Translesion Synthesis by Human DNA Polymerase η across Thymine Glycol Lesions†

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ABSTRACT: The XP-V (xeroderma pigmentosum variant) gene product, human DNA polymerase η (pol η), catalyzes efficient and accurate translesion synthesis (TLS) past *cis*−*syn* thymine−thymine dimers (TT dimer). In addition, recent reports suggest that pol η is involved in TLS past various other types of lesion, including an oxidative DNA damage, 8-hydroxyguanine. Here, we compare the abilities of pol α and pol η to replicate across thymine glycol (Tg) using purified synthetic oligomers containing a 5*R*- or 5*S*-Tg. DNA synthesis by pol α was inhibited at both steps of insertion of a nucleotide opposite the lesion and extension from the resulting product, indicating that pol α can weakly contribute to TLS past Tg lesions. In contrast, pol η catalyzed insertion opposite the lesion as efficient as that opposite undamaged T, while extension was inhibited especially on the 5*S*-Tg template. Thus, pol η catalyzed relatively efficient TLS past 5*R*-Tg than 5*S*-Tg. To compare the TLS abilities of pol η for these lesions, we determined the kinetic parameters of pol η for catalyzing TLS past a TT dimer, an *N*-2-acetylaminofluorene-modified guanine, and an abasic site analogue. The possible mechanisms of pol η-catalyzed TLS are discussed on the basis of these results.

During replication, a variety of unrepaired lesions in template DNA can block replicative DNA polymerases. Cells contain specialized polymerases to overcome such replication blockage. Recently, a new family of DNA polymerases that catalyze translesion synthesis (TLS)[1] past DNA lesions has been discovered (*1*−*6*). This group has been described in the literature as the UmuC/DinB/Rev1/RAD30 family based on the discovery of genes that were originally shown to encode enzymes with DNA-dependent DNA polymerase activity. Now this group is referred to as the Y-family of DNA polymerases (*7*). Genetic studies have revealed that there are two subpathways of mutagenic and relatively accurate TLS. UmuC with UmuD′ (UmuD′$_2$C; *Escherichia coli* pol V) (*8*−*10*), DinB (*E. coli* pol IV, eukaryotic pol κ) (*10*−*18*), eukaryotic Rev1 (*19*−*21*), and Rev3 with Rev7 (pol ζ) (*22*−*25*) are required for the mutagenic pathway. In contrast to these, the product of the *RAD30* gene, pol η, is unique among eukaryotic DNA polymerases in its ability to catalyze efficient and accurate translesion synthesis. Eukaryotic pol ι, another *RAD30* gene family protein, is also likely to be involved in the mutagenic pathway (*26*−*28*).

Loss of human pol η leads to the XP-V syndrome (*29*−*31*). XP-V patients exhibit sunlight sensitivity and a concomitant increase in the rate of cancer, and cultured cells from these patients exhibit an altered mutation spectrum after UV irradiation (*32*−*38*). These results indicate that the *XPV* gene product, human pol η, is at least involved in the accurate translesion pathway for UV-induced lesions in vivo. Consistent with these observations, biochemical studies revealed that pol η catalyzes relatively accurate TLS past the TT dimer (*30*, *31*, *39*, *40*). In addition to the TT dimer, pol η was reported to be able to bypass various other lesions, including AAF-G, cisplatin-GG, AP site analogues, O^6-methylguanines, 1,N^6-ethenodeoxyadenosines, N^2-guanine adducts of 1,3-butadiene metabolites, and (+)-*trans*−*anti*-benzo[*a*]pyrene-N^2-guanines (*40*−*46*). Importantly, Haracska et al. (*47*) demonstrated that pol η is involved in TLS past 8-hydroxyguanine in yeast cells with the *OGG1* minus background. 8-Hydroxyguanine is one of the major DNA lesions produced by oxidative stress and is efficiently repaired by the base excision repair pathway that comprises Ogg1. The lesion partially blocks DNA replication and often induces mutations (*48*, *49*). These findings strongly suggest that pol η contributes to the cell's damage tolerance by catalyzing TLS past a wide range of lesions, including oxidative DNA lesions.

Tg is also a major DNA lesion induced by reactive oxygen species (*50*−*52*). Tg lesions are mainly repaired by the base

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[1] Abbreviations: TLS, translesion synthesis; XP-V, xeroderma pigmentosum variant; TT dimer, *cis*−*syn* thymine−thymine dimer; AAF-G, *N*-2-acetylaminofluorene-modified guanine; cisplatin-GG, cisplatin-induced intrastrand cross-link between two guanines; AP, abasic; Tg, 5,6-dihydro-5,6-dihydroxythymine.

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excision repair pathway which comprises Nth1 in human cells (*53*). The lesions can also be repaired by components of the nucleotide excision repair pathway, at least in vitro (*54*). Although mutation frequency is low (*55*, *56*), Tg is known to block DNA replication by T4 DNA polymerase, *E. coli* Klenow fragment, or eukaryotic DNA polymerase α (*57*−*63*), suggesting that a specialized DNA polymerase is required for efficient DNA replication across the lesion. In fact, SOS-induced TLS past Tg is reported to be involved in the damage tolerance in *E. coli* (*64*), although the responsible DNA polymerase has not been identified yet. In eukaryotic cells, little is known about TLS across Tg lesions. Here, we decided to examine the TLS ability of human pol η, a prominent member of the recently discovered Y-family polymerases, across Tg lesions. Recently, the chemical synthesis of oligonucleotides containing 5*R*- or 5*S*-Tg has been successfully achieved (*65*, *66*). Using these oligomers, we examined the abilities of pol η to catalyze TLS past thymine glycols. In addition, we also determined the kinetic parameters for TLS past different types of lesions, including TT dimers, AAF-G, and AP site analogues to understand the molecular mechanism of pol η-catalyzed TLS.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human pol η tagged with His_6 at its C-terminal side was expressed in Sf9 insect cells using the baculovirus expression system and purified by sequential column chromatography on HiTrap Q, Ni-NTA agarose, and MonoS as described previously (*40*).

The 30-mer oligomers containing *5R*- and *5S*-thymine glycols, the TT dimer, and the AP site analogue were chemically synthesized as described (*65*−*68*). The AAF-modified template was prepared by treating the 30-mer oligonucleotide with *N*-acetoxy-AAF as described (*40*). Deoxyribonucleotide triphosphates (ultrapure grade) were purchased from Amersham Pharmacia.

