcome were analysed. As a null hypothesis, we considered a model in which the slope (coefficient of the linear term) and any potentially required curvature (quadratic term) had the same value shared by the three pols. This was evaluated against a model which allowed different values of these regression coefficients for the separate pols using the *F* statistics for nested models and two-tailed *t* statistics for the appropriate contrasts between the pols. The soundness of the model assumptions were examined by conventional residual plots. The computations were performed using the R statistical language, especially its `lm` function designed to fit linear models.

#### 4.12 Immunoprecipitation (III)

For preparation of whole cell extract for immunoprecipitation, the HeLa or T98G cells were washed with PBS and scraped into lysis buffer (100 mM Tris-HCl, pH 7.5, 80 mM NaCl, 10 % glycerol, 0.1 % Nonidet P-40, 1x complete, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF). Cells were broken by sonication and the extract was further incubated on ice for 15 min and centrifuged. For immunoprecipitations, 0.2-3 mg cell extract and 2-4 µg antibody were used per sample. After preclearing, the antibody protein complexes were allowed to form overnight at 4°C. GammaBind protein G Sepharose was used to collect the immunocomplex. Immunoprecipitates were washed five times with modified lysis buffer (without inhibitors, 100 mM NaCl). Samples were eluted with SDS loading buffer, separated through 6 % SDS-PAGE and transferred to a PVDF membrane. Proteins were detected using appropriate antibodies and ECL reagents.

To study for protein-DNA interactions, some immunoprecipitations were performed in the presence of 50 µg/ml ethidium bromide. To remove phosphate from precipitated proteins, immunoprecipitated proteins were washed with calf intestinal phosphatase(CIP)-buffer (Amersham Biosciences) and treated with CIP prior to elution with SDS-PAGE loading buffer. Cells were treated with 20 µg/ml α-amanitin, 40 µM roscovitine, 50 µM DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) or 5 µg/ml aphidicolin for 1.5 h prior to preparation of cell extract to study the effect of inhibitors on the interaction.

### 4.13 Immunofluorescence microscopy (III)

T98G cells were grown on glass slides to the logarithmic phase and washed with hypotonic KM buffer on ice followed by 30 min permeabilization in the KM buffer containing 0.5 % NP-40, and complete protease inhibitors. The bulk of DNA bound to proteins was removed by digestion of DNA with *HindIII* and *PstI*. Cells were fixed with 3 % paraformaldehyde in PBS followed by quenching with 50 mM NH<sub>4</sub>Cl in PBS.

For UV radiation, T98G cells were grown to the logarithmic phase on glass slides, washed once with PBS and partially exposed to 60 J/m<sup>2</sup> UV light using an 8 μm micropore filter. Cells were allowed to recover from a UV pulse for one hour in balanced growth medium and were then permeabilized with 0.2 % Triton X-100, fixed with 3 % paraformaldehyde, and quenched with 50 mM NH<sub>4</sub>Cl. Samples to be stained with H3 antibody later on were treated with 2 M HCl for 7 minutes at room temperature to denature DNA.

Prior to antibody treatment, cells were treated with 0.2 % cold water fish skin gelatin in PBS for one hour. Primary antibodies were diluted to 5 μg/ml and secondary antibodies to 4 μg/ml with 0.2 % gelatin in PBS. Cells were sequentially stained for 30 min at 37°C. DNA was stained with 1 μg/ml bisbenzimidazole for 5 min at room temperature followed by mounting to objective glass with Immu-Mount. Images were captured with a cooled CCD camera and Olympus BX-61 microscope with an 100x objective followed by image processing with Adobe Photoshop.

## 5 Results

### 5.1 Characterisation of non-homologous end joining *in vitro* and *in vivo* (I)

An assay was developed to study the mechanism of NHEJ *in vitro*. Three different linear substrates that were generated by digestion of M13mp18 derived plasmid were utilised. Two of the substrates were double digested resulting in DNA ends that could not be joined by simple ligation of complementary ends. The S substrate had blunt ends, the XM substrate had 5' protruding ends, and the AK substrate had 3' protruding ends.

Treatment of the substrate with HeLa cell extract resulted in conversion of the substrate into mono- and multimeric products. Multimeric products were mainly formed by simple ligation of complementary ends. For the study of NHEJ, the circular monomer products were the most interesting since they arose by self-joining of non-homologous ends. These circular monomers could be detected after transformation of isolated end joining products into *E. coli* since the circular form is transformed much more efficiently than other forms. Furthermore, additional information on end joining products was gained since the cleavage site of the DNA substrate was located in the  $\alpha$ -complementing *lacZ* reporter gene fragment. Transformation of the repair products in the presence of IPTG and X-gal resulted in either blue or white plaques depending on the reading frame and possible insertions and deletions.

DNA-PK is a central player in mammalian NHEJ. Therefore, we tested whether the NHEJ activity described here was dependent on DNA-PK activity. Two chemicals, wortmannin and LY2940002, that inhibit DNA-PK and other enzymes of the phosphatidylinositol 3-kinase family, efficiently inhibited NHEJ in a dose-dependent manner. Furthermore, a polyclonal antibody against the Ku80 subunit of DNA-PK inhibited NHEJ. These results indicate that formation of circular monomers by the NHEJ activity described here is dependent on DNA-PK and represents the relevant repair pathway in mammals.

To study whether the cell-free NHEJ assay *in vitro* reflected the situation *in vivo* the ability of cycling HeLa cells to join incompatible ends was studied. The substrate XM was electroporated to HeLa cells and after different time points, plasmid was recovered

and analysed by transformation. During the four hour time period studied, a relatively high proportion of DNA (approximately 90%) was repaired within 30 minutes, compared to the 2 hour peak value that was given for 100 % NHEJ activity. A longer incubation time did not increase the NHEJ activity. The repair products after *in vitro* and *in vivo* NHEJ did not vary noticeably as judged by plaque colour distribution and DNA sequencing of products indicating that the *in vitro* NHEJ assay simulated well the situation *in vivo*.

