Highly error-free role of DNA polymerase η in the replicative bypass of UV-induced pyrimidine dimers in mouse and human cells

Jung-Hoon Yoon, Louise Prakash, and Satya Prakash[1]

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch at Galveston, 301 University Boulevard, Galveston, TX 77555-1061

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Cyclobutane pyrimidine dimers (CPDs) constitute the most frequent UV-induced DNA photoproduct. However, it has remained unclear how human and other mammalian cells mitigate the mutagenic and carcinogenic potential of CPDs emanating from their replicative bypass. Here, we examine in human cells the roles of translesion synthesis (TLS) DNA polymerases (Pols) in the replicative bypass of a *cis-syn* TT dimer carried on the leading or the lagging strand DNA template in a plasmid system we have designed, and we determine in mouse cells the frequencies and mutational spectra generated from TLS occurring specifically opposite CPDs formed at TT, TC, and CC dipyrimidine sites. From these studies we draw the following conclusions: (*i*) TLS makes a very prominent contribution to CPD bypass on both the DNA strands during replication; (*ii*) Pols η, κ, and ζ provide alternate pathways for TLS opposite CPDs wherein Pols κ and ζ promote mutagenic TLS opposite CPDs; and (*iii*) the absence of mutagenic TLS events opposite a *cis-syn* TT dimer in human cells and opposite CPDs formed at TT, TC, and CC sites in mouse cells that we observe upon the simultaneous knockdown of Pols κ and ζ implicates a highly error-free role of Polη in TLS opposite CPDs in mammalian cells. Such a remarkably high in vivo fidelity of Polη could not have been anticipated in view of its low intrinsic fidelity. These observations have important bearing on how mammalian cells have adapted to avoid the mutagenic and carcinogenic consequences of exposure to sunlight.

DNA damage and repair | translesion synthesis | UV damage

UV light induces the formation of two major photoproducts, *cis-syn* cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts [(6-4) PPs]. Of the two lesions, (6-4) PPs are removed rapidly and efficiently by nucleotide excision repair (NER), whereas the removal of CPDs by NER occurs at a much slower rate (1); hence, CPDs constitute a much more important premutagenic lesion than (6-4) PPs, and it has been estimated that CPDs account for ≈80% of UV-induced mutations in mammalian cells (2). Because of their relatively high abundance, slow repair, and the fact that CPDs account for a large majority of UV-induced mutations (3, 4), in this study we determine the roles that human and mouse translesion synthesis (TLS) Pols η, ι, κ, and ζ play in TLS opposite CPDs and the relative contributions they make to their error-free vs. mutagenic bypass.

For TLS studies in human cells, we have designed a duplex plasmid system in which bidirectional replication ensues from a replication origin and proceeds through a site-specific *cis-syn* TT dimer carried on the leading or the lagging DNA strand template. Studies with this plasmid system in human cells have allowed us to make several unanticipated observations, some of which we note here: (*i*) opposite a *cis-syn* TT dimer, TLS makes an important contribution to lesion bypass and is predominantly error-free; (*ii*) different TLS Pols contribute similarly to lesion bypass on the leading and lagging DNA strands; (*iii*) Pols κ and ζ provide alternate means for TLS opposite the TT dimer, including for the mutagenic component of TLS; and (*iv*) Polη performs highly error-free TLS opposite TT dimer.

In addition to the formation of cyclobutane dimers at TT sites, UV induces the formation of dimers at 5′-TC-3′ and 5′-CC-3′ dipyrimidine sites, and both in yeast and humans, UV-induced mutations occur predominantly by a C-to-T transition at the 3′ base (5–8). To obtain a more comprehensive understanding of the error-free vs. mutagenic roles of TLS Pols in promoting replication through CPDs formed at various dipyrimidine sequences, we have analyzed UV mutagenesis in the c*II* transgene carried in a mouse cell line that additionally harbors a (6-4) PP photolyase gene so that (6-4) photoproducts can be selectively removed. From these mutational analyses we draw two conclusions: (*i*) whereas Pols κ and ζ promote mutagenic TLS, Polη carries out error-free TLS at an unexpectedly high rate opposite CPDs formed at various dipyrimidine sequences; (*ii*) Pols κ and ζ display a surprisingly high degree of specificity for the dipyrimidine sites upon which they respectively act. We discuss the implications of these results and various other observations for TLS opposite CPDs in human and mouse cells.

Results

Construction of Heteroduplex Target Vectors Containing a Site-Specific *cis-syn* TT Dimer. We have constructed duplex plasmid vectors containing a site-specific *cis-syn* TT dimer (Fig. S1*A Left*), where bidirectional replication initiates from an SV40 replication origin. In vivo studies with SV40 in mammalian cells and in vitro studies with reconstituted mammalian cell-free systems with circular plasmids that harbor an SV40 origin of replication sequence and require the SV40 T antigen, have shown that replication ensues from the SV40 origin and proceeds bidirectionally terminating approximately half way around the circular molecule from the initiation point (9–12).

