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Translesion synthesis by yeast DNA polymerase ζ from templates containing lesions of ultraviolet radiation and acetylaminofluorene

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, DNA polymerase ζ (Polζ) is required in a major lesion bypass pathway. To help understand the role of Polζ in lesion bypass, we have performed *in vitro* biochemical analyses of this polymerase in response to several DNA lesions. Purified yeast Polζ performed limited translesion synthesis opposite a template TT (6-4) photoproduct, incorporating A or T with similar efficiencies (and less frequently G) opposite the 3′ T, and predominantly A opposite the 5′ T. Purified yeast Polζ predominantly incorporated a G opposite an acetylaminofluorene (AAF)-adducted guanine. The lesion, however, significantly inhibited subsequent extension. Furthermore, yeast Polζ catalyzed extension DNA synthesis from primers annealed opposite the AAF-guanine and the 3′ T of the TT (6-4) photoproduct with varying efficiencies. Extension synthesis was more efficient when A or C was opposite the AAF-guanine, and when G was opposite the 3′ T of the TT (6-4) photoproduct. In contrast, the 3′ T of a *cis–syn* TT dimer completely blocked purified yeast Polζ, whereas the 5′ T was readily bypassed. These results support the following dual-function model of Polζ. First, Polζ catalyzes nucleotide incorporation opposite AAF-guanine and TT (6-4) photoproduct with a limited efficiency. Secondly, more efficient bypass of these lesions may require nucleotide incorporation by other DNA polymerases followed by extension DNA synthesis by Polζ.

INTRODUCTION

DNA can be damaged by a variety of physical and chemical agents, such as UV radiation and acetylaminofluorene (AAF). DNA repair constitutes an important defense system by removing the lesions from DNA. However, some DNA lesions can persist in the genome during replication due to limited cellular repair and/or newly formed damage at the S phase of the cell cycle. Since many lesions block replicative DNA polymerases, cells have evolved a damage tolerance response to enable replication of the damaged DNA templates. Lesion bypass represents one of the damage tolerance mechanisms, and requires a DNA polymerase to copy the damaged DNA template. DNA synthesis (nucleotide incorporation) opposite a template lesion is also referred to as translesion synthesis. Depending on the outcome, translesion synthesis is further divided into error-free and error-prone translesion syntheses. While the former predominantly incorporates the correct nucleotide opposite the lesion, thus is a mutation-avoiding mechanism, the latter frequently incorporates an incorrect nucleotide opposite the lesion, thus is a mutation-generating mechanism.

In *Escherichia coli*, DNA polymerase V is a major translesion synthesis polymerase, which functions in the UmuDC mutagenesis pathway (1,2). *In vitro*, DNA polymerase V is capable of error-prone translesion synthesis opposite several lesions (1–3). In eukaryotes, DNA polymerase η (Polη) is involved in error-free lesion bypass of TT dimers (4–6). Thus, Polη functions as an important anti-mutagenesis mechanism in response to UV radiation in humans. Deficiency in Polη activity will lead to the human hereditary disease xeroderma pigmentosum variant (XPV) (6,7), which is characterized by photosensitivity and a predisposition to skin cancer (8). Error-prone translesion synthesis by purified Polη has also been observed *in vitro* in response to certain lesions such as (+)-*trans-anti*-benzo[*a*]pyrene-N^2-dG adducts (9). Most likely, Polη functions as both an error-free and error-prone translesion synthesis polymerase in eukaryotic cells, depending on the specific lesion (10). Consistent with its translesion synthesis activity, Polη is an extraordinarily low fidelity polymerase with a low processivity and lacks 3′→5′ proofreading exonuclease activity (10–12).

Polζ is another eukaryotic DNA polymerase required in a major error-pone lesion bypass pathway (13), which is also referred to as the Polζ mutagenesis pathway. Rev3 is the catalytic subunit of Polζ (13). Rev7 forms a complex with Rev3 protein and is considered a subunit of Polζ (13). Additionally, the Polζ mutagenesis pathway also requires Rad6, Rad18 and Rev1 (14–18). Rad6 is a ubiquitin conjugating enzyme (E2) and forms a complex with Rad18 protein (19,20). Rev1 is a dCMP transferase capable of incorporating a C opposite a template G or an abasic site (21). The human *RAD6* (*HHR6A* and *HHR6B*), *RAD18*, *REV1*, *REV3* and *REV7*

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genes have been isolated (22–29). The human REV3 protein is about twice the size of its yeast counterpart, as a result of additional sequences at the N-terminal 2/3 regions of the human protein (25,27). The significance, if any, of this size difference between the yeast and the human REV3 proteins is not known. The RAD6–RAD18 and REV3–REV7 interactions, and the dCMP transferase activity of REV1 are all conserved in humans (23,24,26,29). Furthermore, UV-induced mutagenesis requires both *REV1* and *REV3* gene expression in cultured human cells (27,28). Thus, the Polζ mutagenesis pathway is probably functional in humans. In addition to Polζ and Polη, it appears that Polκ and Polι are also translesion synthesis polymerases in humans (30–36). Homologs of these two DNA polymerases are not found in *Saccharomyces cerevisiae*.