## **5.2 DNA synthesis in non-homologous end joining *in vitro* (I)**

Since the role of DNA synthesis in NHEJ was under investigation, the nucleotide requirement of NHEJ *in vitro* was studied. ATP was absolutely required for NHEJ as expected since a DNA ligation step is required. On the other hand, dNTPs were not absolutely required, and depending on the substrate, 50 % to 80 % NHEJ activity remained when nucleotides were omitted. Especially interesting was the XM substrate where a subpopulation of end products giving rise to white plaques was considerably reduced after omission of dNTPs. These results suggest that DNA synthesis is involved in NHEJ *in vitro*.

Since a stimulatory effect of dNTPs on NHEJ suggested the involvement of DNA synthesis in NHEJ, the effect of the DNA polymerase inhibitor aphidicolin was studied to help in identification of the pol used in NHEJ. Aphidicolin is an inhibitor for pol  $\alpha$ , pol  $\delta$ , and pol  $\epsilon$ . Aphidicolin inhibited end joining with all substrates, the highest inhibition being for the XM substrate where the inhibition was up to 50 %. Again, in case of the XM substrate, the formation of end products giving rise to white plaques was especially reduced.

Analysis of repair products by sequencing revealed that DNA synthesis indeed influenced NHEJ. Two major modes of repair were detected and microhomology of the overhangs was utilised in both modes. In the direct mode, the reading frame was sustained and this resulted in blue plaques after transformation. In the overlap mode, DNA synthesis was involved and the reading frame was changed, resulting in white plaques after transformation. Omission of nucleotides or addition of aphidicolin reduced the amount of repair products where DNA synthesis was required. It can be concluded that the white plaques represented mainly NHEJ where DNA synthesis was involved.

## **5.3 Pol $\alpha$ is involved in non-homologous end joining *in vitro* (I)**

Studies with the NHEJ *in vitro* assay suggested that polymerase activity is involved in NHEJ. Therefore, to identify the pol involved in NHEJ, the effect of antibodies that neutralise polymerase activity was studied. Neutralising antibodies against pols  $\alpha$ ,  $\beta$ , and  $\epsilon$  were available and as a control, mouse IgG was used. HeLa cell extract was incubated with the antibodies prior to the repair reaction.

Antibodies against pol  $\alpha$  decreased NHEJ activity remarkably in a concentration dependent manner. The formation of white and blue repair products was inhibited to the

same extent, and no effect on the distribution of end joining products was detected as judged by sequencing. The repair products were analysed with Southern analysis, and this revealed that formation of multimers that arise by ligation of complementary ends was not inhibited but rather stimulated. Antibodies against pol  $\beta$  or pol  $\epsilon$  and the control IgG had no effect on NHEJ. Altogether these results indicate that pol  $\alpha$  is required for joining of non-complementary DNA ends *in vitro*.

#### **5.4 PCNA influences non-homologous end joining *in vitro* (I)**

PCNA is a ring-shaped trimeric protein that functions in DNA replication, repair, and cell cycle control (Maga & Hübscher 2003). Two approaches were utilised to study the involvement of PCNA in NHEJ. First, a HeLa cell extract was fractionated through two ion exchange columns to separate PCNA from most cellular proteins (Shivji *et al.* 1992). The fractionation resulted in three fractions, and the ability of different combinations of fractions to support NHEJ *in vitro* was studied. All three fractions were required for optimal NHEJ and this activity was defined as 100 %. After omission of the PCNA containing fraction 79 % NHEJ activity remained, and purified PCNA restored NHEJ activity completely.

An alternative approach was employed to study the involvement of PCNA in NHEJ. PCNA was depleted from a HeLa cell extract with the p21-derived peptide (Warbrick *et al.* 1995). Removal of PCNA was efficient but not complete, because PCNA is an abundant protein its complete depletion is difficult. After depletion of PCNA 85 % NHEJ activity remained and addition of purified PCNA restored the repair activity. A mutated peptide neither precipitated PCNA nor decreased NHEJ activity, confirming that the observed effect is specific for PCNA depletion. The repair products after PCNA removal did not vary noticeably as judged by plaque colour distribution and DNA sequencing of products. Taken together, PCNA influences NHEJ *in vitro*, but its effect is only modest.

#### **5.5 UV-crosslinking of proteins to nascent DNA during S phase (II)**

The DNA polymerase trap technique tags the pols with their DNA product and has been utilised to study the roles of pols in DNA replication (Insdorf & Bogenhagen 1989, Zlotkin *et al.* 1996). The DNA from a monolayer of mimosine synchronised HeLa cell nuclei was pulse labelled with radioactive dATP and BrdUTP facilitating the subsequent UV-crosslinking of DNA to proteins. Following DNase digestion, the photolabelled proteins were purified and the pols were immunoprecipitated. Although this method did not reveal the absolute level of the pols engaged in DNA synthesis, the relative changes in their level as a function of S phase progression could be determined.

Pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were efficiently and specifically labelled with radioactive dATP. The photolabelling intensities of pol  $\alpha$  and pol  $\epsilon$  slightly increased as S phase progressed. For pol  $\delta$  the pattern was different. Pol  $\delta$  was less efficiently photolabelled during early S phase, but at later stages of S phase the labelling intensity of pol  $\delta$  was greatly enhanced.

These results indicate that there are temporal differences between the activities of the pols.

## **5.6 Chromatin association of pols during S phase (II)**

In order to further clarify the roles of pols during replication, the association of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  with chromatin during S phase was investigated. A high salt extraction scheme was employed to separate cellular proteins into different fractions. HeLa cells were lysed in hypotonic buffer in the presence of NP-40 detergent to release detergent-soluble protein. The remaining monolayer of open nuclei was extracted with high-salt buffer resulting in a release of chromatin-associated proteins, this fraction was named the “bound fraction”. The remaining material was solubilised in SDS and was named the “rest fraction”. The success of fractionation was studied with marker proteins from asynchronous cells and their distribution suggests that the fractionation method represents the subcellular localisation of the proteins well. Pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were distributed between the soluble and high salt fractions.

