Human DNA Polymerase κ Bypasses and Extends beyond Thymine Glycols during Translesion Synthesis in Vitro, Preferentially Incorporating Correct Nucleotides*

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From the §Laboratory of Molecular Pathology, Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9072 and the ¶¶Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics, University of Vermont, Burlington, Vermont 05405-0068

Human polymerase κ (polκ), the product of the human POLK (DINB1) gene, is a member of the Y superfamily of DNA polymerases that support replicative bypass of chemically modified DNA bases (Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) Mol. Cell 8, 7–8; Gerlach, V. L., Aravind, L., Gotway, G., Schultz, R. A., Koonin, E. V., and Friedberg, E. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11922–11927). Polκ is shown here to bypass 5,6-dihydro-5,6-dihydroxythymine (thymine glycol) generated in two different DNA substrate preparations. Polκ inserts the correct base adenine opposite thymine glycol in preference to the other three bases. Additionally, the enzyme correctly extends beyond the site of the thymine glycol lesion when presented with adenine opposite thymine glycol at the primer terminus. However, steady state kinetic analysis of nucleotides incorporated opposite thymine glycol demonstrates different misincorporation rates for guanine with each of the two DNA substrates. The two substrates differ only in the relative proportions of thymine glycol stereoisomers, suggesting that polκ distinguishes among stereoisomers and exhibits reduced discrimination between purines when incorporating a base opposite a 5R thymine glycol stereoisomer. Polκ is insensitive to inhibition by aphidicolin or dideoxynucleotides in both nonmutagenic and mutagenic bypass of oxidative damage.

Many types of base damage in DNA cause structural modifications that can result in the stalling or complete arrest of high fidelity DNA synthesis during DNA replication (3, 4). However, the potential for cell death attendant on arrested DNA replication can be mitigated by a mechanism called translesion DNA synthesis (TLS) (5–7). This process effects the replicative bypass of sites of base damage, allowing high fidelity semiconservative DNA synthesis to continue. Important new insights into the biochemical mechanism of TLS have recently been gained by the discovery of a number of new DNA polymerases, all of which share the properties of limited fidelity and processivity when copying undamaged DNA, as well as a lack of 3′ → 5′ proofreading exonuclease activity (1, 5–9). Multiple DNA polymerases of this class have been shown to support TLS of one or more types of base damage in vitro. In some instances, this role is supported by genetic or other biological evidence. Hence, a general theme is beginning to emerge that the redundancy for error-prone DNA polymerases in prokaryotic and especially in eukaryotic cells reflects a requirement for the bypass of multiple types of base damage that can arrest normal DNA replication (5). Recent structural studies on a number of these polymerases suggest that translesion synthesis is effected by a less constrictive, more solvent-accessible active site, which allows for productive interactions with a wider range of template structures, including chemically modified bases (10–12). The increased error rates observed when copying undamaged DNA in vitro (1, 8, 9, 13) are presumably a direct reflection of this relaxed fidelity for nucleotide incorporation.

Among the many recently discovered specialized DNA polymerases is one called DNA polymerase κ (polκ) from human cells, a highly conserved structural ortholog of a bacterial polymerase called DNA polymerase IV (2). Polκ is encoded by the POLK (DINB1) gene and has a predicted molecular mass of ~100 kDa (2). In previous studies, polκ was fused to glutathione S-transferase and expressed in insect cells (14). The purified fusion protein was shown to be a template-directed DNA polymerase with limited processivity and fidelity (15). GST-polκ protein lacks detectable 3′ → 5′ proofreading exonuclease activity and is not stimulated by recombinant human proliferating cell nuclear antigen (PCNA) in vitro (14). However, in the presence of the three replicative accessory factors, PCNA, replication factor C, and replication factor A, polκ exhibits a 50–200-fold stimulation in efficiency but no increase in processivity (16). Additionally, polκ interacts physically with PCNA (16). Human polκ has optimal activity at 37 °C over the pH range 6.5–7.5 and is insensitive to inhibition by aphidicolin or dideoxynucleotides.

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** The abbreviations used are: polκ, -κ; -λ, -η, and -δ, polymerase κ, λ, η, and δ, respectively; PCNA, proliferating cell nuclear antigen; Tyg, thymine glycol; DPAGE, denaturing polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; TLS, translesion DNA synthesis.

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or by NaCl up to 50 mm. Either Mg\textsuperscript{2+} or Mn\textsuperscript{2+} can satisfy a metal cofactor requirement for polk activity (14). In vitro polk extends DNA oligonucleotide primers to a position one base short of the end of the DNA template (14).

