

Error-free replicative bypass of thymine glycol by the combined action of DNA polymerases κ and ζ in human cells

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Thymine glycol (Tg) is the most common DNA lesion of thymine induced by interaction with reactive oxygen species. Because of the addition of hydroxyl groups at C5 and C6 in a Tg lesion, the damaged base loses its aromatic character and becomes nonplanar; consequently, the C5 methyl group protrudes in an axial direction and that prevents the stacking of the 5' base above the Tg lesion. Because Tg presents a severe block to continued synthesis by replicative DNA polymerases, we determine here how human cells manage to replicate through this lesion. Using a duplex plasmid system where bidirectional replication ensues from an origin of replication, we show that translesion synthesis (TLS) makes a prominent contribution to Tg bypass and that it occurs in a predominantly error-free fashion. Also, we provide evidence that Pol κ and Pol ζ function together in promoting error-free replication through the lesion, and based on structural and biochemical information, we propose a role for Pol κ at the insertion step and of Pol ζ at the extension step of Tg bypass. We discuss the implications of these observations and suggest that human cells have adapted the TLS machinery to function in a much more error-free fashion than could have been predicted from the intrinsic catalytic efficiencies and fidelities of TLS polymerases.

DNA polymerases kappa and zeta | error-free bypass of thymine glycol | replicative lesion bypass | thymine glycol bypass in humans

Thymine glycol (Tg) is the most common oxidation product of thymine (1–3). Hydroxyl radicals, the principal DNA damaging species formed from aerobic respiration or from exposure to chemical oxidizing agents or ionizing radiation, interact directly with thymine primarily at the 5,6 double bond, generating a thymine glycol. Although Tg lesions in humans can be removed by base excision repair (BER) involving the action of the DNA glycosylase/AP lyase NTH1 or by nucleotide excision repair (NER) (4, 5), if the damaged base is not removed by these repair processes and goes through the replication fork, that presents a severe block to continued synthesis by eukaryotic replicative DNA polymerases (Pols) (6, 7).

Because of the addition of hydroxyl groups at C5 and C6 in a Tg, the damaged base loses its aromatic character and becomes nonplanar (8–10). As a consequence, the C5 methyl group protrudes in an axial direction that prevents the base 5' to the Tg lesion from stacking above it. Hence a Tg lesion presents a block not only for nucleotide (nt) insertion opposite it but also for the subsequent extension step. Steady-state kinetic analyses of Tg bypass by human translesion (TLS) Pols have indicated that Pol η can insert an A opposite Tg with the same catalytic efficiency as for insertion of an A opposite undamaged T; moreover, Pol η can also extend from the Tg:A base pair with only an ~15-fold reduction in efficiency (7). However, Pol η incorporates the wrong nts opposite Tg rather frequently as the misincorporation of G or a C residue occurs with only an ~8-, and 20-fold reduction in efficiency compared with the efficiency for A incorporation. Pol η can also extend from these base pairs with the same magnitude of reduction in efficiency as that seen for their insertion (7). Hence

although Pol η can promote efficient TLS through a Tg lesion by promoting both the insertion and extension steps, it does so in quite an error-prone manner. Pol κ can promote error-free TLS opposite Tg by inserting an A and extending from there, but its efficiency at the insertion step is reduced ~50-fold, and at the extension step it is inhibited ~250-fold (11). Interestingly, even though Pol κ is less efficient at both steps of TLS than Pol η , it performs TLS in a more accurate manner—as it incorporates nts other than an A with greatly reduced catalytic efficiency (11). The other two human Y family Pols, Rev1 and Pol ι , are unlikely to function in TLS opposite Tg because of their highly specialized roles in DNA synthesis as Rev1 specifically incorporates a C opposite template G and Pol ι is highly inefficient and error-prone opposite pyrimidine template bases, particularly opposite a template T (12).