The behaviour of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  during the S phase was studied by using mimosine synchronised HeLa cells. Pol  $\alpha$  association with chromatin increased slightly during S phase, whereas the association of pol  $\delta$  with chromatin increased more rapidly. The association of pol  $\epsilon$  with chromatin appeared to be largely constant or slightly decreasing during S phase. The detected changes are only moderate, but statistical analysis revealed the differences between the pols to be significant. During S phase the change in high salt extractable pol  $\epsilon$  appears to be different from those of pols  $\alpha$  and  $\delta$ .

## **5.7 Effect of neutralising antibodies on DNA synthesis in the DNA replication assay (II)**

The temporal differences between the contributions of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  during DNA replication was further studied by employing neutralising antibodies in the replication assay in isolated nuclei (Krude *et al.* 1997). The effect of antibodies against the three pols as a function of S phase progression was studied by using nuclei from mimosine synchronised HeLa cells or from serum stimulated T98G cells.

The antibody against pol  $\alpha$  inhibited consistently about half of the replicative DNA synthesis and the inhibition did not vary greatly during S phase. In contrast, the inhibition pattern of the antibody neutralising the polymerase activity of pol  $\delta$  was different, inhibition by this antibody increased almost three times as cells progressed from the G1/S boundary to the late S phase. Pol  $\epsilon$  behaved differentially, the inhibition of DNA replication with an antibody against pol  $\epsilon$  decreased as S phase proceeded.

Mimosine causes DNA damage, and more specifically DNA double strand breaks (Szüts & Krude 2004). However, the method of cell synchronisation did not affect the results, since a similar inhibition pattern was detected for both mimosine synchronised HeLa nuclei and serum stimulated T98G nuclei. For pol  $\delta$  the increase in inhibition was

even more pronounced. From these results it can be concluded that pol  $\alpha$  is active throughout the S phase, pol  $\epsilon$  activity contributes to DNA replication more at early S phase and its relative importance decreases during S phase progression. In contrast, pol  $\delta$  activity tends to increase as S phase proceeds.

## 5.8 Localisation of pols during S phase (II)

The nuclear localisation of pols  $\alpha$ ,  $\delta$ ,  $\epsilon$ , and PCNA during S phase progression was studied by using immunoelectron microscopy and human IMR-90 fibroblasts synchronised with mimosine. In addition, serum stimulated T98G cells were employed for the detection of pol  $\delta$  and pol  $\epsilon$ . For detection of the pol  $\delta$ , an antibody against p50, the B subunit of human pol  $\delta$ , was utilised. p50 has been previously shown to colocalise with the catalytic subunit (Szekely *et al.* 2000). The stainings were specific as judged by very weak background staining after omission of primary antibodies.

Pol  $\alpha$  localised to ring-shaped focal structures or to more dispersed nuclear regions that were particularly visible at later stages of S phase. The ring-shaped foci of pol  $\alpha$  have been previously shown to coincide with sites of DNA synthesis (Lattanzi *et al.* 1998, Jaunin *et al.* 2000). Pol  $\alpha$  colocalised at a near molecular level with pol  $\epsilon$ . For analysis, clusters containing 3 or more gold particles were considered as foci. As determined from pol  $\alpha/\epsilon$  double staining, 41 % of pol  $\alpha$  localised to foci and the relative level of pol  $\alpha$  in foci was rather constant until the late S phase/G<sub>2</sub> transition where the levels decreased. In focal staining of pol  $\alpha$ , there were both foci with pol  $\alpha$  alone or pol  $\alpha$  colocalising with pol  $\epsilon$ .

Pol  $\delta$  localised mainly outside the ring-shaped foci typical for pols  $\alpha$  and  $\epsilon$ . As judged from double stainings with pol  $\epsilon$ , only 30 % of pol  $\delta$  was found in foci of 3 or more gold particles, compared to 74 % focal localisation of pol  $\epsilon$ . Pol  $\delta$  staining was rather dispersed but still restricted to certain regions of the nucleus. Pol  $\delta$  often adopted a “beads-on-a-string” structure. Comparison of overall staining intensities of pol  $\delta$  and pol  $\epsilon$  revealed that the staining intensity of pol  $\delta$  relative to pol  $\epsilon$  increased as S phase advanced.

Pol  $\epsilon$  staining was concentrated in foci. In pol  $\alpha/\epsilon$  double staining, 75 % of pol  $\epsilon$  was focal, and with pol  $\delta/\epsilon$  double staining, 74 % of pol  $\epsilon$  was focal. Focal staining of pol  $\epsilon$  was most pronounced from G<sub>1</sub> up to early S phase. Pol  $\epsilon$  levels detected by staining decreased relative to pol  $\alpha$  and pol  $\delta$  as S phase advanced.

Pol  $\delta/\epsilon$  double labelling was repeated with serum stimulated T98G cells. The major features of the staining were conserved when the results from the two synchronisation methods were compared. Pol  $\epsilon$  staining was mainly focal (65 %) whereas pol  $\delta$  staining was predominantly dispersed (30 % focal). Pol  $\epsilon$  staining was strongest in early S phase and as S phase progressed relative pol  $\epsilon$  staining decreased. Dispersed staining of pol  $\delta$  prevailed also in T98G cells after serum stimulation, and part of pol  $\delta$  was concentrated in foci, which consisted either of pol  $\epsilon$  and  $\delta$ , or solely of pol  $\delta$ .