Full-length purified polk fusion protein is unable to extend a DNA primer past bulky base adducts such as thymine-thymine dimers or [6-4]-pyrimidine-pyrimidone photoproducts generated by exposure of cells or DNA to UV radiation (17). Similarly, the enzyme does not support TLS past cisplatin intratand cross-links in template DNA (14). In contrast, the enzyme can support TLS past acetylaminofluorene-guanine, any of the four stereoisomer adducts resulting from reaction of benzo[a]pyrene-7,8-diol 9,10-epoxide at C-10 with the exocyclic N\textsubscript{2} of guanine (BPDE-G) in template DNA, a biologically important form of DNA lesion. The enzyme does not support TLS past cisplatin intrastand dimer or [6-4]-pyrimidine-pyrimidone photoproducts generated by exposure of cells to either doxorubicin or UV radiation (18). Polk additionally supports extension synthesis on primer-template substrates terminating in a 3’- mispaired base, incorporating nucleotides with a high error rate (20).

**POLK** mRNA and polk protein are highly expressed in the adrenal cortex of adult mice, beginning in early embryonic life (2)\textsuperscript{3}. Indeed, at embryonic day 15.5 this is the only tissue in which **POLK** expression can be detected by in vitro hybridization.\textsuperscript{2} Furthermore, this expression pattern appears to be relatively specific, since genes encoding two other recently discovered specialized DNA polymerases, polk and poln are not uniquely or highly expressed in the adrenal cortex.\textsuperscript{2} Steroid biosynthesis in the adrenal cortex is known to involve the generation of large amounts of reactive oxygen species, which may result in an unusual burden of oxidative DNA damage in adrenal cortical cells (23, 24).\textsuperscript{3} Consistent with a possible role in the replicative bypass of oxidative base damage to DNA, the Polk (DinB1) gene is up-regulated in mouse embryo fibroblasts following exposure of cells to either doxorubicin or UV radiation.\textsuperscript{2} Both of these agents are known to generate reactive oxygen species that can damage DNA (25–28). Furthermore, mouse embryo fibroblasts from a mutant mouse strain defective in polk activity manifest increased sensitivity to killing following exposure to UV radiation (29).

In the present studies, we have investigated the ability of human polk to support primer extension in vitro past thymine glycol (Tg) residues in DNA, a biologically important form of oxidative base damage that potently inhibits DNA replication by many high fidelity polymerases (30).\textsuperscript{4} Recently, another Y-family DNA polymerase, human polγ was reported to bypass Tg lesions in vitro (31). Polγ synthesizes DNA past Tg with an efficiency nearly equal to that of undamaged DNA but with an extremely high rate of error (31). Additionally, polγ exhibits a stereochemical preference for the R stereoisomer at C-5 of Tg (31). For the present studies, we employed primer-template substrates in which a single Tg residue in the template DNA strand was generated by two different methods (Fig. 1A). Both procedures result in a mixture of the four possible stereoisomers of Tg in different relative proportions (Fig. 1B)\textsuperscript{19, 32, 33}. We show by both qualitative and quantitative steady-state kinetic analysis that polk supports TLS across both of these substrates. During this replicative bypass, the base A is preferentially incorporated opposite Tg. Additionally, polk is able to extend the primer template beyond the lesion, preferentially incorporating the correct next base. However, differences are observed both in the efficiency of A incorporation and in the misincorporation rate of G opposite Tg when comparing the two templates. TLS appears to be more efficient and specific opposite the substrate putatively containing a larger proportion of 5S stereoisomers.

**EXPERIMENTAL PROCEDURES**

**Biological Reagents**—Terminal deoxynucleotransferase and T4 DNA polymerase were obtained from Invitrogen. The Klenow fragment of *E. coli* DNA polymerase I (exo-) was obtained from New England Biolabs. T4 DNA polymerase was obtained from U.S. Biochemical Corp. Deoxynucleoside triphosphates were from Promega. Osmium tetroxide (OsO\textsubscript{4}) was purchased from Aldrich.

**Expression and Purification of GST-Polk**—GST-polk fusion protein was purified as previously described (14).

**DNA Substrates**—The primer used for running start experiments was P4-0X-RS (5’-dGAATTCCTGACGCCAGGAT)\textsuperscript{31}; the primer for standing start experiments was P5-0X-SS (5’-dGAATTCCTGACGCCAGGATCGACGCCAGGATCGACGGTCG)	extsuperscript{32}. The primer for steady state extension experiments (k\textsubscript{cat}) was P5-0X-SSA (5’-dGAATTCCTGACGCCAGGATCGACGGTCG)	extsuperscript{33}. The template DNA for these experiments was the sequence 5’-dATTCGACAGTCCATAAAACGCGTTGGACGGTGGTGGATCC	extsuperscript{32} (the site of thymine glycol modification is underlined). The control template was of the same sequence, except the underlined base was simply thymine. DNA oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (DPAGE). Five pmol of each primer was 5’-end-labeled with T4 polynucleotide kinase (Invitrogen) in the presence of [γ\textsuperscript{32}P]ATP, and the unincorporated radiolabeled was removed using a Sephadex G-25 spin column equilibrated with STE (100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM Na\textsubscript{2}EDTA). Primers were annealed to template strands in a stoichiometric ratio of 1:1.5 (primer-template) by heating (90 °C, 5 min, 1 × STE buffer) and cooling on the bench top (15 min). Native polyacrylamide gels showed altered gel mobility for the labeled primers under these conditions when compared with single-stranded primers, indicating duplex DNA character for the template-annaeled primers under these conditions.

**Thymine Glycol Template 1 (TgBr\textsubscript{2}) Has a Lower Proportion of 5R Stereoisomers**—5,6-Dihydro-5,6-dihydroxythymidine-5’-triphosphate (thymidine glycol-5’-triphosphate) was synthesized according to a previously published protocol (34) and characterized by HPLC (one peak), \textsuperscript{31}P NMR (three lines), \textsuperscript{1}H NMR, and ESI mass spectrometry (M+H\textsuperscript{+} = 515 atomic mass units). Additionally, the stereochemical composition of this sample was assayed by digestion of the deoxynucleoside triphosphate with alkaline phosphatase and separation of the deoxynucleosides by HPLC according to a previously published method (35). The parent peak was separated into two subpeaks (5R,5S), and the three cis isomers, which elute together (55,6R) and (55,6S) with nearly identical retention times and peak area ratios, as had previously been reported for oxidation of thymidine deoxynucleoside (data not shown). Hence, stereochemical composition is identical to previous studies up to the point of incorporation of the nucleoside triphosphate.

The product nucleotide triphosphate was reacted with the 5’-end of a DNA oligonucleotide enzymatically, 5’-dATTCCAGACTGCTACTAA-CACGG), by incubation with terminal deoxynucleotransferase ([DNA oligonucleotide] = 1 μM, [thymine glycol triphosphate] = 100 μM, [terminal deoxynucleotransferase] = 60 nU, 100 mM sodium cacodylate, 2 mM Mg\textsubscript{2+} (0.2 mM dithiothreitol, pH 7.0, 1 h, 30 °C). The product containing the addition of a single 3’-thymidine glycol nucleotide was purified by HPLC and gel electrophoresis, desalted by SepPak C\textsubscript{18} chromatography, and characterized by ES mass spectrometry, which indicated the addition of a single thymidine glycol nucleotide residue to the unreacted oligonucleotide. Ligation to a 3’-flank oligonucleotide was accomplished by annealing the thymine glycol oligonucleotide to the flanks strand, 5’-phosphoryl-(dGGACACGATCGATCTGGGCGCGGAGTGGTGGCAG-GAATTC), and to a splinting strand, 5’-dAGGACATCGGCTGGCCACCCG-GTGTATTGCACGATCT, and incubating with T4 DNA ligase (manufacturer’s buffer, 16 °C, 12 h). The full-length, ligated thymine glycol substrate (TgBr\textsubscript{2}), was purified by DPAGE, quantitated by UV, and annealed to appropriate radiolabeled primers for primer extension analysis.

**Thymine Glycol Template 2 (TgOsO\textsubscript{4}, Higher Proportion of 5R Stereoisomers)**—A DNA oligonucleotide of the sequence 5’-dAACACGCCGTGACGGGCCGACG-GACC) (2.4 mg) was incubated in aqueous OsO\textsubscript{4} (3 stoichiometric references


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Piperidine cleavage analysis of a 5’ DNA oligonucleotide. The purified sample was further characterized by ESI mass spectrometry, which indicated the addition of 34 atomic mass units (M + H+) to the unreacted DNA oligonucleotide. The purified sample was further characterized by piperidine cleavage analysis of a 5’-32P-end-labeled sample (~1 pmol, 100 µl, 30 min, 90 °C), which revealed 100% truncation of the DNA oligonucleotide at the thymine position, confirming that the site of modification was exclusively thymine and that 100% of thymines were oxidized. Additionally, the thymine glycol-modified oligonucleotide had distinct gel mobility from the unmodified strand, which aided in both purification and characterization. Background piperidine cleavage at positions corresponding to deoxycytidines was also observed in incompletely purified samples.

The thymine glycol oligonucleotide was phosphorylated with T4 polynucleotide kinase and ATP, purified using a Sephadex G-25 spin column, and ethanol-precipitated. The resulting phosphorylated, thymine glycol oligonucleotide was ligated to flanker sequences analogously to the preparation of the TgBr2 substrate by heating in the presence of 5’-dAATCCAGACTGTCAAT and 5’-phosphoryl-dTCGATCCTGGGCTGAGGAAATCC and a splintering DNA with the sequence 5’-dAGGATCTGGTCCACCCGTTATGACGTC. The full-length, DPAGE-purified, ligated thymine glycol substrate (TgOsO4), which has the same sequence as the TgBr2 substrate (described above), was quantitated by UV and annealed to appropriate primers for primer extension analysis.

DNA Polymerase Assays—Running start and standing start assays shown in Figs. 2–4 were performed as previously described except that gels were phosphorimaged and quantitated using Amersham Biosciences software (14). Steady-state kinetics experiments (Fig. 5) were performed according to previously published methods (35, 36). Radiolabeled primer-templates (20 nM) were incubated (10 min) with GST-pol (5 mM) and the indicated concentration of a single deoxynucleotide 5’-triphosphate. The resulting primers were resolved by DPAGE, and bands corresponding to unextended primer were quantitated by phosphorimaging. Numerical data corresponding to these gels are shown in Table I. Data were fit to the Michaelis-Menten equation as described by a hyperbolic curve using SigmaPlot 2001. Apparent Km and Vmax values were calculated from the plots, and kcat values were subsequently calculated from Vmax. In all cases, data points resulting in greater than 25% primer turnover were not used in plots calculating the Vmax and Kmax. Steady state experiments were performed in triplicate, and results were averaged to obtain the values reported in Table I along with the corresponding standard deviations.

RESULTS
Preparation of Two Substrates Containing Different Relative Amounts of Thymine Glycol Stereoisomers—We generated a single Tg lesion at a defined position in a DNA oligonucleotide template using two different procedures. One substrate (TgBr2) was generated by incorporation of Tg from thymidine glycol-5’-triphosphate prepared by bromination and oxidation as previously described (33). This procedure has been shown to yield a stereochemical mixture containing 64.3% of the two 5R stereoisomers, and 35.7% of the 5S stereoisomers (33). Following oxidation, the stereochemistry at C-5 is fixed, but epimerization occurs about the C-6 center to yield 87% of the 5S stereoisomer and 13% of the 5R stereoisomer (33). Epimerization equilibrates at room temperature within a few hours (33), yielding an equilibrium distribution of ~55.9% (5R, 6S), 8.4% (5R, 6R), 28.6% (5S, 6S), and 7.1% (5S, 6R) (Fig. 1B). When the thymidine glycol triphosphate used to prepare this substrate was digested to the nucleoside with alkaline phosphatase and analyzed by HPLC under conditions previously described (33), the same ratios of the two trans and cis isomers were obtained as reported in the earlier study (data not shown). 4 A second substrate of identical nucleotide sequence, 5’-AGGATCTGGTCCACCGTGTTATTGACAGTC-3’, was extracted with chloroform and ethanol-precipitated before purifying by HPLC and DPAGE. The purified thymine glycol DNA oligonucleotide was characterized by ESI mass spectrometry, which indicated the addition of 34 atomic mass units (M + H+) to the unreacted DNA oligonucleotide. The purified sample was further characterized by piperidine cleavage analysis of a 5’-32P-end-labeled sample (~1 pmol, 100 µl, 30 min, 90 °C), which revealed 100% truncation of the DNA oligonucleotide at the thymine position, confirming that the site of modification was exclusively thymine and that 100% of thymines were oxidized. Additionally, the thymine glycol-modified oligonucleotide had distinct gel mobility from the unmodified strand, which aided in both purification and characterization. Background piperidine cleavage at positions corresponding to deoxycytidines was also observed in incompletely purified samples.

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4 It should be noted that, following preparation of the thymidine glycol triphosphate, the glycol nucleotide was incorporated enzymatically to create the site-specifically modified substrate, a process that could in principle proceed with stereochemical selection. Hence, although there is no reason to believe otherwise, we cannot be certain that the stereochemical composition of the final substrate is identical to that in the triphosphate.
quencing (TgOsO4) was generated by direct oxidation of the single T residue in the template DNA using osmium tetroxide (OsO4). With this procedure, the 5R and 5S stereoisomers are formed in a ratio of 6:1 (32), and following epimerization the relative percentages of the four stereoisomers are expected to be 74.7% (5R,6S), 11.3% (5R,6R), 11.1% (5S,6R), and 2.9 (5S,6R) (Fig. 1B). Thus, in the TgOsO4 template, the 5R stereoisomers are expected to be in slightly greater relative abundance than in the TgBr2 template (Fig. 1B).

**TLS across Thymine Glycol by Pol θ**—We previously reported that the GST-pol fusion protein used in the present studies does not support TLS across cisplatin adducts in DNA (14). In contrast, incubation of a primer-TgBr2 template with purified GST-pol fusion protein revealed progressively more efficient TLS past Tg as a function of increasing enzyme concentration (Fig. 2). The high fidelity replicative enzyme DNA polymerase δ from calf thymus did not support bypass across the Tg lesion at the enzyme concentrations tested, although it exhibited primer extension of the undamaged control template DNA that was characteristically stimulated by the accessory factor PCNA (Fig. 2, lanes 10–14). Like all of the replicative polymerases, pol δ exhibits a 3′→5′ exonuclease activity, which yielded faint primer degradation bands in lanes 10–14 below the portion of the gel shown in Fig. 2 (data not shown). Consistent with the results of previous studies (37), comparable levels of the Klenow exo− form (defold of 3′→5′ exonuclease activity) of E. coli DNA polymerase I also bypassed Tg in this substrate (Fig. 2). Essentially identical results were observed with the primer-TgOsO4 template (Fig. 3A). Once again, bypass was observed in the presence of *Escherichia coli* Klenow exo− fragment (Fig. 3B). However, comparable amounts of the high fidelity replicative DNA polymerases from phage T4 (Fig. 3B) and phage T7 (data not shown) characteristically did not support bypass of Tg. Additionally, when we performed side-by-side standing start experiments in which the two templates were compared directly, we observed essentially identical levels of bypass by GST-pol (Fig. 3C). In all reactions with or without the presence of Tg primer, extension terminated one nucleotide short of the end, a previously described intrinsic property of pol θ (14).

**Fidelity of Nucleotide Incorporation Opposite Tg by GST-pol**—To determine the fidelity of TLS across Tg by the GST-pol fusion protein, we performed standing start primer extension reactions in the presence of each of the four individual deoxynucleoside triphosphates. As shown in Fig. 4, the correct complementary base A is qualitatively preferentially incorpor-
rated opposite Tg. However, the incorrect bases C, T, and especially G are also misincorporated. To compare the efficiency and fidelity of nucleotide incorporation in undamaged template DNA and that of DNA containing a single Tg residue at the identical position but prepared using two different protocols, we performed experiments under steady state conditions (Fig. 5 and Table I).

A comparison of the parameter $k_{\text{cat}}/K_m$ for the incorporation of A opposite T and Tg shows a 20-fold reduced efficiency for the TgBr2 template and a 50-fold reduced efficiency for the TgOsO4 template relative to T (Table I and Fig. 6A). When copying the undamaged template, the GST-polκ fusion protein misincorporates nucleotides opposite the T residue with frequencies of $2.7 \times 10^{-3}$ (G), $9.6 \times 10^{-4}$ (C), and $2.7 \times 10^{-4}$ (T) relative to the correct nucleotide A (Table I). The same comparison for nucleotide incorporation opposite Tg in either substrate (i.e., the frequency of misincorporation of G, C, and T relative to the correct incorporation of A) reveals ~1.2–3-fold reduced discrimination between the correct and incorrect nucleotide in all cases except G misincorporation opposite the TgOsO4 template lesion (Table I and Fig. 6B). Nonetheless, the preference for A opposite Tg is 2–3 orders of magnitude greater than for any other nucleotide. After A, the base most frequently incorporated opposite either T and Tg is G.

Whereas the relative preference for incorporating G compared with the other bases opposite T in the undamaged template or Tg in TgBr2 template is similar (Table I and Fig. 6B), this preference is increased ~5–20-fold in the TgOsO4 template (Table I and Fig. 6B). This may relate to the fact that the TgOsO4 template is expected to contain a greater proportion of the 5R stereoisomers. This stereochemically based difference presumably interferes with discrimination of the purine versus pyrimidine character of the incoming nucleotide. A myriad of models are tenable to explain this phenomenon, especially since the site of hydrogen bonding is on the opposite end of the template base. It is important to note that the relative percentage of each of the stereoisomers is not anticipated to be greatly different between the two templates tested. The 5R stereoisomers are expected to be only ~25% more abundant in the TgOsO4 template. Hence, one might predict even greater stereochemical effects on TLS by polκ in a stereochemically pure 5R or 5S sample. Accordingly, in vivo the 5S stereoisomers may be the preferred substrate for this polymerase.

