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Strand displacement synthesis by yeast DNA polymerase ε

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ABSTRACT

DNA polymerase ε (Pol ε) is a replicative DNA polymerase with an associated 3′–5′ exonuclease activity. Here, we explored the capacity of Pol ε to perform strand displacement synthesis, a process that influences many DNA transactions *in vivo*. We found that Pol ε is unable to carry out extended strand displacement synthesis unless its 3′–5′ exonuclease activity is removed. However, the wild-type Pol ε holoenzyme efficiently displaced one nucleotide when encountering double-stranded DNA after filling a gap or nicked DNA. A flap, mimicking a D-loop or a hairpin structure, on the 5′ end of the blocking primer inhibited Pol ε from synthesizing DNA up to the fork junction. This inhibition was observed for Pol ε but not with Pol δ, RB69 gp43 or Pol η. Neither was Pol ε able to extend a D-loop in reconstitution experiments. Finally, we show that the observed strand displacement synthesis by exonuclease-deficient Pol ε is distributive. Our results suggest that Pol ε is unable to extend the invading strand in D-loops during homologous recombination or to add more than two nucleotides during long-patch base excision repair. Our results support the hypothesis that Pol ε participates in short-patch base excision repair and ribonucleotide excision repair.

INTRODUCTION

DNA polymerase ε (Pol ε) is a replicative polymerase that carries out leading-strand synthesis at the eukaryotic replication fork (1–7). Pol ε is a high-fidelity polymerase with high nucleotide selectivity and an associated 3′–5′ exonuclease activity (8). In addition to its role at the replication fork, Pol ε is engaged in many different cellular functions such as the initiation of DNA replication, S-phase checkpoint activation and DNA repair (9). Participation in DNA repair processes often requires that a DNA polymerase is able to extend 3′-OH ends on nicked substrates, fill in short gaps and/or carry out strand displacement synthesis. Strand displacement synthesis is a process in which the DNA polymerase simultaneously synthesizes DNA and separates the two strands ahead of the DNA polymerase. A single-stranded DNA flap is created after the DNA polymerase has dissociated, and the flap must be removed by a nuclease before a ligase can seal the remaining nick. The commercially available φ29 DNA polymerase has a very high strand displacement activity that is utilized to create PCR-free templates for DNA sequencing (10). However, replicative DNA polymerases are in general very inefficient in strand displacement synthesis. It was recently shown that the regression pressure imposed by the upstream double-stranded DNA shifts the primer terminus from the polymerase active site to the exonuclease active site (11), thus suppressing strand displacement synthesis. It has also recently been shown in the T4 and T7 bacteriophage replication systems that there is a functional coupling between the helicase and polymerase that promotes strand displacement synthesis. In one of the studies, the authors showed that the helicase relieves the regression pressure of the downstream DNA and thus promotes strand displacement synthesis (12). In another study, it was shown that T7 DNA polymerase opens up the double-stranded DNA, and the helicase translocates along and traps the unwound bases (13). In the T4 and T7 bacteriophage, the helicase resides on the lagging strand and thus the leading strand polymerase is on the opposite strand. However, the properties of the leading strand DNA polymerase in eukaryotes might be different because in eukaryotes both Pol ε and the helicase reside on the same strand, the leading strand (3).

Previous studies where strand displacement synthesis by Pol ε were discussed suggested that Pol ε was very inefficient in performing strand displacement synthesis (3,14,15). Here, we show that the strand displacement synthesis by Pol ε is limited to only one nucleotide. We further examined the ability of Pol ε to carry out strand displacement synthesis on different model substrates resembling the intermediate

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substrates of excision repair, ribonucleotide excision repair and extension of the invading strand in D-loops during recombination. Our results support the hypothesis that Pol ε participates in short patch base excision repair and ribonucleotide excision repair.

MATERIALS AND METHODS

Purification of proteins

Saccharomyces cerevisiae Pol ε, Pol ε exo$^-$, Pol2$_{core}$ (aa 1–1228) and Pol2$_{core}$ exo$^-$ were overexpressed in *S. cerevisiae* strain Py116 and purified as previously described (16). The overexpression plasmid for RB69 gp43 was a kind gift from Dr Susan Wallace (University of Vermont), and RB69 gp43 was over-expressed in the *E. coli* BL21 (DE3) strain as previously described (17). Briefly, an overnight primary inoculum was sub-cultured into 1 l LB-media and later induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). BL21 (DE3) cell lysate was clarified by centrifugation, and the supernatant was passed over a nickel column. The column was thoroughly washed and the protein was eluted with 500 mM imidazole. The fractions containing RB69 gp43 were pooled and dialysed to remove the imidazole. The protein was snap frozen in liquid nitrogen and stored at −80°C. Pol η, Pol δ, PCNA and RFC was a kind gift from Dr Peter Burgers (Washington University School of Medicine, University in St. Louis, MO, USA). Proteins for the D-loop extension assay were purified as described (18–21). Klenow DNA polymerase was purchased from New England Biolabs.

DNA substrates

Oligonucleotides were purchased from MWG Operon (Germany). The oligonucleotides were gel purified before primer-templates were prepared by mixing 6 μM primer strand, 7.2 μM template strand and 8.6 μM of blocking primer in a buffer containing 100 mM Tris-HCl (pH 7.5) and 100 mM NaCl and heating to 85°C for 5 min in a heating block followed by slow cooling to room temperature. The oligonucleotides are listed in Table 1. DNA for the D-loop extension, pUC19 DNA (2686 bp) and pBluscript II KS(+) (2961 bp) was prepared by Triton/SDS lysis and purified by isopycnic density centrifugation on CsCl/Ethidium bromide gradients (22).

Strand displacement synthesis

All experiments were performed in RQ buffer containing 20 mM Tris-HCl (pH 7.8), 100 μg/ml bovine serum albumin (BSA) and 1 mM dithiothreitol (DTT). Reaction mixture A contained 10 nM DNA substrate (primer/template annealed to a blocking primer) and 22 nM enzyme in RQ buffer, and reaction mixture B contained 200 μM dNTP and 16 mM magnesium acetate in RQ buffer. Primer extension assays were performed by mixing 10 μl of reaction mixture A with 10 μl of reaction mixture B. The final concentrations of the reactants were 11 nM enzyme, 5 nM DNA, 8 mM magnesium acetate and 100 μM dNTP. Reactions were incubated at 30°C for 0.5, 2, 5 and 10 min and were stopped by the addition of 20 μl of 95% formamide, 20 mM EDTA and 0.1% bromophenol blue. A total of 8 μl of this reaction mixture was loaded onto a 10% polyacrylamide gel containing 8 M urea and 25% formamide in 1x TBE. The gel was scanned with a Typhoon Scanner 9400 (GE Healthcare) at the Alexa 532 nm setting to excite the fluorophore, tetrachlorofluorescein, that was covalently linked to the 5′ end of the primer.

Proofreading limits strand displacement synthesis

Reaction mixture A contained 10 nM of DNA substrate (primer/template annealed to a 22-mer blocking primer) and 22 nM enzyme in RQ buffer, and reaction mixture B contained 2000 μM dNTPs, 16 mM magnesium acetate and 2 μg/ml heparin in RQ buffer. Primer extension assays were performed by mixing 10 μl of reaction mixture A with 10 μl of reaction mixture B. The final concentrations of the reactants were 11 nM enzyme, 5 nM DNA, 8 mM magnesium acetate, 1 μg/ml heparin and 1000 μM dNTPs. Reactions were incubated at 30°C for 0.5, 2, 5 and 10 min and were subsequently stopped by the addition of 20 μl of 95% formamide, 20 mM EDTA, and 0.1% bromophenol blue. A total volume of 8 μl of this reaction mixture was loaded onto a 10% polyacrylamide gel containing 8 M urea and 25% formamide in 1x TBE. The gel was scanned with a Typhoon Scanner 9400 (GE Healthcare) at the Alexa 532 nm setting to excite the fluorophore, tetrachlorofluorescein, that was covalently bound to the 5′ end of the primer.

D-loop extension assay with 95-mer

The D-loop extension assay was performed essentially as described (23) with the following specific conditions. The reaction was carried out at 30°C in SEB buffer (30 mM Tris-acetate pH 7.5, 1 mM DTT, 50 μg/ml BSA, 1.5 mM ATP, 5 mM magnesium acetate, 20 mM phosphocreatine, 20 U/ml creatine kinase) supplemented with dNTPs at 100 μM each. The 95-mer (1 μM nt, 10 nM molecule; oWDH1473 5′-ACTGGCAGCAGCCACTGGTAACAGGATTAGCA GAGCGAGGTATGTAGGCGGTGCTAC AGAGTT CTTGAAGTGGTGGCCTAACTACGGCTACACTA-3′) was incubated with Rad51 (0.34 μM) for 10 min to assemble nucleoprotein filaments. RPA (0.05 μM) was then added to the reaction for an additional 10 min. The formation of D-loops was catalyzed by addition of Rad54 (28 nM) and pUC19 plasmid (84.9 μM bp, 30 nM molecule) to the reaction for 5 min. PCNA (5 nM) and RFC-1D complex (5 nM) were added to the D-loop reaction for an additional 2 min. The initiation of D-loop extension was started by the addition of either Pol δ (20 nM) or Pol ε (10, 20, 30, 32 nM). At each time point (0, 2, 5, 10 min), aliquots were mixed with stop buffer (1% SDS, 0.1 M EDTA, 4 mg/ml Proteinase K) and incubated for 10 min at 30°C. The DNA was separated on 1% agarose gels at 6 V/cm for 150 min. The gels were dried and analyzed by a phosphoImager.

D-loop extension assay with 37-mer

The D-loop extension assay was performed essentially as described (23) with the following specific conditions. The

Table 1. Oligonucleotides

Primer (50-mer)	5′GATCAGACTGTCCTTAGAGGATACTCGCTCGCAGCCGTCCACTCAACTCA 3′
Template (80-mer)	5′CAGCTTGATAGTCAGTGACGTTGTTCTGGATGAGTTGAGTGGACGGCTGCGA GCGAGTATCCTCTAAGGACAGTCTGATC 3′
22-mer blocking primer	5′AACGTCACTGACTATCAAGCTG 3′
Phosphorylated 22-mer blocking primer	5′ P-AACGTCACTGACTATCAAGCTG 3′
1-nucleotide flap (23-mer)	5′GAACGTCACTGACTATCAAGCTG 3′
5-nucleotide flap (27-mer)	5′ TCCTGAACGTCACTGACTATCAAGCTG 3′
10-nucleotide flap (32-mer)	5′ AGGTGTCCTGAACGTCACTGACTATCAAGCTG 3′
20-nucleotide flap (42-mer)	5′AGTAGAGCTCAGGTGTCCTGAACGTCACTGACTATCAAGCTG 3′
30-mer blocking primer	5′ P-TCCAGAACAACGTCACTGACTATCAAGCTG 3′
30-mer RiboU blocking primer	5′ P-UCCAGAACAACGTCACTGACTATCAAGCTG 3′
Phosphorylated 22-mer Tetrahydrofuran blocking primer	5′ P-THFACGTCAC TGACTATCAAGCTG 3′

reaction was carried out at 30°C in SEB buffer (30 mM Tris-acetate pH 7.5, 1 mM DTT, 50 μg/ml BSA, 1.5 mM ATP, 5 mM magnesium acetate, 20 mM phosphocreatine, 20 U/ml creatine kinase) supplemented with dNTPs at 100 μM each. The 37-mer (185 nM nt, 5 nmol molecule; pBluSKP-1254 5′-CGACGCTCAAGTCAGAGGTGGCGAAACC CGACAGGAC-3′) was incubated with Rad51 (150 nM) for 10 min to assemble nucleoprotein filaments. RPA (25 nM) was then added to the reaction for an additional 10 min. The formation of D-loops was catalyzed by addition of Rad54 (17 nM) and pBluescript II KS (+) plasmid (59.2 μM bp, 20 nM molecule) to the reaction for 5 min. PCNA (10 nM) and RFC-1D complex (10 nM) or buffer were added to the D-loop reaction for an additional 2 min. The initiation of D-loop extension was started by the addition of either Klenow DNA polymerase (2.5 U), Pol δ (10 nM) or Pol ε (10 nM). At each time point (0, 2, 5, 10 min; Klenow 0, 5, 10 min), aliquots were mixed with stop buffer (1% SDS, 0.1 M EDTA, 4 mg/ml Proteinase K) and incubated for 10 min at 30°C. The DNA was separated on 1% agarose gels at 6 V/cm for 150 min. The gels were dried and analyzed by a phosphoImager.

Primer extension assay with 37-mer

The 37-mer (pBluSKP-1254)) was annealed to pBluescript II KS (+) single-stranded circular DNA and 5 nM (molecule) substrate was per reaction. The buffer conditions and temperature were the same as above. RPA (488 nM) was added to the annealed substrate and incubated for 10 min, after which PCNA (10 nM) and RFC (10 nM) or buffer were added for another 2 min incubation. DNA synthesis was initiated by the addition of either Klenow DNA polymerase (2.5 U), Pol δ (10 nM) or Pol ε (10 nM). At each time point (0, 2, 5, 10 min; Klenow 0, 5, 10 min), aliquots were mixed with stop buffer (1% SDS, 0.1 M EDTA, 4 mg/ml Proteinase K) and incubated for 10 min at 30°C. The DNA was separated on 1.5% denaturing alkaline agarose gels as described (22). The gels were dried and analyzed by a phosphoImager.

RESULTS

Strand displacement synthesis by Pol ε

To examine if Pol ε has the capacity to carry out strand displacement synthesis, we designed a gapped DNA substrate in which a 50-mer primer and a downstream 22-mer blocking primer were annealed to an 80-mer template. The two primers were separated by a single-stranded gap of 8 nucleotides that can be filled in by a DNA polymerase (Figure 1A). Thus, primer extension products greater than 58 nucleotides would require that Pol ε carry out strand displacement synthesis. Wild-type Pol ε was unable to carry out extended strand displacement synthesis, but it was able to efficiently insert a single nucleotide and displace one nucleotide that formed a flap (Figure 1A). In contrast, exonuclease-deficient Pol ε (Pol ε exo$^-$) efficiently displaced the blocking primer and performed strand displacement synthesis to the end of the template. In this experiment, a blocking primer was used with a hydroxyl group at the 5′ end. To test a biologically relevant substrate, we repeated the experiment with a 22-mer blocking primer that was phosphorylated at the 5′ end (Figure 1B). Again wild-type Pol ε stalled at position +1 after adding one nucleotide past the junction at position 0. Pol ε exo$^-$ was again able to efficiently carry out strand displacement synthesis to the end of the template.

Effect of accessory subunits and salt concentration on strand displacement synthesis

Strand displacement synthesis by DNA polymerases has been suggested to be enhanced by accessory subunits that form complexes surrounding the DNA (24), and we have previously shown that accessory subunits contribute to the processivity and loading of Pol ε onto DNA (25–27). To analyze whether the accessory subunits influence strand displacement synthesis by Pol ε, we compared the ability of Pol ε holoenzyme (which consists of the Pol2 subunit and three accessory proteins) and the Pol2 catalytic domain, $Pol2_{core}$ (aa 1–1228), to carry out strand displacement. We found that the accessory subunits and the C-terminus of Pol2 did not influence the strand displacement synthesis when compared to four-subunit Pol ε (Figure 1B).

It was previously shown that high concentrations of salt inhibit strand displacement synthesis by Pol δ exo$^-$ (28). To ask whether the salt concentration also influences strand displacement synthesis by Pol ε, we performed assays in the presence of various concentrations of NaAc (Figure 2). We observed that Pol ε exo$^-$ was no longer able to carry out extensive strand displacement synthesis at 62.5 mM NaAc and that strand displacement synthesis at 250 mM NaAc was as poor as that of Pol ε. However, the addition of salt had no significant effect on the limited strand displacement

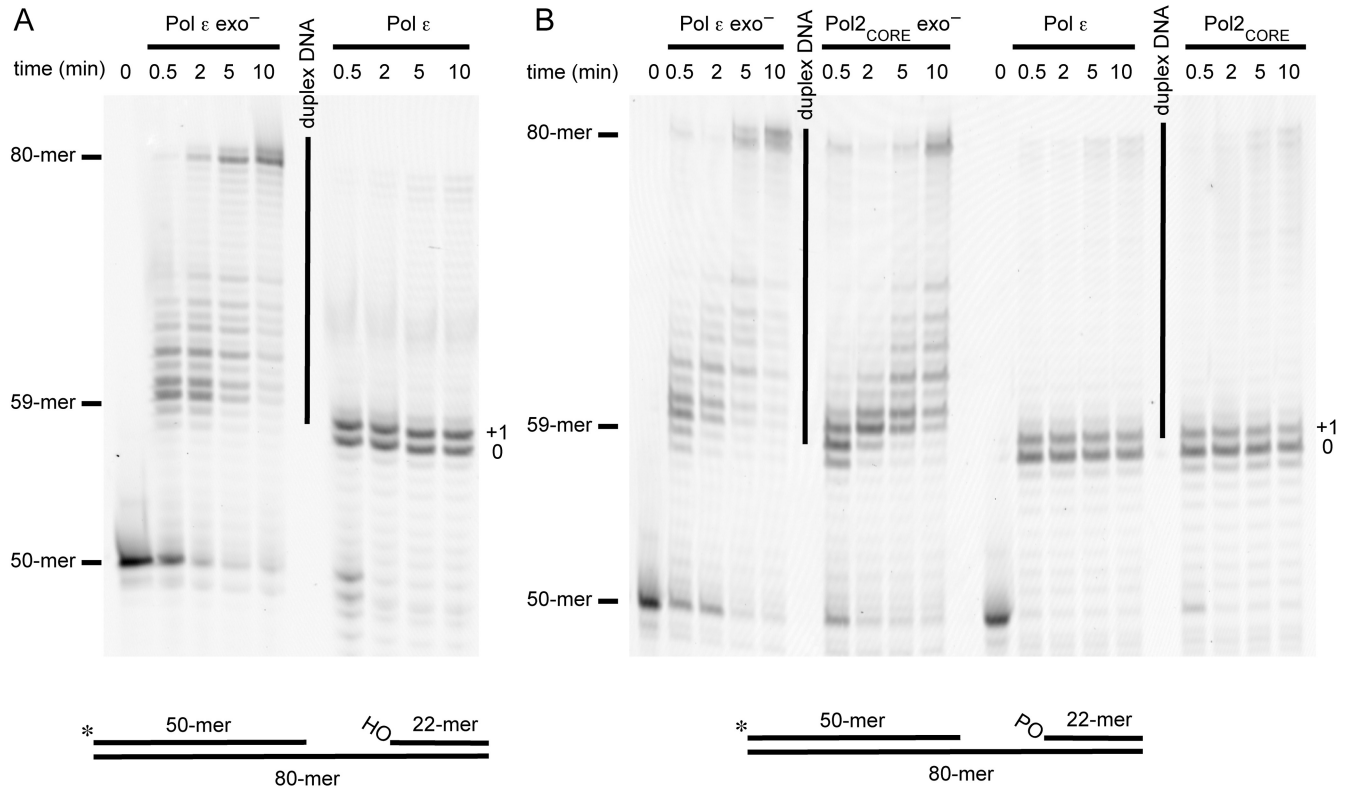

Figure 1 (**A**) Strand displacement synthesis by Pol ε. A 22-nucleotide blocking primer was annealed to a primer-template (50/80-mer) resulting in the formation of a DNA substrate with a gap of 8 nucleotides. (**B**) Comparison of strand displacement synthesis between Pol ε and $Pol2_{core}$. Strand displacement synthesis was examined by mixing preformed enzyme-DNA complexes with dNTP and magnesium acetate. The reactions were stopped at the indicated time points and loaded onto an acrylamide gel. The 0 sign indicates the incorporation of 8 nucleotides to fill the gap up to the 5′ end of the blocking primer, and +1 indicates the strand displacement synthesis of a single nucleotide.

synthesis by Pol ε (position +1 in Figure 2). The polymerase activities of both Pol ε and Pol ε exo⁻ were unaffected under these conditions because the 8-nucleotide gap was still readily filled by both enzymes (Figure 2A).

Strand displacement synthesis on nicked substrates

The integrity of the genome is under constant challenge, and there are many DNA repair pathways that protect the genome from alterations that can affect the function of genes (29). Base excision repair (BER) corrects minor alterations of bases and removes abasic sites that result from depurination events. The first step in BER involves an endonuclease that cleaves the DNA strand on the 5′-end of the damage. This generates a nick with a 3′-OH that will be extended by a DNA polymerase in the next step of the repair process. To ask if Pol ε has the ability to extend a 3′-OH on a nicked substrate during BER, we designed a 30-nucleotide blocking primer that was annealed to the 50/80-mer primer-template. This left a nick (a missing phosphodiester bond) between the 50-mer extendable primer and 30-mer blocking primer (Figure 3A). We used either a 5′-phosphorylated (Figure 3A) or an unphosphorylated blocking primer (data not shown). In both cases, Pol ε exo⁻ efficiently displaced the downstream primer and wild-type Pol ε still only displaced the blocking primer by just one nucleotide (Figure 3A). The ability to open up the phosphorylated blocking primer shows that Pol ε is able to recognize a nick and displace one nucleotide.

To determine whether Pol ε can displace more than one nucleotide when encountering the kind of structure that would result from cleavage at an abasic site by an AP endonuclease, we designed a substrate with a 5′-phosphorylated tetrahydrofuran moiety on the blocking primer (Figure 3B). The missing base in the tetrahydrofuran moiety results in a substrate similar to a 1-nucleotide flap (Figure 4A). Pol ε added a nucleotide in place of the tetrahydrofuran moiety when filling in the gapped substrate, but it was unable to efficiently extend the synthesis beyond the position of the tetrahydrofuran moiety in the blocking primer.

Ribonucleotides are frequently incorporated into DNA during the synthesis of a new DNA strand (30,31). In fact, ribonucleotides constitute the most common form of DNA damage in cycling cells, even surpassing the numbers of abasic sites. The presence of ribonucleotides in DNA makes the DNA susceptible to strand cleavage, which leads to replication fork arrest. If not repaired, ribonucleotides in the template strand can lead to 2–5 base pair deletions (31). Ribonucleotide excision repair (RER) also provides a substrate that is similar to the short BER substrate with a nick (32). In this case, there is a ribonucleotide at the 5′ position of the downstream strand. Again we found that Pol ε displaces the riboU on a nicked substrate and extends the 3′

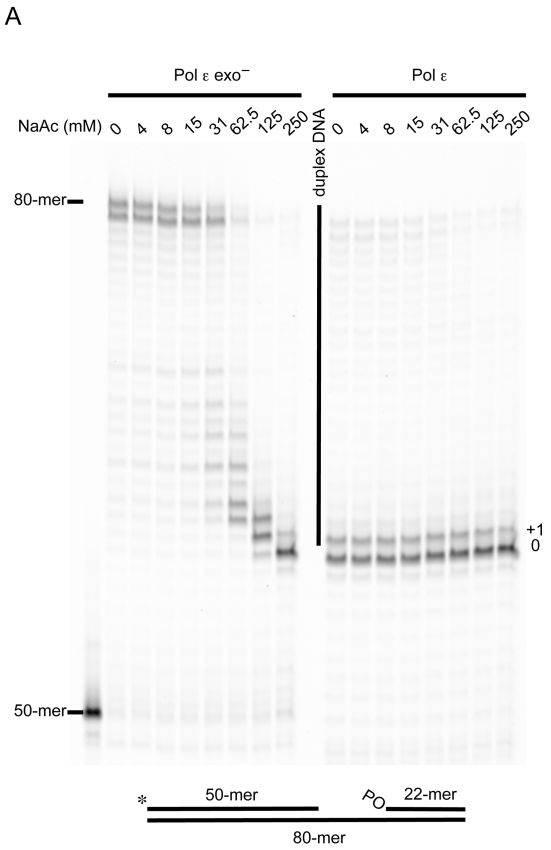

Figure 2. (**A**) Salt dependence of strand displacement synthesis. Strand displacement synthesis was performed by mixing preformed enzyme-DNA complexes with dNTP, magnesium acetate and varying concentrations of sodium acetate as indicated in the figure. The reactions were stopped after 10 min and loaded onto a polyacrylamide gel. The 0 sign indicates the incorporation of 8 nucleotides to fill the gap up to the 5′ end of the blocking primer, and +1 indicates the strand displacement synthesis of a single nucleotide. (**B**) Quantification of replication products in panel A. The intensity of replication products at position 0, +1 and products longer than +1 were quantified and divided by the sum of all extension products.

end by one nucleotide before stalling (Figure 3C). This is in agreement with reconstitution experiments showing that Pol ε can participate in RER, although less efficiently than Pol δ (32).

Strand displacement synthesis is blocked by a 5-nucleotide 5′ flap on the blocking primer

Long-patch BER, D-loop extensions and hairpin structures on the template strand all provide a similar forked substrate that can be mimicked by annealing a blocking primer with a long flap at the 5′ end that is not complementary with the template DNA. Pol ε was earlier suggested to participate in long-patch BER (33), so we first asked if a 1-nucleotide flap at the 5′ end of the blocking primer would give the same result as when a tetrahydrofuran moiety was present in the flap. The presence of a mismatched nucleotide allowed Pol ε to add a nucleotide under the flap (position 0 in Figures 3B and 4A) and then to displace the next properly paired nucleotide with a higher efficiency than with a tetrahydrofuran moiety in the flap (compare position +1 in Figures 3B and 4A). However, we must emphasize that the sequence context varies between these two experiments, and this might be the reason for the small difference that is observed. The relationships of incorporation products at the properly paired primer terminus (0) and one displaced nucleotide (+1) were similar for blocking primers with no flap and primers with a 1-nucleotide flap (compare positions 0 and +1 in Figures 1B and 4A). In contrast, Pol ε was inhibited when a 5-nucleotide flap was introduced in the blocking primer (Figure 4A), and the presence of a 5-nucleotide flap appeared to stall Pol ε up to 4 nucleotides from the fork junction (position −3, Figure 4A).

We next determined whether the dNTP concentration influences the ability of Pol ε to reach the junction because DNA damage can induce checkpoint activation leading to increased dNTP concentrations. We found no effect on the ability of Pol ε to carry out strand-displacement synthesis even at dNTP concentrations up to 1 mM (Supplementary Figure S1), which is more than 10-fold above the estimated *in vivo* concentrations during S-phase (34). Nor did the addition of single-stranded DNA binding protein (RPA) facilitate strand-displacement synthesis by Pol ε (data not shown).

We then asked if the length of the 5′ flap affects the ability of Pol ε to reach the fork junction or of Pol ε exo⁻ to carry out extensive strand displacement synthesis. We designed substrates containing flaps with a length of 1, 5, 10 or 20 nucleotides. It was recently shown that strand displacement synthesis by Pol δ exo⁻ (pol3-5DV) is strongly inhibited when the blocking primer contains longer flaps (28). In contrast to Pol δ exo⁻, our results show that Pol ε exo⁻

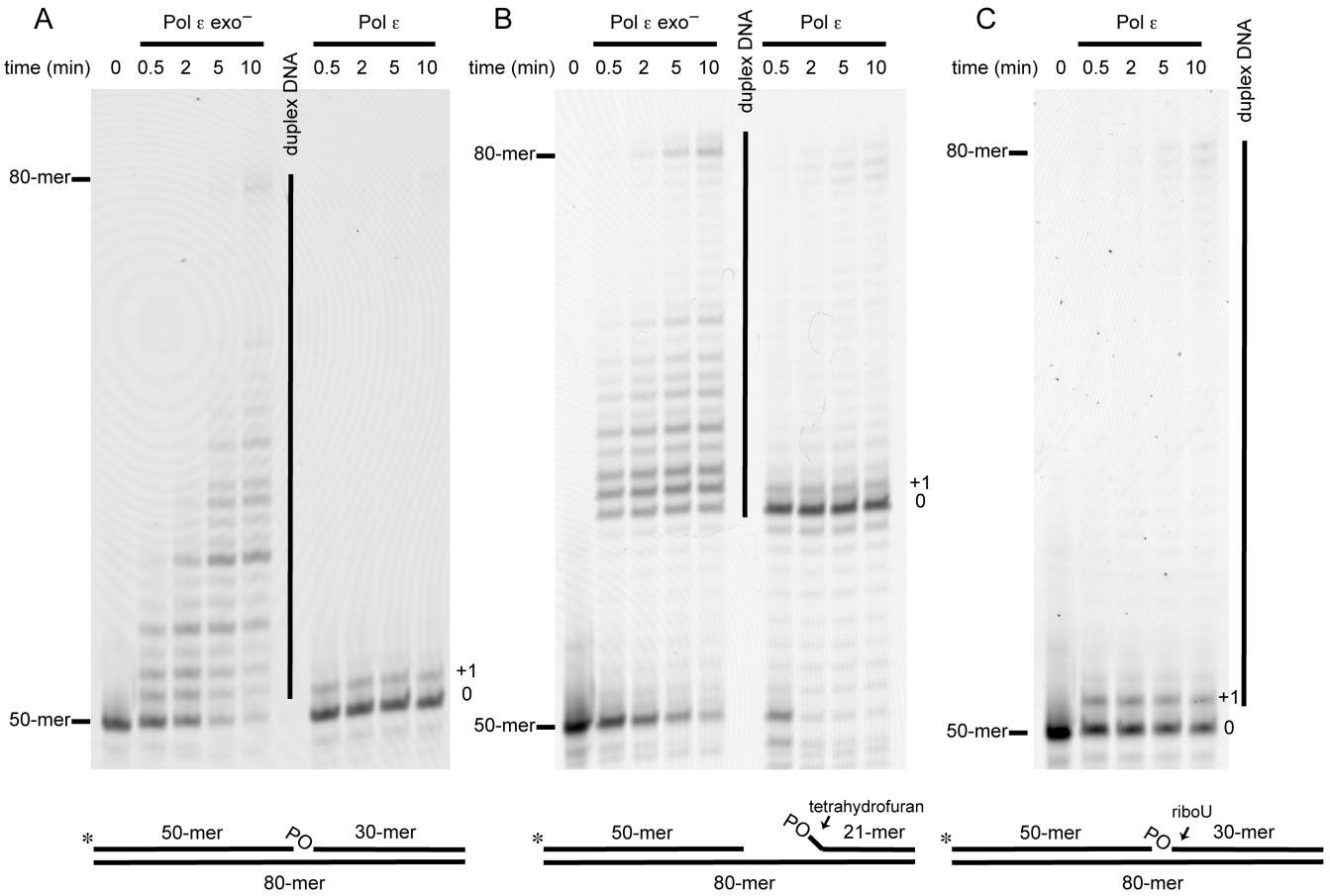

Figure 3. Strand displacement synthesis on nicked substrates. Primer extension reactions were carried out by mixing preformed enzyme–DNA complexes with dNTP and magnesium acetate. The reactions were stopped at the indicated time points and loaded onto a polyacrylamide gel. (**A**) Displacement of a nicked substrate. A 5′ phosphorylated 30-nucleotide blocking primer was annealed to a primer-template (50/80-mer) resulting in the formation of a nicked substrate. The 0 sign indicates the length of the primer, and +1 indicates the strand displacement synthesis. (**B**) Displacement of a tetrahydrofuran moiety. Primer extension reactions were carried out on a substrate containing a 5′ phosphorylated 22-nucleotide blocking primer with a 5′ end tetrahydrofuran moiety annealed to a primer-template (50/80-mer). The 0 sign indicates the position opposite where the tetrahydrofuran is located in the blocking primer, and +1 indicates the strand displacement synthesis. (**C**) Displacement of a 5′ ribonucleotide. Primer extension reactions were carried out on a substrate containing a phosphorylated ribonucleotide at the 5′ end of the 30-mer blocking primer, which resembles a substrate nicked by RNAse H2. The 0 sign indicates the length of the primer, and +1 indicates the strand displacement synthesis.

is not hindered by the flaps and is able to synthesize DNA to the end of the template by displacing the entire blocking oligo irrespective of the flap size (Figure 4D). We next examined whether longer flaps could affect strand displacement synthesis by Pol ε. We found that Pol ε was increasingly inhibited by increasing lengths of the 5′ flap on the blocking primer (Figure 4B, C and D). The greatest effect was seen when increasing the length from 1 to 5 nucleotides. A further increase in flap length enhanced the shift of products from the fork junction toward position −4.

To ask whether the inhibitory effect of the 5-nucleotide flap was unique for Pol ε, we compared the ability of several different polymerases to extend the primer when encountering the same 5′ flap. In contrast to Pol ε, yeast Pol δ extended the primer one or two nucleotides into the double-stranded DNA and was unaffected by the flap (Figure 5). RB69 gp43, another family B polymerase with 3′–5′ exonuclease activity, gave replication products similar to Pol δ but with even more products at position +2. In contrast, Pol η, which lacks exonuclease activity, carried out extensive strand displacement synthesis and was not inhibited by the 5′ flap.

In vitro reconstitution of D-loop extension

Both, Pol δ and Pol ε were implicated by genetic experiments in recombination-associated DNA synthesis (35). Yeast and human Pol δ are capable of extending the invading strand of Rad51-mediated D-loops (23,36,37), which involves displacement synthesis (Figure 6A). The role of Pol ε in recombination-associated DNA synthesis has never been examined. We directly compared the ability of Pol δ and Pol ε in reconstituted D-loop reactions, using a 95-mer invading single-stranded DNA with a pUC19 dsDNA partner and the *S. cerevisiae* HR proteins Rad51, RPA and Rad54. As expected from previous results (23,37), Pol δ vigorously extended D-loops, whereas Pol ε displayed a very significantly reduced activity (Supplementary Figure S2B). To exclude any possible effects of DNA sequence context, we recapitulated this experiment using a different oligonucleotide