The system we have designed is based on pBluescript (+/−) vector, where the sense (+) and the antisense (−) single stranded circular DNAs can be separately purified. The bacterial *lacZ′* gene in pBluescript is used for selection in the TLS assays. The multiple cloning site within the LacZ′ gene in pBluescript vector was replaced with a specific target sequence, one strand of that contains an *Spe*I site and the other strand carries a *cis-syn* TT dimer (Fig. S1*B Left*). Using such a plasmid system, we can obtain information not only on different aspects of TLS during replication in human cells, but can also distinguish any differences in TLS on the leading vs. the lagging DNA strand. For this reason, we constructed two vectors, one in which the lesion is located on the leading strand template (pBS vector), and the other where the lesion is on the lagging strand template (pSB vector) (Fig. S1*B Left*). The basic strategy for the construction of heteroduplex plasmid containing a site-specific

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[1]To whom correspondence should be addressed. E-mail: s.prakash@utmb.edu.

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Table 1. Effects of siRNA knockdowns of Pols η, ι, κ, and ζ on TLS opposite a *cis-syn* TT dimer carried on the leading or lagging strand DNA template in NER defective XPA human fibroblast (HF) cells

	Leading strand			Lagging strand		
siRNA	No. *Kan*$^+$ colonies	No. blue colonies among *Kan*$^+$	TLS, %*	No. *Kan*$^+$ colonies	No. blue colonies among *Kan*$^+$	TLS, %
NC siRNA	669	276	41.3	546	161	27.5
Polη siRNA	444	78	17.6	584	74	12.6
Polι siRNA	530	210	39.6	533	152	27.5
Polκ siRNA	526	155	29.5	596	113	18.8
Rev3 siRNA	632	197	31.2	769	166	21.6
Rev7 siRNA	740	228	30.8	455	82	18.0
Polη + Polι siRNA	548	94	17.2	552	72	13.0
Polη + Polκ siRNA	434	51	11.8	568	56	9.9
Polη + Rev3 siRNA	541	62	11.5	613	56	9.1
Polη + Rev7 siRNA	584	69	11.8	636	59	9.3
Polκ + Rev3 siRNA	559	121	21.6	664	89	13.4
Polκ + Rev7 siRNA	771	168	21.8	654	87	13.3

*Because the lesion-containing DNA strand in the duplex plasmid is *kan*$^+$ (the other strand being *kan*$^-$), and because TLS through the lesion would produce a blue colony, the TLS frequencies are determined from the number of blue colonies relative to the total colonies on LB + kan plates containing IPTG and X-gal.

cis-syn TT dimer on the leading or the lagging strand template is shown in Fig. S1*B* and described in the *SI Text*.

Contributions of Pols η, ι, κ, and ζ to TLS Opposite a *cis-syn* TT Dimer. To determine the roles of different TLS Pols in promoting replication through a *cis-syn* TT dimer in human cells, we used duplex siRNAs to inhibit their expression (13). That the siRNA knockdown was highly efficient was verified by RT-PCR (Fig. S2) and by Western blotting analysis (Fig. S3). To avoid the possibility of TT dimer removal by NER that would occur in normal human cells, we used XPA human fibroblast (HF) cells so that we could obtain a good estimate of the frequency with which TLS operates opposite this lesion in human cells. XPA(HF) cells were first transfected with siRNA for 48 h to inhibit the expression of the TLS Pol and then the site-specific lesion-containing plasmid and the siRNA were cotransfected into these pre-siRNA treated cells (see step IV in Fig. S1*B*). Cells were then incubated for ≈30 h to allow for the replication of plasmid, the plasmid was isolated (step V in Fig. S1*B*), and the frequencies of TLS (steps VI and VII in Fig. S1*B*), determined from the number of blue colonies relative to the total *Kan*$^+$ colonies, and the identity of the nucleotide, incorporated during TLS, were analyzed (Fig. S1*C*). As expected, with undamaged plasmid, the frequency of blue colonies among total *Kan*$^+$ colonies that result from the replication of undamaged TT-containing DNA strand was not altered in different experiments and with siRNA knockdowns of different TLS Pols regardless of whether the undamaged TT was on the leading or the lagging strand template. Hence, any reduction in the frequency of blue colonies among the total *Kan*$^+$ colonies with damaged plasmid upon depletion of a TLS Pol represents the contribution of that Pol to TLS opposite the lesion.