Having established that GST-polκ inserts a base opposite Tg in a largely correct fashion, we examined the fidelity of extension beyond the lesion. Recent reports have documented the ability of polκ to extend mispaired primer-termini promiscuously (20). Additionally, extension of the Tg-A base pair is believed to be the arresting substrate for most of the polymerases arrested by Tg, since a number of them insert A correctly opposite Tg but are unable to incorporate the next base (38–43). We determined steady state values for the extension of a primer terminating with correctly base-paired deoxyadenosine (P5-ox-ss-A) opposite the Tg lesion (in TgOsO4), using each of the four deoxyribonucleoside triphosphates. The next correct base, C, was incorporated in clear preference to the other bases (Table I).

We also measured $k_{\text{cat}}$ for C incorporation using the TgBr2 template. This returned $k_{\text{cat}}$ and $K_m$ values very similar to those observed with TgOsO4, suggesting little or no stereochem-
Table I

<table>
<thead>
<tr>
<th>DNA substrate</th>
<th>dNTP added</th>
<th>(k_{\text{cat}}) (\text{min}^{-1})</th>
<th>(K_m) (\mu\text{M})</th>
<th>(k_{\text{cat}}/K_m) (\mu\text{M}^{-1} \text{min}^{-1})</th>
<th>(f_{\text{inc}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertion opposite T</td>
<td>dATP</td>
<td>0.68 ± 0.04</td>
<td>0.015 ± 0.004</td>
<td>4.5 \times 10^1</td>
<td>1</td>
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<tr>
<td>5'-TCC</td>
<td>dGTP</td>
<td>0.84 ± 0.02</td>
<td>7.0 ± 0.2</td>
<td>1.2 \times 10^{-1}</td>
<td>2.7 \times 10^{-3}</td>
</tr>
<tr>
<td>-AGGTTG-</td>
<td>dCTP</td>
<td>0.73 ± 0.09</td>
<td>16.7 ± 1</td>
<td>4.4 \times 10^{-2}</td>
<td>9.6 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>dTTP</td>
<td>0.53 ± 0.3</td>
<td>43 ± 9</td>
<td>1.2 \times 10^{-2}</td>
<td>2.7 \times 10^{-4}</td>
</tr>
<tr>
<td>Insertion opposite Tg</td>
<td>(T_{\text{Br2}})</td>
<td>dATP</td>
<td>0.36 ± 0.1</td>
<td>0.16 ± 0.01</td>
<td>2.3 \times 10^{-1}</td>
</tr>
<tr>
<td>5'-TCC</td>
<td>(T_{\text{OsO4}})</td>
<td>dATP</td>
<td>0.67 ± 0.01</td>
<td>0.79 ± 0.2</td>
<td>8.5 \times 10^{-1}</td>
</tr>
<tr>
<td>-AGGTTgG-</td>
<td>(T_{\text{OsO4}})</td>
<td>dGTP</td>
<td>1.0 ± 0.2</td>
<td>130 ± 30</td>
<td>7.8 \times 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>dCTP</td>
<td>0.35 ± 0.3</td>
<td>111 ± 90</td>
<td>3.2 \times 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>dTTP</td>
<td>0.56 ± 0.4</td>
<td>675 ± 625</td>
<td>8.3 \times 10^{-4}</td>
</tr>
<tr>
<td>Extension from A opposite T</td>
<td>dATP</td>
<td>1.0 ± 0.1</td>
<td>6800 ± 700</td>
<td>1.5 \times 10^{-4}</td>
<td>2.4 \times 10^{-5}</td>
</tr>
<tr>
<td>5'-TCCA</td>
<td>dGTP</td>
<td>0.32 ± 0.03</td>
<td>1200 ± 100</td>
<td>2.7 \times 10^{-4}</td>
<td>4.3 \times 10^{-5}</td>
</tr>
<tr>
<td>-AGGTTG-</td>
<td>dCTP</td>
<td>0.74 ± 0.1</td>
<td>12 ± 0.03</td>
<td>6.2 \times 10^{-4}</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dTTP</td>
<td>0.72 ± 0.1</td>
<td>5900 ± 600</td>
<td>1.2 \times 10^{-4}</td>
<td>2.0 \times 10^{-5}</td>
</tr>
<tr>
<td>Extension from A opposite (T_{\text{OsO4}})</td>
<td>dATP</td>
<td>0.16 ± 0.02</td>
<td>280 ± 30</td>
<td>5.7 \times 10^{-4}</td>
<td>2.2 \times 10^{-2}</td>
</tr>
<tr>
<td>5'-TCCA</td>
<td>dGTP</td>
<td>0.24 ± 0.03</td>
<td>330 ± 30</td>
<td>7.3 \times 10^{-4}</td>
<td>2.8 \times 10^{-2}</td>
</tr>
<tr>
<td>-AGGTTG-</td>
<td>dCTP</td>
<td>0.16 ± 0.02</td>
<td>6.2 ± 1</td>
<td>2.6 \times 10^{-2}</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dTTP</td>
<td>0.60 ± 0.07</td>
<td>1100 ± 100</td>
<td>5.5 \times 10^{-4}</td>
<td>2.1 \times 10^{-2}</td>
</tr>
</tbody>
</table>

* \(f_{\text{inc}}\) is defined as \((k_{\text{cat}}/K_m)_{\text{inc}}/(k_{\text{cat}}/K_m)_{\text{correct}}\).

\(\text{inc} \) Obtained by comparison of indicated dCTP or TTP \(k_{\text{cat}}/K_m\) values with the dATP \(k_{\text{cat}}/K_m\) value obtained by averaging results from both \(T_{\text{Br2}}\) and \(T_{\text{OsO4}}\) experiments.

**Fig. 6.** Graphical representation of select kinetic parameters from Table I. A, \(k_{\text{cat}}/K_m\) values for nucleotide insertion opposite thymine glycol in the \(T_{\text{OsO4}}\) template (black bars), the \(T_{\text{Br2}}\) template (blue bars), and the undamaged template (yellow bars). The left graph shows directly plotted \(k_{\text{cat}}/K_m\) values obtained for incorporation of the bases A, C, G, or T, emphasizing the difference in incorporation efficiency of A between the control and Tg templates. The right graph is a y axis blown up version of the same graph to emphasize that A is incorporated in great preference to the other bases in all three of the templates and that GST-poly may exhibit stereochemical preference during insertion of A opposite of thymine glycols. B, comparison of the \(f_{\text{inc}}\) values obtained for each template, measuring the degree of preference for incorporation of the correct base. \(f_{\text{inc}}\) is defined as \((k_{\text{cat}}/K_m)_{\text{inc}}/(k_{\text{cat}}/K_m)_{\text{correct}}\). The left graph is a direct plot of the \(f_{\text{inc}}\) values for nucleotide insertion opposite thymine glycol in the \(T_{\text{OsO4}}\) template (black bars), the \(T_{\text{Br2}}\) template (blue bars), and the undamaged template (yellow bars), emphasizing the high degree of preference for A incorporation in each template. The graph on the right is a y axis blown up version of the same graph to emphasize that the misincorporation frequency of G increases in template \(T_{\text{OsO4}}\), suggesting that thymine glycol stereochemistry influences the ability of GST-poly to discriminate between the purines during incorporation opposite the lesion.
ical influence in the ability of polκ to extend beyond the Tg lesion (Table I). Measurement of $k_{\text{cat}}$ revealed that the other three bases, A, G, and T were misincorporated with about the same relative efficiency, approximating the high level of G misincorporation opposite Tg in the TgOsO$_4$ template. However, the levels of base misincorporation observed were considerably lower than those reported for extension performed with undamaged but mispaired substrates in previous studies (20). In summary, GST-polκ clearly prefers the correct base both opposite and at least 1–2 bases beyond the Tg residue in the DNA sequence context tested here.

**DISCUSSION**

Tg has been associated with potent blocking of DNA replication by high fidelity polymerases, in particular full-length *E. coli* DNA polymerase I or the Klenow exo$^+$ fragment (38–43). Our results (and those reported elsewhere (37)) indicate that at DNA/enzyme stoichiometric equivalents approximating those generally employed for *in vitro* TLS by Y family polymerases, the Klenow exo$^+$ fragment of *E. coli* polymerase I does indeed support limited bypass of Tg. This presumably derives from the absence of the 3$'$ → 5$'$ proofreading exonuclease activity and from our use of a highly purified Tg template DNA devoid of background oxidation at neighboring deoxyxycytidine residues. Previous studies utilizing Tg templates were prepared with a vast stoichiometric excess of OsO$_4$ without removal of secondarily oxidized DNA (see “Experimental Procedures”). We have observed reduced Tg bypass by both Klenow exo$^+$ and GST-polκ using DNA prepared in this manner (data not shown). Hence, we suggest that the replicative arrest observed in previous studies reflects, at least in part, the presence of multiple oxidative lesions rather than a single Tg residue. More relevant to the present studies, Tg is unambiguously an arresting lesion in experiments using various eukaryotic DNA polymerases (18, 31).