Localisation of pols  $\delta$  and  $\epsilon$  relative to PCNA was studied to clarify the functional significance of the observed staining pattern of pols  $\delta$  and  $\epsilon$ . PCNA is the processivity factor of both pol  $\delta$  and pol  $\epsilon$ , and a widely used marker for active replication (Bravo &



MacDonaldo-Bravo 1987). The PCNA staining pattern resembled pol  $\alpha$  by being partly focal and partly disperse or “beads-on-a-string”-like. This kind of PCNA staining has been reported to coincide at least partly with sites of DNA synthesis in previous electron microscopy studies (Raska *et al.* 1991, González-Melendi *et al.* 2000). Focal staining is most obvious from G<sub>1</sub> to early S phase, and these foci often contained also pol  $\epsilon$ , but less often pol  $\delta$ . In double staining of pol  $\delta$  and PCNA intimate colocalisation was detected, since pairs of small and large gold particles were found, especially at late S phase. The levels of pol  $\delta$  relative to PCNA slightly increased during S phase, whereas for pol  $\epsilon$ , staining decreased relative to PCNA.

Altogether the IEM stainings showed that pol  $\delta$  localisation was mostly dispersed or beads-on-a-string-like that prevailed in late S phase, whereas pol  $\epsilon$  localised mainly to a ring-shaped clusters that dominated during early S phase. The staining patterns of two markers for DNA replication, pol  $\alpha$  and PCNA, had both focal and dispersed features.

### 5.9 Pol $\epsilon$ is labelled with RNA (III)

A UV-crosslinking technique was utilised to study the association of pols with nascent nucleic acids. HeLa cells were labelled with radioactive UTP or dATP, and pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were immunoprecipitated. All three pols were labelled with dATP as expected since they are required for mammalian DNA replication (Zlotkin *et al.* 1996). In addition, pol  $\alpha$  and pol  $\epsilon$  were labelled with UTP.

To test whether the RNA labelling of pols  $\alpha$  and  $\epsilon$  was linked to lagging strand synthesis during DNA replication, a panel of controls was tested. Either DNA replication or transcription was inhibited and in one sample, BrdUTP that facilitates crosslinking, was omitted. For pol  $\alpha$ , inhibition of replication and omission of BrdUTP abolished the labelling whereas inhibition of transcription had no effect, suggesting that the labelling of pol  $\alpha$  was linked to DNA replication. In contrast, pol  $\epsilon$  behaved differentially. Inhibition of replication or omission of BrdUTP did not remove the UTP labelling of pol  $\epsilon$ , whereas inhibition of transcription completely abolished the labelling. These results suggest that the labelling of pol  $\alpha$  is linked to DNA replication whereas pol  $\epsilon$  labelling is linked to transcription and that pol  $\epsilon$  associates with nascent RNA during transcription.

### 5.10 Pol $\epsilon$ interacts with RNA pol II (III)

UV-crosslinking studies of human replicative pols with freshly synthesised nucleic acids suggested that pol  $\epsilon$  was crosslinked to nascent RNA not related to DNA replication. Therefore, the association of pol  $\epsilon$  with the transcription machinery was studied. Immunoprecipitations from HeLa cell extract with antibodies against RNA pol II, pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were conducted. An antibody against RNA pol II coprecipitated pol  $\epsilon$  but not pols  $\alpha$  and  $\delta$ . Reciprocal immunoprecipitations with antibodies against pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  confirmed the observation since only the antibody against pol  $\epsilon$  coimmunoprecipitated RNA pol II. The effect of ethidium bromide was tested to study whether the interaction was mediated through DNA (Lai & Herr 1992). Ethidium bromide did not

influence the interaction, indicating that the interaction between RNA pol II and pol  $\epsilon$  was not mediated through DNA.

Pol  $\epsilon$  has been previously found in the RNA pol II holoenzyme complex (Maldonado *et al.* 1996). Therefore we studied whether RNA pol II copurified with pol  $\epsilon$  purified with a standard protocol (Syväoja & Linn 1989). The presence of RNA pol II in the fractions containing peak activity of pol  $\epsilon$  was studied with Western analysis. Pol  $\epsilon$  and part of the RNA pol II copurified through three columns. However, during purification RNA pol II was degraded, especially in the last steps of the purification. The copurification is consistent with the observed interaction between pol  $\epsilon$  and RNA pol II.

### **5.11 Pol $\epsilon$ interacts with a hyperphosphorylated elongation isoform of RNA pol II (III)**

RNA pol II is regulated through phosphorylation. During the initiation of transcription, RNA pol II is hypophosphorylated (IIA isoform) and subsequently, during elongation, the IIA isoform is hyperphosphorylated to the IIO isoform. The IIO form has reduced electrophoretic mobility compared to the IIA form. Anti-pol  $\epsilon$  antibodies coprecipitated only the slowly migrating form of RNA pol II. The phosphorylation status of the RNA pol II associated with pol  $\epsilon$  was studied by treating the immunoprecipitates with phosphatase. Treatment of pol  $\epsilon$  and RNA pol II immunoprecipitates with phosphatase resulted in an increased mobility of the RNA pol II that corresponded to the molecular weight of the isoform IIA. These results indicate that pol  $\epsilon$  specifically interacts with the hyperphosphorylated, transcriptionally active isoform of RNA pol II.

During the transcription cycle, RNA pol II is sequentially phosphorylated at serines 2 and 5 in the C-terminal domain heptapeptide repeat. Phosphospecific antibodies were employed to study the interaction between RNA pol II and pol  $\epsilon$  more carefully during the transcription cycle. Antibodies recognising hyperphosphorylated RNA pol II with a certain phosphorylation pattern were utilised. All the tested antibodies against RNA pol II coimmunoprecipitated pol  $\epsilon$ . The RNA pol II coprecipitating with pol  $\epsilon$  was recognised by all antibodies as the IIO form. These results suggest that although pol  $\epsilon$  interacts only with hyperphosphorylated RNA pol IIO, the interaction is not dependent on phosphorylation of specific residue.