Translesion Synthesis Assays. Standard reactions of 10 μL contained 40 mM Tris-HCl (pH 8.0), 10 mM DTT, 250 μg/mL BSA, 60 mM KCl, 2.5% glycerol, 5 mM $MgCl_2$, 40 nM 5′-^{32}P-labeled primer-template DNA, 100 μM each of the four dNTPs, and pol α or pol η. Reactions were performed at 37 °C for 15 min and terminated by addition of 10 μL of formamide followed by boiling. Products were electrophoresed on a 20% polyacrylamide/7 M urea gel and autoradiographed.

Steady-State Kinetics. Steady-state kinetic parameters K_m and k_{cat} were measured using 30-mer templates hybridized to 16-, 17-, or 18-mer primers labeled at their 5′-ends at a molar ratio of 1:1. The sequences of the oligomers are shown in Figure 1B−E. Reactions (10 μL) contained 40 mM Tris-HCl (pH 8.0), 10 mM DTT, 250 μg/mL BSA, 100 mM KCl, 2.5% glycerol, 5 mM $MgCl_2$, 100 nM 5′-^{32}P-labeled primer-template DNA, 0.045 nM pol η, and dATP, dCTP, dGTP, or dTTP at concentrations of 0.3−3000 μM. In the experiments for elongation of the AP site, 1 nM pol η was included to increase products to a quantifiable level. Before the addition of pol η the reaction mixture was preincubated at 37 °C for 1.5 min. Reactions were initiated by addition of pol η, and incubation was continued at 37 °C. Aliquots were removed at three appropriate time points from 1 to 20 min followed by the addition of an equal volume of formamide and chilling on ice. The products were denatured by boiling, electrophoresed on a 20% polyacrylamide/7 M urea gel, and quantified by the BAS-2500 Bio Imaging analyzer (Fujifilm).

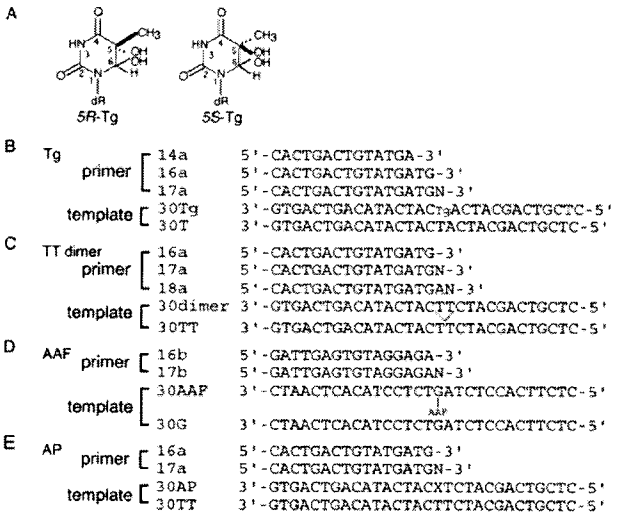

FIGURE 1: DNA substrates used in this study. (A) Structures of the 5*R*- and 5*S*-thymine glycols; dR = deoxyribose. (B−E) Sequences of oligonucleotides. (B) The 30Tg template contained 5*R*- or 5*S*-thymine glycol at Tg. (C) The 30dimer template contained TT dimer at the bridged TT. (D) The 30AAF template contained AAF-G at the indicated site. (E) The 30AP template contained the AP site analogue at X.

Kinetic constants were derived as described (*69*). The velocity of each deoxynucleotide incorporation, v, was determined by dividing the amount of reaction product by the reaction time. The relationship between v and dNTP concentration conformed to a Michaelis−Menten equation, as indicated by the linearity in a Hanes−Woolf plot of [dNTP]/v versus [dNTP]. V_{max} (the maximum value of reaction velocity) and K_m (dNTP concentration at which the reaction velocity is half-maximal) were determined from a Hanes−Woolf plot by linear least-squares fit. These parameters were used to calculate the frequency of deoxynucleotide insertion (f_{ins}) and extension (f_{ext}) using the equation $f_{ins\ or\ ext} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$. Duplicate or triplet determinations were performed for each template−dNTP combination at six different dNTP concentrations. Less than 20% of the primers were extended under the steady-state conditions ensuring single hit conditions. As expected in steady state, V_{max} values were proportional to enzyme concentration (data not shown). Here, k_{cat} was presented by utilizing the equation $k_{cat} = [V_{max}$(mol of primer-template)]/[(mol of polymerase)·min].

RESULTS

Human DNA Polymerase η Is Able To Replicate past Thymine Glycol. Thymine glycol is one of the major DNA lesions produced by oxidative stress. Two *cis* isomers (5*R*,6*S* and 5*S*,6*R*) are known to be produced on DNA in equal amounts by γ irradiation (*70*), while the *cis*-5*R*,6*S* form is produced preferentially by the oxidation of thymidine or oligonucleotides (*71*, *72*). The *cis* forms are in equilibrium with the *trans* isomers in solution (*73*). Recently, Iwai (*65*, *66*) succeeded in the chemical synthesis of oligonucleotides containing 5*R*- (mixture of 5*R*,6*S* and 5*R*,6*R*) or 5*S*- (mixture of 5*S*,6*S* and 5*S*,6*R*) thymine glycols. Using these oligomers,