Pol ζ , a B-family Pol, comprised of Rev3 catalytic subunit and Rev7 accessory subunit (13), is highly specialized for extending from nts inserted opposite a lesion site by another DNA Pol (12). Because it has not been possible to purify human Pol ζ , all the inferences for the TLS role of Pol ζ in human cells have been drawn from biochemical studies done with yeast Pol ζ . These studies have indicated that Pol ζ can extend efficiently opposite from a diverse array of DNA lesions, as for example, a *cis-syn* TT dimer (14), a (6-4) photoproduct (14, 15), an abasic site (16), 1,N⁶-etheno deoxyadenosine (edA) (17), and others (18); however, Pol ζ is inhibited at inserting nts opposite these lesions. Steady-state kinetic analyses have indicated that Pol ζ can also extend from the Tg:A base pair as efficiently as from the T:A base pair (6).

These biochemical observations with the TLS Pols would suggest a role of Pol η , Pol κ , and Pol ζ in promoting replication through a Tg lesion, in which Pol η could carry out efficient but highly mutagenic TLS, whereas Pol κ could act primarily at the insertion step with the subsequent extension step being performed by Pol ζ . To elucidate the roles of various Pols in promoting replication through a Tg lesion in human cells, here we examine TLS using a duplex plasmid in which bidirectional replication ensues from a replication origin (19, 20) and proceeds through a site-specific Tg lesion carried on the leading or the lagging DNA strand template. From these analyses, we make the following observations: (i) On both the DNA strands, TLS makes a prominent contribution to the replicative bypass of Tg lesion; (ii) in normal human cells, TLS through the lesion occurs in a predominantly error-free fashion; and (iii) Pols κ and ζ function together in promoting error-free TLS through the Tg lesion. We

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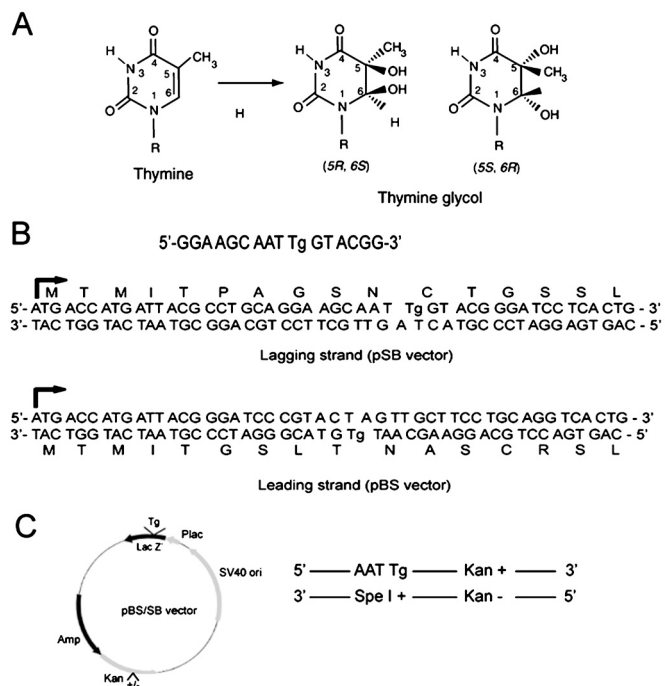


Fig. 1. Strategy for examining TLS opposite a Tg lesion carried on the leading or lagging strand DNA template of a duplex plasmid. (A) Structures of 5R,6S and 5S,6R Tg. (B) The target 16-mer sequence containing a Tg, and the sequences of the N-terminal portion of the *lacZ* gene in the lagging strand (pSB) and the leading strand (pBS) vector with a Tg lesion are shown. (C) Strategy for TLS assays. (Left) The locations of the Tg, the *lacZ* gene, SV40 origin, and the *kan* gene are shown on the vector. (Right) In the duplex plasmid, the DNA strand containing the Tg also carries the wild type kanamycin gene (*Kan*⁺) so that TLS through the Tg will produce a blue colony on LB/Kan plates with IPTG and X-gal. The other DNA strand harbors a *SpeI* sequence opposite the Tg and carries the *kan*⁻ gene. Because the *SpeI* sequence puts the *lacZ* sequence out of frame, replication of the Tg-containing strand by template switching would generate white colonies.

discuss the implications of these observations for the role of TLS in promoting error-free lesion bypass during replication and we consider the contributions of TLS Pols to Tg bypass in relation to their intrinsic efficiency and fidelity opposite this lesion.