It has been proposed that Polζ is generally responsible for error-prone translesion synthesis step during lesion bypass, and Rev1 is responsible for nucleotide incorporation opposite template apurinic/apyrimidinic (AP) sites (13,21,37). However, two *in vitro* studies with yeast Polζ have yielded conflicting results. Nelson *et al.* (13) reported that purified yeast Polζ is able to bypass a template *cis–syn* TT dimer, whereas Johnson *et al.* (34) reported that yeast Polζ is unable to incorporate a nucleotide opposite this lesion. In an earlier study we found that, following one nucleotide incorporation by purified yeast Polη opposite a template AP site, yeast Polζ is able to extend DNA synthesis from opposite the lesion (10). Thus, AP site bypass was achieved *in vitro* by the combined activities of Polη nucleotide incorporation and subsequent Polζ DNA extension (10). Based on this two-polymerase two-step model of Yuan *et al*. (10), Johnson *et al.* (34) later observed *in vitro* bypass of an AP site and a TT (6-4) photoproduct by combined actions of human Polι and yeast Polζ. Johnson *et al.* (34) further concluded that Polζ is an 'extender' rather than an 'inserter' during lesion bypass. These limited studies did not yield a clear understanding of Polζ in lesion bypass. To define the precise role of Polζ in lesion bypass, many more biochemical analyses of this polymerase in response to additional DNA lesions are needed.

To help understand the role of Polζ in lesion bypass, we have performed *in vitro* biochemical analyses of this polymerase in response to several DNA lesions. In this report, we show that (i) purified yeast Polζ is able to perform error-prone translesion synthesis opposite a template TT (6-4) photoproduct and an AAF-adducted guanine (AAF-G) to a limited extent, but it is unresponsive to a template *cis–syn* TT dimer due to Polζ blockage by the modified 3′ T and (ii) yeast Polζ is capable of extension DNA synthesis from primers annealed opposite these lesions. These results led to a dual-function model of Polζ, in which Polζ functions both as a nucleotide incorporation polymerase opposite some lesions and as an extension DNA synthesis polymerase during lesion bypass by the two-polymerase two-step mechanism.

MATERIALS AND METHODS

Materials

A mouse monoclonal antibody against the His_6 tag was obtained from Qiagen (Valencia, CA). Alkaline phosphatase conjugated anti-mouse IgG was obtained from Sigma Chemical Co (St Louis, MO). *Taq* DNA polymerase was purchased from BRL (Bethesda, MD). *N*-acetoxy-*N*-2-acetylaminofluorene (AAAF, the activated form of AAF) was obtained from the Midwest Research Institute (Kansas City, MO).

Damaged DNA templates

A 49-nt DNA template containing a site-specific *cis–syn* TT dimer or a TT (6-4) photoproduct was prepared as previously described (38). Its sequence was 5′-AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT-3′, where the modified TT is underlined. AAF-adducted DNA template was prepared by incubating 2 nmol of the oligonucleotide 5′-CCTTCTTCATTCGAACATACTTCTTCTTCC-3′ with 200 nmol of AAAF at 37°C in the dark for 3 h in 100 µl of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) containing 20% ethanol, followed by purification as previously described (31).

Overexpression plasmids of the yeast *REV3* and *REV7* genes

The yeast *REV3* gene was obtained by polymerase chain reaction (PCR) amplification from *S.cerevisiae* DNA using *Taq* DNA polymerase and two primers, 5′-CGGGATCCATGTCGAGGGAGTCGAACGAC-3′ and 5′-CGCGTCGACCCAATCATTTAGAGATATTAATGCTTCTTCC-3′. The resulting 4.5 kb PCR product was then cloned into the *Bam*HI and *Sal*I sites of the vector pEGTh6, yielding pEGTh6-REV3. The yeast *REV7* gene was obtained by PCR amplification from *S.cerevisiae* DNA using *Taq* DNA polymerase and two primers, 5′-GCTCTAGAATGAATAGATGGGTAGAGAAGTGGCTG-3′ and 5′-CCGCTCGAGCCATGGCTGCAGAAACAAAGATCCAAAAATGCTCTC-3′. The resulting 740 bp PCR product was then cloned into the *Xba*I and *Pst*I sites of the vector pEGUh6, yielding pEGUh6-REV7. The cloned yeast *REV3* and *REV7* genes in the expression constructs were confirmed by functional complementation of the deficient UV-mutagenesis of yeast *rev3* and *rev7* deletion mutant strains, respectively. Yeast expression vectors pEGTh6 and pEGUh6 were derived from Yeplac112 and Yeplac195, respectively (39), which contained the yeast *TRP1* gene and the yeast *URA3* gene, respectively, for plasmid selection. Additionally, both vectors contained the 2 µm origin for multicopy plasmid replication, the yeast *GAL1/10* promoter for inducible gene expression and six histidine codons for N-terminal protein tagging.

Purification of yeast DNA Polζ

Yeast Polζ is composed of the Rev3 (the catalytic subunit) and Rev7 proteins in a complex (13). Yeast cells harboring both pEGTh6-REV3 and pEGUh6-REV7 were grown to stationary phase in minimal medium containing 2% sucrose. Induction of Polζ expression was achieved by diluting the culture 10-fold in 16 l of YPG (2% bacto-peptone, 1% yeast extract and 2% galactose) medium supplemented with 0.5% sucrose and growth for 15 h at 30°C. Cells were collected by centrifugation and washed in water. After resuspending in an extraction buffer containing 50 mM Tris–HCl, pH 7.5, 1 M KCl, 10% sucrose, 5 mM β-mercaptoethanol, and protease inhibitors (23), cells were disrupted by Zirconium beads in a bead-beater (Biospec Products, Bartlesville, OK) for 15 pulses of 30 s each on ice. The clarified extract (~120 ml) was loaded onto two