In XPA cells treated with control (NC) siRNA (Table 1), TLS contributes to lesion bypass on the leading strand to a higher extent (≈41%) than on the lagging strand (≈27%), and these differences for the two DNA strands are highly significant ($P < 0.0001$). Template switching using copy choice type of DNA synthesis or recombinational repair presumably accounts for the remainder of lesion bypass. For both the DNA strands in XPA cells, Polη knockdown conferred ≈50% reduction in the frequency of TLS compared with that in control cells treated with NC siRNA. Upon Polι knockdown, we observed no reduction in TLS frequencies opposite the TT dimer on either DNA strand, and even the simultaneous knockdown of Pols η and ι caused no further reduction in TLS frequencies over that seen upon the knockdown of Polη alone (Table 1). Interestingly, Polκ knockdown resulted in ≈30% reduction in the frequency of TLS opposite the TT dimer carried on either of the DNA strands, and the knockdown of either Polζ subunit—Rev3 or Rev7—conferred a reduction in TLS frequencies similar to that from Polκ knockdown (Table 1). The simultaneous knockdown of Polζ and Polκ led to a further reduction in TLS frequencies over that seen upon the knockdown of either Pol alone. TLS on both the DNA strands was reduced by ≈50% upon Polζ plus Polκ knockdown compared with the 20–30% reduction seen upon the knockdown of either Pol alone (Table 1). To determine whether Pols κ and ζ function independently of Polη, we examined TLS frequencies opposite the TT dimer upon the simultaneous knockdowns of Polη with Polκ and of Polη with Polζ. An approximate 30% further reduction in the frequencies of TLS occurs in cells upon the simultaneous knockdown of Polη with Polκ and of Polη with Rev3 compared with the results seen for Polη knockdown alone (Table 1). We verified that for both the DNA strands, the reductions observed in TLS frequencies upon Polη knockdown or upon the simultaneous knockdown of Pols κ and ζ were in fact statistically highly significant ($P \ll 0.0001$). From these observations we infer that Pols η, κ, and ζ provide alternative means of promoting replication through a *cis-syn* TT dimer.

To further ascertain the inference that Pols η, κ, and ζ provide three alternate means of TLS opposite a *cis-syn* TT dimer and that Polι makes no contribution to TLS opposite this lesion, we examined the effects of knockdowns of these Pols in an XPV cell line. As shown in Table 2, we found that whereas the knockdown of Polι has no effect on TLS for either DNA strand, the knockdown of either Polκ or Polζ conferred a decrease in TLS frequencies, and a further decrease in TLS occurred upon their simultaneous knockdown. For both the DNA strands, the reduction in TLS frequencies that we observe in XPV cells upon the simultaneous knockdown of Pols κ and ζ, compared with that for control (NC) siRNA-treated cells, is very highly significant ($P \ll 0.0001$), and it is very likely the much reduced level of TLS that still remains after the knockdowns of both Pols κ and ζ in XPV cells derives from the residual levels of these Pols that persist in such cells.

Mutational Analyses of Products Resulting from TLS Through a *cis-syn* TT Dimer. Sequence analyses of TLS products resulting from TT dimer bypass indicate that on both DNA strands, replication through this UV lesion occurs predominantly in an error-free manner, because in XPA cells transfected with control (NC) siRNA only ≈2% of the TLS products carry mutations resulting from a G or a T insertion opposite the 3′T or the 5′T (Tables S1 and S2). Compared with control XPA cells, the mutation frequency rises

Table 2. Effects of siRNA knockdowns of Pols η, ι, κ, and ζ on TLS opposite a *cis-syn* TT dimer carried on the leading or lagging strand DNA template in Polη defective XPV (XP30R0) human fibroblast cells

	Leading strand			Lagging strand		
siRNA	No. *Kan*$^+$ colonies	No. blue colonies among *Kan*$^+$	TLS, %	No. *Kan*$^+$ colonies	No. blue colonies among *Kan*$^+$	TLS, %
NC siRNA	440	55	12.5	514	52	10.1
Polι siRNA	430	54	12.6	452	47	10.4
Polκ siRNA	434	35	8.1	338	20	5.9
Rev3 siRNA	419	34	8.1	343	21	6.1
Rev7 siRNA	384	30	7.8	397	25	6.3
Polκ + Rev3 siRNA	369	15	4.1	296	10	3.4
Polκ + Rev7 siRNA	339	13	3.8	332	10	3.0

approximately 2-fold for TLS on both DNA strands upon Polη knockdown, but the mutational pattern remains approximately the same as in control XPA cells, except for the incorporation of a C opposite the 3′T that is observed only in cells deficient for Polη (Tables S1 and S2).