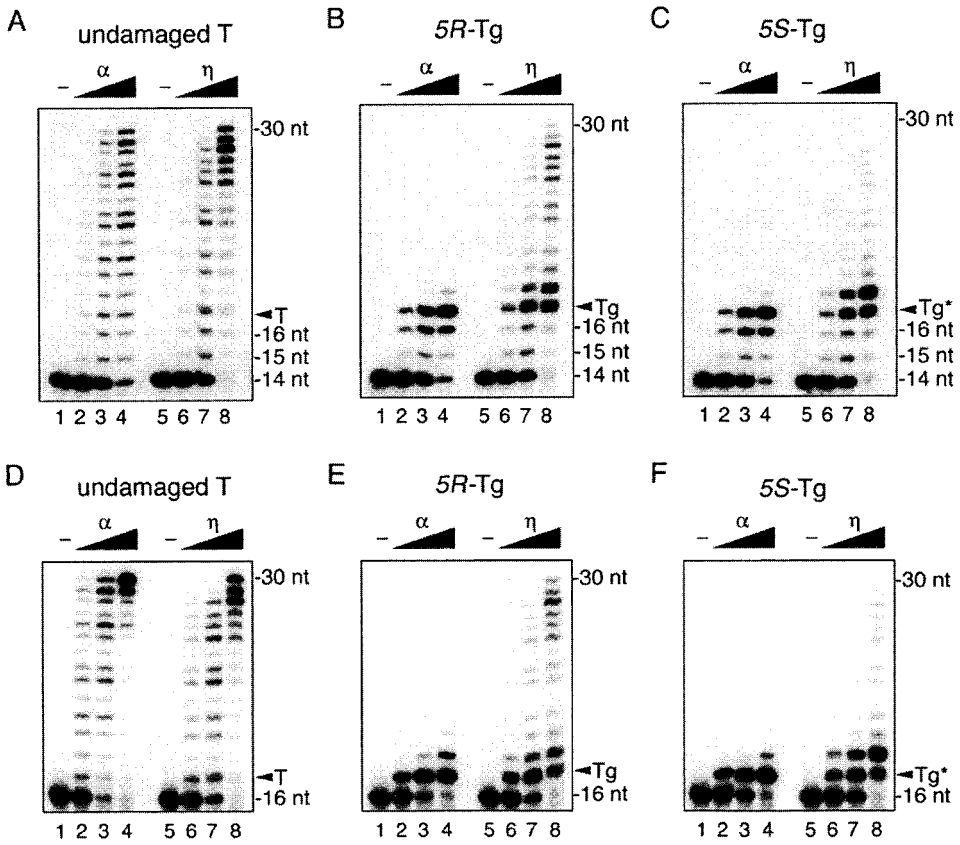

FIGURE 2: Translesion synthesis across Tg by pol η and pol α. Increasing amounts of pol α or pol η were incubated with the 5′-^{32}P-labeled primer-templates at 37 °C for 15 min in the standard reaction mixture. The amounts of pol α were 0.94 fmol in lane 2, 4.7 fmol in lane 3, and 23.6 fmol in lane 4. The amounts of pol η were 0.2 fmol in lane 6, 1 fmol in lane 7, and 5 fmol in lane 8. Lanes 1 and 5 contained no enzyme. The products were subjected to polyacrylamide gel electrophoresis under denaturing conditions. The autoradiograms of the gels are shown. The primer-templates used were 14a-30T (A), 14a-30Tg (5*R*) (B), 14a-30Tg (5*S*) (C), 16a-30T (D), 16a-30Tg (5*S*) (E), and 16a-30Tg (5*S*) (F).

we examined the ability of human pol η to replicate across 5*R*- or 5*S*-Tg in template DNA (Figure 1A,B). To consider the effects of surrounding sequences around the lesion, we performed experiments with templates containing T as well as A on the immediate 5′ side of the Tg. In this paper, however, we show the results with templates containing A immediately 5′ to the 5*R*- or 5*S*-Tg, because no remarkable difference was observed between these templates.

First, we performed running-start experiments using a 30-nt DNA template containing a single Tg lesion located 17 nt from its 3′ end and primed with a 5′-^{32}P-labeled 14-nt oligomer (Figure 2A−C). The bypass activity of mouse pol α, a replicative DNA polymerase, was also examined. Pol α and pol η could synthesize DNA products up to 30 nt in length equally well on the undamaged template, if enough enzyme was added (Figure 2A). When the DNA template contained a 5*R*- or 5*S*-Tg, reactions driven by pol α were clearly inhibited by the lesion, with small amounts of bypass products only being observed in reactions containing excess enzyme (Figure 2B,C, lane 4). Under conditions where template-primers were in excess of enzyme (Figure 2B,C, lanes 2 and 3), pol α stopped 30−37% and 48−59% of the time just before the lesion (at 16 nt) and just after the incorporation of a nucleotide opposite the lesion (at 17 nt), respectively. Thus, both 5*R*- and 5*S*-thymine glycols in template DNA inhibit DNA replication by pol α. Pol η replicated through a 5*R*-Tg more efficiently than pol α, although a portion of the reaction was inhibited after the incorporation of a nucleotide opposite the lesion (17 nt) or after the incorporation of the next nucleotide (18 nt) (Figure 2B, lanes 6−8). On the template containing 5*S*-Tg, pol η was also able to bypass the lesion, but the reaction was much less efficient than that on the 5*R*-Tg template (Figure 2C, lanes 6−8).

We also performed standing-start experiments using the 30-nt templates primed with a 5′-^{32}P-labeled 16-nt oligomer (Figure 2D−F). On this template-primer, a large excess of pol α gave small amounts of bypass products, but most reactions stopped after the incorporation of a nucleotide opposite the lesion. Some minor reaction products were the result of pol α stalling after the incorporation of another nucleotide opposite the A next to the lesion (Figure 2E,F, lanes 2−4). The results indicate that both 5*R*- and 5*S*-thymine glycols constitute major obstacles for DNA synthesis by pol α. Pol η bypassed 5*R*-Tg under the standing-start conditions (Figure 2E, lanes 6−8), although a portion of the reaction was inhibited after the incorporation of one or two nucleotides, in agreement with the running-start experiments (Figure 2B). Under these conditions, TLS by pol η past 5*S*-Tg was again less efficient than with 5*R*-Tg. A larger portion of the reactions stopped after the incorporation of a nucleotide opposite the lesion (17 nt) and especially after the incorporation of a nucleotide opposite A right after the lesion (18 nt) (Figure 2F, lanes 6−8). These results suggest that 5*R*-thymine glycol is a better substrate for pol η bypass than the 5*S* form. It should be mentioned here that the products

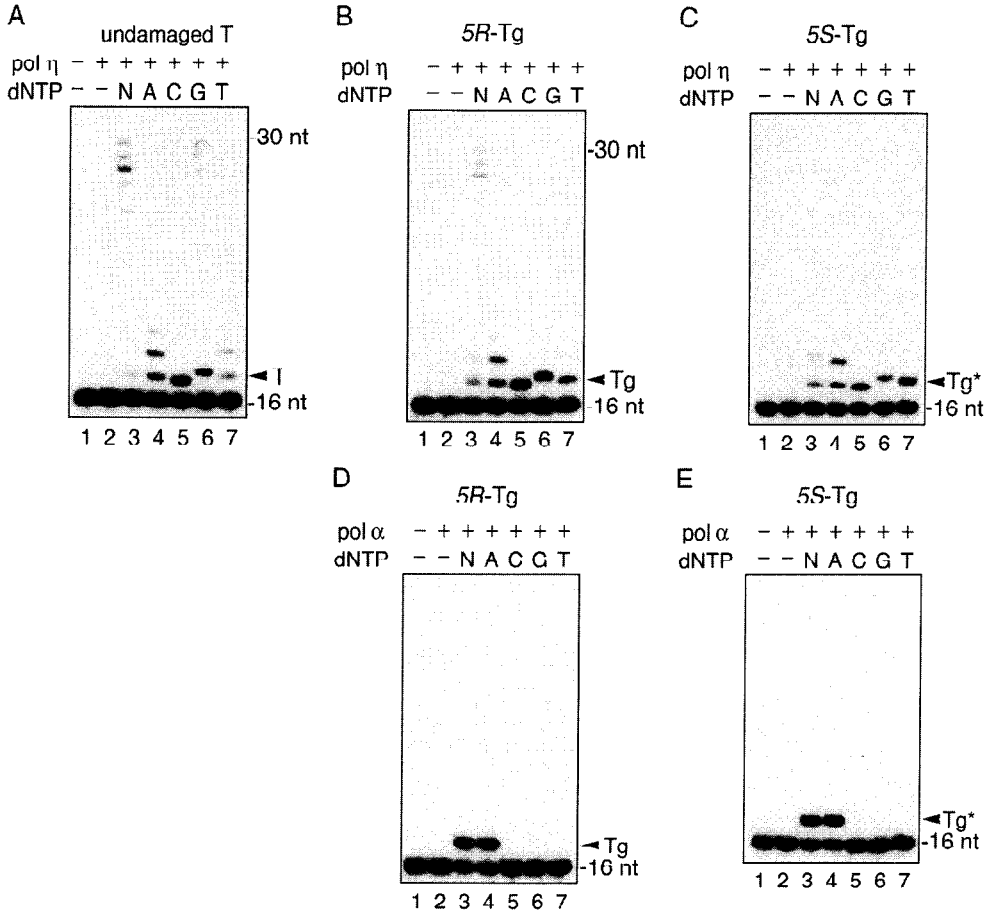

FIGURE 3: Nucleotide selectivities of pol η and pol α incorporation opposite Tg. Pol η (0.2 fmol in panels A−C, lanes 2−7) or pol α (0.94 fmol in panels D and E, lanes 2−7) was incubated with 30-mer DNA annealed to a 5′-^{32}P-labeled 16-mer DNA in the presence of each of the four dNTPs (100 μM) (lane 3) or of one of the indicated dNTPs (100 μM) (lanes 4−7) or in the absence of dNTPs (lane 2). Lane 1 contained no enzyme. The autoradiograms of the gels are shown. The primer-templates were 16a-30T (A), 16a-30Tg (5*R*) (B and D), and 16a-30Tg (5*S*) (C and E).

of pol η stalling opposite Tg lesions were observed as doublet bands, suggesting that pol η incorporated at least two kinds of nucleotides opposite the lesions.

Deoxyribonucleotide Inserted Opposite the 5R- or 5S-Thymine Glycol by Human DNA Polymerase η. To identify the deoxyribonucleotide inserted opposite a 5*R*- or 5*S*-Tg by DNA polymerases η and α, polymerization reactions using the 30-nt template DNA containing the 5*R*- or 5*S*-Tg primed with a 5′-^{32}P-labeled 16-nt oligomer were performed in the presence of only one kind of deoxyribonucleotide (Figure 3). In agreement with our previous reports, pol η incorporated one nucleotide opposite undamaged T and stopped polymerization when only dCTP or dGTP was supplied (Figure 3A, lanes 5 and 6). In the presence of dATP, pol η occasionally incorporated another dAMP opposite the A next to the lesion (Figure 3A, lane 4). Two nucleotides were also incorporated in the presence of dTTP (Figure 3A, lane 7), probably because the next nucleotide to T on the template was A. On the templates containing Tg, pol η incorporated one nucleotide opposite the lesion and stopped synthesis in the presence of dCTP, dGTP, or dTTP (Figure 3B,C, lanes 5−7). The second nucleotide was incorporated only when dATP was supplied (Figure 3B,C, lane 4), suggesting that pol η preferentially continues DNA synthesis only when the correct nucleotide, A, is incorporated opposite Tg. As for pol α, only dAMP was incorporated opposite 5*R*- and 5*S*-thymine glycols (Figure 3D,E).

Ability of Human DNA Polymerase η To Elongate DNA Chains past 5R- or 5S-Thymine Glycol. For error-free translesion synthesis, it is important that the polymerase only extends the primer that has the correctly incorporated nucleotide at its 3′-end opposite the lesion. To test whether pol η has this activity for Tg bypassing, we designed sets of 17-nt primers that had different sequences at their 3′-ends. Annealing of these primers to a 30-nt DNA template containing a 5*R*- or 5*S*-Tg placed the 3′ nucleotide of the primer opposite the lesion. When the template contained an undamaged T, pol η and pol α elongated DNA more efficiently from a paired primer terminus than from mispaired primer termini (Figure 4A). However, pol η could synthesize DNA chains from mispaired primer termini when excess enzyme was added to the reaction. In particular, a G·T mismatch at the primer terminus was elongated more efficiently than other mismatches (Figure 4A, lane 11), which is in good agreement with our previous observations (*40*). When the template contained the 5*R*- or 5*S*-Tg, pol η could synthesize DNA chains more efficiently from a primer that had A opposite the Tg (Figure 4B,C), although most of the reactions stopped after the insertion of one more nucleotide, especially on a template containing 5*S*-Tg (Figure 4C, lane 3). This is consistent with the results shown in Figure 2C where one of the major products of arrested DNA synthesis was observed to result from the incorporation of a nucleotide opposite A next to the lesion. Pol α was unable to elongate

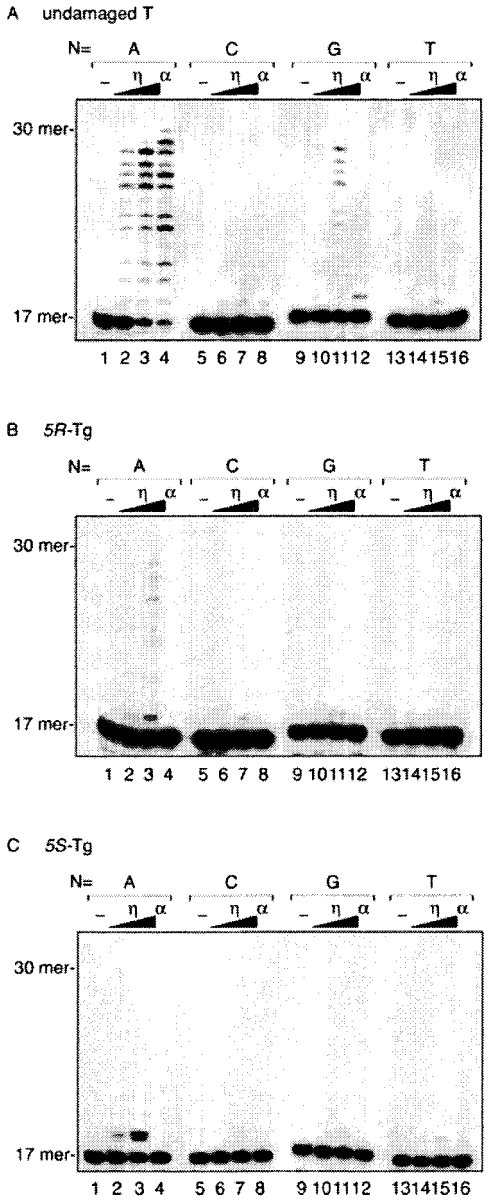

FIGURE 4: Ability of pol η to elongate DNA chains past Tg. Sets of 5′-^{32}P-labeled 17-mer primers, which contained different sequences at their 3′ ends (indicated by N, where N was A, C, G, and T in lanes 1−4, 5−8, 9−12, and 13−16, respectively), were annealed to the 30-mer template containing Tg. Increasing amounts of pol η or pol α were incubated with these primed templates. The autoradiograms of the gels are shown. The amounts of pol η were 0.13 fmol in lanes 2, 6, 10, and 14 and 0.63 fmol in lanes 3, 7, 11, and 15. Pol α (lanes 4, 8, 12, and 16) was present at 4.7 fmol. Lanes 1, 5, 9, and 13 contained no enzyme. The primer-templates used were 17a-30T (A), 17a-30Tg (5*R*) (B), and 17a-30Tg (5*S*) (C).

DNA chains from any of these template-primers containing Tg under these conditions (Figure 4B,C, lanes 4, 8, 12, and 16), indicating that the critical bypass activity of pol η relies on its ability to continue elongation when the correct nucleotide has been incorporated opposite the thymine glycol.

Steady-State Kinetic Analyses of Nucleotide Insertion Opposite 5R-Thymine Glycol and of Subsequent Extension by Human DNA Polymerase η. To assess the efficiency and fidelity of Tg bypass replication by human pol η quantitatively, we measured the kinetics of nucleotide insertion and extension during DNA synthesis past Tg. We employed a template containing 5*R*-Tg, which was a better substrate for pol η than 5*S*-Tg as shown in Figures 2 and 4. The kinetics of insertion of a single deoxynucleotide opposite 5*R*-Tg and the kinetics of addition of the next correct nucleotide to various 3′-primer termini situated across from 5*R*-Tg were determined as a function of deoxynucleotide concentration under steady-state conditions. Pol η was incubated with the DNA substrate and with increasing concentrations of one of the four deoxyribonucleotides for the three time points (see Experimental Procedures). The K_m and k_{cat} parameters were determined and used to calculate the efficiency of the reaction (k_{cat}/K_m), the frequency of nucleotide misincorporation (f_{ins}), and the frequency of mismatch extension (f_{ext}) (Table 1).

As indicated by the k_{cat}/K_m values, human pol η incorporates A opposite the 5*R*-Tg lesion ($k_{cat}/K_m = 21$) as efficiently as opposite the undamaged T ($k_{cat}/K_m = 19$). We also determined kinetic parameters for dAMP incorporation by pol α opposite undamaged T and 5*R*-Tg. The parameters of the pol α reaction on undamaged and 5*R*-Tg templates were 5.4 ± 1.1 and 69 ± 1 for the K_m and 31 ± 2 and 24 ± 5 for k_{cat}, respectively. Thus the efficiency of pol α to incorporate A opposite Tg ($k_{cat}/K_m = 0.35$) was about 16-fold lower than that opposite undamaged T ($k_{cat}/K_m = 5.7$). This is consistent with the results shown in Figure 2B where more than 30% of pol α-catalyzed DNA synthesis stopped before incorporation of a nucleotide opposite 5*R*-Tg. This incomplete but nonetheless clear inhibition of a replicative DNA polymerase by Tg suggests that a specialized polymerase is involved in TLS on Tg-containing templates.

Although pol η incorporated dAMP opposite 5*R*-Tg very efficiently, other nucleotides were also incorporated to various extents (Table 1). This was particularly true for dGMP, which was incorporated with an efficiency of 12% compared to dAMP. This indicates that the doublet bands observed in the reactions by pol η on Tg templates (Figure 2B,C,E,F) were due to misincorporation of dGMP in addition to the normal incorporation of dAMP. Extension by pol η from the A·Tg base pair ($k_{cat}/K_m = 0.54$) was about 16-fold less efficient than extension from the normal A·T base pair ($k_{cat}/K_m = 8.4$). This is consistent with the observation that a portion of pol η-catalyzed DNA synthesis on the Tg template stopped after the incorporation of a nucleotide opposite Tg (Figure 2B,E). Although the efficiency of strand extension was reduced on the Tg-containing template, pol η preferentially elongated from A·Tg base pairs.

The frequency (f_{ins}) at which deoxyribonucleotides are inserted opposite lesion, coupled with the frequency (f_{ext}) at which various base pairs are extended from the 3′ primer terminus, provides kinetic parameters that can be used to predict miscoding by DNA lesions during translesion synthesis. The most frequent error produced by pol η-catalyzed TLS past 5*R*-Tg lesions was the T to C transition via G·Tg mispairing, and the frequency of this error is estimated to be about 1% of correct incorporation.

Efficiency and Fidelity of TLS by Human DNA Polymerase η across a Cis−Syn Thymine−Thymine Dimer, an AAF-G, and an AP Site Analogue. Previously, we have shown that human pol η catalyzes translesion synthesis past a *cis−syn* TT dimer, an *N*-2-acetylaminofluorene-modified G (AAF-G), a cisplatin-induced intrastrand cross-link between two guanines, and an abasic (AP) site analogue (*40*). Here we have shown that pol η is able to bypass the 5*R*-Tg lesion.

Table 1: Fidelity of Pol η at 5*R*-Tg by Steady-State Kinetics

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ins}		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ext}
	incorporation opposite undamaged T					extension from primer-template termini at undamaged T[a]			
A	10 ± 2	190 ± 10	19	1	A·T	32 ± 2	270 ± 20	8.4	1
C	950 ± 60	360 ± 50	0.38	2.0×10^{-2}	C·T	2200 ± 500	110 ± 20	0.05	6.0×10^{-3}
G	57 ± 7	84 ± 7	1.5	7.9×10^{-2}	G·T	580 ± 90	240 ± 40	0.41	4.9×10^{-2}
T	1100 ± 100	270 ± 30	0.25	1.3×10^{-2}	T·T	980 ± 10	78 ± 2	0.080	9.5×10^{-3}
	incorporation opposite 5*R*-Tg					extension from primer-template termini at 5*R*-Tg[a]			
A	14 ± 2	290 ± 10	21	1	A·T	820 ± 80	440 ± 10	0.54	1
C	440 ± 10	440 ± 50	1.0	4.8×10^{-2}	C·T	1300 ± 200	20 ± 1	0.015	2.8×10^{-2}
G	74 ± 15	190 ± 40	2.6	1.2×10^{-1}	G·T	2900 ± 400	130 ± 30	0.045	8.3×10^{-2}
T	730 ± 170	330 ± 30	0.45	2.1×10^{-2}	T·T	2900 ± 1800	20 ± 11	0.0069	1.3×10^{-2}

[a] Extension was examined in the presence of dTTP, the correct nucleotide for template A.

Table 2: Fidelity of Pol η at the TT Dimer by Steady-State Kinetics

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ins}		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ext}
	incorporation opposite 3′T of undamaged TT					extension from primer-template termini at 3′T of undamaged TT[a]			
A	4.7 ± 0.6	170 ± 10	36	1	A·T	15 ± 3	230 ± 20	15	1
G	48 ± 6	71 ± 2	1.5	4.2×10^{-2}	G·T	140 ± 20	150 ± 10	1.1	7.3×10^{-2}
	incorporation opposite 3′T of TT dimer					extension from primer-template termini at 3′T of TT dimer[a]			
A	3.9 ± 0.3	85 ± 9	22	1	A·T	30 ± 1	180 ± 10	6.0	1
C	250 ± 40	130 ± 20	0.52	2.3×10^{-2}	C·T	64 ± 2	3.3 ± 0.6	0.052	8.7×10^{-3}
G	210 ± 50	46 ± 2	0.22	1.0×10^{-2}	G·T	170 ± 20	69 ± 8	0.41	6.8×10^{-2}
T	450 ± 30	110 ± 10	0.24	1.1×10^{-2}	T·T	200 ± 40	6.5 ± 2.3	0.033	5.5×10^{-3}
	incorporation opposite 5′T of undamaged TT					extension from primer-template termini at 5′T of undamaged TT[b]			
A	15 ± 3	230 ± 20	15	1	A·T	3.4 ± 1.4	200 ± 30	59	1
G	52 ± 17	90 ± 17	1.7	1.1×10^{-1}	G·T	34 ± 11	81 ± 9	2.4	4.1×10^{-2}
	incorporation opposite 5′T of TT dimer					extension from primer-template termini at 5′T of TT dimer[b]			
A	30 ± 1	180 ± 10	6.0	1	A·T	3.2 ± 0.7	110 ± 30	34	1
C	530 ± 90	21 ± 3	0.040	6.7×10^{-3}	C·T	75 ± 3	17 ± 5	0.23	6.8×10^{-3}
G	420 ± 10	5.4 ± 0.1	0.013	2.2×10^{-3}	G·T	31 ± 9	26 ± 8	0.84	2.5×10^{-2}
T	1800 ± 100	51 ± 1	0.028	4.7×10^{-3}	T·T	11 ± 3	6.7 ± 2.1	0.61	1.8×10^{-2}

[a] Extension was examined in the presence of dATP, the correct nucleotide for template T. [b] Extension was examined in the presence of dGTP, the correct nucleotide for template C.

To compare the TLS abilities of pol η for these lesions, we determined the kinetic parameters of pol η for catalyzing TLS past a TT dimer, AAF-G, and an AP site analogue. The sequences of the templates and primers used are shown in Figures 1C−E.

The kinetic parameters for deoxyribonucleotide incorporation and for chain extension opposite 3′- and 5′-T of the TT dimer are presented in Table 2. We also examined the kinetic parameters of dAMP and dGMP incorporation opposite undamaged T and the efficiency of chain extension from the resulting base pairs. The previous report has shown that a base substitution, T to C, was the most common error made by pol η within the 275-base *LacZ* sequence, indicating that the most frequent misincorporation event involves the insertion of G opposite T (*74*). As indicated by the k_{cat}/K_m values, pol η incorporated A opposite the 3′T of the dimer ($k_{cat}/K_m = 22$) about 1.6-fold less efficiently than opposite undamaged T ($k_{cat}/K_m = 36$). Pol η incorporated A opposite the 5′T of the dimer ($k_{cat}/K_m = 6$) about 2.5-fold less efficiently than opposite T of the undamaged template ($k_{cat}/K_m = 15$). These incorporation efficiencies opposite the 5′T also are extension efficiencies from A·3′T base pairs. Extension from the A·5′T of the dimer ($k_{cat}/K_m = 34$) was about 1.7-fold less efficient than extension from the normal A·T base pair ($k_{cat}/K_m = 59$). These large k_{cat}/K_m values indicate that pol η can catalyze efficient TLS past TT dimers. As for fidelity, f_{ins} and f_{ext} values for misincorporation and extension from mispairs ranged from 10^{-2} to 10^{-3} relative to correct base pairs on TT dimer templates, whereas the values on undamaged templates were between 10^{-1} and 10^{-2}. Especially, pol η misincorporated G opposite 5′T on the undamaged template with a frequency of 11% compared to that of A incorporation. However, the misincorporation frequency of G opposite 5′T on the TT dimer was 2 orders of magnitude lower than that on the undamaged TT template. Thus, pol η catalyzed more accurate DNA synthesis on the TT dimer template than on the undamaged TT template.

The kinetic parameters for nucleotide incorporation and chain extension on the AAF-G template are shown in Table 3. The efficiency of correct nucleotide (dCMP) incorporation opposite AAF-G ($k_{cat}/K_m = 0.91$) was about 34-fold lower than that on the undamaged template ($k_{cat}/K_m = 31$). The chain extension efficiency from a primer having a correct nucleotide opposite AAF-G ($k_{cat}/K_m = 0.34$) was also about 18-fold lower than that on the undamaged template ($k_{cat}/K_m = 6.2$). Thus, TLS past AAF-G by pol η is less efficient than TLS past a TT dimer, a Tg, or DNA synthesis on undamaged templates, indicating that nucleotide incorporation and primer extension by pol η are severely affected by the presence of an AAF adduct. The misincorporation frequency (f_{ins}) of dAMP, dGMP, and dTMP opposite AAF-G was $(3.3-4.4) \times 10^{-2}$ relative to that of dCMP incorporation. The frequencies (f_{ext}) of chain extension from mispaired primers were 1.5×10^{-2} to 4.1×10^{-3}. Thus, pol

Table 3: Fidelity of Pol η at AAF-G by Steady-State Kinetics

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ins}		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ext}
	incorporation opposite undamaged G					extension from primer-template termini at undamaged G[a]			
A	590 ± 170	45 ± 15	0.076	2.5×10^{-3}	A·G	1400 ± 500	29 ± 8	0.021	3.4×10^{-3}
C	13 ± 4	400 ± 30	31	1	C·G	55 ± 20	340 ± 70	6.2	1
G	360 ± 40	82 ± 1	0.23	7.4×10^{-3}	G·G	89 ± 17	17 ± 7	0.19	3.1×10^{-2}
T	180 ± 30	61 ± 1	0.34	1.1×10^{-2}	T·G	240 ± 10	23 ± 2	0.096	1.5×10^{-2}
	incorporation opposite AAF-G					extension from primer-template termini at AAF-G[a]			
A	300 ± 10	9.7 ± 2.5	0.032	3.5×10^{-2}	A·G	440 ± 70	2.2 ± 0.2	0.005	1.5×10^{-2}
C	110 ± 40	100 ± 20	0.91	1	C·G	470 ± 40	160 ± 10	0.34	1
G	310 ± 40	9.2 ± 0.2	0.030	3.3×10^{-2}	G·G	550 ± 150	2.2 ± 0.7	0.0040	1.2×10^{-2}
T	1300 ± 300	51 ± 7	0.039	4.4×10^{-2}	T·G	1200 ± 400	1.7 ± 0.2	0.0014	4.1×10^{-3}

[a] Extension was examined in the presence of dTTP, the correct nucleotide for template A.

Table 4: Fidelity of Pol η at the AP Site by Steady-State Kinetics

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ins}		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	f_{ext}
	incorporation opposite AP site					extension from primer-template termini at AP site[a]			
A	710 ± 20	120 ± 20	0.17	1	A·X	530 ± 240	14 ± 7	0.026	1
C	940 ± 100	34 ± 2	0.036	2.1×10^{-1}	C·X	1400 ± 400	1.4 ± 0.2	0.0010	3.8×10^{-2}
G	220 ± 20	22 ± 3	0.1	5.9×10^{-1}	G·X	740 ± 180	3.4 ± 0.5	0.0046	1.8×10^{-1}
T	2800 ± 1500	73 ± 3	0.026	1.5×10^{-1}	T·X	1200 ± 400	1.9 ± 0.7	0.0016	6.2×10^{-2}

[a] Extension was examined in the presence of dATP, the correct nucleotide for template T.

η can catalyze relatively accurate TLS past a lesion, although the reaction still remains inefficient. These results suggest that pol η can utilize the base pairing characteristics of AAF-G despite the geometric distortions of the DNA at the site of the lesion, which make the reaction inefficient.

The kinetic parameters for nucleotide incorporation and chain extension on the AP site analogue-containing template are shown in Table 4. Pol η incorporated dAMP more frequently than any other nucleotide. The incorporation of other dNMPs was 1.7−6.5-fold lower than the efficiency of dAMP incorporation. It has been recently reported that yeast and human pol η incorporated dGMP more efficiently than other nucleotides opposite an AP site analogue (*41*). This difference may result from the altered sequence context around the lesion. Kinetic parameters for chain extension revealed that pol η extended DNA chains from a primer having A opposite AP more efficiently than others. However, the chain extension efficiencies (k_{cat}/K_m) of pol η reactions on AP templates were less efficient than those on any other templates used in this study, suggesting that the AP site is a difficult lesion for bypass by pol η. As for fidelity, pol η can catalyze TLS past the AP site analogue at best by incorporating dAMP opposite the lesion, and dGMP was incorporated and extended opposite the lesion with a frequency of about 59% and 18% relative to that of dAMP, respectively.

DISCUSSION

TLS across 5R- and 5S-Thymine Glycols. In cells, a major source of spontaneous damage to DNA is attack by reactive oxygen species. Various lesions, such as 8-hydroxyguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy), 5-hydroxyhydantoin, hydroxyadenines, and others are induced by radicals (*75*). Tg is one of the most frequent lesions caused by oxidation of the thymine base. Efficient base excision repair mechanisms to remove Tg lesion are known (*49*, *76*), and nucleotide excision repair is also reported to be capable of removing the lesion (*54*). However, it is apparently impossible to remove all lesions from the genome because living cells continuously produce radicals. Thus, there is a high risk that the DNA replication machinery will often encounter Tg lesions during genome replication even in healthy cells. Recent findings strongly suggest that when the replicative DNA polymerases α, δ, or ϵ encounter a harmful lesion, the specialized DNA polymerases ζ, η, ι, κ, and/or Rev1 take over and catalyze TLS.

Two isoforms of 5*R*- and 5*S*-thymine glycols are known. Recent advances in the chemical synthesis of the building block for each Tg allowed us to examine the effect of each isoform on DNA polymerase synthesis in vitro (*65*, *66*). DNA synthesis by pol α was almost equally inhibited by either Tg isomer, suggesting that TLS polymerases must be involved in the replication of Tg lesions. Major inhibition of the pol α reaction on Tg-containing templates occurred after incorporation of dAMP opposite the lesion. In this situation, a polymerase that can elongate DNA chains from inadequate primer ends would be a good candidate for efficient TLS. Pol ζ has been reported to be able to efficiently extend DNA chains from nucleotides opposite lesions, although it is deficient in inserting nucleotides opposite lesions (*77*, *78*). It is possible that pol ζ participates in replication across Tg lesions by extending DNA chains whose elongation has been arrested after the incorporation of a nucleotide opposite the lesion. As the replicative polymerase α preferentially incorporated dAMP opposite Tg lesions, TLS by pol α and pol ζ may be accurate.

While pol α could incorporate dAMP opposite Tg lesions, more than 30% of the pol α reaction was inhibited before insertion of a nucleotide opposite the lesion. Kinetic analyses also revealed that pol α incorporates dAMP opposite 5*R*-Tg about 16-fold less efficiently than on an undamaged template. These observations suggest that a DNA polymerase that can insert a nucleotide opposite a lesion would be a good candidate for the efficient replication of Tg-containing

templates. As for 5*R*-Tg, pol η would be the most likely candidate because pol η was able to catalyze TLS past this lesion (Figure 2B,E). Kinetic analyses also indicate that pol η can insert a nucleotide opposite 5*R*-Tg as efficiently as opposite an undamaged sequence (Table 1). Although pol η misincorporated incorrect nucleotides, especially dGMP, opposite 5*R*-Tg, it preferentially elongated from A·Tg pairs, and hence the majority (about 99%) of TLS past Tg by pol η was accurate. However, if another polymerase, such as pol ζ, was also included in the reaction, the reaction could become mutagenic.

Compared to TLS past 5*R*-Tg, the 5*S*-Tg template presented a much more formidable barrier for pol η-catalyzed TLS (Figure 2C,F). This finding suggests that there exist differences in the conformation of DNA around these isomers. It is probable that positions and/or orientations of N^3 and/or O^4 (which will pair with an incorporated nucleotide) of 5*R*-Tg in the pol η−template complex are better for TLS than those of 5*S*-Tg. In fact, our steady-state kinetic analyses revealed that pol η incorporated A opposite 5*S*-Tg with almost the same efficiency ($k_{cat}/K_m = 25$) as opposite 5*R*-Tg ($k_{cat}/K_m = 21$), while extension by pol η from the A·5*S*-Tg base pair ($k_{cat}/K_m = 0.13$) was about 4-fold less efficient than extension from the A·5*R*-Tg base pair ($k_{cat}/K_m = 0.54$). These findings also raise a possibility that another polymerase, such as pol ι or κ, might assist in TLS past the 5*S* isomer. To our knowledge, this is the first report showing the differential biological effects of two Tg isomers.

It was reported that XP-V cells have a reduced repair capacity for some types of γ-ray-induced DNA damage (*79*). To determine whether pol η is involved in tolerance to DNA damage induced by oxidative stresses in cells, we examined the sensitivities of XP-V cells to peroxide. However, it was not clear whether XP-V cells were more sensitive or not to peroxide than normal cells (unpublished observation). Efficient repair mechanisms for oxidative lesions may be more important for damage tolerance than TLS, which may only make a relatively minor contribution. In fact, Haracska et al. (*47*) succeeded in detecting the contribution of yeast pol η to TLS past 8-hydroxyguanine only when the base excision repair mechanism for the lesion was deficient. Furthermore, Swanson et al. (*80*) reported that at least four pathways of repair, that is, base excision repair, nucleotide excision repair, recombination, and TLS (pol ζ dependent), are involved in damage tolerance against peroxide-induced lesions in yeast. In mammals, the availability of knockout animals for the various components of these pathways would provide useful information about the relative importance of these complicated mechanisms.

TLS by Human Pol η. The abilities of a DNA polymerase to insert nucleotides opposite a lesion and to extend the DNA chain from the resulting primer terminus are two important parameters that affect the efficiency and fidelity of translesion synthesis. In this paper, we determined these kinetic parameters for pol η under steady-state conditions with various lesions. The analyses clearly demonstrated that the most efficient target for pol η is the TT dimer. Pol η inserted nucleotides in front of the TT dimer and extended the resulting primer end with almost the same efficiency as on undamaged DNA. Furthermore, the pol η-catalyzed polymerization on DNA containing a TT dimer was more accurate than on undamaged DNA. Together with previous in vivo observations, which showed that XP-V cells were more sensitive to, and more mutatable by, UV irradiation than wild-type cells (*32*−*38*), the optimum substrate for pol η appears to be the TT dimer, the most frequently occurring lesion induced by UV irradiation. It is suspected that pol η may have a catalytic center whose conformation is well suited to accommodating the thymines of TT dimers.

Compared to the TT dimer, TLS past AAF-G by pol η was less efficient for both insertion and extension. However, though the efficiencies were low, pol η preferentially incorporated the correct nucleotide, C, opposite the lesion and preferentially extended the DNA chain from the C·AAF-G pair. Similar results demonstrating relatively accurate TLS by pol η have already been reported for various lesions (*43*−*46*), although the efficiency was found to differ from lesion to lesion. These observations suggest that pol η can bypass various lesions and that TLS efficiency depends on the type of lesion, perhaps reflecting the structural distortion of the template DNA induced by the lesion. In other words, pol η can deal with a lesion efficiently when the lesion induces a relatively small distortion of the DNA structure but cannot deal efficiently with lesions that induce large structural distortions of DNA. This property may constitute the crossover point between pol η-catalyzed TLS and nucleotide excision repair for which a large structural distortion of the DNA by a lesion provides a powerful trigger for the repair reaction (*81*). Thus, pol η can catalyze efficient TLS past lesions that are inefficient substrates for nucleotide excision repair.

Although pol η could bypass an AP site analogue mainly by incorporating dAMP, the efficiency of insertion and especially elongation in front of the AP site were the worst among the other lesions examined in this study. The poor efficiency on the AP template suggests that pol η requires the presence of a template base for both the insertion and especially the elongation steps, as demonstrated previously by Haracska et al. (*42*). The comparatively high efficiency of Tg bypass by pol η is interesting because thermodynamic analyses indicate that, like AP site analogues, Tg (both isomers) lesions cannot engage in base pairing with any nucleobase on the complementary strand (*66*). Since almost all of the terminal products of pol η reactions had mismatched 3′-ends, as observed under conditions when the correct nucleotide was absent (Figure 3) or when the lesion was difficult to bypass these (6-4) photoproducts (*30*, *40*), it is likely that base pair formation is not a major requirement for nucleotide insertion opposite a lesion but is required for the extension reaction. These properties of pol η confirm and extend the data showing that pol η is especially ineffective in extending DNA from an A·AP pair. In contrast, the observation that pol η could catalyze moderately efficient extension from the A·Tg pair suggests that Tg in the template DNA can form a base pair with incorporated dAMP in the DNA−pol η complex, despite the observed lack of base pair formation on naked double-stranded DNA. Pol η occasionally stopped after incorporation of a nucleotide opposite the A next to the Tg (Figures 2 and 4). The 5*R*-Tg lesion has been reported to induce a significant and localized structural change with the lesion largely extrahelical in duplex oligomers (*72*). It is plausible that the nucleotide next to Tg in the template cannot form a base pair easily if Tg is already engaged in base pairing with a nucleotide that blocks further

DNA synthesis by pol η bound to the primer terminus. As we showed previously, arrest of DNA synthesis after the incorporation of a nucleotide opposite the next nucleotide to a lesion was also observed on AAF-G templates (*40*). Thus, such unusual arrest of DNA synthesis caused by a DNA lesion is thought to reflect the intrinsic nature of the catalytic site of pol η. To address these conjectures, structural analysis of the DNA−pol η complex will be required.

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