Results

The Plasmid System Containing a Site-Specific Tg Lesion and TLS Analyses. Tg is one of the major stable products of thymine modification produced by cellular oxidative damage or by exposure to ionizing radiation. Tg exists in two *cis* isomer forms: 5R,6S and 5S,6R (21) (Fig. 1A), and both of these isomers block synthesis by eukaryotic replicative Pols. We have incorporated each of these isomers in the duplex plasmid in the leading strand or the lagging

strand DNA template (Fig. 1B). Because the DNA strand that has the Tg lesion also carries the *kan*⁺ gene and because the *lacZ* sequence on this strand is in-frame (Fig. 1B and C), TLS through the Tg will produce a blue colony on LB/kan plates with IPTG and X-gal. The other DNA strand without the lesion is marked with the *kan*⁻ gene, and it carries a *SpeI* sequence opposite from the lesion on the other strand, which puts the *lacZ* gene out of frame (Fig. 1B and C). Hence replication through the Tg lesion via template switching will produce white colonies. Removal of the lesion by BER or NER before replication also would generate a white colony because that would result from repair synthesis using the nondamaged *SpeI* DNA strand as the template. The TLS frequencies are determined from the number of blue colonies among the total *kan*⁺ colonies.

For determining the roles of various Pols in TLS through the Tg lesion, we carried out parallel experiments with undamaged and damaged plasmid where the levels of particular Pol(s) were depleted by siRNA knockdowns, and as expected, whereas with the undamaged plasmid the frequency of blue colonies among the *kan*⁺ colonies remained the same regardless of which Pol had been depleted, with the Tg containing plasmid, a reduction in the frequency of blue colonies occurred upon the siRNA knockdown of some but not of other Pols, indicative of the role of particular Pol(s) in mediating TLS through the Tg lesion.

Requirement of Pols κ and ζ for TLS Opposite Tg. As shown in Table 1, in normal human cells treated with control (NC) siRNA, TLS through the Tg lesion accounts for ~23% of lesion bypass on the leading strand and for ~19% on the lagging strand. Hence the contribution of TLS to the bypass of the 5R,6S lesion on both the DNA strands is quite similar. The TLS frequency for the lesion carried on either the leading DNA strand or the lagging strand template remained about the same in Pol η siRNA treated cells as in cells treated with control siRNA. We also verified that TLS on both the DNA strands in xeroderma pigmentosum variant (XPV) cells occurred as frequently as in normal human cells. The siRNA knockdown of Pol η also conferred no reduction in TLS frequencies. To exclude the possibility that Pols η and ι provide alternate means of TLS opposite the 5R,6S Tg lesion, we examined whether the simultaneous knockdown of both these Pols has an effect on TLS frequencies. However, because TLS occurs as frequently upon depletion of both these Pols as in normal cells (Table 1), Pols η and ι do not provide alternative competing pathways for TLS opposite this DNA lesion. On the other hand, the siRNA depletion of Pol κ led to an ~50% reduction in TLS frequencies opposite the 5R,6S lesion carried on either DNA strand, and because the simultaneous depletion of either Pol κ and Pol η or of Pol κ and Pol ι caused no further reduction in TLS frequencies than that observed upon the knockdown of Pol κ alone (Table 1), we conclude that Pol η and ι play no significant role in TLS opposite the 5R,6S Tg lesion whereas Pol κ