In XPA cells with Polι knockdown, we observed no significant change in mutation frequencies or in mutational pattern for TLS on either DNA strand (Tables S1 and S2). We also determined whether simultaneous knockdown of Pols η and ι causes any change in mutation frequencies or in mutational pattern from that observed upon Polη knockdown alone. As shown in Table S1, however, we found no additional effect of Polι knockdown in cells with diminished Polη expression. To further verify the lack of involvement of Polι in TT dimer bypass, we sequenced the TLS products from XPV cells transfected with Polι siRNA. However, the mutational analyses of approximately 300 TLS products showed no differences in either the frequency or the pattern of mutations generated upon Polι knockdown in XPV cells from that in XPV cells transfected with control siRNA. Thus, all our observations indicate that Polι plays no significant role in promoting replication through a *cis-syn* TT dimer.

We find that the knockdown of Polκ or the Rev3 or Rev7 subunits of Polζ confers ≈50–70% reduction in mutation frequency for TLS opposite the TT dimer on both the DNA strands (Tables S1 and S2). The simultaneous knockdown of Polκ with Rev3 or with Rev7 resulted in a further decline in the incidence of mutagenic TLS; and most interestingly, among almost 2,000 TLS products analyzed for both the DNA strands, we could find no evidence of a mutational change (Tables S1 and S2). A comparison of pooled data for both the DNA strands in XPA cells treated with control siRNA vs. in XPA cells, where both Pols κ and ζ have been knocked down, shows that the observed differences between them are very highly significant ($P \ll 0.0001$).

In summary, our findings that Pols κ and ζ provide alternate pathways for TLS opposite a TT dimer and that these two Pols are responsible for all of the mutagenic TLS through the TT dimer have allowed us to deduce the important conclusion that Polη functions in TLS opposite TT dimer in humans with a very high fidelity, and as we discuss in the next section, from studies in mouse cells we are able to verify and extend this conclusion to CPDs not only at TT dipyrimidine sites, but at TC and CC sites as well.

Contributions of TLS Polymerases to UV Mutagenesis Resulting from Bypass of CPDs at TT, TC, and CC Sites in Mouse Cells. Although our analyses with a site-specific *cis-syn* TT dimer carried on the leading or the lagging strand DNA template are highly informative for the relative contributions of TLS to lesion bypass and for the roles different Pols play in TLS opposite this lesion in human cells, the plasmid system does not allow us to examine TLS opposite CPDs at TC and CC sites. Because UV-induced mutations occur predominantly by a C-to-T transition at the 3′C of CPDs, it becomes important to examine TLS through such sites as well. Therefore, to obtain a more comprehensive view of the roles of various Pols in TLS opposite CPDs formed at various dipyrimidine sites, we used the big blue mouse embryonic fibroblast (BBMEF) cell line in which 40 copies of λLIZ shuttle vector have been integrated into the genome. This vector carries two reporter genes—*lacI* and c*II*—for mutational analyses (2). We chose to examine UV mutations in the c*II* gene because it is only 294-bp long and hence more convenient for sequence analyses (14). This system has been used extensively for mutational analyses in mammalian cells and it shows similar mutational responses to different DNA damaging agents as those observed with endogenous chromosomal genes (2, 8, 15). To measure the frequencies and types of mutations generated from TLS opposite CPDs, we selectively removed the (6-4) photoproducts (PPs) from the genome by expressing a (6-4) photolyase gene in the mouse embryonic fibroblast cell line, as described in ref. 2. As has been reported (2), the experimental protocol we have used allows for the complete removal of (6-4) PPs and we have independently verified this fact.

To examine the role of various Pols in promoting error-free vs. error-prone TLS opposite CPDs formed at TT, TC, and CC sites, we used siRNA knockdown in big blue mouse embryonic fibroblasts expressing the (6-4) PP photolyase and determined the frequencies of UV-induced mutations in the c*II* gene. The highly efficient siRNA knockdown of mouse Pols η, ι, κ, and Rev3 was verified by RT-PCR (Fig. S4), and this knockdown efficiency was maintained for at least 4 days. The frequency of mutations in the c*II* gene in unirradiated cells treated with control (NC) siRNA and with or without exposure to photoreactivating light was $\approx 16 \times 10^{-5}$ (Table 3), and in non-UV-irradiated cells, the siRNA knockdown of any of the Pols η, ι, κ, or Rev3 singly or in combinations had no effect on this mutation frequency.

Table 3. UV-induced mutation frequencies in the c*II* gene in (BBMEF) mouse cells expressing a (6-4)PP photolyase and treated with siRNAs for different TLS Pols

siRNA	UV*	PR†	Mutation frequency,‡ × 10^{-5}
NC§	−	−	16.5 ± 2.8
NC	−	+	15.2 ± 3.4
NC	+	−	56.8 ± 3.2
NC	+	+	45.5 ± 4.3
Polη	+	+	106.2 ± 5.8
Polι	+	+	44.6 ± 2.4
Polκ	+	+	29.8 ± 3.2
Rev3	+	+	30.2 ± 4.3
Polκ + Rev3	+	+	16.8 ± 2.6

*5 J/m^2 of UVC (254 nm) light.
†Photoreactivation with UVA (360 nm) light for 3 h.
‡Mutation frequency data were obtained from averages of 7 independent experiments.
§NC, negative control siRNA.