As previously mentioned, polκ is highly expressed in cells in the mouse adrenal cortex. The adrenal cortex is a site of active steroidogenesis, and the covalent adduction of estrogen metabolites with DNA has been demonstrated experimentally (44). Additionally, steroidogenesis is associated with the potential for generating reactive oxygen species (45–47). This tissue-specific expression pattern of polκ suggests a role in the response to DNA damage from reactive oxygen species and/or aromatic hydrocarbons. Support for the former role derives from the observation that the mouse *Dinb1* gene is up-regulated after exposure of cells to agents known to promote oxidative DNA damage and from the present studies demonstrating that GST-polκ can support bypass of a well-characterized form of oxidative DNA damage, Tg, incorporating A opposite Tg almost as efficiently as it does opposite T in undamaged DNA but with a level of overall misincorporation of ~7% (31). The authors of this study did not report misincorporation rates for extension, but even in the unlikely case that those were modest, the overall yield would decrease with each extension event, yielding an overall rate much lower than that exhibited by GST-polκ.

Interestingly, like GST-polκ, polη mis-incorporates G with the highest frequency relative to the other bases (31). Polη synthesizes DNA more rapidly than polκ, with $k_{\text{cat}}/K_m$ values approximately an order magnitude higher, but is more error-prone when synthesizing past Tg. Most provocatively, however, polη apparently prefers the 5R Tg stereoisomers over the 5S forms. Steady state kinetics results were not reported for 5S; however, running start synthesis clearly demonstrated less robust bypass. In the present study, we observed the opposite behavior for GST-polκ, which exhibits small, but reproducible differences in the efficiency of incorporation of A opposite Tg in templates differing only in stereochemical composition. The template that exhibits more efficient turnover and greater accuracy of incorporation by GST-polκ may contain a greater relative abundance of the 5S stereoisomer. Whereas polη is approximately an order of magnitude more efficient than polκ in vitro, the role of accessory proteins may influence the $k_{\text{cat}}/K_m$ values reported here and elsewhere. Additionally, up-regulation of the *POLK* gene in tissues with DNA oxidative damage-prone environments may promote higher levels of polκ.

Our observation that the misincorporation rate for G by GST-polκ is increased in the template with a putatively greater proportion of 5R stereoisomers may reflect important features of the polκ active site. The conformation of the Tg base is calculated to be the “half-chair” in which the most abundant 5R stereoisomer, (5R,6S) has been calculated to reside largely in a conformation that places the methyl group of C-5 pseudoaxial and the hydroxyl group of C-6 pseudoaxial (49). It has been proposed that such a structure could generate a G-T wobble base pair (49). Perhaps the Tg base preference for the half-chair “down” conformation in 5R,6S adjusts the plane of the opposite edge of the base (the hydrogen bonding surface), allowing for a G-T wobble base pair, whereas in the opposite stereoisomer (5S,6R) a half-chair “up” conformer would predominate, which may leave the hydrogen bonding edge of the 5S,6R.
base more closely aligned with that of a normal thymine template base (Fig. 7). Interestingly, human Rev1 polymerase, another Y family member, has been shown to be a dCMP nucleotidyl transferase that preferentially incorporates C opposite a number of DNA lesions but incorporates T next most frequently (50). Thus, while polδ and polκ apparently prefer incorporating purines opposite template lesions, Rev1 inserts pyrimidines preferentially. This contrast suggests that some Y family polymerases may be more specialized for purine-based lesions and others for pyrimidine-based lesions.

Importantly, GST-polκ is able to extend beyond sites of Tg damage. The enzyme exhibits an increase in overall misincorporation beyond the Tg lesion, echoing previous reports on damage. The enzyme exhibits an increase in overall misincorporation opposite a lesion (18, 29). These observations have prompted formation. We also thank Dr. Ulrich Hubscher for the gift of calf thymus GST-polκ, which preliminary experiments were performed with the generous gift of a thymine glycol-containing oligonucleotide in a point of nucleotide incorporation opposite a lesion (38—43).

Polκ has also been shown to efficiently extend mispaired bases, BPDE-G adducts, acetylaminofluorene-guanine adducts, and AP sites (18, 29). These observations have prompted the notion that polκ has a primary role in extension beyond sites of base damage. However, GST-polκ manifests a significant preference for the correct base, incorporating C greater than 97% of the time immediately beyond the site of the Tg lesion. This observation is significant, because Tg lesions are typically arresting to polymerases following the point of nucleotide incorporation opposite a lesion (38—43).

Acknowledgments—We gratefully acknowledge Dr. Tom Kunkel for the generous gift of a thymine glycol-containing oligonucleotide in a different sequence with which preliminary experiments were performed. We also thank Dr. Ulrich Hubscher for the gift of calf thymus DNA polymerase δ.