Table 1. Effects of siRNA knockdowns of TLS Pols on the replicative bypass of 5R,6S Tg lesion carried on the leading or lagging strand DNA template in human cells

siRNA	Leading strand			Lagging strand		
	# <i>Kan</i> ⁺ colonies	# blue colonies among <i>Kan</i> ⁺	TLS, %	# <i>Kan</i> ⁺ colonies	# blue colonies among <i>Kan</i> ⁺	TLS, %
NC	648	152	23.4	626	121	19.4
Pol η	546	110	20.1	645	118	18.3
Pol ι	536	134	25.0	542	123	22.7
Pol κ	602	66	10.9	624	58	9.4
Rev3	620	77	12.4	584	60	10.3
Rev7	586	74	12.6	623	66	10.6
Pol η + Pol ι	526	128	24.4	621	132	21.2
Pol η + Pol κ	572	59	10.4	584	57	9.8
Pol ι + Pol κ	610	66	10.8	526	54	10.2
Pol κ + Rev3	642	75	11.7	589	62	10.6
Pol κ + Rev7	516	59	11.4	612	56	9.2

Table 2. Effects of siRNA knockdowns of TLS Pols on the replicative bypass of 5S,6R Tg lesion carried on the leading strand template in human cells

siRNA	# <i>Kan</i> ⁺ colonies	# blue colonies among <i>Kan</i> ⁺	TLS, %
NC	475	115	24.2
Polη	326	77	23.6
Polι	312	79	25.3
Polκ	512	57	11.1
Rev3	612	65	10.6
Rev7	586	60	10.2

makes a prominent contribution to promoting replication through this lesion.

To determine whether Polκ acts in TLS opposite 5R,6S Tg alone or together with another DNA Pol, next we examined whether depletion of Polζ had an effect on TLS frequencies. As shown in Table 1, depletion of either the Rev3 catalytic subunit or the Rev7 accessory subunit conferred a reduction in TLS frequencies similar to that seen upon Polκ depletion, which suggested that Pols κ and ζ collaborate in promoting TLS opposite the Tg lesion. To verify this possibility, we examined the effects of simultaneous depletion of Polκ with Rev3 and of Polκ with Rev7. Because the reduction in TLS frequencies observed upon the simultaneous depletion of Pols κ and ζ is very similar to those observed upon depletion of either Pol, this epistatic interaction signifies that the two Pols act together in promoting replication through the 5R,6S Tg lesion.

Biochemical studies have suggested that human TLS Pols can react differently to the 5R,6S vs. 5S,6R isoforms of Tg; for example, the 5R,6S stereoisomer is less inhibitory to TLS by Polη than the 5S,6R form (7), and Polκ misincorporates a G with a higher frequency opposite the 5R,6S form (11). Hence, we verified whether our conclusion for the combined role of Polκ and Polζ in TLS opposite the 5R,6S form applies also to the 5S,6R form. As shown in Table 2, TLS opposite the 5S,6R form carried on the leading strand template occurs as frequently as opposite the 5R,6S isoform and the various TLS Pols operate very similarly opposite both forms of Tg lesion. Thus, whereas depletion of Polη or Polι has no effect, depletion of either Polκ or Polζ confers an over 50% reduction in TLS frequencies opposite the 5S,6R form as well.

Pols κ and ζ Mediate Error-Free Bypass of a Tg Lesion. Next, we determined the mutagenicity of TLS opposite Tg in normal human cells. We find that opposite the 5R,6S isoform TLS occurs in a predominantly error-free manner regardless of whether the lesion is on the leading or on the lagging strand DNA template, as only ~2% of TLS events had incurred mutational changes that resulted from an insertion of a G, C, or T opposite the Tg site

rather than the correct A (Table 3). As expected from the lack of involvement of Polη or Polι in TLS opposite Tg, the incidence of mutational TLS events was not affected upon knockdown of either of these Pols. Interestingly, the knockdowns of either Polκ or of Rev3 or of Rev7 conferred an over 2-fold increase in mutagenic TLS events as the mutation frequencies rose to ~5% (Table 3). In particular, a T is incorporated somewhat more frequently in cells depleted of Polκ or Polζ than in normal cells (Table 3). Because the frequency and pattern of TLS events (mutagenic or nonmutagenic) are very similar for the lesion carried on either the leading or the lagging template strands, pooling the data for the two strands from control (NC) siRNA treated cells shows 8 TLS events resulting from T insertion among a total of 852 TLS, whereas in Polκ-depleted cells 22 T insertions were observed among a total of 888 TLS events, and in cells depleted of either of the subunits of Polζ, 22 T insertions occurred among the 861 TLS events. A χ^2 comparison of these data for control cells vs. Polκ-depleted cells or for control cells vs. Polζ-depleted cells indicates that the increase in the frequency of T insertion in Polκ- or Polζ-depleted cells is significantly different ($p < 0.025$) from that in normal cells.

We also tested the roles of TLS Pols in mutagenic vs. error-free lesion bypass opposite the 5S,6R isoform carried on the leading strand template. As shown in Table 4, opposite this stereoisomer also only ~2% of TLS events were mutagenic, and the frequency of mutagenic TLS rose to ~5% upon siRNA knockdown of either Polκ or Polζ. Hence we conclude that TLS Pols κ and ζ mediate predominantly error-free TLS opposite both the 5R,6S and 5S,6R isoforms of Tg.

Discussion

Role of TLS in Promoting Replication Through a Tg Lesion. Here we show that TLS in normal human cells can account for ~20–25% of replicative bypass of a Tg lesion. The overall contribution of TLS to Tg bypass, however, may be significantly higher because in repair proficient cells, the lesion could have been removed from the duplex plasmid by BER or NER before its transit through the replication fork. Previously, from our studies with *cis-syn* TT dimer and a (6-4) TT photoproduct, we showed that whereas in NER defective human cells TLS opposite both these lesions contributes to ~30–40% of lesion bypass, in NER proficient human cells TLS accounts for ~20% of lesion bypass (19, 20, 22).

Opposite the Tg lesion, TLS on the leading DNA strand occurs only somewhat more frequently than on the lagging strand; however, the differences in TLS frequencies on the two strands are rather small and not statistically significant. Although TLS opposite a *cis-syn* TT dimer and (6-4) TT photoproduct was observed to occur somewhat more frequently on the leading strand than on the lagging strand, opposite both these lesions also

Table 3. Effects of siRNA knockdowns of TLS Pols on mutation frequencies and nucleotides inserted opposite a 5R,6S Tg carried on the leading or the lagging strand DNA template in human cells

DNA strand	siRNA	# of <i>Kan</i> ⁺ blue colonies sequenced*	Nucleotide inserted					Mutation frequency (%)
			A	G	C	T	Other [†]	
<u>Leading</u>	NC	356 (8)	348	2	2	3	1	2.2
	Polη	164 (4)	160	1	1	2	0	2.4
	Polι	192 (5)	187	1	1	3	0	2.6
	Polκ	392 (17)	375	4	2	9	2	4.3
	Rev3	213 (10)	203	2	3	4	1	4.7
	Rev7	268 (14)	254	3	2	8	1	5.2
	NC	496 (10)	486	2	2	5	1	2.0
<u>Lagging</u>	Polη	224 (5)	219	1	1	3	0	2.2
	Polι	144 (3)	141	0	0	2	1	2.1
	Polκ	496 (23)	473	4	3	13	3	4.6
	Rev3	208 (9)	199	1	2	5	1	4.3
	Rev7	172 (8)	164	1	2	5	0	4.7

*Numbers of mutational TLS events are shown in parenthesis.

[†]Mutation at the flanking 5'T to C.

Table 4. Effects of siRNA knockdowns of TLS Pols on mutation frequencies and nucleotides inserted opposite a 5S,6R Tg carried on the leading strand DNA template in human cells

siRNA	# of <i>Kan</i> ⁺ blue colonies sequenced*	Nucleotide inserted					Mutation frequency (%)
		A	G	C	T	Other [†]	
NC	380 (9)	371	2	2	4	1	2.4
Polη	188 (4)	184	1	0	3	0	2.1
Polι	190 (4)	186	1	1	2	0	2.1
Polκ	238 (12)	226	2	1	7	2	5.0
Rev3	192 (9)	183	2	2	4	1	4.7
Rev7	240 (11)	229	1	2	6	2	4.6

*Numbers of mutational TLS events are shown in parenthesis.

[†]Mutation at the flanking 5'T to C.