connected HiTrap chelating columns (5 ml each) charged with $NiSO_4$ (Amersham Pharmacia Biotech, Piscataway, NJ), followed by washing the columns sequentially with 100 ml Ni buffer A (20 mM phosphate buffer, pH 7.4, 0.5 M NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and protease inhibitors) containing 10 mM imidazole and 100 ml Ni buffer A containing 35 mM imidazole. Bound proteins were eluted with a linear gradient (250 ml) of 35–108 mM imidazole in Ni buffer A. Pooled Polζ fractions were concentrated by dialysis against solid polyethylene glycol 10 000 overnight and desalted through five connected HiTrap Sephadex G-25 columns (5 ml each) (Amersham Pharmacia Biotech) in FPLC buffer A (50 mM Tris–HCl, pH 7.5, 10% glycerol, 5 mM β-mercaptoethanol) containing 80 mM KCl. The resulting sample (~50 ml) was loaded onto an FPLC Mono S HR5/5 column (Amersham Pharmacia Biotech) equilibrated with FPLC buffer A containing 80 mM KCl. Bound proteins were eluted with 30 ml linear gradient of 80–500 mM KCl in FPLC buffer A. Pooled Mono S fractions (~5 ml) were concentrated by polyethylene glycol 10 000 and loaded onto an FPLC Superdex 200 gel filtration column equilibrated with FPLC buffer A containing 300 mM KCl. During Polζ purification, the yeast Rev3 and Rev7 proteins were identified by western blot analyses using a mouse monoclonal antibody against the His_6 tag.

DNA polymerase assays

The standard DNA polymerase reaction (10 µl) contained 25mM KH_2PO_4 pH 7.0, 5 mM $MgCl_2$, 5 mM dithiothreitol, 100 µg/ml bovine serum albumin, 10% glycerol, 50 µM each dATP, dCTP, dTTP and dGTP, 50 fmol of a primed DNA template and purified yeast Polζ. DNA primers were labeled at their 5′ ends by [γ-^{32}P]ATP and T4 polynucleotide kinase. After incubation at 30°C for 30 min, reactions were terminated with 7 µl of a stop solution (20 mM EDTA, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol). Reaction products were separated by electrophoresis on a 20% polyacrylamide gel containing 8 M urea and visualized by autoradiography.

Kinetic measurements of primer extension by yeast Polζ

Kinetic analysis of primer extension by yeast Polζ was performed using a previously described method (30,40,41). Briefly, DNA polymerase assays were performed using 50 fmol of a primed DNA template, 10 ng (50 fmol) of purified yeast Polζ and increasing concentrations of dGTP (0.1–300 µM). Four DNA primers, 5′-GGAAGAAGAAGTATGTTC-3′, 5′-GGAAGAAGAAGTATGTTA-3′, 5′-GGAAGAAGAAGTATGTTT-3′ and 5′-GGAAGAAGAAGTATGTTG-3′, were labeled at their 5′ ends with ^{32}P and separately annealed to the AAF-adducted DNA template with the primer 3′ nucleotide opposite the template AAF-G. The next template base was a C. After incubation for 10 min at 30°C under standard DNA polymerase assay conditions, reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. The percentage of primers extended by the polymerase was calculated following scanning densitometry of the extended DNA band and the remaining primer band on the autoradiogram. Product formed (P) was derived from the calculation P = fraction of primer extension × 50 fmol. Observed enzyme velocity (v) was obtained from the calculation v = P/10 min.

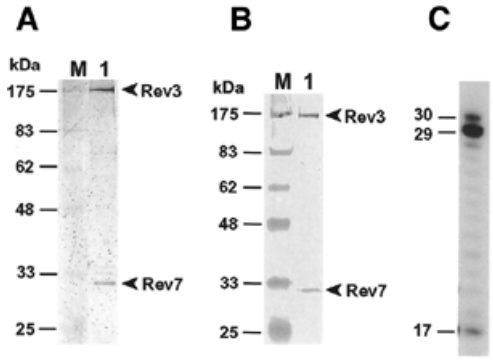

Figure 1. Analyses of purified yeast Polζ. (**A**) Purified yeast Polζ (300 ng) was analyzed by electrophoresis on a 10% SDS–polyacrylamide gel and visualized by silver staining. Protein size markers (lane M) are indicated on the left. (**B**) Purified yeast Polζ (300 ng) was analyzed by a western blot using a mouse monoclonal antibody against the His_6 tag. (**C**) A DNA polymerase assay was performed with purified yeast Polζ (26 ng, 129 fmol) using the 30mer DNA template 5′-CCTTCTTCATTCGAACATACTTCTTCTTCC-3′ annealed with the 5′-^{32}P-labeled 17mer primer 5′-GGAAGAAGAAGTATGTT-3′. DNA size markers in nucleotides are indicated on the left.

Then, the observed enzyme velocity was plotted as a function of dGTP concentration. The plotted data reflected the Michaelis–Menton kinetics of enzyme reaction, and thus were fitted by a non-linear regression curve to the Michaelis–Menton equation, $v = (V_{max} \times [dNTP])/(K_m + [dNTP])$, using the SigmaPlot software. V_{max} and K_m values for the primer extension were obtained from the fitted curves. Relative efficiency of extension (f_{ext}) of matched primer versus mismatched primer from opposite the AAF-G was finally calculated from the equation $f_{ext} = (V_{max}/K_m)_{matched}/(V_{max}/K_m)_{mismatched}$.

RESULTS

Purification of yeast DNA Polζ

To facilitate protein purification, we tagged both Rev3 and Rev7 with six histidine residues at their N-termini and co-overexpressed both proteins in yeast cells. Plasmids coding for the His-tagged Rev3 and Rev7 fully complemented the yeast *rev3* and *rev7* mutants, respectively, for UV-induced mutagenesis (data not shown), indicating that the His_6 tag did not affect Rev3 and Rev7 functions. Polζ was purified as the Rev3–Rev7 protein complex by liquid chromatography through an affinity Ni–agarose column, an FPLC Mono S column and an FPLC gel filtration column. The most pure fraction of the Rev3–Rev7 complex was analyzed by electrophoresis on a 10% SDS–polyacrylamide gel. As shown in Figure 1A, two major bands of ~175 and ~30 kDa, respectively, were detected by silver staining, consistent with the calculated molecular weight of yeast Rev3 (173 kDa) and Rev7 (29 kDa), respectively. Western blotting analysis using a monoclonal antibody against the His_6 tag confirmed that the two bands were Rev3 and Rev7, respectively (Fig. 1B). As expected, a DNA polymerase activity was detected with the purified yeast Polζ (Fig. 1C). We consistently observed that the last template base was inefficiently copied by yeast Polζ (Fig. 1C, 30mer DNA band).

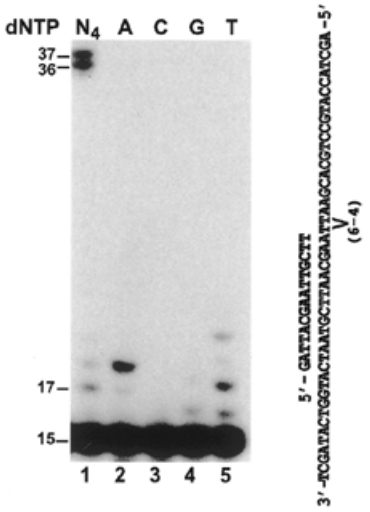

Figure 2. Bypass of a template TT (6-4) photoproduct by yeast Polζ. A 15mer primer was labeled with ^{32}P at its 5′ end and annealed to a DNA template containing a TT (6-4) photoproduct, right before the lesion. DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (lane 1), or a single deoxyribonucleoside triphosphate, dATP (lane 2), dCTP (lane 3), dGTP (lane 4) or dTTP (lane 5). DNA size markers in nucleotides are indicated on the left.

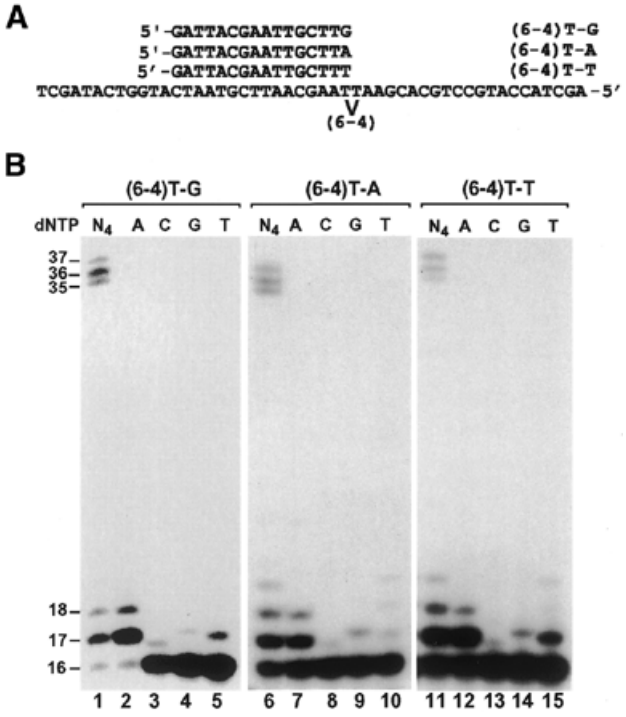

Figure 3. Nucleotide incorporation by yeast Polζ opposite the 5′ T of the TT (6-4) photoproduct. (**A**) Three 16mer primers that differed by 1 nt at the 3′ end were labeled with ^{32}P at their 5′ ends and separately annealed to the indicated DNA template containing a TT (6-4) photoproduct, right before the 5′ T of the lesion. (**B**) DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (N_4), or a single deoxyribonucleoside triphosphate, dATP (A), dCTP (C), dGTP (G) or dTTP (T), as indicated. DNA size markers in nucleotides are indicated on the left.

Lesion bypass of a template TT (6-4) photoproduct by yeast Polζ

Cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts are the major lesions induced by UV radiation (42). To ask whether Polζ can replicate UV-damaged DNA, we first examined translesion synthesis activity of purified yeast Polζ opposite a template TT (6-4) photoproduct. A ^{32}P-labeled 15mer primer was annealed to a 49mer DNA template containing a TT (6-4) photoproduct, right before the 3′ T of the lesion (Fig. 2). DNA synthesis assays were then performed with purified yeast Polζ at 30°C for 30 min. As shown in Figure 2 (lane 1), some TT (6-4) photoproducts were bypassed by yeast Polζ. In a separate experiment, we found that purified human Polκ was completely blocked by the TT (6-4) photoproduct in this same DNA template (31), confirming that the DNA substrates used were not contaminated by unmodified templates. To identify the nucleotide incorporated opposite the 3′ T of the TT (6-4) photoproduct, we performed DNA synthesis assays in the presence of only one deoxyribonucleoside triphosphate, dATP, dCTP, dTTP or dGTP individually. As shown in Figure 2 (lanes 2–5), yeast Polζ incorporated A or T with similar efficiencies opposite the 3′ T of the TT (6-4) photoproduct. Less frequently, G was also incorporated (Fig. 2, lane 4). In the presence of dATP alone, translesion synthesis stopped opposite the undamaged template A 5′ to the TT (6-4) photoproduct (Fig. 2, lane 2). The misincorporation of A opposite the undamaged template A suggests that yeast Polζ is not a highly accurate polymerase, consistent with its ability to perform translesion synthesis.

To identify the nucleotide incorporated opposite the 5′ T of the TT (6-4) photoproduct, three 16mer primers were synthesized, labeled with ^{32}P at their 5′ ends, and separately annealed to the damaged DNA template right before the 5′ T of the lesion (Fig. 3A). DNA synthesis assays were then performed with purified yeast Polζ at 30°C for 30 min. As shown in Figure 3B (lanes 1, 6 and 11), the primers were extended by yeast Polζ regardless of whether the primer 3′ end was an A, G or T opposite the 3′ T of the TT (6-4) photoproduct. Since C incorporation by yeast Polζ was not detected opposite the 3′ T of this lesion (Fig. 2, lane 3), extension DNA synthesis from C-terminated primer was not examined. Extension from G opposite the 3′ T of the TT (6-4) photoproduct was most efficient (Fig. 3B, compare lanes 1, 6 and 11). It is also apparent that nucleotide incorporation by yeast Polζ opposite the 5′ T was more efficient than that opposite the 3′ T of the TT (6-4) photoproduct (compare Fig. 2, lane 1 with Fig. 3B, lanes 1, 6 and 11). Furthermore, purified yeast Polζ predominantly incorporated the correct A opposite the 5′ T of the TT (6-4) photoproduct with all three primers (Fig. 3B, lanes 2, 7 and 12). These results indicate that yeast Polζ is capable of bypassing a template TT (6-4) photoproduct with a low efficiency, and that nucleotide incorporation by this polymerase is more error-prone and less efficient opposite the 3′ T compared to the 5′ T of the lesion.

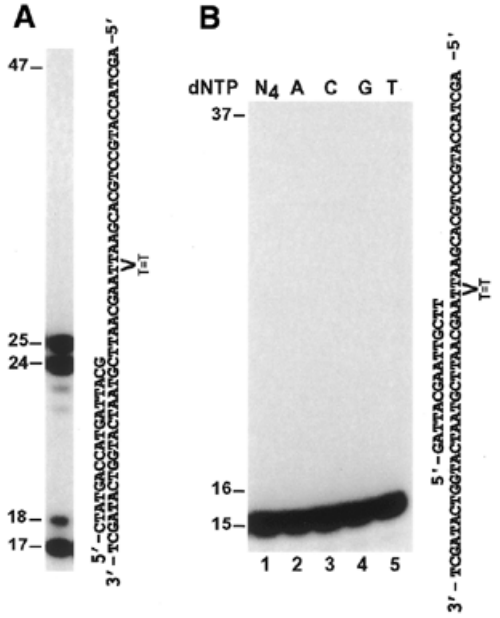

Figure 4. Response of yeast Polζ to a *cis–syn* TT dimer in template DNA. (**A**) A 17mer primer was labeled with ^{32}P at its 5′ end and annealed 8 nt before a template TT dimer as indicated. A DNA polymerase assay was performed with 150 ng (743 fmol) of purified yeast Polζ in the presence of all four dNTPs. (**B**) A 15mer primer was labeled with ^{32}P at its 5′ end and annealed right before the TT dimer as indicated. DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (lane 1), or a single deoxyribonucleoside triphosphate, dATP (lane 2), dCTP (lane 3), dGTP (lane 4) or dTTP (lane 5). DNA size markers in nucleotides are indicated on the left.

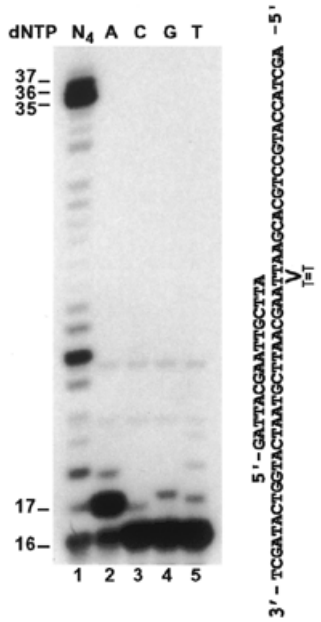

Figure 5. Efficient bypass of the 5′ T of the *cis–syn* TT dimer by yeast Polζ. A 16mer primer was labeled with ^{32}P at its 5′ end and annealed right before the 5′ T of the TT dimer as indicated. DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (lane 1), or a single deoxyribonucleoside triphosphate, dATP (lane 2), dCTP (lane 3), dGTP (lane 4) or dTTP (lane 5). DNA size markers in nucleotides are indicated on the left.

Strong blockage of yeast Polζ by the 3′ T of a template *cis–syn* TT dimer

Following UV radiation, *cis–syn* TT dimers are among the CPDs formed in DNA. To examine translesion synthesis activity of purified yeast Polζ opposite a template *cis–syn* TT dimer, we annealed a ^{32}P-labeled 17mer primer to a 49mer DNA template, 8 nt before the 3′ T of the dimer (Fig. 4A). DNA synthesis assays were then performed with purified yeast Polζ at 30°C for 30 min. As shown in Figure 4A, DNA synthesis by yeast Polζ stopped right before the 3′ T of the TT dimer (25mer DNA band). Additionally, nucleotide incorporation opposite the undamaged template base 3′ to the TT dimer was also inhibited by the lesion, as evidenced by accumulation of the 24mer DNA band (Fig. 4A). DNA synthesis opposite or beyond the TT dimer was not detected even when excessive amounts (150 ng, 743 fmol) of yeast Polζ was used (Fig. 4A). Translesion synthesis was also not detected using a primer annealed right before the lesion (Fig. 4B). In separate experiments, the TT dimer in this same DNA template was efficiently bypassed by purified yeast and human Polη as expected (9,10), confirming the integrity of the template used.

To examine if the 5′ T of the *cis–syn* TT dimer also blocks yeast Polζ, we annealed a ^{32}P-labeled 16mer primer right before the 5′ T of the dimer (Fig. 5). DNA synthesis assays were then performed with purified yeast Polζ. As shown in Figure 5 (lane 1), yeast Polζ efficiently bypassed the 5′ T of the *cis–syn* TT dimer. To identify the nucleotide incorporated opposite the 5′ T of the *cis–syn* TT dimer, we performed DNA synthesis assays in the presence of only one deoxyribonucleoside triphosphate: dATP, dCTP, dTTP or dGTP individually. As shown in Figure 5 (lanes 2–5), yeast Polζ predominantly incorporated the correct A opposite the 5′ T of the *cis–syn* TT dimer. These results show that yeast Polζ is unable to perform translesion synthesis opposite the 3′ T of a template *cis–syn* TT dimer, leading to DNA synthesis stop right before the lesion.

Translesion synthesis opposite AAF-adducted guanine by yeast Polζ

AAF-Gs are bulky lesions in DNA, which block many DNA polymerases (10,43). To examine whether yeast Polζ is able to perform translesion synthesis opposite the bulky AAF-G, we annealed a ^{32}P-labeled 13mer primer 4 nt before the template AAF-G (Fig. 6A). DNA synthesis assays were then performed with purified yeast Polζ. As shown in Figure 6A (lane 2), some AAF-damaged DNA templates were bypassed by yeast Polζ. However, a significant amount of DNA synthesis stopped right before the lesion (17mer DNA band) (Fig. 6A, lane 2). Major DNA synthesis stop at this position was not observed with the undamaged control template (Fig. 6A, lane 1). Thus, nucleotide incorporation opposite the AAF adduct by yeast Polζ was inhibited by the lesion. Translesion synthesis was also observed by using a 17mer primer annealed right before the template AAF-G (Fig. 6B, lane 1). Apparently, following 1 nt incorporation opposite the lesion, the subsequent extension by yeast Polζ was also significantly inhibited by the AAF adduct, as indicated by the accumulation of the 18mer DNA band (Fig. 6B, lane 1). In a separate experiment, this same AAF-damaged template completely blocked yeast Polα (10),

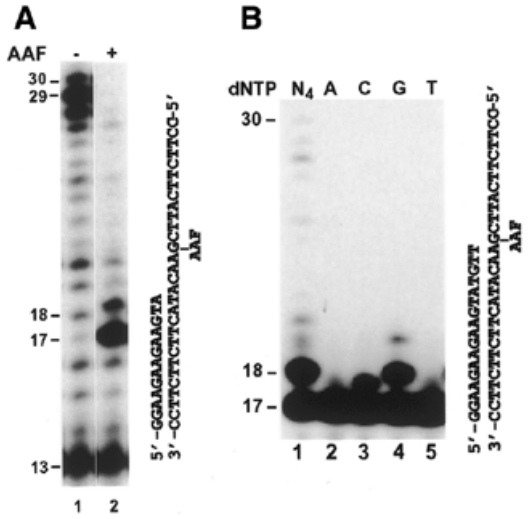

Figure 6. Translesion synthesis by yeast Polζ opposite an AAF-G. (**A**) A 13mer primer was labeled with ^{32}P at its 5′ end and annealed 4 nt before the template G without (lane 1) or with (lane 2) an AAF adduct. DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs. Nucleotide incorporation opposite the template AAF-G would form an 18mer DNA band. (**B**) A 17mer primer was labeled with ^{32}P at its 5′ end and annealed right before the template AAF-G as indicated. DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (lane 1), or a single deoxyribonucleoside triphosphate, dATP (lane 2), dCTP (lane 3), dGTP (lane 4) or dTTP (lane 5). DNA size markers in nucleotides are indicated on the left.

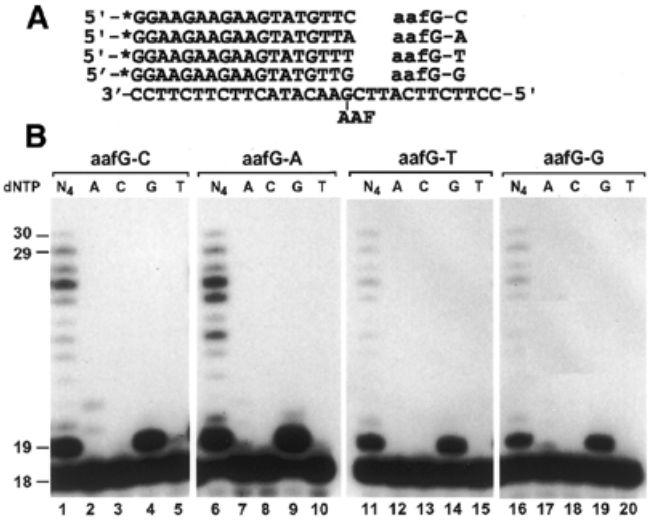

Figure 7. Primer extension from opposite the AAF-G by yeast Polζ. (**A**) Four 18mer primers that differed by 1 nt at the 3′ end were labeled with ^{32}P at their 5′ ends and separately annealed to the indicated DNA template, with the primer 3′ end opposite the template AAF-G. (**B**) DNA polymerase assays were performed with 39 ng (193 fmol) of purified yeast Polζ in the presence of all four dNTPs (N_4), or a single deoxyribonucleoside triphosphate, dATP (A), dCTP (C), dGTP (G) or dTTP (T) as indicated. DNA size markers in nucleotides are indicated on the left.

confirming that the DNA substrates used were not contaminated by unmodified templates.

To identify the nucleotide incorporated opposite the AAF-guanine, we performed DNA synthesis assays in the presence of only one deoxyribonucleoside triphosphate. As shown in Figure 6B (lanes 2–5), yeast Polζ most frequently incorporated a G opposite the AAF-guanine. These results show that yeast Polζ is capable of error-prone nucleotide incorporation opposite an AAF-G, although the lesion is inhibitory to this polymerase at both the nucleotide incorporation and subsequent extension steps.