As shown in Table 3, when mouse cells expressing the (6-4) PP photolyase were UV irradiated (5 J/m^2) but not exposed to photoreactivating light, the c*II* mutation frequency increased ≈4-fold to ≈57.0 × 10^{-5}. To estimate the contribution of CPDs to this mutation frequency, the UV-irradiated cells were exposed to photoreactivating light for 3 h to remove (6-4) PPs by the action of the (6-4) PP photolyase. Photoreactivation reduced the mutation frequency to ≈45 × 10^{-5}, indicating that under these experimental conditions almost 80% of UV mutations result from TLS opposite CPDs (Table 3). In the cell line that was transfected with the vector only control instead of the plasmid harboring the (6-4) PP photolyase gene, the mutation frequency remained the same (≈57 × 10^{-5}) with or without photoreactivating treatment.

Next, we examined the effects of knockdowns of Pols η, ι, κ, or Rev3, and of the simultaneous knockdown of Polκ and Rev3 on UV-induced c*II* mutation frequencies, resulting from TLS opposite CPDs. Whereas compared with the mutation frequency of ≈45 × 10^{-5} in NC siRNA-treated cells, the knockdown of Polη conferred an >2-fold increase in mutation frequency to ≈105 × 10^{-5}, the knockdown of Polι had no effect on mutation frequencies opposite CPDs (Table 3). The knockdown of either Polκ or Rev3, the catalytic subunit of Polζ, reduced mutation frequency from ≈45 × 10^{-5} by >30% to ≈30 × 10^{-5}. Interestingly and most importantly, the simultaneous knockdown of Polκ and Rev3 led to a drastic decline in the mutation frequency to ≈17 × 10^{-5}, which is very similar to mutation frequencies (≈16 × 10^{-5}) observed in unirradiated cells (Table 3). We draw two conclusions from these observations: (*i*) Pols κ and ζ contribute almost equally to TLS opposite CPDs formed at various dipyrimidine sites, and they are responsible for almost all of the mutagenic component of TLS opposite CPDs. (*ii*) Our observation that the simultaneous inactivation of Pols κ and ζ reduces mutation frequency to a level that is very similar to that in undamaged cells leads us to conclude that in vivo, Polη carries out TLS opposite CPDs formed at TT, TC, or CC dipyrimidine sites in a highly error-free manner.

Next, we determined the types of mutations that were formed in UV-irradiated BBMEF cells expressing (6-4) PP photolyase and exposed to photoreactivating light. As shown in Table S3, in cells treated with control (NC) siRNA ≈65% of UV-induced mutations were C-to-T transitions, and the preponderance of C-to-T transitions remained approximately the same in cells treated with siRNAs for Pols η, κ, or Rev3; by contrast, the simultaneous knockdown of Pols κ and Rev3 led to a reduction in the frequency of C-to-T transitions, similar to that in unirradiated cells.

In Fig. 1, we compare the pattern of mutations resulting from TLS opposite CPDs in the c*II* gene after the knockdown of different Pols. In control cells treated with NC siRNA, the preponderance of UV-induced mutations are seen to occur at particular dipyrimidine sequences that we have labeled with nos. 1–11 (Fig. 1*A*). Although the frequency of UV-induced mutations is elevated upon Polη knockdown (Table 3), the pattern of mutational hotspots upon Polη knockdown resembles that in control cells (Fig. 1*A*). In striking contrast, a remarkably distinct pattern of UV-induced mutations is observed upon Polκ vs. Rev3 knockdown, given that a very different set of hotspots remain after the knockdown of Polκ (nos. 4, 5, 7, 8) vs. the knockdown of Rev3 (nos. 1, 2, 3, 6); whereas the incidence of mutations at hotspot positions 9–11 is greatly diminished upon the knockdown of either Polκ or Rev3 (Fig. 1*B*). Remarkably, none of the UV-induced mutational hot spots that occur in cells treated with control siRNA or with Polη siRNA remain when both Polκ and Rev3 have been simultaneously knocked down, and the overall distribution and pattern of mutations in these cells becomes very similar to that seen in non-UV-irradiated cells (Fig. 1*C*).