TLS accounted for a very significant fraction of lesion bypass on both the DNA strands (19, 20). Furthermore, from the observations that the genetic control of TLS on the two DNA strands is almost identical for the Tg lesion, and a similar phenomenon is observed for TLS opposite a *cis-syn* TT dimer and a (6-4) TT photoproduct (19, 20), we conclude that TLS makes a prominent contribution to lesion bypass on both the DNA strands and that similar genetic mechanisms modulate TLS on the two strands.

From studies of replication products of SV40 DNA in UV irradiated human cells, and from the analysis of replication products from human cell-free extracts replicating SV40-derived plasmids, it has been inferred that a UV lesion located on the leading strand template presents a severe block to replication fork movement, whereas a UV lesion on the lagging strand does not significantly inhibit replication fork progression but inhibits the completion of Okazaki fragments (23, 24). Because the gap remaining in the Okazaki fragment opposite the DNA lesion could be filled in by template switching mechanisms using the newly synthesized leading strand as the template for synthesis, one might have anticipated TLS to be more active on the leading strand than on the lagging strand, because that could have provided for a more rapid completion of replication through the lesion, whereas a gap on the lagging strand could have been subsequently filled in by template switching. However, our observations that TLS occurs nearly equally frequently and has the same genetic control on the two DNA strands raises the possibility that when the replication fork stalls at a lesion site on either DNA strand, the action of the TLS Pols is somehow coordinated with the stalled replisome, irrespective of whether the lesion is located on the leading or the lagging DNA strand.

TLS Promotes Predominantly Error-Free Bypass Through a Tg Lesion.

Here we show that in human cells replicative bypass of a Tg lesion occurs in a predominantly error-free fashion as only ~2% of TLS events incur mutations that result from the incorporation of a C, a G, or a T instead of an A. Although because of the low fidelity of TLS Pols it has been assumed that lesion bypass by them would be a highly mutagenic process, and this idea may be generally valid for *Escherichia coli* and to a lesser extent for yeast (25), our studies in human cells have pointed to a more error-free role of TLS in promoting lesion bypass during replication. Similar to the observations of predominantly error-free TLS through a Tg lesion, we showed previously that human TLS Pols promote predominantly error-free replication through a *cis-syn* TT dimer as well as a (6-4) TT photoproduct (19, 20). Although different sets of TLS Pols promote replication through these UV induced lesions and through the Tg lesion, our observations that mutations occur in only ~2% of TLS events opposite any of these lesions would suggest that in human and other mammalian cells, mechanisms have evolved that adapt TLS Pols to act in a more highly error-free fashion than could have been predicted from their intrinsic fidelities.

Pols κ and ζ Function Together in Promoting Error-Free Replicative Bypass of a Tg Lesion in Human Cells.

From studies with siRNA

knockdowns of Pols η, ι, κ, and ζ, singly and in combinations, we conclude a role for Pols κ and ζ in promoting replication through a Tg lesion. However, because depletion of either Polκ, or of the Rev3 or Rev7 subunit of Polζ confers an ~50%–60% reduction in TLS opposite the lesion carried on either the leading or the lagging DNA strand, we are led to infer the additional involvement of as yet unidentified Pol(s) in TLS opposite this lesion (Fig. 2). Hence, we propose that TLS through a Tg lesion occurs via a Polκ/ζ-dependent pathway and via another pathway mediated by Pols that remain to be identified.

Because biochemical studies with human Polκ have indicated that it is more proficient at the insertion step than at the extension step (11), and biochemical studies with yeast Polζ have shown that it is highly proficient at extending from an A placed opposite Tg (6), we propose a role for Polκ at the insertion step and of Polζ at the extension step of Tg bypass (Fig. 2). A Tg lesion is a block to replication at both the insertion and extension steps because the C5 methyl group protrudes in the axial direction that prevents the base 5' to the Tg lesion from stacking above it and that weakens the ability of the 5' template base for Watson–Crick hydrogen bonding (8–10). The structure of Polκ bound to DNA and dNTP suggests that it is well suited for inserting an A opposite the Tg lesion (26). The axial orientation of the C5 methyl group is unlikely to impinge on the 5' base in the Polκ active site because the 5' unpaired template strand is directed out of the active site cleft and is stabilized via interactions with the residues in the N-clasp of Polκ; additionally, the Tg lesion could be stabilized via interactions between its C5 methyl group and Met135 emanating from the fingers domain in Polκ active site (26). Because Polζ promotes proficient extension from nts opposite a variety of DNA lesions that distort the DNA helix and/or impair Watson–Crick base pairing, we presume that the inability of the 5' template base pair to properly stack above the Tg:A base pair and to form Watson–Crick hydrogen bonding is also not inhibitory for synthesis by Polζ.

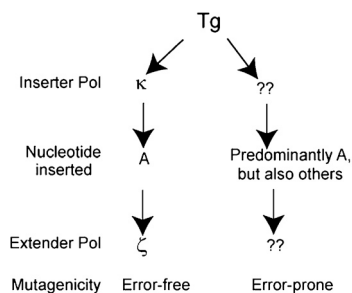


Fig. 2. Pathways for replicative bypass of a Tg (5R,6S or 5S,6R) lesion in human cells. The combined action of Polκ and Polζ, in which Polκ inserts an A opposite Tg and Polζ extends from the Tg:A base pair, promotes error-free replication through the lesion. In the alternative pathway controlled by TLS Pols that remain to be identified, although A is the predominant nt incorporated, other nts, in particular a T, are also inserted but with a low frequency; hence the alternative pathway is somewhat more mutagenic.

Although TLS opposite a Tg lesion generates only ~2% mutational events, our observations that this error frequency increases by over 2-fold in cells depleted of either Polk or Pol ζ would suggest that Pols κ and ζ mediate a more error-free mode of TLS than that mediated by the other as yet unidentified pathway (Fig. 2). The identification of Pols involved in the alternative TLS pathway could be important to establish the degree of error-free lesion bypass by Pols κ and ζ , because then one could determine whether upon the inactivation of the alternative pathway, TLS becomes so highly error-free that almost no mutational events are recovered.

Other Considerations. Our observations that Pols κ and ζ promote predominantly error-free replication through a Tg lesion raise a number of important issues. Because biochemical studies have indicated that Pol η is highly efficient at inserting an A opposite the Tg (5R,6S) lesion and it extends from the Tg:A base pair with only an ~15-fold reduction in efficiency (7), whereas Polk inserts an A opposite the Tg (5R,6S) lesion with an ~50-fold reduction in efficiency and is inhibited at extending from the Tg:A base pair by ~250-fold (11), one would have expected Pol η to function in TLS opposite the Tg lesion because then this Pol alone could have carried out both the insertion and extension steps. What then might have been the underlying basis for human cells to have evolved a strategy whereby they utilize two different polymerases—Polk and Pol ζ , instead of the highly efficient Pol η to perform TLS opposite the Tg lesion. These considerations might suggest that human and other mammalian cells have adapted the TLS machinery such that a more error-free Pol or a combination of Pols is utilized over a more efficient but also more error-prone Pol. Thus, although Pol η is very efficient at both the insertion and extension steps of TLS opposite the Tg lesion, it also is quite error-prone as it inserts a G opposite Tg and extends from the Tg:G base pair with only an ~8–10 fold reduction in efficiency (7). Polk on the other hand is reduced by ~3,000-fold in inserting a G opposite the 5R,6S isomer compared to insertion of an A opposite undamaged T (11). Hence, we presume that mechanisms exist in human cells that enable Polk and not Pol η to function in TLS opposite the Tg lesion. Also, because Polk is inhibited at the extension step, Pol ζ -mediated extension from the Tg:A base pair would lead to error-free lesion bypass.

How could an inherently mutagenic process such as TLS be made to act in a predominantly error-free manner in human cells? Whereas the basic structural features could account for the specificity, and to a certain degree for the efficiency and fidelity of lesion bypass by human TLS Pols, as for example, the ability of Pol η to accommodate the two templating residues of a *cis-syn* TT dimer in its active site (27, 28), or the ability of Pol η to push the templating purine bases into a *syn* conformation and the resultant Hoogsteen base pairing (17, 29, 30), or the structural features of Polk that could allow it to insert an A opposite Tg (26), the catalytic efficiencies and fidelities of these TLS Pols may be further modulated by proteins that these Pols associate with to carry out TLS in conjunction with the replication ensemble. Hence we presume that whereas such protein–protein interactions improve the catalytic efficiency and fidelity of Polk opposite Tg, they might have a more negative impact upon Pol η 's ability to act opposite Tg. Such a conjecture implies that in humans and

presumably in other mammalian cells, a complex network of protein–protein interactions affects the roles of TLS Pols such that an intrinsically less efficient but more error-free Pol is selected to carry out lesion bypass over a Pol that is more efficient but also more error-prone.

In summary, in spite of their intrinsic error-proneness, the mutagenic contributions of TLS Pols to the replicative bypass of DNA lesions are minimized not just by limiting their access to the replication ensemble only to when the replisome stalls at a lesion site and proliferating cell nuclear antigen (PCNA) is ubiquitinated (12), but also by their structural features providing for a high degree of specificity in lesion bypass. Protein–protein interactions and other modifications could additionally contribute to enhance their catalytic efficiency and fidelity opposite DNA lesions.

Materials and Methods

Construction of Plasmid Vectors Containing a Thymine Glycol. The 16-mer oligonucleotides containing the (5R, 6S) or the (5S, 6R) isomer of thymine glycol (Fig. 1A) were synthesized and purified. (for detailed information on method see *SI Text*). The in-frame target sequences of *lacZ'* gene in the resulting vectors are shown in Fig. 1B. Because in the DNA strand containing the Tg lesion, the *lacZ'* gene sequence is in-frame (Fig. 1B) and this strand carries also the *kan^r* gene (Fig. 1C), TLS through the Tg lesion will produce a blue colony on LB/*kan* plates with IPTG and X-gal. Because the other DNA strand has the *SpeI* sequence opposite the lesion and carries the *kan^r* gene (Fig. 1C), and the *lacZ'* sequence is out of frame in this strand (Fig. 1B), replication by template switching would generate white colonies.

Translesion Synthesis Assays and Mutational Analyses of TLS Products from Human Cells.

Human fibroblast cells were plated in 6-well plates at 70% confluence (approximately, 3×10^5 cells per well) and transfected with 100 pmole duplex siRNAs and the efficiency of siRNA inhibition of TLS Pols (Fig. S1) determined as described (19). For the simultaneous siRNA knockdown of two genes, 100 pmole siRNAs for each gene were mixed and transfected. After 48 h incubation, the heteroduplex target vector DNA (1 μ g) and 50 pmole of siRNA (second transfection) were cotransfected with Lipofectamine 2000 (Invitrogen). After 30 h incubation, plasmid DNA was rescued from cells by the alkaline lysis method and digested with *DpnI* to remove unreplicated plasmid DNA. The plasmid DNA was then transformed into *E. coli* XL1-Blue super competent cells (Stratagene). Transformed bacterial cells were diluted in 1 mL super optimal broth with catabolite repression (SOC) media and plated on both LB/amp (50 μ g/mL ampicillin, Sigma) and LB/*kan* (25 μ g/mL kanamycin, Sigma) plates containing 1 μ M isopropyl-1-thio- β -D-galactopyranoside (IPTG) (Roche) and 100 μ g/mL of X-Gal (Roche). After 16 h incubation at 37 $^{\circ}$ C, blue and white colonies were counted from kanamycin plates. The TLS frequency was determined from the number of blue colonies out of total colonies growing on LB/*kan* plates. Plasmid DNA obtained from blue colonies was analyzed to determine the mutation frequency and the mutational changes incorporated during TLS. The details of methods for construction of lesion-containing duplex plasmids, for siRNA knockdowns and for checking that the depletion of various TLS Pols was highly efficient, and for TLS and mutational analyses in human cells have been described previously (19).

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