Chemicals known to inhibit transcription were employed to study the nature of the interaction with RNA pol II and pol  $\epsilon$  more carefully. Roscovitine and DRB inhibit phosphorylation of RNA pol II and, thereby, prevent transition to the elongation complex. Treatment of the cells with these inhibitors strongly reduced the hyperphosphorylation of RNA pol II. Treatment with DRB or roscovitine abolished the coimmunoprecipitation of RNA pol II with pol  $\epsilon$ .  $\alpha$ -amanitin inhibits transcription both in the initiation and elongation phase (Gong *et al.* 2004). Treatment of the cells with  $\alpha$ -amanitin prior to preparation of cell extract had no effect on the interaction between RNA pol II and pol  $\epsilon$ . These results indicate that pol  $\epsilon$  associates specifically with elongation competent RNA pol II, and the interaction persists even when transcription is stalled.

### **5.12 Pol $\epsilon$ and RNA pol II colocalise (III)**

The localisation of pol  $\epsilon$  and RNA pol II in cells was studied with immunofluorescence and immunoelectron microscopy. T98G cells were stained with RNA pol II antibody recognising the transcriptionally active form of RNA pol II. In immunofluorescence, pol  $\epsilon$  localised to numerous foci whereas RNA pol II showed rather even staining with very few distinct foci. Focal staining of RNA pol II was facilitated by hypotonic permeabilization and removal of bulk DNA prior to fixation. These RNA pol II foci colocalised with pol  $\epsilon$ .

IEM of T98G cells revealed that pol  $\epsilon$  and RNA pol II colocalise also when studied with higher resolution technique. Quantitative analysis of stainings indicated that, on average, 52 % of pol  $\epsilon$  and 66 % of RNA pol II localised to centers containing 3 or more gold particles. Approximately 40 % of RNA pol II and pol  $\epsilon$  colocalised.

### **5.13 The interaction between pol $\epsilon$ and RNA pol II is not linked to nucleotide excision repair (III)**

Pol  $\epsilon$  and components of the RNA pol II holoenzyme complex have been implicated in nucleotide excision repair (de Laat *et al.* 1999, Araújo *et al.* 2000). Therefore, we studied whether the observed interaction between RNA pol II and pol  $\epsilon$  was linked to NER. T98G cells were partially UV irradiated through a micropore filter, stained with antibodies against RNA pol II, pol  $\epsilon$  or markers for nucleotide excision repair. The marker proteins XPA and XPG localised to the irradiated area indicating that the cells responded to UV damage. However, pol  $\epsilon$  and most of the RNA pol II did not show a difference in staining intensity between damaged and control areas.

Coimmunoprecipitation studies from UV damaged cells were conducted to study the nature of the interaction further. None of the marker proteins for NER, namely XPA, XPB or XPG, were found to coimmunoprecipitate with RNA pol II or pol  $\epsilon$  before or after UV-damage. The results from immunofluorescence stainings and coimmunoprecipitation studies suggest that the interaction between RNA pol II and pol  $\epsilon$  is not linked to NER.

### **5.14 The interaction between pol $\epsilon$ and RNA pol II persist through the cell cycle (III)**

In order to determine whether the interaction between pol  $\epsilon$  and RNA pol II was linked to DNA replication, their behaviour during cell cycle was studied. IEM was carried out on T98G cells that were serum deprived and stimulated creating samples from the G<sub>0</sub> until the late S/G<sub>2</sub> phases of the cell cycle. In IEM pol  $\epsilon$  and RNA pol II demonstrated striking colocalisation, but no apparent differences in their localisation pattern during different cell cycle stages was detected.

RNA pol II and pol  $\epsilon$  were immunoprecipitated from cell extracts prepared from serum starved and stimulated T98G cells from G<sub>0</sub> until G<sub>2</sub>/M phases. The immunoprecipitation studies indicated that the interaction between RNA pol II and pol  $\epsilon$  was not restricted to a certain stage of the cell cycle. Furthermore, aphidicolin, an inhibitor of DNA replication, did not prevent the interaction. These results suggest that the interaction is not restricted to S phase and is not dependent on DNA replication. However, this does not exclude the possibility that the interaction is linked to coordination of transcription and DNA replication.

## 6 Discussion

### 6.1 The role of DNA synthesis in non-homologous end joining

We developed an *in vitro* method for studying NHEJ activity in mammalian cell extracts. DNA-PK is a key component in NHEJ. The NHEJ activity measured depended on DNA-PK activity and, thus represents a relevant pathway in mammals. What is more, the substrates utilised in this study could not be joined by simple ligation of complementary ends but required trimming, which indicated that sophisticated repair machinery had to be employed. In addition, the NHEJ measured *in vitro* reflected well the conditions *in vivo*, as judged by plaque color distribution and DNA sequencing of repair products. DNA synthesis, and particularly pol  $\alpha$ , was found to facilitate NHEJ, since omission of dNTPs, addition of aphidicolin or antibodies against pol  $\alpha$  decreased NHEJ. Polymerase activity was recognised as a novel factor contributing to the stability of the DNA ends.

The involvement of DNA repair synthesis in NHEJ has remained controversial. The earliest NHEJ assays were conducted in the absence of nucleotides, and obviously in the absence of DNA synthesis (e.g. North *et al.* 1990). However, it is possible that residual nucleotides from cell extracts may have been sufficient for DNA synthesis. Later, various pols have been suggested to function in NHEJ. Pol  $\alpha$  has been found to perform DNA synthesis on discontinuous, unlinked DNA templates, a process that resembles NHEJ (Islás *et al.* 1998). Islás and coworkers suggested that pol  $\alpha$  may rejoin DSBs occurring during DNA replication. In yeast, pol4 (pol  $\beta/\lambda$ ) was implicated in NHEJ but pol  $\beta$  did not contribute to end joining in mammals (Wilson & Lieber 1999, Ma *et al.* 2004). Furthermore, the recently identified pols, pol  $\mu$ , pol  $\lambda$ , and TdT, have been implicated in NHEJ (Mahajan *et al.* 1999, 2002, Lee *et al.* 2004b). It would be interesting to study the role of recently identified pols in NHEJ with our *in vitro* assay.