Discussion

DNA Polymerases η, κ, and ζ Provide Three Alternate Pathways for TLS Through a *cis-syn* TT Dimer During Replication in Human Cells. From analyses of TLS opposite a *cis-syn* TT dimer in human cells, we make the following observations: (*i*) TLS makes a prominent contribution to CPD bypass on both DNA strands (30–40%); (*ii*) although TLS occurs less frequently on the lagging strand than on the leading strand, the relative contributions and the mutagenic vs. the error-free roles of different TLS Pols are similar for both DNA strands; (*iii*) TLS opposite a TT dimer occurs in a predominantly error-free manner: only ≈2% of TLS events are mutagenic and all of the mutations involve a base change at the 3′T or 5′T of the dimer; and (*iv*) Pols η, κ, and ζ provide three alternate pathways of TLS opposite a TT dimer, with Polη responsible for ≈50% of lesion bypass and Pols κ and ζ contributing about equally and accounting for the remainder of TLS. Although the observations that UV-induced mutation frequencies increase in XPV cells had indicated an error-free role of Polη in replicating through CPDs, from such observations one could not have known how frequently Polη generates errors during TLS in human cells. That is because the increase in mutation frequencies in XPV cells is indicative only of Polη being more error-free than the Pol(s) that replicate through the lesion in the absence of Polη, but that yields no information on the extent by which Polη is error-free in human cells. In this study, the identification of Pols κ and ζ as the alternate mutagenic TLS pols opposite a TT dimer has enabled us to examine this issue in depth, and our observation that we could find no mutagenic events among the almost 2,000 TLS products examined from human cells, where both Pols κ and ζ have been depleted, suggested that the error rate of TLS by Polη opposite a TT dimer could be approximately 1 × 10^{-3}. However, as we discuss this point further in the next section, we estimate that Polη's error rate opposite CPDs is likely to be considerably lower than that.

Polη Carries Out Highly Error-Free TLS Opposite CPDs at TC, CC, and TT Dipyrimidine Sites. From our plasmid studies in human cells, we could infer that Polη carries out TLS opposite a *cis-syn* TT dimer with an error frequency of approximately 1 × 10^{-3}. Because this fidelity estimate was restricted to a TT dimer and because the need for sequencing TLS products greatly limited the number of mutational events that could be analyzed, we resorted to examining the frequency of UV-induced mutations that would result from TLS opposite CPDs formed at TC, CC, and TT dipyrimidine sites in the c*II* transgene in mouse cells. Interestingly and importantly, with this system we found that whereas the knockdown of either Polκ or Polζ led to a reduction in the frequency of UV-induced mutations, the simultaneous knockdown of both Pols κ and ζ lowered the frequency of UV-induced mutations to nearly the same level as that in unirradiated cells. Furthermore, in cells knocked down for both Pols κ and ζ, the increase in the frequency of C-to-T transitions that is so characteristic of mutagenic TLS of CPDs at TC and CC sites was reduced to almost the same level as in unirradiated control cells, and the mutational spectra of UV-induced mutations opposite CPDs in these cells resembled closely the pattern of mutations that occur spontaneously. We conclude from these various observations that Polη from humans and mice carries out TLS opposite CPDs formed at TC, CC, and TT sites with an error frequency that does not confer a significant increase in spontaneous mutation frequency. Such a high fidelity of mammalian Polη opposite CPDs could not have been anticipated because steady-state kinetic studies have indicated that both on undamaged and *cis-syn* TT dimer-containing DNAs, human Polη misincorporates nucleotides with a frequency of ≈10^{-2}, and in an in vitro gap-filling assay, human Polη introduced 1 error in ≈30 nucleotides incorporated (16, 17). Thus, by contrast to its very low intrinsic fidelity indicated from biochemical studies, in mouse and human cells Polη carries out TLS opposite CPDs with a substantially higher fidelity. We presume that Polη achieves its high fidelity opposite CPDs in mammalian cells via its association with protein factors that remain to be identified.

Polι Has No Role in TLS Opposite CPDs. We find that the Polι knockdown has no effect on TLS opposite the *cis-syn* TT dimer in