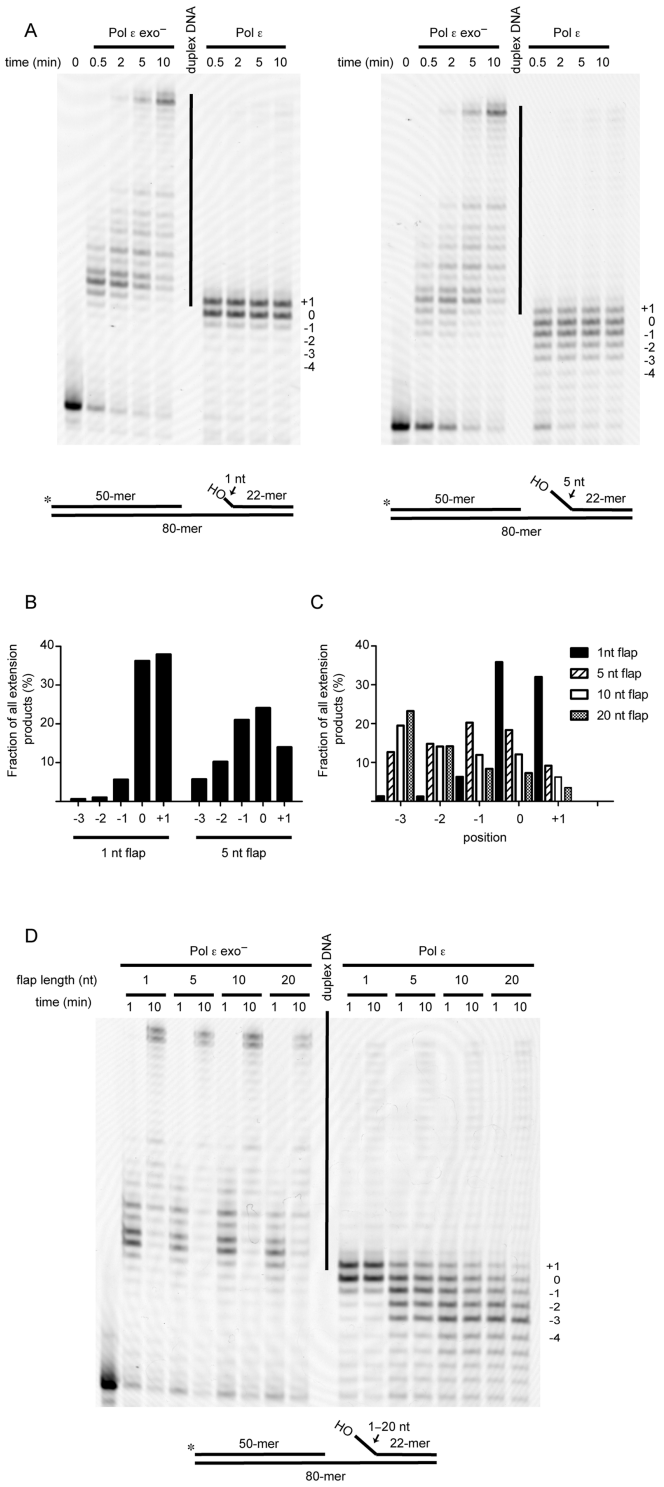

Figure 4 (**A**) Inhibition of DNA synthesis by a downstream flap. Pol ε was preincubated with a substrate either containing a 1-nucleotide flap or a 5-nucleotide flap at the 5′ end of the blocking primer, and the reactions were started by the addition of dNTPs and magnesium acetate. The reactions were stopped at the indicated time points and loaded onto a polyacrylamide gel. (**B**) Quantification of products in reactions with wild-type Pol ε from panel A. The intensity of replication products at position −3, −2, −1, 0 and +1 were quantified and divided by the sum of all extension products for the 10 min time point. (**C**) Quantification of products in reactions with wild-type Pol ε from panel D. The intensity of replication products at position −3, −2, −1, 0 and +1 were quantified and divided by the sum of all extension products. (**D**) Influence of flap length on strand displacement synthesis. To test for the dependence of strand displacement synthesis on the flap length of the blocking primer, primer extension assays were performed by preincubating Pol ε with substrates containing flaps of different lengths ranging from 1 nucleotide to 20 nucleotides as indicated in the figure. The reactions were started by the addition of dNTPs and magnesium acetate and incubated for either 1 min or 10 min. The reaction products were resolved on a 10% polyacrylamide gel. The 0 sign indicates the position opposite the furthest downstream mismatched nucleotide in the blocking primer, +1 indicates the strand displacement synthesis and −4 to −1 indicate the inhibition of DNA synthesis by the downstream flap.

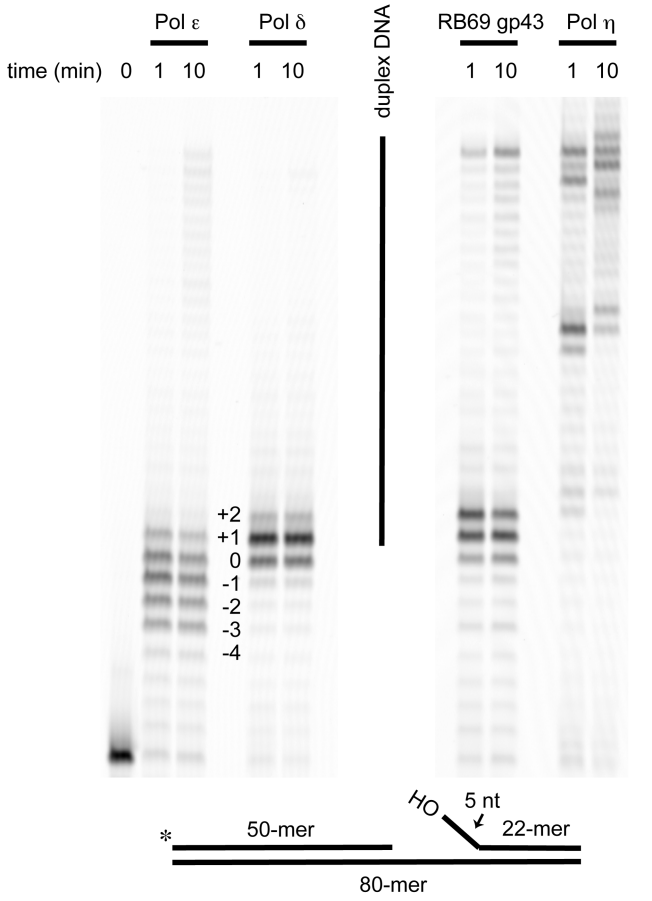

Figure 5. Comparison of strand displacement synthesis between different polymerases. Strand displacement synthesis by Pol ε was compared to that of other polymerases on a substrate containing a 5-nucleotide flap at the 5′ end of the blocking primer. Preformed DNA-polymerase complexes were mixed with dNTPs and magnesium acetate and incubated for the times indicated in the figure. The 0 sign indicates the position opposite the furthest downstream mismatched nucleotide in the blocking primer, +1 indicates the strand displacement synthesis and −4 to −1 indicate the inhibition of DNA synthesis by the downstream flap.

(37-mer) and duplex DNA target (pBluescript) (Figure 6A). Consistent with previous results (23,37), Klenow polymerase and Pol δ were capable of extending D-loop in a PCNA/RFC-dependent fashion (Figure 6B). Instead, Pol ε showed very little activity. To ascertain that the Pol ε preparation was active on the DNA substrates used, we employed the same 37-mer in primer extension assays after annealing the oligonucleotide with single-stranded circular pBluescript DNA (Figure 6C and D). Pol ε showed equivalent activity to Pol δ on this substrate with the expected stimulation by PCNA/RFC. From these experiments we conclude that Pol ε is quite poor at extending Rad51-mediated D-loops.

Processive proofreading limits strand displacement synthesis

To determine why Pol ε displaces only one nucleotide of the blocking DNA, the reactions with a matched blocking primer were followed under single-turnover conditions by including heparin in the reaction. Heparin traps Pol ε molecules that dissociate from the prebound DNA sub-

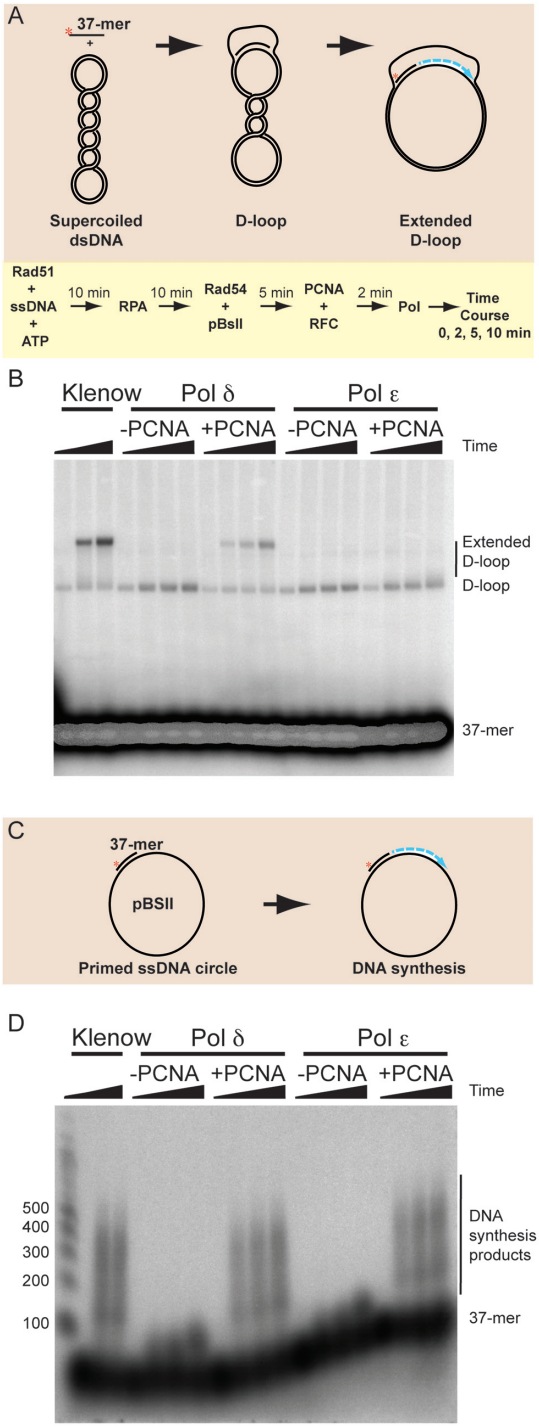

Figure 6. Pol ε poorly extends D-loops in comparison to Pol δ but is proficient to extend primed single-stranded DNA using the same substrates under the same conditions. (**A**) *In vitro* D-loop reactions using a 37-mer oligonucleotide were reconstituted using purified *S. cerevisiae* proteins as described in Materials and Methods. (**B**) Product analysis of reconstituted D-loop reactions containing either Klenow polymerase, Pol δ (10 nM) or Pol ε (10 nM) at 0, 2 (not for Klenow), 5 and 10 min extension times. (**C**) Extension of primed single-stranded circular template DNA using a 37-mer oligonucleotide. (**D**) Product analysis of primer extension on denaturing gels of reaction containing Klenow polymerase, Pol δ (10 nM) or Pol ε (10 nM) each plus or minus 10 nM PCNA/RFC at 0, 2 (not for Klenow), 5 and 10 min extension times. A 100 nt size ladder is shown in the left-most lane.

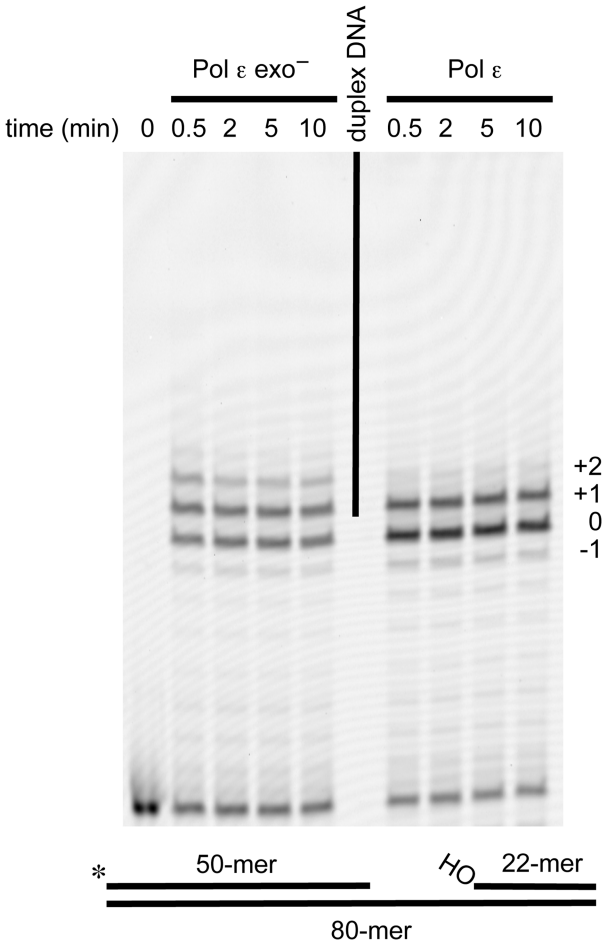

Figure 7. Modulation of strand displacement synthesis by processive proofreading. Strand displacement synthesis was carried out on substrates containing a 22-nucleotide blocking primer and in the presence of heparin. Preformed enzyme-DNA complexes were mixed with 1000 μM dNTPs (final concentration), magnesium acetate and heparin. Heparin was used to study strand displacement synthesis under single turnover conditions. The reactions were stopped at the indicated time points and loaded onto a polyacrylamide gel. The 0 sign indicates the incorporation of 8 nucleotides to fill the gap up to the 5′ end of the blocking primer, and +1 indicates the strand displacement synthesis of a single nucleotide.

strate and thus the products that are visualized are the result of a single binding event (Figure 7). Control experiments were performed previously to verify that mutations in the exonuclease domain did not affect the polymerase activity of Pol ε (16), and the pause sites between wild-type and Pol ε exo⁻ were found to be similar (16). Therefore, any differences are due to the lack of exonuclease activity in Pol ε exo⁻. Pol ε exo⁻ was able to displace 2 nucleotides with greater efficiency than wild-type Pol ε before dissociating from the substrate even though the polymerase activity of the two enzymes is similar. We conclude that the exonuclease activity of Pol ε efficiently removes an incorporated nucleotide at position +2 resulting in products at position +1. We hypothesize that the delay in forward synthesis caused by the blocking primer leads to a transfer of the primer 3′-OH from the polymerase site to the exonuclease site. The experiment also showed that Pol ε exo⁻ is only able to carry out extended strand-displacement synthesis when allowed to engage in multiple binding events.

DISCUSSION

Many DNA repair mechanisms involve strand-displacement synthesis by DNA polymerases. This results in the creation of a flap that is later removed by endonucleases, and the resulting nick is sealed by a DNA ligase. Pol ε has been suggested to play a role in several DNA repair processes, but detailed studies of how Pol ε responds to common DNA repair structures are limited. To explore whether Pol ε might be able to participate in various DNA repair mechanisms, we challenged the enzyme with various substrates mimicking DNA repair intermediates.

First we showed that wild-type yeast Pol ε has strand displacement activity that is limited to one nucleotide, whereas the exonuclease-deficient Pol ε has a robust strand displacement activity over many nucleotides (Figure 1). It was previously suggested that limited strand displacement synthesis is a consequence of regression pressure from the downstream double-stranded DNA that stimulates the 3′–5′ exonuclease activity of the replicative DNA polymerase (11). Our results under single turnover conditions are in agreement with that model (Figure 7). In this context, Pol ε will tend to shift the primer into the exonuclease site, thereby limiting the strand displacement synthesis when encountering duplex DNA. In contrast, Pol η lacks 3′–5′ exonuclease activity and can perform extensive strand displacement synthesis (Figure 5) (38).

The structural basis for limited strand displacement synthesis by Pol ε is not yet understood because no crystal structures of Pol ε in complex with nicked DNA exist. However, the biochemical evidence from studies of *E. coli* DNA Pol I suggests that the motion of the fingers domain associated with pyrophosphate release after nucleotide incorporation helps in the displacement of the downstream DNA (39). The crystal structure of the Family B bacteriophage φ29 DNA polymerase indicates that a threading mechanism is used to pass single-stranded template DNA through a tunnel thereby displacing the blocking complementary strand. The narrow width of this downstream tunnel prevents the passage of double-stranded DNA and only allows template DNA to pass through it, and this strand displacement activity supports the very high processivity of φ29 DNA polymerase (24). Neither Pol ε nor Pol δ has such a tunnel, and both enzymes have lower processivity compared to φ29 DNA polymerase (40–42).

DNA repair synthesis often involves a step where a DNA polymerase fills a gap, and the removal of damaged DNA during nucleotide excision repair (NER) or mismatch repair always generates a gap of several nucleotides. Here, we found that Pol ε efficiently filled a gap of 8 nucleotides and dissociated after leaving a ligatable nick (position 0 in Figure 1) or a 1-nucleotide flap (position +1 in Figure 1), and both products were formed in equal numbers. There is a pathway within NER where FEN1 participates together with DNA ligase 1, thus both of the resulting products from Pol ε can be processed after Pol ε has dissociated from the DNA. In support of these conclusions is recent

work with yeast cell-free extracts containing temperature-sensitive mutants of Pol2 and Pol3 (catalytic subunit of Pol δ) that were defective in NER. The deficiency in NER was rescued by the addition of purified Pol2 and Pol3, respectively (43).

Both BER and RER generate a 1-nucleotide gap that must be filled by a DNA polymerase. BER is either carried out by short-patch BER (filling in one nucleotide) or long-patch BER (filling in 2 or more nucleotides). DNA polymerase β (Pol β) is the primary polymerase involved in BER in mammalian cells (44,45). Cell extracts from Pol β-depleted mouse fibroblasts showed that both Pol δ and Pol ε can act as back-up repair polymerases for BER (33). We observed here that yeast Pol ε has the ability to recognize a nick and can displace one base pair in double stranded DNA when adding one nucleotide (Figure 3), and the presence of an abasic site adjacent to the nick allowed Pol ε to add two nucleotides. On the other hand, Pol ε was unable to extend further into the blocking primer, and longer flaps at the 5′ end of the blocking primer inhibited Pol ε from even reaching the flap junction. These results support a role for Pol ε in short-patch BER, and these findings are further supported by the observed interactions of Pol2 with BER intermediates in cell-free extracts of *S. cerevisiae* and complementation of pol2-deficient yeast extracts with Pol ε (43,46). The role of Pol ε in long-patch BER is less clear. Based on the results presented here, it is unlikely that Pol ε participates in long-patch BER with patches that are 3 nucleotides or longer. Earlier *in vitro* studies also suggested that Pol ε does not interact as efficiently with FEN1 when compared to Pol δ, suggesting that Pol ε is not the preferred polymerase during long-patch BER (14). It is frequently stated that Pol β and Pol δ/Pol ε carry out long-patch BER (47,48). However, the original discoveries showed that long-patch BER depends on PCNA (49) and that PCNA is required for Pol δ, FEN1 and DNA ligase 1 to efficiently process their DNA substrates. At that time, it was also assumed that Pol ε depends on PCNA and it was concluded that both Pol ε and Pol δ might participate in long-patch BER despite the fact that the reported reconstitution experiments only included Pol δ (50). Aphidicholin is an inhibitor of long-patch BER, but it is not possible to conclude that Pol ε participates in long-patch BER because aphidicholin inhibits both Pol ε and Pol δ (51). The lack of functional interaction between Pol ε and FEN1 (14), and the inability to carry out longer stretches of strand-displacement synthesis strongly suggest that Pol ε does not have a major role in long-patch BER. However, Pol ε adds two nucleotides at a low frequency when encountering a tetrahydrofuran moiety (Figure 3B), and this will in some assays be considered to be long-patch BER with the current definition of long-patch BER as the addition of two or more nucleotides. All things considered, the role of Pol ε in extended long-patch BER (patches that are three nucleotides or longer) should be revisited and clarified in genetic experiments, in particular because the role of PCNA in Pol ε activity might be much less important than previously assumed.

The inhibition by longer flaps at the 5′ end of the blocking primer suggests that Pol ε is unlikely to carry out any extension of fork-like structures or to bypass secondary structures such as hairpins without the assistance of a helicase. Nicks that evade the BER machinery are known to cause replication fork collapse and subsequent double-stranded breaks. These double-stranded breaks are generally repaired by either homologous recombination or non-homologous DNA end joining (52). DNA synthesis is an integral part of homologous recombination and occurs in two different phases. First, the invading strand of the Rad51-mediated D-loop is extended by displacement synthesis. Second, after second end capture or annealing, the 3′-OH of the second end is extended without necessity for strand displacement synthesis. All DNA polymerases are capable of extending a primed template, similar to what is expected for second end DNA synthesis. Instead, first end DNA synthesis during recombination requires a DNA polymerase capable of displacement synthesis. Biochemical analysis of human cell extracts and biochemical reconstitution experiments with yeast and human proteins identified Pol η as a DNA polymerase capable of performing D-loop extension (23, 36–38). Moreover, it was shown that yeast and human Pol δ are capable of first end DNA synthesis and efficiently extend Rad51-mediated D-loops (23,36,37), while the role of Pol ε remained to be determined. Genetic studies in yeast suggest that Pol δ and to some extent Pol ζ are involved in homologous recombination (53–55). However, single mutants of Pol δ only show a modest reduction in recombination, which indicates possible functional overlap with additional polymerases. There is also genetic evidence that Pol ε is involved in D-loop–mediated recombination during double-stranded repair (56), but the genetic experiments left open, whether Pol ε acts redundantly with Pol δ in first-end synthesis or not. The strong inhibition of Pol ε by longer flaps (Figures 4C and 5) argues against a role for Pol ε during D-loop–mediated extension, since Pol ε was found to be inhibited by the flap before reaching the double-stranded junction. Here, we show that Pol ε has very low activity in extending D-loops compared to Pol δ in reconstituted D-loop reactions with two different DNA substrates (Figure 6B and Supplementary Figure S2B). Although our biochemical experiments suggest that Pol ε does not carry out the extension of the invading strand, we cannot exclude the possibility that Pol ε may play a role in second end DNA synthesis during homologous recombination.

It was recently shown that replicative polymerases frequently incorporate ribonucleotides into the genome (30), and the timely removal of ribonucleotides is important for the maintenance of genomic integrity because ribonucleotides embedded in DNA can lead to short deletions (31). Several pathways, such as those mediated by RNase H2 or topoisomerase I, are involved in removing the incorporated ribonucleotides from the DNA (57). Pol δ is considered to be the primary polymerase involved in RER, however, it can be substituted by Pol ε albeit inefficiently (32). Our results suggest that Pol ε can efficiently displace the ribonucleotide at a nicked rNTP site and simultaneously add a dNTP, which supports a role for Pol ε in RER (Figure 3C).

Recent reports demonstrate that human Exo1 cleaves 1-nucleotide flaps much more efficiently than longer flaps (58). This function coincides with the ability of Pol ε to generate single nucleotide flaps, and this underscores a possible

collaboration between Exo1 and Pol ε in processing gapped DNA structures.

Genetic studies have shown that there is a synergistic effect on +1 frameshift mutations within homonucleotide runs when a mutation in the exonuclease domain of Pol ε is combined with the deletion of either FEN1 or Exo1 (59). This indicates that under certain circumstances Pol ε can collaborate with FEN1 and Exo1 on some DNA substrates. Our results show that Pol ε provides the short flaps that are preferred by FEN1 and Exo1. In principal, Pol ε might functionally interact with FEN1 or Exo1 during DNA repair events because only a single binding event of FEN1 or Exo1 will yield a nick that can be sealed by a ligase. However, Pol ε lacks the ability to idle thus making the removal of RNA primers too inefficient for the Okazaki fragment maturation process and less efficient than Pol δ during long-patch BER and other repair processes where FEN1 is an important factor.

We have shown that Pol ε has limited strand displacement synthesis capacity that restrict the generation of long flaps and this may in turn restrict participation of Pol ε to a limited number of DNA repair processes. In addition, the short flaps created by Pol ε have a smaller risk of causing genomic instability compared to longer flaps. The limited strand displacement synthesis will allow Pol ε to take part in processes such as short-patch BER and RER but not in the extension of the invading strand in D-loops or extended patches in long-patch BER.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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