Many pols are evidently capable of DNA synthesis in NHEJ ensuring efficient repair of DSBs. The polymerase used may depend on the assay conditions utilised, end configuration at the break site or the stage of the cell cycle. It was found that the template affects the pol usage, at least for pol  $\lambda$ , pol  $\mu$ , and TdT, for which a gradient of template dependence was found (Nick McElhinny *et al.* 2005). In addition, Daley and Wilson (2005) found three alternative pathways for rejoining of DSBs: NHEJ, a single strand

annealing-like pathway, and a novel pathway independent of NHEJ proteins and Rad52. Pathway choice and repair efficiency were governed by the base pairing potential of the overhangs. DSBs arise spontaneously during DNA replication (Pastwa & Blasiak 2003). Pol  $\alpha$  is well suited to repair of DSBs arising during DNA replication, since pol  $\alpha$  takes part in DNA replication, and therefore it is readily available in the vicinity of the damage.

## 6.2 PCNA is involved in non-homologous end joining

PCNA was found to enhance NHEJ, but the effect detected was minor. PCNA is a protein with multiple functions. In DNA replication, PCNA serves as a processivity factor for pols  $\delta$  and  $\epsilon$ , it is involved in numerous DNA repair pathways, and it has a role in cell cycle control (Maga & Hübscher 2003). This suggested the possibility that PCNA was involved in NHEJ. First, it was considered to aid in identification of the pol involved in NHEJ, but subsequently the role of PCNA in NHEJ was considered to be independent of polymerase function. PCNA depletion did not affect the product distribution, indicating that PCNA probably does not serve as an auxiliary factor for the pol. This is consistent with results from the assay with neutralising antibodies, where antibodies against pol  $\alpha$  inhibited NHEJ.

In *Drosophila melanogaster*, PCNA, Ku, and Rad54 are required for the repair of DSBs created by P-element excision and X-ray damage in a homology dependent process (Beall & Rio 1996, Henderson & Glover 1998, Kooistra *et al.* 1999). Increased interaction of PCNA with Ku70-Ku80 after  $\gamma$ -radiation in human cells was observed (Balajee & Geard 2001). Furthermore, Nabatiyan and colleagues (2006) found that PCNA has a role in DSBR in human cells. The expression of PCNA was induced upon the generation of DSBs, and this induction was DNA-PK dependent. PCNA was recruited to damaged chromatin undergoing DNA repair of DSBs by a NHEJ pathway in the absence of extensive DNA synthesis. These observations are consistent with our findings.

The removal of PCNA decreased NHEJ activity only moderately in our *in vitro* assay. Three possibilities can explain why a greater effect for removal of PCNA was not observed. It could result from incomplete depletion of PCNA, the residual PCNA could have been sufficient for relatively efficient NHEJ. However, the fractionation results do not support this view, since PCNA was efficiently separated to one fraction. Alternatively, some other component could substitute for PCNA in NHEJ. The Rad1-Rad9-Hus1 complex resembles a PCNA-like sliding clamp, and it has been shown to interact or colocalise with DNA repair proteins e.g. after IR (Meister *et al.* 2003, Parrilla-Castellar *et al.* 2004). It is as well possible that PCNA is involved in an alternative, minor pathway of NHEJ.

## 6.3 The roles of pol $\alpha$ , pol $\delta$ , and pol $\epsilon$ in DNA replication

DNA polymerases are important enzymes in DNA replication where they polymerise long polynucleotide chains. Pol  $\alpha$  synthesises short RNA-DNA primers for the leading and lagging strands. Pol  $\delta$  and pol  $\epsilon$  are both linked to leading and lagging strand

synthesis, but their exact roles in DNA replication remains to be determined conclusively. The study of mammalian DNA replication is challenging because of the large size of the genome and many proteins involved in the process. In this study, versatile biochemical and cell biological methods were utilised to clarify the mechanism of DNA replication; especially the roles of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  during S phase. Results from four independent methods show that there are temporal differences between the activities and localisations of pols. Pol  $\alpha$  is active through S phase, pol  $\delta$  activity increases as S phase advances whereas the contribution of pol  $\epsilon$  to DNA synthesis decreases as S phase progresses. IEM stainings revealed that pols  $\delta$  and  $\epsilon$  localise to mainly different nuclear sites throughout S phase. These results suggest that pol  $\delta$  and pol  $\epsilon$  pursue their functions independently during S phase.

It could be considered that the differences in the behaviour of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  would reflect DNA repair, since mimosine utilised to synchronise cells induces DNA damage (Szüts & Krude 2004). However, we exclude this interpretation since results from serum starved and stimulated cells are in line with results from mimosine synchronised cells.

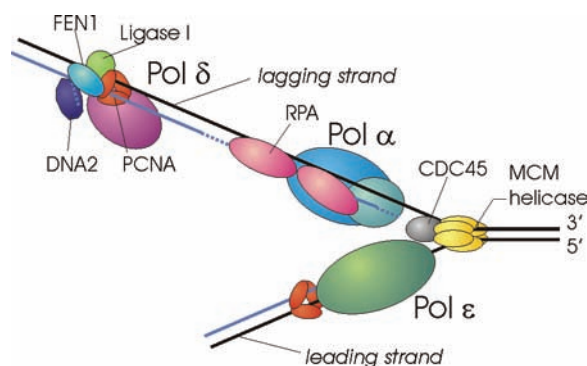
Loading of pols onto a replication origin region during the cell cycle in *S. cerevisiae* was studied by Hiraga and coworkers (2005). Interestingly, they found that pol  $\alpha$  and pol  $\epsilon$  associated with an early firing autonomously replicating sequence ARS305 at almost the same time but before pol  $\delta$  during early S phase. This is consistent with our findings. The localisation of pol  $\epsilon$  and PCNA in proliferating primary fibroblasts during S phase was studied by confocal scanning laser microscopy (Fuss & Linn 2002). Pol  $\epsilon$  formed foci throughout the cell cycle. During early S phase pol  $\epsilon$  foci were adjacent to PCNA foci and these foci colocalised with PCNA and sites of DNA synthesis only in late S phase suggesting a role for pol  $\epsilon$  in DNA replication late in S phase. However, when we studied the localisation of these proteins with higher resolution immunoelectron microscopy, the localisation pattern was different. PCNA foci often contained pol  $\epsilon$  and the colocalisation was highest in early S phase and as S phase advanced, pol  $\epsilon$  staining relative to PCNA decreased. The large foci observed by Fuss & Linn (2002) correspond well in size to the regions of dispersed staining in late S phase in our study.

Three models are considered to explain the observed differences between behaviours of pols  $\delta$  and  $\epsilon$  during DNA replication. First, pol  $\epsilon$  could be involved in initiation of DNA replication as suggested earlier (Aparicio *et al.* 1997, 1999, Mimura *et al.* 2000). Yet, this model does not exclude a role for pol  $\epsilon$  in the elongation phase. It was demonstrated with chromatin immunoprecipitation studies that pols  $\alpha$  and  $\epsilon$  load concurrently onto origins of replication (Aparicio *et al.* 1997, 1999, Masumoto *et al.* 2000). As S phase progressed these pols were transferred from origin to nonorigin DNA with Cdc45 and MCM2-7. This is in agreement with our observations that pol  $\epsilon$  is also involved in elongation of DNA replication since pol  $\epsilon$  was crosslinked to nascent DNA throughout S phase. Furthermore, DNA elongation is significantly impaired without pol  $\epsilon$  in *Xenopus* egg extracts (Waga *et al.* 2001, Fukui *et al.* 2004).

In the second model, pols  $\delta$  and  $\epsilon$  replicate different regions of the genome, e.g., heterochromatic or euchromatic DNA. Increased participation of pol  $\delta$  in DNA synthesis in later S phase could indicate a specific role for this enzyme in heterochromatic DNA replication, and the more pronounced role of pol  $\epsilon$  in early S phase suggest that pol  $\epsilon$  is involved in euchromatic DNA replication. However, Hiraga and coworkers (2005) showed that all three replicative pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  were associated with early-firing origins

in cells arrested early in S phase, suggesting that all three replicases participate in the synthesis at each active origin.

In the third model, the initiating pol  $\alpha$  and the elongating pol ( $\delta$  and/or  $\epsilon$ ) are physically coupled at the fork junction through interactions with the primosome components, notably the fork unwinding helicase and single stranded DNA binding protein RPA (Kaufmann & Nethanel 2004). Still, in this case elongation and maturation of the discontinuous DNA strand could be spatially and temporally separated from the events at the fork junction and especially from leading strand synthesis (Fig. 3). The maturation reactions in lagging strand synthesis where pol  $\delta$  is implicated could be separated considerably in space and time from the events at the fork junction. Earlier observations support this view. It was found that emetine-induced histone depletion preferentially inhibited the synthesis of lagging strand and under these conditions uncoupling of DNA synthesis on both strands could be directly detected (Burhans *et al.* 1991). In addition, uncoupling of DNA synthesis from the helicase was observed in DNA replication in the *Xenopus* egg extracts after DNA damage or in the presence of aphidicolin (Walter *et al.* 2000, Byun *et al.* 2005). Pol  $\delta$ , but not pol  $\epsilon$ , was found to cooperate with Fen1 nuclease in processing of the nicks arising in the lagging strand synthesis (Garg *et al.* 2004). Our observations from IEM fit the model where synthesis of the two strands is not coupled. Pols  $\alpha$  and  $\epsilon$  could be located at the fork junction, the former depositing RNA-DNA primers on the lagging strand and the latter extending the continuous DNA strand. This would explain the observed colocalisation and ring-shaped structures for pols  $\alpha$  and  $\epsilon$ . The Pol  $\delta$  visualised outside these foci would be engaged in lagging stand synthesis and lag behind. This separation may increase as the S phase progresses, especially if Okazaki fragment synthesis would be distributive. Distributive Okazaki fragment synthesis would allow the synthesis of more than one Okazaki fragment at a time. Garg & Burgers (2005) discuss this possibility, and some evidence on distributive Okazaki fragment synthesis has been found from Archaea (Matsunaga *et al.* 2003). This distributive Okazaki fragment synthesis could result in the increased contribution of pol  $\delta$  to replicative DNA synthesis in late S phase. Obviously, further experiments are required to determine the actual roles of the three replicases at the eukaryotic replication fork.



**Fig. 3. DNA replication fork, adapted with modification from Helmut Pospiech.**



## 6.4 The interaction between pol $\epsilon$ and RNA pol II

Pol  $\epsilon$  and RNA pol II were found to associate. Reciprocal immunoprecipitations with multiple antibodies demonstrated strong interaction between RNA pol II and pol  $\epsilon$  but not with pols  $\alpha$  or  $\delta$ . Especially the hyperphosphorylated isoform of RNA pol II, which is involved in transcription elongation, was found to associate with pol  $\epsilon$ . In addition, pol  $\epsilon$  was labelled with the RNA precursor UTP indicating a close association of pol  $\epsilon$  with nascent transcripts. The proteins colocalised in cells as judged by results from immunofluorescence and immunoelectron microscopy.

Our observation on the interaction between pol  $\epsilon$  and RNA pol II are in line with previous reports that RNA pol II associates with other DNA replication and repair factors. An RNA pol II complex associated with several general transcription factors, DNA repair proteins, and pol  $\epsilon$  was purified by Maldonado and coworkers (1996). MCM proteins involved in initiation of DNA replication were found to copurify with the RNA pol II holoenzyme and general transcription factors (Yankulov *et al.* 1999). In addition, antibodies against MCM2 inhibited transcription by RNA pol II. However, we were not able to detect XPB, a subunit of TFIIH, in our RNA pol II and pol  $\epsilon$  precipitates suggesting that pol  $\epsilon$  does not interact with RNA pol II during the early phases of transcription (Zurita & Merino 2003). Still, it is possible that the purification method employed may affect the outcome.

The CTD of RNA pol II is an important regulator of the function of RNA pol II (Palancade & Bensaude 2003). Phosphorylation of the CTD allows the transcription cycle to proceed in a coordinated manner. Since the hyperphosphorylated RNA pol II was found to associate with pol  $\epsilon$ , and kinase inhibitors that prevented the phosphorylation-dependent transition from the initiation to the elongation of transcription almost abolished the interaction, it can be speculated that CTD mediates the interaction between pol  $\epsilon$  and RNA pol II. It is also possible, that the interaction is mediated by another protein or other regions of RNA pol II.

Two explanations are considered to interpret the observed interaction between pol  $\epsilon$  and RNA pol II. First, as both pol  $\epsilon$  and components of RNA pol II holoenzyme are linked to nucleotide excision repair, the observed interaction may facilitate NER (de Laat *et al.* 1999). However, the staining pattern after local UV radiation do not support this view since a concerted response of pol  $\epsilon$  and RNA pol II was not detected. In addition, the observed interaction did not depend on the presence of DNA damage. We see no support for the hypothesis that the observed interaction facilitates NER, still we cannot exclude a positive effect on NER.

Second, the interplay between pol  $\epsilon$  and RNA pol II could mediate DNA replication and transcription, either on a local or a global scale. On a local scale the interaction between pol  $\epsilon$  and RNA pol II could be important for the non-disruptive by-pass of a transcription bubble by a replication fork as reported in bacteria (Liu *et al.* 1993). However, when we studied association and localisation of pol  $\epsilon$  and RNA pol II during the cell cycle we found that the interaction was not limited to S phase but persisted throughout the cell cycle, indicating that the interaction did not depend on DNA replication. This argues for more global implications of the interaction in regulating transcription and DNA replication.

There are direct links between DNA replication and transcription. Actively transcribed genes are replicated early in S phase (Hatton *et al.* 1988). This positive regulation of DNA replication can be either a direct effect of the transcription machinery or due to remodelling of chromatin mediated by transcription factors (Hassan & Cook 1994, Kohzaki & Murakami 2005). DNA replication and transcription sites were found to colocalise in HeLa cells when studied with confocal microscopy (Hassan *et al.* 1994). When electron microscopy was utilised, sites of DNA replication were also found to contain RNA processing components, such as factors involved in RNA splicing (Philimonenko *et al.* 2006). Results from our IEM experiments revealed that pol  $\epsilon$  and RNA pol II colocalised extensively and clustered into centres close to small electron-dense domains of the nucleus. The RNA pol II staining corresponded well to earlier ultrastructural studies, where RNA pol II was concentrated into clusters overlapping with the sites of RNA synthesis (Iborra *et al.* 1996, Cmarko *et al.* 1999).

Pol  $\epsilon$  in yeast has been implicated in transcriptional silencing of ribosomal DNA, the mating type locus, and subtelomeric regions (Ehrenhofer-Murray *et al.* 1999, Smith *et al.* 1999, Iida & Araki 2004). Furthermore, the second largest subunit of mouse pol  $\epsilon$  was found to interact with SAP18, which is known to associate with the transcriptional corepressor Sin3 (Wada *et al.* 2002). The interaction between the pol  $\epsilon$  second largest subunit and SAP18 induced repression of transcription in a reporter plasmid assay.

The present study provides a link between components of two cellular processes; transcription and DNA replication. Earlier findings that replication always takes place at transcriptionally active sites stretch the functional relationship between DNA replication and transcription (Hassan & Cook 1994, Hassan *et al.* 1994). The interaction between pol  $\epsilon$  and RNA pol II may regulate DNA replication and transcription on a global scale. Clearly, further experiments are needed to clarify the significance of the observed interaction between pol  $\epsilon$  and RNA pol II.

## 7 Conclusions

In this study, the contribution of replicative pols on various aspects of nucleic acid metabolism of the cell was studied. Utilising an *in vitro* assay for measuring non-homologous end joining, we found that DNA synthesis plays a role in NHEJ and contributes to the stability of DNA. We provided evidence that pol  $\alpha$  has a role in NHEJ. In addition, PCNA was shown to be involved in NHEJ, albeit the effect detected was small.

We studied the roles of pols  $\alpha$ ,  $\delta$ , and  $\epsilon$  in mammalian DNA replication with four techniques. Results from polymerase trap, chromatin association, replication in isolated nuclei, and immunoelectron microscopy studies indicated that pol  $\epsilon$  was more active during early S phase whereas the activity of pol  $\delta$  increased as S phase advanced. These results support the idea that pol  $\delta$  and  $\epsilon$  pursue their function independently during DNA replication.

We found that pol  $\epsilon$  could be crosslinked with nascent RNA. In addition, pol  $\epsilon$  and RNA pol II coimmunoprecipitated. Only the transcriptionally active form of RNA pol II was found to coimmunoprecipitate with pol  $\epsilon$ . We found that a large proportion of RNA pol II and pol  $\epsilon$  colocalised in the cell. The staining pattern did not depend on cell cycle stage, nor was it associated with nucleotide excision repair markers after UV damage. Although the exact function of the interaction between RNA pol II and pol  $\epsilon$  remains to be clarified, the strong association between pol  $\epsilon$  and RNA pol II suggests that the two proteins may be intimately involved in the coordination of DNA replication and transcription.

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- I Pospiech H\*, Rytkönen AK\* & Syväoja JE (2001) The role of DNA polymerase activity in human non-homologous end joining. *Nucleic Acids Res* 29: 3277-3288.
- II Rytkönen AK, Vaara M, Nethanel T, Kaufmann G, Sormunen R, Läärä E, Nasheuer H-P, Rahmeh A, Lee MYWT, Syväoja JE & Pospiech H (2006) Distinctive activities of DNA polymerases during human DNA replication. *Febs J* 273: 2984-3001.
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