Accurate extension of DNA synthesis from opposite the AAF-adducted guanine by yeast Polζ

Recently, we observed that yeast Polη and human Polι are able to insert a C opposite the template AAF-G before aborting DNA synthesis (10,32). It is possible that the aborted DNA synthesis may be continued by another DNA polymerase to achieve bypass of AAF adducts in cells. We hypothesize that Polζ could play such a role as the second DNA polymerase, as we have observed for AP site bypass by the combined activities of yeast Polη and Polζ (10). To test this possibility, we asked whether purified yeast Polζ can extend matched and mismatched DNA primers from opposite the AAF-G. Four 18mer primers were labeled with ^{32}P at their 5′ ends and annealed separately to the AAF-damaged DNA template, generating four DNA substrates containing primer 3′ C, A, T and G, respectively, opposite the AAF-G (Fig. 7A). DNA synthesis assays were then performed with purified yeast Polζ. As shown in Figure 7B (lanes 1, 6, 11 and 16), yeast Polζ extended the primers from opposite the AAF-G with different efficiencies. Surprisingly, extension from the mismatched A:G^{AAF} was most efficient, followed by the matched C:G^{AAF} primer (Fig. 7B, lanes 1, 6, 11 and 16). Extensions from mismatched T:G^{AAF} and G:G^{AAF} were least efficient among the four substrates (Fig. 7B, lanes 1, 6, 11 and 16). To identify the nucleotide incorporated opposite the undamaged template C 5′ to the AAF-G, we performed DNA synthesis assays in the presence of only one deoxyribonucleoside triphosphate. As shown in Figure 7B (lanes 4, 9, 14 and 19), yeast Polζ incorporated the correct G during extension from opposite the AAF-G to the next template base C, regardless of which nucleotide had resided opposite the lesion.

To quantitatively compare the relative extension efficiencies among the four different primers, we measured the kinetic parameters of primer extension by yeast Polζ from opposite the AAF-G to the next template C. Based on the V_{max}/K_m values (Table 1), primer extension by purified yeast Polζ followed the order, from most efficient to most inefficient, A:G^{AAF} > C:G^{AAF} > T:G^{AAF} > G:G^{AAF}. This conclusion agreed with that obtained from the experiment shown in Figure 7B. These results show that yeast Polζ is capable of accurate extension DNA synthesis from opposite the AAF-G, especially when the primer 3′ end is an A or a C opposite the lesion.

DISCUSSION

Yeast genetic studies revealed that Polζ is required for UV- and AAF-induced mutagenesis *in vivo* (37,44,45). In this study, we have examined the ability of purified yeast Polζ in translesion synthesis opposite an AAF-G, a TT (6-4) photoproduct and a *cis–syn* TT dimer. Yeast Polζ is able to perform translesion synthesis opposite the first two DNA lesions

Table 1. Kinetic measurements of primer extension from opposite the G^{AAF} to the next template C by yeast Polζ

Primer: template	V_{max} (fmol/min)	K_m (μM)	V_{max}/K_m	f_{ext}[a]
A:G^{AAF}	1.10 ± 0.06	2.3 ± 0.6	0.480	1.3
C:G^{AAF}	0.77 ± 0.07	2.1 ± 1.1	0.370	1.0
T:G^{AAF}	0.71 ± 0.07	6.0 ± 2.6	0.120	3.2×10^{-1}
G:G^{AAF}	0.78 ± 0.02	36.0 ± 4.3	0.022	5.9×10^{-2}

[a]$f_{ext} = (V_{max}/K_m)_{matched\ primer}/(V_{max}/K_m)_{mismatched\ primer}$. The correct nucleotide G was incorporated opposite the template C 5′ to the lesion. Thus, dGTP was used for the kinetic measurements of extension.

in vitro. However, both lesions significantly inhibited the DNA synthesis activity of yeast Polζ (compare Fig. 1C with Figs 2 and 6B, lane 1). Thus, the efficiency of translesion syntheses by yeast Polζ is low opposite both the TT (6-4) photoproduct and the AAF-G.

Recently, several lesion bypass DNA polymerases have been described in eukaryotes. We have tested purified human Polη, Polι and Polκ for *in vitro* bypass of TT (6-4) photoproduct. Remarkably, none of the polymerases alone is able to incorporate two nucleotides opposite the two modified Ts (5,9,31,32). Thus, yeast Polζ is the only eukaryotic DNA polymerase that can perform translesion synthesis opposite both the modified Ts of the TT (6-4) photoproduct and subsequently extend DNA synthesis further downstream, at least under our *in vitro* reaction conditions.

Opposite the 3′ T of the TT (6-4) photoproduct, yeast Polζ incorporates either an A or a T with similar efficiencies, and less frequently a G. Opposite the 5′ T of the lesion, yeast Polζ incorporates the correct A much more frequently than other nucleotides. Therefore, the *in vitro* results predict that the 3′ T is more mutagenic than the 5′ T of the TT (6-4) photoproduct during its bypass by yeast Polζ. When A is incorporated opposite the damaged 3′ T, subsequent extension DNA synthesis by yeast Polζ occurs more efficiently than when T is incorporated opposite the damaged 3′ T. Together, the incorporation and extension results predict that bypass of TT (6-4) photoproduct by yeast Polζ would mainly result in two correct A incorporations opposite the lesion. The most efficient extension DNA synthesis by yeast Polζ occurs when a G is first incorporated opposite the 3′ T of the TT (6-4) photoproduct, which is expected to enhance T→C mutations at the damaged 3′ T. These biochemical properties of Polζ and the resulting predictions are consistent with and can provide explanations for the *in vivo* results of TT (6-4) photoproduct bypass in yeast cells reported by Nelson *et al.* (46). After replicating a plasmid containing a site-specific TT (6-4) photoproduct in yeast cells, the majority of bypassed products (69%) contained two correctly inserted As opposite the damaged Ts, and the mutagenic bypass events occurred mainly as G (25%) and T (4%) misinsersions opposite the 3′ T of the lesion (46). Based on the agreement between the *in vitro* and the *in vivo* results, we conclude that Polζ directly participates in the bypass of TT (6-4) photoproducts in yeast cells in that this polymerase catalyzes both nucleotide incorporations opposite the lesion and subsequent extension DNA synthesis.

It is possible that *in vivo* bypass of TT (6-4) photoproduct may be stimulated by other proteins of the Polζ mutagenesis pathway. Nevertheless, the inefficient *in vitro* bypass by purified yeast Polζ is also consistent with the observation that the efficiency of TT (6-4) photoproduct bypass is only 19% *in vivo* (46). *In vitro*, human Polη and Polι are able to preferentially incorporate a G and an A, respectively, opposite the 3′ T of the TT (6-4) photoproduct before aborting DNA synthesis (9,32,35). Since yeast Polζ is capable of extension DNA synthesis from opposite the 3′ T of the TT (6-4) photoproduct, Polζ may additionally function to extend DNA synthesis following Polη or Polι action. Participation by additional DNA polymerases in translesion synthesis opposite the 3′ T of the TT (6-4) photoproduct would enhance the overall efficiency of replication of this lesion in cells compared to the inefficient bypass by Polζ alone. Whether Polη and Polι indeed contribute to error-prone and error-free bypass, respectively, of TT (6-4) photoproduct in cells remains to be tested by genetic experiments.

Unlike a TT (6-4) photoproduct, a template *cis–syn* TT dimer completely blocked purified yeast Polζ, even when a large excess of the polymerase was used. This is in dramatic contrast to yeast Polη, which efficiently bypasses the TT dimer (4). The 3′ T of the dimer is responsible for blocking yeast Polζ. In contrast, the 5′ T can be effectively bypassed by yeast Polζ from a primer with a 3′ A annealed opposite the 3′ T of the dimer. Our results do not support the conclusion of Nelson *et al.* (13) that yeast Polζ is able to bypass a template *cis–syn* TT dimer. It is possible that the different results between our studies and those of Nelson *et al.* (13) may reflect different yeast Polζ preparations. While only one column chromatography was used for yeast Polζ purification in the studies of Nelson *et al.* (13), our Polζ was purified by three column chromatography steps. Thus, our yeast Polζ preparation may be more pure.

Our *in vitro* results suggest that lesion bypass activities of Polζ and Polη are complementary to each other in response to UV radiation. While Polη can bypass TT dimers but not TT (6-4) photoproducts (4–6,9), Polζ can bypass TT (6-4) photoproducts but not TT dimers. Such complementary functions of Polη and Polζ predict that both polymerases are important for translesion synthesis in response to UV radiation. Genetic studies demonstrate that this is indeed the case. Either Polη or Polζ mutant cells are sensitive to UV radiation (37,44,47,48). Furthermore, *in vivo* studies show that replication of DNA containing *cis–syn* TT dimers is not affected in the absence of Polζ in yeast cells (46). The inability of Polζ to bypass TT dimers provides a molecular basis for this *in vivo* observation.

With a low efficiency, yeast Polζ is able to bypass a template AAF-G in an error-prone manner. Our *in vitro* results agree with *in vivo* results of Baynton *et al.* (45) that lesion bypass of AAF-G in yeast cells requires Polζ and occurs with a low efficiency. However, it was observed that 96% of the bypassed products are error-free (45). Error-free bypass of AAF-G in yeast cells could be significantly contributed by Polη, since this polymerase can incorporate a C opposite the AAF-G *in vitro* (10). If this indeed occurs *in vivo*, Polζ is likely required to continue the aborted DNA synthesis following C incorporation by Polη opposite AAF-G. Supporting this model, purified yeast Polζ is capable of extension DNA synthesis from a C-terminated primer opposite a template AAF-G. Surprisingly,

such extension is most efficient when the primer 3′ end is an A opposite the AAF-G. An implication of this observation is that misincorporated A opposite the AAF-G would be more likely to be fixed into mutations. Consistently, G→T transversion has been reported as a major mutation event at the AAF-G in mammalian systems (49). It should be noted that the human DINB1 protein (Polκ) can effectively incorporate an A opposite the AAF-G (31,33), which may further enhance the AAF-induced G→T transversions in mammalian cells. The yeast *S.cerevisiae*, however, does not contain a DINB1 homolog.

In eukaryotes, it appears that multiple enzymes are involved in translesion DNA synthesis, including Rev1, Polζ, Polη, Polι and Polκ. While some lesions such as AAF-G may be responded to by multiple polymerases, other lesions such as TT dimer may require a specific polymerase for translesion synthesis. It is perhaps this functional specificity that underscores the importance of evolving multiple DNA polymerases for translesion synthesis. Missing one lesion bypass polymerase may trigger severe health problems in humans, such is the case of XPV, a sunlight-sensitive and skin cancer-prone disease, in which Polη activity is lost (6,7). Even more dramatically, mouse embryonic development cannot be completed without Polζ (50–52).

Based on the biochemical properties of yeast Polζ in response to TT (6-4) photoproduct and AAF-guanine *in vitro*, we propose a dual-function model for Polζ in bypassing these two DNA lesions in cells. One function of Polζ is to directly catalyze nucleotide incorporation (translesion synthesis) opposite the lesion and subsequently extend DNA synthesis past the lesion. Since nucleotide incorporation by Polζ is inefficient opposite TT (6-4) photoproduct and AAF-G, their more efficient bypasses may require other DNA polymerases to additionally catalyze nucleotide incorporation opposite the lesion. The second function of Polζ is to catalyze the extension DNA synthesis following nucleotide incorporation opposite the lesion by other polymerases, thus serving as the second polymerase and acting at the second step in the two-polymerase two-step lesion bypass mechanism as originally postulated by Yuan *et al.* (10).

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