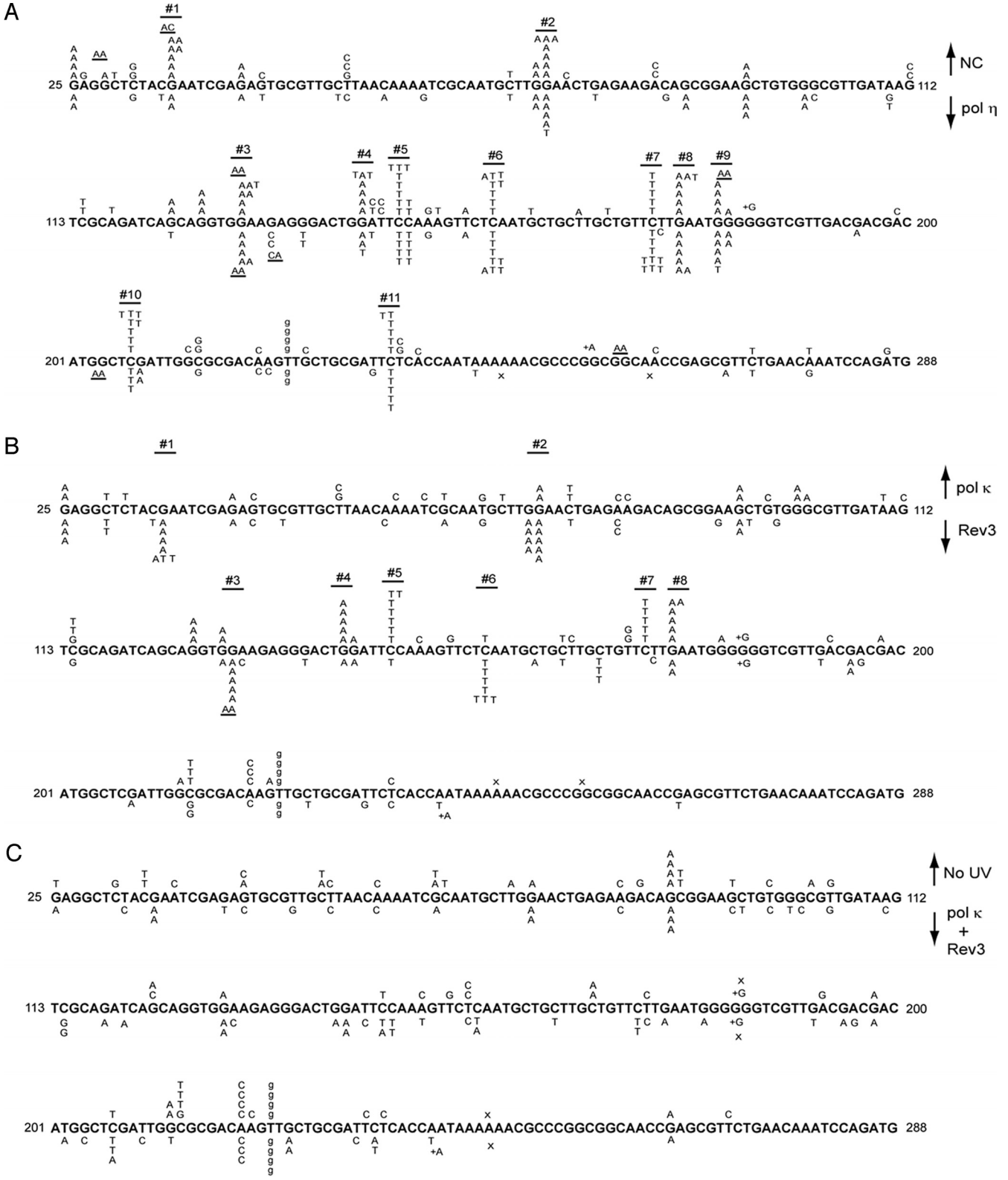

Fig. 1. Mutational spectra resulting from TLS opposite CPDs in (6-4) PP photolyase expressing (BBMEF) mouse cells treated with siRNAs for different TLS Pols. Mutational spectrum was determined for the *cII* gene in cells exposed to 5 J/m^2 of UV light. Because the UV-induced mutational hotspots are clustered in the *cII* gene from nucleotide positions 25–288, only this region of the sequence is shown. The positions of UV-induced hot spots (nos. 1–11) are indicated. A frequently occurring spontaneous mutational hot spot is indicated by lowercase letter 'g' at position 223. (*A*) Mutations in UV-irradiated cells treated with control (NC) siRNA (above the sequence) and treated with Polη siRNA (below the sequence). (*B*) Mutations in UV-irradiated cells treated with Polκ siRNA (above the sequence) and with Rev3 siRNA (below the sequence). Note the strong demarcation of mutational hotspots generated by Polκ vs. Polζ (Rev3) action. (*C*) Mutational changes in UV-irradiated cells treated with both Polκ and Rev3 siRNAs (below the sequence) and in non-UV-irradiated cells (above the sequence). Note the absence of any significant UV-induced hotspots 1–11 in cells treated with Polκ and Rev3 siRNAs, and note also the similarity of the mutational pattern in UV-irradiated cells treated with Polκ and Rev3 siRNAs with that in unirradiated cells.

plasmid assays in human cells or on the frequency of mutations resulting from TLS through CPDs formed at various dipyrimidine sites in the c*II* gene in mouse cells. The lack of any involvement of Polι in CPD bypass is in keeping with the biochemical studies indicating that this Pol is strongly inhibited in synthesizing DNA opposite a *cis-syn* TT dimer (18, 19). Also, structural studies have indicated that the two covalently linked pyrimidine residues of a CPD could not be accommodated in the Polι active site (20–22). And moreover, even opposite undamaged T and C residues, Polι inserts nucleotides with a very low efficiency and fidelity (18, 19). Hence, from all of these different observations, we can infer that Polι plays no significant role in TLS opposite CPDs.

TLS Through CPDs in Mammalian Cells. From considerations of our analyses of TLS through a *cis-syn* TT dimer in plasmid assays in human cells and of c*II* mutational assays in mouse cells, we propose a scheme for TLS through the CPDs in human and other mammalian cells (Fig. S5). In this scheme, Polη carries out predominantly error-free TLS through the CPDs, and Pols κ and ζ provide alternate pathways of TLS in which mutations could arise at a frequency of $\approx 5 \times 10^{-2}$. Because Pols κ and ζ would act at the extension step, there must be an as yet unidentified DNA polymerase that is able to insert nucleotides opposite CPDs, and we presume that even though this polymerase inserts a wrong nucleotide opposite CPDs, it does so with an error frequency of $\approx 5 \times 10^{-2}$.

The roles of Pols κ and ζ in extending from the nucleotide inserted opposite the 3′ residue of a CPD by another Pol have been indicated from biochemical studies (19, 23); whereas both these Pols are strongly inhibited from inserting a nucleotide opposite the 3′T of the lesion (19, 24, 25). Furthermore, structural studies with Polκ have shown that its active site is very constrained, and it could not accommodate the two Ts of a TT dimer; hence Polκ is unable to act at the insertion step. However, there are no structural constraints for Polκ performing the extension step (26).

Although the involvement of Polη in error-free TLS through CPDs has been suggested from the increase in UV mutation frequencies observed in XPV cells (27, 28), and biochemical evidence and structural studies have indicated a specialized role of Polη in efficient CPD bypass because of its unique ability to accommodate both the pyrimidine residues of a CPD in its active site (16, 29–31), the high fidelity of CPD bypass by Polη in mouse and human cells that our studies have revealed could not have been anticipated from any of the previous observations. This conclusion was made possible from our finding that Pols κ and ζ provide alternate means of mutagenic TLS and that in their absence, the TLS that is carried out by Polη is virtually error-free.

Materials and Methods

For TLS studies in human cells, we constructed a duplex plasmid system that carries an SV40 replication origin and in which a *cis-syn* TT dimer is present on the leading or the lagging DNA strand; hence, TLS through the DNA lesion can be separately analyzed for the two strands during replication. We used siRNA treatment to achieve high efficiency depletion of various TLS Pols in human cells and determined the effects of their depletion on the frequency of TLS and analyzed whether TLS occurred in an error-free or mutagenic manner. To examine the roles of various Pols in mutagenic vs. error-free TLS opposite CPDs formed at TT, TC, and CC dipyrimidine sites, frequencies of UV-induced mutations were examined in the *cII* gene carried in big blue mouse embryonic fibroblast (BBMEF) cells in which (6-4) photoproducts were specifically removed from the genome by expressing a (6-4) photolyase gene and where the TLS Pols had been depleted by siRNA treatment.

The detailed methods for plasmid constructions, TLS assays, and mutational analyses of TLS opposite a *cis-syn* TT dimer in human cells are given in *SI Text* and shown in Fig. S1. The methods for siRNA knockdown of TLS Pols and for verifying their knockdown efficiency in human cells are also described in *SI Text*, and the related information is provided in Tables S4 and S5 and Figs. S2 and S3. The detailed methods for mutational studies opposite CPDs in mouse cells, including the description of mutational analysis of the c*II* gene, siRNA knockdowns of TLS Pols, and the verification of their knockdown efficiency are also given in *SI Text* and the related information is included in Tables S6 and S7 and Fig. S4.

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1. Sancar A (1996) DNA excision repair. *Ann Rev Biochem* 65:43–81.
2. You Y-H, Lee D-H, Yoon J-H, Nakajima S, Yasui A, Pfeifer GP (2001) Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. *J Biol Chem* 276:44688–44694.
3. Pfeifer GP (1997) Formation and processing of UV photoproducts: Effects of DNA sequence and chromatin envirnoment. *Photochem Photobiol* 65:270–283.
4. Yoon J-H, Lee CS, O'Connor TR, Yasui A, Pfeifer GP (2000) The DNA damage spectrum produced by simulated sunlight. *J Mol Biol* 299:681–693.
5. Armstrong JD, Kunz BA (1990) Site and strand specificity of UVB mutagenesis in the *SUP4-o* gene of yeast. *Proc Natl Acad Sci USA* 87:9005–9009.
6. Brash DE (1997) Sunlight and the onset of skin cancer. *Trends Genet* 13:410–414.
7. Canella KA, Seidman MM (2000) Mutation spectra in supF: Approaches to elucidating sequence context effects. *Mutat Res* 450:61–73.
8. You Y-H, Pfeifer GP (2001) Similarities in sunlight-induced mutational spectra of CpG-methylated transgenes and the *p53* gene in skin cancer point to an important role of 5-methylcytosine rsidues in solar UV mutagenesis. *J Mol Biol* 305:389–399.
9. