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Enzymatic switching for efficient and accurate translesion DNA replication

Scott D. McCulloch, Robert J. Kokoska, Olga Chilkova[1], Carrie M. Welch[2], Erik Johansson[1], Peter M. J. Burgers[2] and Thomas A. Kunkel*

Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, NIH, DHHS, Research Triangle Park, NC 27709, USA, [1]Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87 Umeå, Sweden and [2]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St Louis, MO 63110, USA

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ABSTRACT

When cyclobutane pyrimidine dimers stall DNA replication by DNA polymerase (Pol) δ or ε, a switch occurs to allow translesion synthesis by DNA polymerase η, followed by another switch that allows normal replication to resume. In the present study, we investigate these switches using *Saccharomyces cerevisiae* Pol δ, Pol ε and Pol η and a series of matched and mismatched primer templates that mimic each incorporation needed to completely bypass a *cis–syn* thymine–thymine (TT) dimer. We report a complementary pattern of substrate use indicating that enzymatic switching involving localized translesion synthesis by Pol η and mismatch excision and polymerization by a major replicative polymerase can account for the efficient and accurate dimer bypass known to suppress sunlight-induced mutagenesis and skin cancer.

INTRODUCTION

Faithful DNA replication depends on repair processes to provide undamaged templates for accurate and processive copying by DNA polymerases (Pol). Consistent with their roles in replicating large eukaryotic genomes, DNA polymerases δ and ε processively synthesize DNA with high fidelity [reviewed in (1)] resulting from high nucleotide selectivity at the polymerase active site and proofreading of rare errors by their intrinsic 3′ exonucleases. However, some DNA lesions that escape repair can stall DNA replication. One solution to replication blockage by lesions is translesion synthesis (TLS) catalyzed by specialized DNA polymerases. Among many template-dependent eukaryotic DNA polymerases [reviewed in (2)], at least five have been implicated in TLS, Pol ζ (B family) and four Y family members, Rev1p, Pol η, Pol ι and Pol κ. Because multiple DNA polymerases may participate in TLS, and because a wide variety of structurally distinct and potentially mutagenic lesions are produced by chemical and physical insults to DNA, it is of interest to understand how polymerase switches are coordinated for efficient and accurate TLS at a replication fork. Here we investigate this issue using the best-understood example of TLS in eukaryotes, bypass of a *cis–sin* thymine–thymine (TT) dimer by DNA polymerase η.

TT dimers are among several types of DNA photoproducts generated by exposure to the ultraviolet (UV) light component of sunlight. As implied by the name, a TT dimer contains two covalently linked thymine bases. The most common form is a *cis–syn* dimer, which is repaired by nucleotide excision repair more slowly than are other UV-induced lesions (3). Moreover, *cis–syn* TT dimers distort the DNA helix [see (4) and references therein] and when present in DNA templates, they strongly impede synthesis by most DNA polymerases, including Pol δ (5). However, DNA polymerase η, the product of the yeast *RAD30* gene (6) and the human *POLH* gene (7,8), can bypass TT dimers. The importance of this bypass is indicated by the fact that humans with mutations in the *POLH* gene that inactivate Pol η suffer from *Xeroderma pigmentosum variant* (XPV), one symptom of which is greatly increased susceptibility to sunlight-induced skin cancer (9). The current hypothesis to explain this striking phenotype is that Pol η suppresses the UV-induced mutagenesis that underlies skin cancer by participating in efficient TLS that is more accurate than is the bypass conducted by other polymerases when Pol η is inactivated [reviewed in (10,11)].

One idea we are using to investigate enzymatic switching during translesion replication is that on–off switches occur during transitions from preferential to disfavored use of damaged primer templates, and that among multiple possibilities, the polymerase called upon following each successive nucleotide incorporated is the one whose properties ultimately result in the highest efficiency and fidelity of bypass. With this hypothesis in mind, we recently developed experimental approaches to quantify the efficiency of TLS during a single cycle of processive synthesis, and also to measure the fidelity of complete TLS reactions (12). Using these approaches, we unexpectedly observed that human Pol η preferentially copies thymine dimers and one or two flanking bases with higher processivity than it copies undamaged DNA, and then it switches to less processive synthesis (13). This implies that Pol η senses the location of the dimer as synthesis proceeds. Termination following incorporation of 1 or 2 nt beyond the dimer places the two damaged bases at the second, third and

*To whom correspondence should be addressed. Tel: +1 919 541 2644; Fax: +1 919 541 7613; Email: kunkel@niehs.nih.gov

fourth base pairs of the duplex primer template. Based on structural studies of homologous *Sso* Dpo4 (14,15), these base pairs should interact with the little finger domain that is present only in Y family polymerases and differs among family members. We suggested that such interactions might result in conformational changes that trigger the switch to another polymerase.

Just as for the other four polymerases implicated in TLS, Pol η lacks a 3′ exonuclease activity and therefore cannot proofread the errors it frequently makes. We recently found that Pol η bypasses a TT dimer with very low fidelity, e.g. one dGMP was misincorporated opposite the 3′ T of a TT dimer for every 26 correct incorporations of dAMP (13). This error rate is consistent with the low fidelity with which Pol η copies undamaged DNA (16) and with the ability of Pol η to misinsert certain incorrect nucleotides opposite damaged and undamaged template bases at high rates (16–18). The possibility has been considered that even such low fidelity synthesis by Pol η may be sufficient to suppress UV-induced mutagenesis and skin cancer, because dimers may rarely be encountered by the replication machinery in repair proficient humans (19). In addition to that possibility, we (16) discussed several ways by which the fidelity of bypass *in vivo* could be improved despite the intrinsically low fidelity of Pol η. One of these ideas is enzymatic switching during low processivity bypass to allow proofreading of Pol η errors by the 3′ exonuclease activity of Pol δ or Pol ε. That one polymerase may proofread errors made by another was suggested in an earlier study involving primer extension by Pol α and Pol δ (20). More recently, we applied this hypothesis to the action of a TLS polymerase during bidirectional replication of SV40 origin containing undamaged DNA by the multiprotein replication machinery in human cell extracts that are primarily catalyzed by Pol δ. Replication errors in that system were induced by human Pol η, and the Pol-η-dependent error rate was found to be affected by changing the dNTP concentration or adding dGMP to the reaction (21). These effects are classical hallmarks of exonucleolytic proofreading and suggest that Pol δ can proofread errors made by Pol η during replication.

In the present study of TT dimer bypass by yeast polymerases, we first show that primer templates containing TT dimers that are poor substrates for extension by Pol δ and Pol ε are good substrates for Pol η. We then show that the reverse is also true, i.e. poor substrates for extension by Pol η are good substrates for Pol δ and Pol ε. This complementary pattern, involving both primer extension and primer digestion, suggests that efficient and accurate TT dimer bypass can be accomplished by multiple switching involving a 3′ exonuclease and two DNA polymerase activities with very different properties.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase and restriction enzymes were purchased from New England Biolabs. Unlabeled dNTPs, [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Amersham Biosciences. Full-length human Pol η was purified as described previously (18). Yeast Pol η, purified as described in (22) was purchased from Enzymax. Wild-type and exonuclease-deficient forms of *Saccaromyces cerevisiae* Pol δ and Pol ε holoenzymes were purified as described in (23,24). Materials for fidelity assays were from previously described sources (25).

DNA substrates

Primer templates are shown in Figure 1A. Undamaged, gel-purified oligonucleotides were purchased from Invitrogen. Templates containing a *cis–syn* TT dimer, synthesized as described in (26), were kindly provided by S. Iwai (Osaka University). Primers were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The primer and template strands were mixed in a 1:1.5 molar ratio, heated to 85°C and then slowly cooled to room temperature.

Primer extension/excision reactions

Primer extension reactions with human Pol η were performed as described in (13). Reaction mixtures (30 µl) with yeast Pol η contained 40 mM Tris pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 60 mM KCl, 100 µM dNTPs, 2.5% glycerol and 0.1 mg/ml BSA. Extension/excision reaction mixtures with yeast Pol δ (exo$^+$ and exo$^-$) (30 µl) contained 40 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM NaCl, 25 µM dNTPs and 0.1 mg/ml bovine serum albumin. Yeast Pol ε (exo$^+$ and exo$^-$) reaction mixtures (30 µl) contained 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 2 mM dithiothreitol, 25 mM NaCl, 10% glycerol, 25 µM dNTPs and 0.1 mg/ml bovine serum albumin. All reactions contained 133 nM labeled DNA substrate and 0.33 nM yeast Pol η, 0.53 nM yeast Pol δ or 0.13 nM yeast Pol ε. Reaction mixtures were incubated at 37°C and products (6 µl) were removed after 3, 6 and 9 min and diluted in 6 µl of formamide stop buffer (95% deionized formamide, 25 mM EDTA, 0.01% bromophenol blue and 0.01% xylene cyanol). Products were heated to 95°C for 3 min and separated on a 12% denaturing polyacrylamide gel. Product bands were quantified by phosphoimagery and the values were used to calculate the probability of termination of processive synthesis and the insertion efficiencies at each template nucleotide, as described in detail previously (12). The termination probability at any position (N) is defined as the band intensity at (N) divided by the total intensity for all bands $\geq N$. The insertion efficiency at any position (N) is defined as the intensity at bands $\geq N$ divided by the intensity at bands $\geq N-1$. Here we report values only for polymerization reactions wherein these probabilities remained constant over the short incubation times used, i.e. the DNA products result from a single cycle of processive synthesis. Results using this experimental approach are similar to those obtained when processivity is measured in the presence of a trap (27).

Assays for proofreading at TT dimers

Proofreading reactions followed by primer extension utilized unlabeled primer template substrates consisting of the 45mer template strand annealed to primer strands of 25, 28 or 31 nt (primers -3, $3'T_m$, and $+2_m$, respectively). The latter two substrates contained a G–T mispair at the 3′ T of the dimer. Reactions containing 267 nM DNA substrate were performed with the conditions described above for human Pol η in the presence of 50 µCi (3000 Ci/mmol) of [α-^{32}P]dCTP. Reactions (30 µl) contained 130 nM human Pol η alone or in combination with 13 nM yeast Pol δ (exo$^+$ or exo$^-$) or yeast Pol ε (exo$^+$ or exo$^-$) at 37°C for 15 min. Full-length

A.

	Primer Name	Position of first: Incorporation	Excision
5′- 23 mer-GG	-3	-2	-4
5′- 23 mer-GGC	-2	-1	-3
5′- 23 mer-GGCT	-1	3′ T	-2
5′- 23 mer-GGCT**A**	3′ T	5′ T	-1
5′- 23 mer-GGCT**A**A	5′ T	+1	3′ T
5′- 23 mer-GGCT**A**AC	+1	+2	5′ T
5′- 23 mer-GGCT**A**ACC	+2	+3	+1
5′- 23 mer-GGCT**A**ACCC	+3	+4	+2
5′- 23 mer-GGCT**A**ACCCG	+4	+5	+3
5′- 23 mer-GGCT**A**ACCCGG	+5	+6	+4
3′- 23 mer-CCGATTGGGCCATGGCTCGACC-5′		45-mer template	
5′- 23 mer-GC	-3	-2	-4
3′- 23 mer-CGAATTAAGCACGTCCGTACCATCGA-5′		49-mer template	

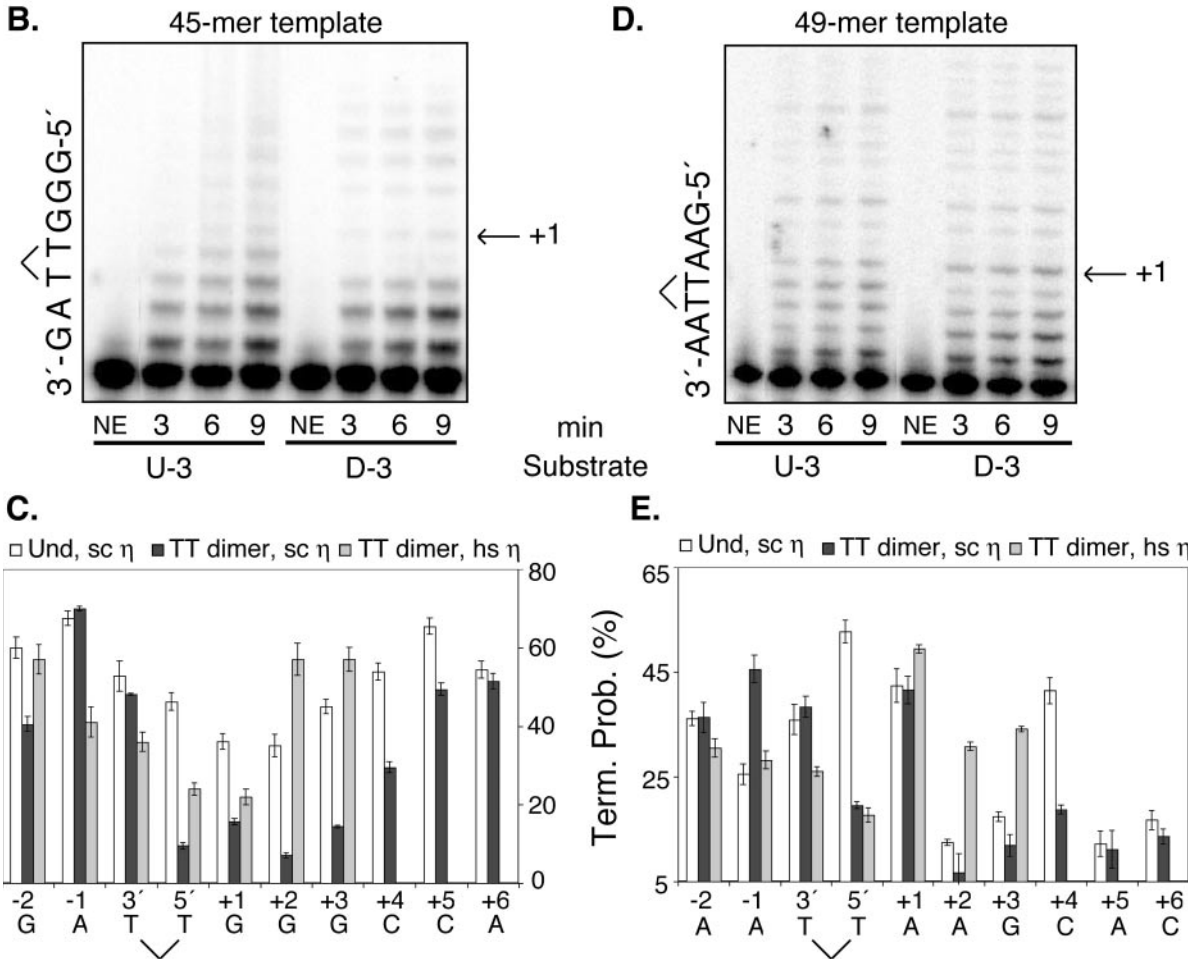

Figure 1. TT dimer bypass by yeast Pol η. (**A**) Partial sequences of the 25–34 nucleotide primers and the 45mer and 49mer templates. A subset of these primers (28–34 nt in length) contained a G at the position (indicated in boldface) opposite the 3′ T of the template. Primers are named based on the location of the 3′ terminal base relative to the TT dimer (negative numbers are upstream and positive numbers downstream of the dimer). The site of the TT dimer is indicated by a caret. Substrates are named by combining either a U (undamaged template) or D (damaged template) with the primer name. For example, 'D+1' describes a damaged template with a primer that ends opposite the +1 site. Substrates containing a G:T mispair are further designated with a subscript m (i.e. $D+1_m$). (**B**) Products generated with undamaged (U-3) and damaged (D-3) primer templates using the 45mer template. For reference, +1 indicates the product of one nucleotide insertion beyond the dimer. (**C**) Termination probabilities at positions along the 45mer template during dimer bypass by *S.cerevisiae* Pol η (sc η; white and black bars, undamaged and damaged templates, respectively) and human Pol η (hs η, gray bars, damaged template). Data for human Pol η comes from reference (13). Termination probability is defined as the total product at any position divided by the amount of that product plus all longer products (12). Values are averages from 3–10 data points at reaction intervals from 3–9 min with error bars representing standard deviations. (**D** and **E**) are as described for (B and C), respectively, using the 49mer template.

products were digested and recovered and the single-stranded labeled primer strand was annealed to gapped M13*lac* molecules that were introduced into *Escherichia coli* and scored for plaque color as described in (12).

RESULTS

TT dimer bypass parameters for yeast Pol η

We first compared the ability of yeast Pol η to bypass a *cis–syn* TT dimer or two undamaged thymines in the same sequence context. A 400-fold excess of primer template over enzyme and short reaction times were used. Under these conditions, quantitative analyses performed as described previously (12,13) revealed that the probability of termination of processive synthesis remains constant with time, demonstrating that the observed DNA products are generated during a single cycle of processive DNA synthesis. When copying undamaged 45mer or 49mer templates, the results (left lanes in Figure 1B and D, respectively) indicate that yeast Pol η is not a highly processive polymerase [open bars in Figure 1C and E, also see (28)]. The results are different with damaged DNA (right lanes in Figure 1B and D), where, with one exception (the +1 position of the 49mer), yeast Pol η incorporates nucleotides opposite the 5′ T of the dimer and a few additional positions with higher processivity, i.e. lower termination probability (black bars in Figure 1C and E, respectively). For incorporation beyond the +4 position, termination probabilities by yeast Pol η are similar for the damaged (black bars) and undamaged templates (open bars).

For comparison, termination probabilities during dimer bypass by human Pol η using the same substrates are also shown [gray bars in Figure 1C and E, from (13)]. This comparison indicates that yeast and human Pol η share an ability to copy certain template nucleotides in the TT dimer-containing DNA with higher processivity than with undamaged DNA, and that both enzymes switch to less processive synthesis after dimer bypass. Interestingly, human Pol η (gray bars) switches after fewer nucleotides are incorporated beyond the dimer as compared to yeast Pol η (black bars). Moreover, termination probability patterns during dimer bypass differ depending on the DNA sequence (Figure 1B and C versus Figure 1D and E) and the polymerase (black versus gray bars, e.g. +2 template position in Figure 1C and E).

When the DNA product distributions were used to calculate nucleotide insertion efficiencies by yeast Pol η opposite each template position, the values were higher for the dimer-containing substrate than for undamaged DNA at the 5′ T of the dimer and several subsequent positions. We also calculate [as described in (12)] that yeast Pol η bypassed the TT dimer in both sequence contexts with an efficiency that is 240% of that seen when copying the equivalent two undamaged thymines. Thus, like human Pol η (13), yeast Pol η actually prefers to copy a *cis–syn* TT dimer as compared to undamaged DNA.

TT dimer bypass parameters for yeast Pol δ and Pol ε with correctly paired substrates

Next, we compared the ability of yeast Pol δ and Pol ε to use primer templates representing the successive incorporations needed for complete dimer bypass. Three-subunit Pol δ (23) and four-subunit Pol ε (24) were used, and results with the wild-type enzymes were compared to those obtained with derivatives lacking 3′ exonuclease activity. Template primers were used that contained a *cis–syn* TT dimer or two undamaged thymines in the 45mer template (Figure 1A), in each case correctly paired with adenines. Primers of increasing length were used to test the ability of each replicative enzyme to either extend or digest the primer depending on the location of the terminus relative to the dimer. Again, a large excess of DNA over enzyme and short incubation times were used, such that DNA product bands result from a single encounter between the protein and substrate.

The product distributions for copying the undamaged template (Figure 2A) indicate that all four DNA polymerases extended all 10 undamaged primer templates. The processivity of these reactions was higher than observed with yeast Pol η, and Pol ε appears to be somewhat more processive than Pol δ. Moreover, Pol ε is capable of completely copying the template, whereas Pol δ does not do so during a single cycle of synthesis. Under these reaction conditions with dNTPs present, the exonuclease activities of the wild-type enzymes (lanes 2 and 4) only digested a small amount of correctly paired primers (e.g. see U5′T, U+1).

The results with the dimer-containing primer templates (Figure 2B) reveal that Pol δ and Pol ε can insert nucleotides opposite the two template nucleotides preceding the dimer (primers D-3 and D-2), but they do not insert nucleotides opposite the 3′ T of the dimer, the 5′ T of the dimer, or the next template base (primers D-1, D3′T and D5′T, respectively). Lack of insertion at these positions (quantified in Figure 3) is not due to the inability of Pol δ and Pol ε to bind these primer templates, because both wild-type polymerases digest the primers to remove bases opposite the 5′ T, the 3′ T and the −1 template position. This excision of correctly paired bases is much more robust than seen with undamaged DNA, indicating that it is largely dimer-dependent. Interestingly, the digestion generates primers that are not extended by Pol δ or Pol ε, while primer termini at these positions are preferentially used by yeast Pol η. The fact that even shorter products are not seen could reflect termination of dimer–dimer-dependent digestion because Pol δ and Pol ε no longer senses a dimer in the single-stranded template strand of those substrates, or perhaps additional digestion may occur followed by DNA re-synthesis up to the dimer by the polymerase activities of Pol δ and Pol ε (i.e. idling).

The results are quite different for primers that would be made available to Pol δ and Pol ε when Pol η terminates correct synthesis after the dimer is bypassed. These include primers D+1 through D+5, each of which is extended by Pol δ and Pol ε. The proportion of primers extended by the wild-type enzymes increases as the number of correct base pairs beyond the dimer increases, and this is accompanied by a corresponding decrease in excision of correct base pairs (Figures 2B and 4A and B). Both the polymerization and excision reactions appear to be more processive for Pol ε than for the Pol δ (e.g. compare extension and excision band patterns in lanes two and four for primers D+1 through D+5 in Figure 2B). With both enzymes, dimer-dependent excision proceeds to the −2 position (bands marked with asterisks in Figure 2B), i.e. the −1 primer nucleotide is removed. Some excision is observed with the D+5 primer, indicating that even when the dimer is embedded at the sixth and seventh base pairs in the duplex,

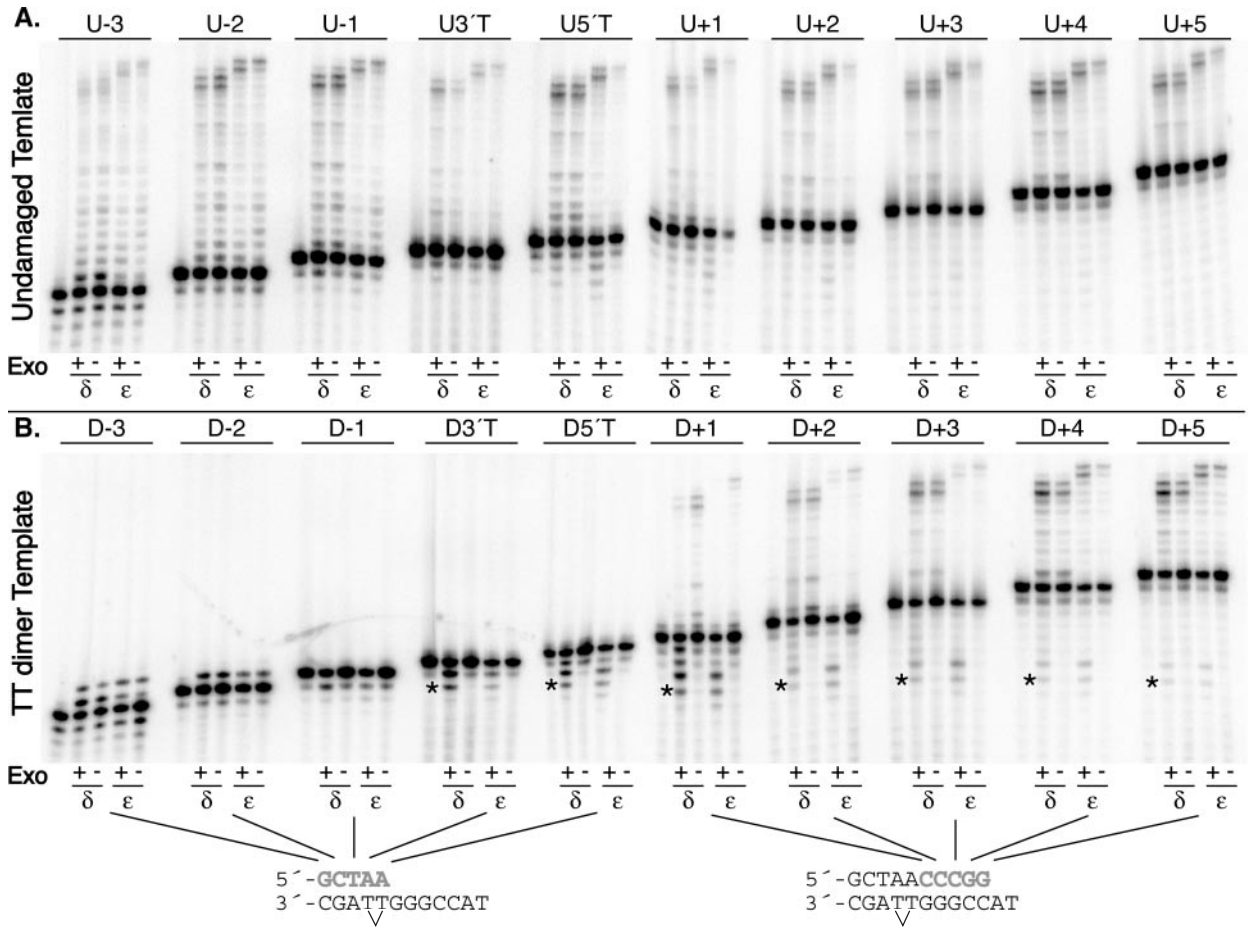

Figure 2. Yeast Pol δ and Pol ε activity on matched undamaged and damaged substrates. (**A**) Gel image of primer extension/excision products for a series of substrates containing undamaged templates. (**B**) Gel image of primer extension/excision products for a series of substrates containing a TT dimer in the positions indicated by a caret. A diagram of the substrates used is given below (B), with the lines pointing to where the primer terminus is for each set of lanes. Each panel of five lanes is further identified by the substrate name described in the Figure 1A legend. The products shown in each lane are 9 min time points from reactions containing DNA polymerase Pol δ (exo$^+$ and exo$^-$) and Pol ε (exo$^+$ and exo$^-$). All reactions contain all four dNTPs. The leftmost lane in each set is from a reaction containing no enzyme. The most intense band in each lane represents the unreacted primer strand. All reactions were also analyzed at 3 and 6 min with similar results. Bands marked with an asterisk are primers excised back to the −2 position.

Pol δ and Pol ε can still detect its presence and excise eight correctly paired bases. The extension product distributions were also used to calculate nucleotide insertion efficiencies with the damaged templates during processive synthesis by wild-type Pol δ and Pol ε (Figure 3). (Note that, because the first nucleotide incorporated by Pol δ and Pol ε with the damaged template at +2 does not reflect processive synthesis, insertion efficiencies at this position were not calculated.) The overall results with the dimer-containing primer templates reveal a complementary pattern of insertion by Pol η/δ and Pol η/ε (Figure 3), whereby substrates not extended by Pol δ and Pol ε are preferentially extended by Pol η, and as this preference fades, Pol δ and Pol ε can use the extended primers and partition in favor of polymerization over excision as the number of correct base pairs increases (Figure 4A and B).

Bypass parameters for yeast Pol δ and Pol ε with substrates containing a mismatch

Next, we examined the ability of yeast Pol δ and Pol ε to extend or digest a series of seven primer templates of increasing length that contain a G opposite the 3′ T of a TT dimer or the corresponding undamaged T in the 45mer template. This mismatch was chosen because it results from the most frequent misincorporation by human Pol η during TT dimer bypass (13). With the undamaged template, exonuclease-deficient Pol δ and Pol ε do not efficiently extend the primer with the mismatch at the terminus (Figure 5A, lanes 3 and 5 with the U3′T_m primer). However the adjacent primer (U5′T_m) containing one correct base pair at the terminus is extended by both polymerases to some extent, and extension efficiencies increase as the mismatch is increasingly embedded into the duplex. Wild-type Pol δ and Pol ε (lanes 2 and 4 for each primer) engage all the mismatched primers hybridized to undamaged templates (U3′T_m through U+5_m), and perform both excision and extension reactions that increasingly partition in favor of polymerization as the mismatch is located deeper in the duplex.

The results with the dimer-containing mismatched primer templates (Figure 5B) reveal that none of the four polymerases insert nucleotides opposite the 5′ T of the dimer or the next template base. For example, with substrates containing a

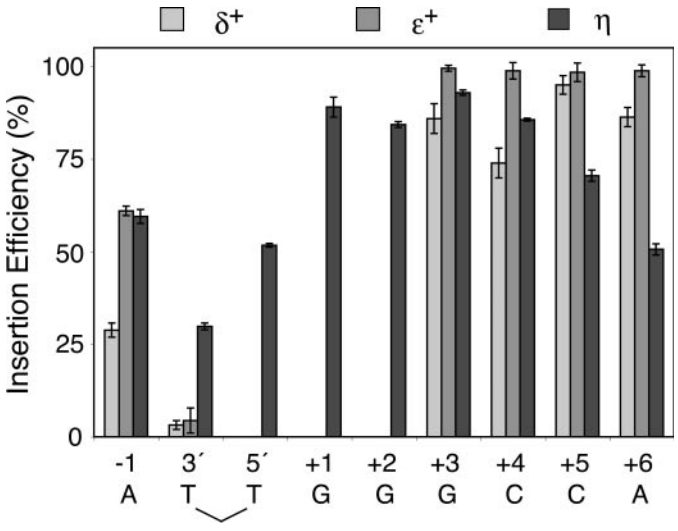

Figure 3. Insertion efficiencies of Pols δ, ε and η. Insertion efficiencies at positions along the 45mer TT dimer containing template are shown for wild-type yeast Pol δ (light gray bars), Pol ε (dark gray bars) and Pol η (black bars). Values are based on the distribution of extension products such as those shown in Figures 1B and 2B. The insertion efficiency at any given position is defined as the sum total of the amount of reaction product at that position and longer divided by that total plus the amount of product at the position immediately preceding it (see Materials and Methods). This measurement is only valid for reactions that incorporate at least 2 nt and reflect a single cycle of polymerase use on the substrate. Thus, incorporations at the +2 template site by Pol δ and Pol ε using substrate D+1 (see Figure 2B) are not shown.

terminal mispair, the exonuclease proficient form of both Pol δ and Pol ε were at least 90 times less efficient at incorporating a nucleotide opposite the first undamaged template base following the dimer compared to the same site on the undamaged template. That Pol δ and Pol ε can nonetheless bind to primer templates containing a dimer and a mismatch is indicated by the fact that the wild-type enzymes excise the mismatched primers up to and including the −1 base (Figure 5B, asterisks). The longer mismatched primers are used by Pol δ and Pol ε for both excision and extension, with the balance between the two activities again shifting towards extension as the mismatch is located deeper in the duplex. When this partitioning by Pol δ (Figure 4C) and Pol ε (Figure 4D) is compared to results with correctly paired substrates (Figure 4A and B, respectively), it is clear that the presence of the mismatch shifts both wild-type enzymes to partition even more in favor of mismatch excision than is seen with a correct base pairs at the 3′ T of the dimer (e.g. compare D+1 versus D+1_m, D+2 versus D+2_m, D+3 versus D+3_m and D+5 versus D+5_m).

Evidence for extrinsic exonucleolytic proofreading during TT dimer bypass

The above results suggest that if yeast Pol η misinserts a base during preferential copying of a TT dimer and a few undamaged template bases beyond the dimer (Figure 1), then that mismatch should be subject to proofreading by Pol δ and Pol ε. To test this hypothesis, we first performed five different polymerization reactions (Table 1) to completely copy the 45mer template containing a TT dimer, each primed with a 28 base oligonucleotide having a 3′ terminal G opposite the 3′ T of the dimer (D3′T_m, Table 1). Full-length DNA product were recovered from denaturing polyacrylamide gels, processed and hybridized to gapped M13lac DNA molecules, which were then introduced into *E.coli* to obtain M13 plaques (see Materials and Methods). In this 45mer template 5′-***TT***AG sequence context, base substitutions resulting from mispairs at the 3′ T of the dimer (indicated by bold italics) are easily scored with ~60% expression in *E.coli*, as dark blue plaque revertants of the TAG amber codon (underlined) in the *LacZ* α-complementation gene, which encodes a faint blue plaque phenotype (12). As expected for complete dimer bypass by exonuclease-deficient Pol η without the possibility of excising the terminal mismatch, primer extension generated products that yielded the expected high percentage of dark blue plaques (Table 1, 62%). When reactions were performed with combinations of Pol η plus exonuclease-deficient Pol δ or Pol ε (10:1 molar ratio of Pol η to Pol δ or Pol ε), those products also yielded 60% dark blue plaques. However, when similar reactions were performed with Pol η plus wild-type Pol δ or Pol ε, those extension products yielded only 9.3% and 8.3% dark blue plaques, respectively. Thus, despite the fact that wild-type Pol δ and Pol ε were present at 10-fold lower concentration than Pol η and cannot extend that dimer containing mismatched primer (Figure 5B), their exonucleases can excise >85% of the terminal G residues, either prior to the initial extension by Pol η after copying of the dimer by Pol η but prior to the complete extension of the template. Consistent with the lower mismatch excision by Pol δ and Pol ε observed when the mismatch is embedded in duplex DNA (Figure 5B), reactions in which Pol η plus wild-type Pol δ or Pol ε were used to extend a mismatched 31mer primer containing three correct base pairs at the terminus (D+2_m) yielded much higher proportions of dark blue plaques (Table 2, 52 and 29%, respectively, for Pol η/δ^+ and Pol η/ε^+). The lower percentage of blue plaques obtained in both experiments with wild-type Pol ε (8.3 and 29%) as compared to wild-type Pol δ (9.3 and 52%) indicates that Pol ε competes more effectively with Pol η for mismatched primer termini than does Pol δ.

Finally, we performed an experiment to determine if wild-type Pol δ or Pol ε could proofread errors made by Pol η during dimer bypass, as opposed to preformed errors introduced by the primer. Reactions similar to those just described were conducted but now using a perfectly matched 25mer (D-3) to allow Pol η to commit errors during complete bypass (Table 2). Pol η alone generated 4.5% dark blue plaques during dimer bypass, 75% of which reflect misincorporation of dGMP opposite the 3′ T of the dimer (13). However, inclusion of wild-type Pol δ or Pol ε reduced the dark blue plaque frequency to 1.1% and 1.0%, respectively. Thus, even though Pol η is present in 10-fold excess over Pol δ and Pol ε, about four of five misinsertions by Pol η are proofread by the exonuclease activities of wild-type Pol δ and Pol ε.

DISCUSSION

Eukaryotic DNA replication may require seven or more DNA polymerases for chromosomal DNA replication. These include Pol α-primase to initiate replication, Pol δ and Pol ε to perform the bulk of chain elongation on the leading and lagging strands, and Pol ζ, Pol η, Pol ι, Pol κ and Rev1p for replication

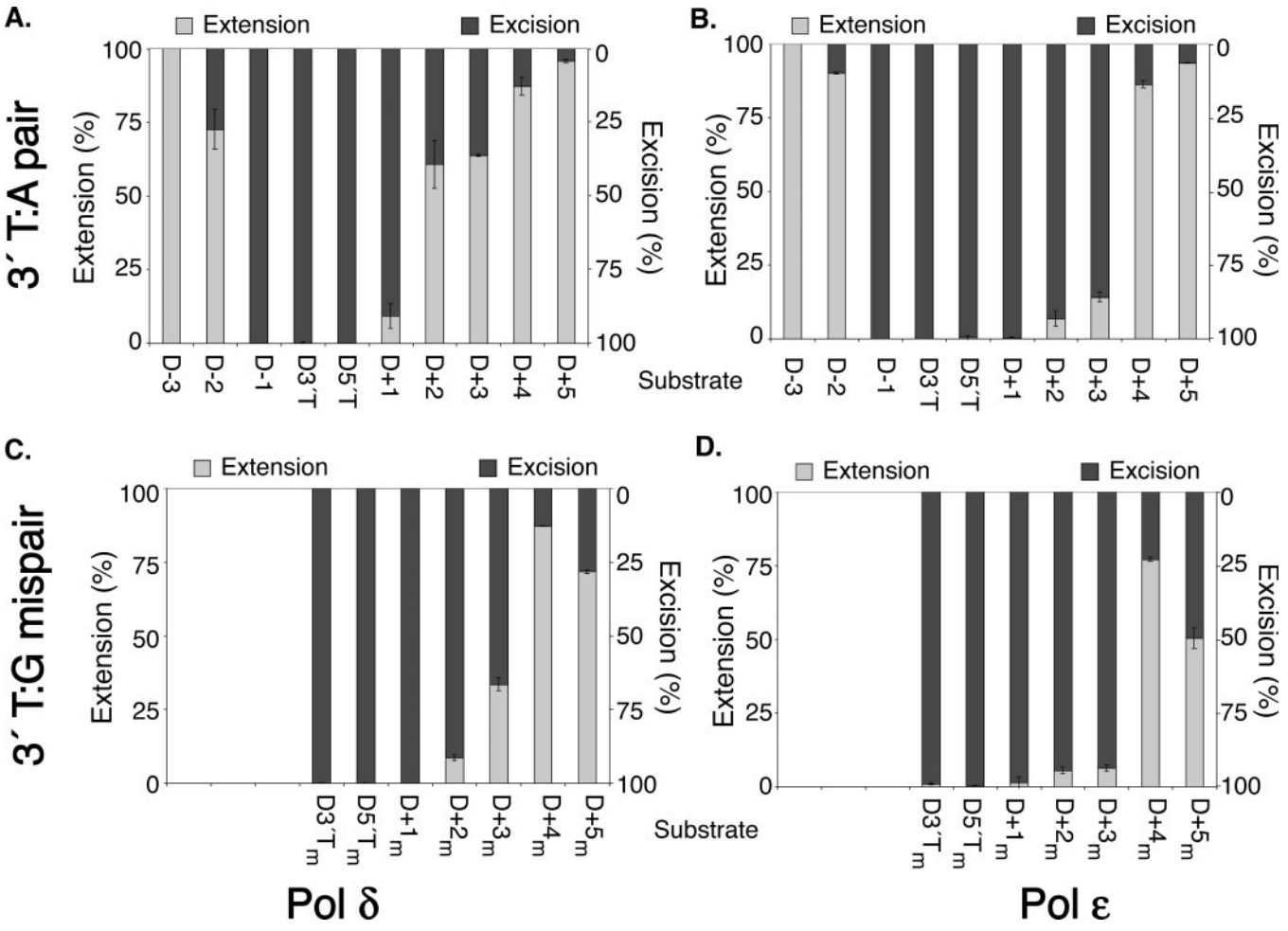

Figure 4. Polymerase/exonuclease partitioning using TT dimer-containing substrates. (**A**) Bar graphs indicating the relative level of extension products (gray sections, quantified on left *y*-axis) compared to excision products (black sections, quantified on right *y*-axis) resulting from activity of wild-type Pol δ on TT dimer containing substrates with matched primers. (**B**) Pol ε partitioning on TT dimer containing substrates and matched primers. (**C**) Pol δ partitioning on TT dimer containing substrates with a 3′ T:G mispair. (**D**) Pol δ partitioning on TT dimer containing substrates with a 3′ T:G mispair. The values shown are calculated from Figures 2B and 5B as the amount of extension products (bands longer than the primer) or excision products (bands shorter than the primer) relative to all reaction products. All reactions were run under conditions that produce a single cycle of interaction between DNA and protein.

bypass of a wide variety of lesions. Replication of large eukaryotic genomes must be efficient and complete to maintain cell viability; yet, certain lesions strongly impede synthesis by the major replicative polymerases. Replication must be accurate to maintain genome stability, yet Pol α, Pol ζ, Rev1p, Pol η, Pol ι and Pol κ lack intrinsic proofreading activity, the nucleotide selectivity of the latter three polymerases is particularly low, and many lesions have altered base-coding properties and are potentially mutagenic when bypassed. Thus, efficient and accurate replication is expected to require multiple switches among polymerases, and possibly between polymerases and proofreading exonucleases. This study provides information on how these enzymatic switches may be coordinated during complete bypass of a TT dimer.

We report here a complementary pattern of primer template use by three yeast polymerases that is comprised of five key features. First, just at the point where wild-type, multisubunit forms of Pol δ and Pol ε are no longer capable of extending TT dimer-containing primer templates (Figure 2), yeast Pol η can do so, and with a preference for more processive synthesis with damaged as compared to undamaged DNA. This preference continues for several incorporations beyond the dimer (Figure 1). Second, as Pol η termination probabilities rise after dimer bypass (Figure 1), Pol δ and Pol ε can bind to and use the resulting primers for a combination of extension (Figure 2) and excision (Figures 3 and 4). Third, as the number of correct base pairs beyond the dimer increases, Pol δ and Pol ε increasingly partition in favor of polymerization over excision (Figure 4). Fourth, the presence of a mismatched base pair at the 3′ T of the dimer shifts this partitioning by Pol δ and Pol ε towards excision (Figure 4C and D). Fifth, when present in a TT dimer bypass reaction with Pol η, the exonuclease activities of Pol δ and Pol ε can excise a terminal mismatch that is provided (Tables 1 and 2) or a mismatch actually created by Pol η during complete bypass (Table 3). These data support the suggestion made by us (16,21), and later by others (29), that errors made by Pol η may be proofread by Pol δ and/or Pol ε. They are also consistent with a report demonstrating that several correct base pairs 5′ to a *trans-anti*-BPDE-N^2-dG lesion are required for primer extension by the catalytic

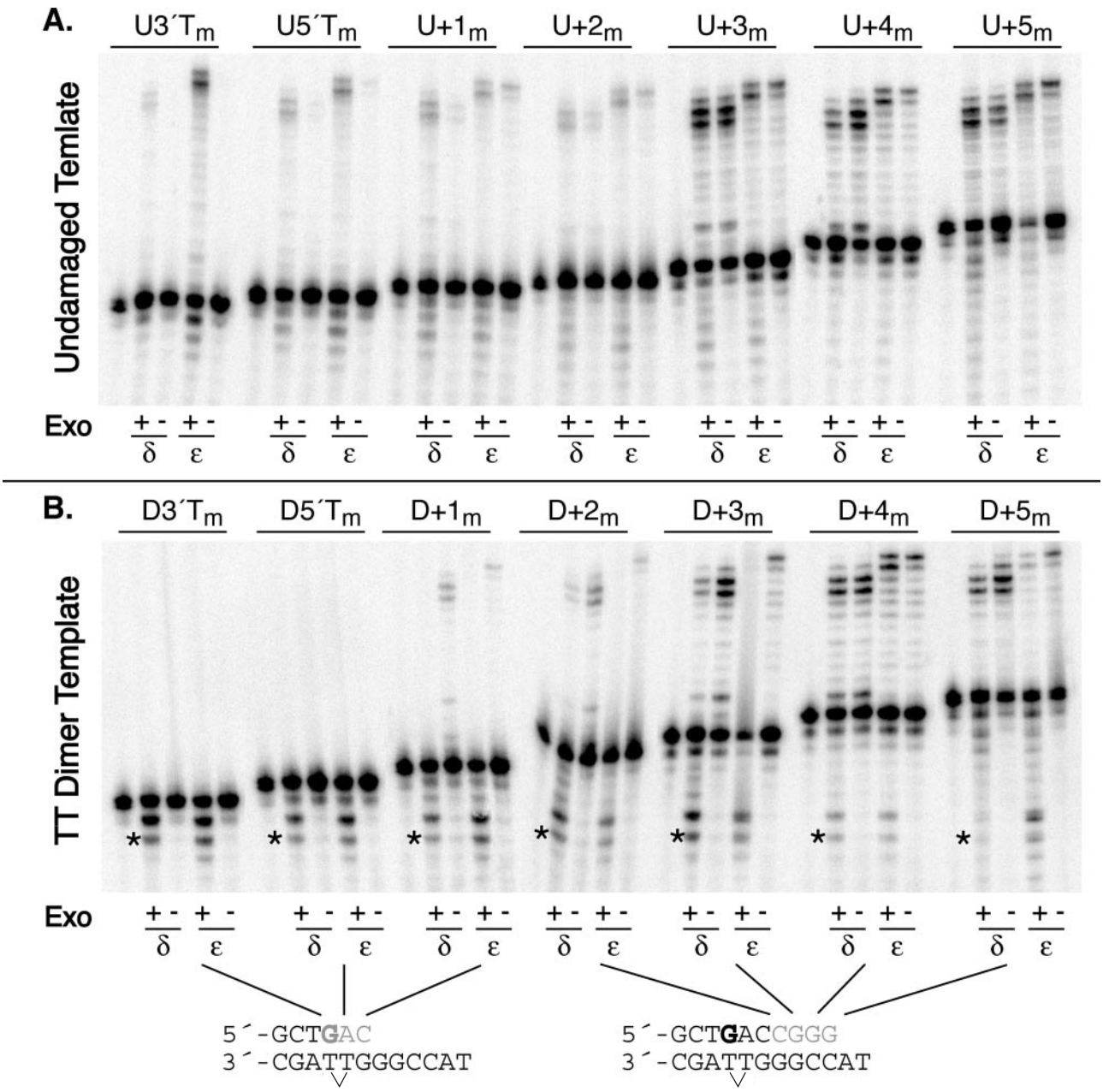

Figure 5. Pol δ and Pol ε activity on undamaged and damaged substrates containing a 3′ T:G mispair. (**A**) Gel image of primer extension/excision products for a series of substrates containing undamaged templates. (**B**) Gel image of primer extension/excision products for a series of substrates containing a TT dimer in the positions indicated by carets. All substrates contain a T:G mispair at the 3′ T of the di-thymine sequence. A diagram of the substrates used is given below (B), with the lines indicating where the primer terminus is for each set of lanes. Each panel of five lanes is further identified by the substrate name described in the legend to Figure 1A. Reactions were performed as described in the legend to Figure 2. Bands marked with an asterisk are primers excised back to the −2 position.

subunit of Pol δ, and also that these primers are subjected to degradation to a position immediately prior to the lesion (30).

Given that Pol ε plays a major role in replicating eukaryotic genomes, it is remarkable that this is the first study of TT dimer bypass by Pol ε. It follows that this provides the first direct comparison of how Pol δ and Pol ε process TT-dimer-containing DNA templates. Their behavior with these substrates is similar but not identical. The results (asterisks in Figures 2B and 5B) suggest that both enzymes recognize the presence of a dimer in the duplex fully, 7 bp upstream of the polymerase active site, and that they can both digest the primer strand containing either exclusively correct base pairs or containing a mismatch at the dimer. However, with certain substrates (D+1 through D+3), Pol ε partitions more in favor of excision over polymerization than does Pol δ (Figure 4), and excision by Pol ε appears to be somewhat more processive than does excision by Pol δ (Figures 2 and 5). These difference may relate to the results in Table 1, which suggest that in a two-polymerase bypass reaction, Pol ε may proofread Pol η errors somewhat more efficiently than does Pol δ. Given that Pol ε and Pol δ

Table 1. Proofreading during TT dimer bypass using substrate D3′T_m

		Plaques Dark blue	Total	Mutation frequency (%)
D3′T_m	η	576	936	62
	η/δ^+	73	788	9.3
5′-GCT**G**	η/δ^-	495	832	60
3′-CGATTGGGCC	η/ε^+	99	1187	8.3
∨	η/ε^-	508	843	60

Fidelity assays were performed as described in (12,13) using the substrate indicated. Reactions were run as described in Materials and Methods for human Pol η and yeast Pol δ or Pol ε (+/− indicating status of exonuclease activity). The substrate contains a 3′ T:G mispair that codes for a dark blue plaque phenotype with 60% expression (12) unless proofread by the exonuclease activity of Pol δ or Pol ε.

Table 2. Proofreading during TT dimer bypass using substrate D+2_m

		Plaques Dark blue	Total	Mutation frequency (%)
D+2_m	η	724	1316	55
	η/δ^+	758	1455	52
5′-GCT**G**ACC	η/δ^-	382	613	62
3′-CGATTGGGCC	η/ε^+	167	582	29
∨	η/ε^-	382	669	57

Fidelity assays were performed as described in (12,13) using the substrate indicated. Reactions were run as described in Materials and Methods for human Pol η and yeast Pol δ or Pol ε ($^{+/-}$ indicating status of exonuclease activity). The substrate contains a 3′ T:G mispair that codes for a dark blue plaque phenotype with 60% expression (12) unless proofread by the exonuclease activity of Pol δ or Pol ε.

Table 3. Proofreading during TT dimer bypass using substrate D-3

		Plaques Dark blue	Total	Mutation frequency (%)
D-3	η	131	2930	4.5
	η/δ^-	36	3156	1.1
5′-G	η/δ^+	80	2613	3.1
3′-CGATTGGGCC	η/ε^+	46	4481	1.0
∨	η/ε^-	94	2490	3.8

Substrate D-3 allows for detection of proofreading of errors introduced by Pol η at either the 3′ T or at the two preceding undamaged positions. Previous studies have shown that ~75% of the dark blue plaques reflect misinsertion of G opposite the 3′ T of the TT dimer (13).

may operate on different strands and/or at different times during replication in S phase, their non-identical properties in processing damaged DNA substrates may have interesting implications for damage-induced mutagenesis. This study also provides the first comparison of the wild-type forms of Pol ε and Pol δ to their exonuclease-deficient derivatives. The results show that, despite inactivation of their exonuclease activities, neither Pol δ nor Pol ε can bypass a dimer, and they cannot even extend dimer-containing template primers unless the primer terminus is located beyond the dimer. Thus, it is not the exonuclease activity itself that prevents bypass of a TT dimer. This differs from a recent report suggesting that the exonuclease activity of replicative T4 DNA polymerase is the major determinant of bypass of an abasic site (31).

The results presented here lead to a model (Figure 6) wherein multiple switching events involving a 3′ exonuclease and two DNA polymerase activities with very different properties (Pol δ/η or Pol ε/η) can achieve efficient and accurate TT dimer bypass. For correct synthesis, the second switch may occur as Pol η termination probabilities increase, which may reflect the ability of Pol η to monitor the location of the lesion as synthesis proceeds (13), perhaps through interactions with the little finger domain that is unique to TLS polymerases. Exactly where termination increases appears to depend on which Pol η is used. With the 45mer template (Figure 1B), termination by human Pol η increases at +2 and +3 (gray bars), whereas termination by yeast Pol η increases at +4, +5 and +6 (black bars). That yeast and human Pol η differ in this bypass feature is not necessarily surprising, since they also differ in other properties, such as kinetic parameters for nucleotide insertion and mismatch extension (22,29,32,33). The template positions where termination probabilities increase also depend on the sequence surrounding the dimer. With yeast Pol η and the 45mer template with the TT in a largely G–C-rich environment, the increase in termination is greater at +4 through +6 than at +1 (black bars in Figure 1C). However, the reverse is true with the 49mer template, where the dimer is in an A—T-rich environment (black bars in Figure 1E). At the same time with human Pol η, the increase in termination with the 45mer template is greater at +2 and +3 than at +1 (gray bars in Figure 1C), whereas the reverse is true with the 49mer template (gray bars in Figure 1E). Thus, the location of switching during TLS *in vivo* may vary depending on the sequence in which the lesion is present.

Figure 6 depicts a baseline model derived from these studies with the catalytic subunit of Pol δ plus two accessory proteins, and the catalytic subunit of Pol ε plus three accessory proteins. This is a starting point to determine the effects of other accessory proteins on polymerase switching during lesion bypass. For example, two other polymerases implicated in TLS, Pol ι (34) and Rev1p (35), interact with Pol η. Also, PCNA interacts with Pol η (36) and Pol δ (37) and Pol η mutants lacking the PCNA interaction domain are non-functional (36). In initial experiments performed as in Figure 1, unmodified PCNA did not change Pol η TT dimer bypass efficiency, the presence or location of the switchpoint or the processivity of polymerization (data not shown). This is consistent with the observation that PCNA does not enhance Pol η processivity when copying undamaged DNA (38). In contrast, PCNA is well known to increase the processivity of Pol δ on undamaged DNA (37). However, this effect does little to overcome the strong impediment to dimer bypass, because TT dimer bypass by Pol δ in the presence of PCNA is inefficient and has only been observed when excess polymerase and long incubation times are used (5). Nonetheless, evidence now suggests that post-translational modifications of PCNA, such as monoubiquitination and sumoylation, are important for TLS (39–41). Thus, it will be interesting to investigate whether modified forms of PCNA influence the polymerase and exonuclease activities of Pol δ, Pol ε and Pol η. These experiments await the availability of appropriately modified derivatives of PCNA.

For incorrect TT dimer bypass synthesis, the ability of Pol δ and Pol ε to bind to and excise primers reflecting only partial

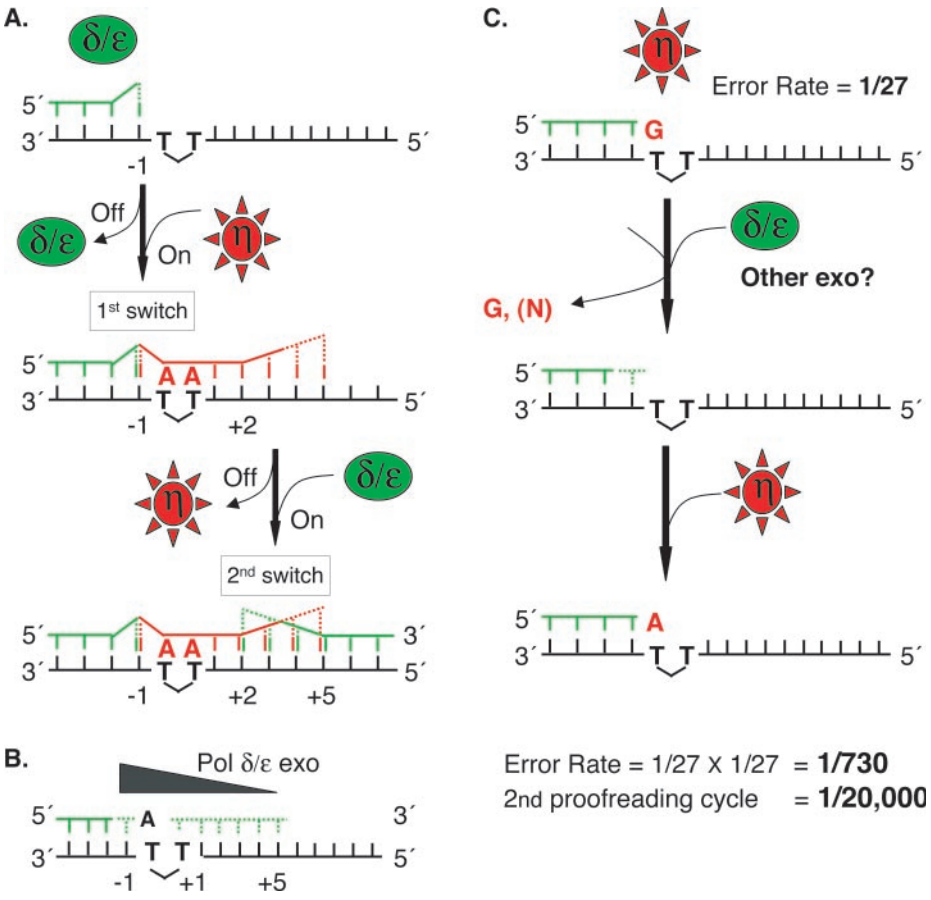

Figure 6. Models for enzymatic switching and proofreading during TT dimer bypass. (**A**) Both Pol δ and Pol ε can synthesize up to a TT dimer, indicated by solid green lines, with less efficient synthesis at the −1 site, indicated by dashed lines. After a polymerase 'switch', preferential copying by Pol η (indicated by red lines) of the −1 site (if needed), both dimer Ts, and at least one undamaged template base occurs, with further synthesis occurring with lower probability (dashed lines). The exact number of undamaged bases copied is both species- and sequence-context-dependent. Complementary to this decreasing Pol η synthesis, forward polymerization by Pol δ or Pol ε increases as the primer terminus is further from the TT dimer. The second switch between polymerases appears to occur within a broader zone than the first. (**B**) The partitioning between exonuclease and forward polymerization activities by Pol δ and Pol ε is dependent on the distance between the primer terminus and TT dimer, even with matched primers. (**C**) Human Pol η misinserts a G opposite the 3′ T of a TT dimer with a frequency of 1/27 (13). The fidelity of TT bypass can be greatly increased by proofreading of these errors by the exonucleases activities of Pol δ, Pol ε or other 3′→5′ exonucleases, allowing Pol η a second chance for insertion of the correct nucleotide. For example, adding a second round of dimer bypass would theoretically have an error rate of $(1/27)^2$, while adding yet a third round of synthesis would be predicted to have an error rate of $(1/27)^3$.

bypass implies that if Pol η generates a mismatch that slows elongation (21,29), switching may occur even earlier to facilitate excision at any point in the bypass reaction, even opposite the dimer. Proofreading of Pol η errors by Pol δ and/or Pol ε is one possible explanation for why TT dimer bypass in yeast cells occurs with a lower error rate [1/690 at the 3′ T; (42)] than Pol η error rates *in vitro*. For example, the rate of misincorporation of G opposite the 3′T of a TT dimer during complete bypass by human Pol η is 1/27 (13), and kinetic studies show that certain single nucleotide insertion rates by yeast and human Pol η are in this same high range [e.g. up to 1/43 in (28); up to 1/28 in (43); up to 1/44 in (44)]. In fact, multiple cycles of proofreading are theoretically possible and could potentially allow very accurate TLS (Figure 6). Errors at sites beyond the dimer would also presumably be proofread, since they would be closer to the primer terminus. Proofreading during TLS is an alternative to the idea that the fidelity of Pol η alone may be sufficient to suppress UV-induced mutagenesis and skin cancer (19). It is also worth noting the mice deficient in proofreading by DNA polymerase δ have increased susceptibility to several types of cancer, including skin cancer (45). At least in theory, the inability to proofread errors made during TLS may contribute to this phenotype.

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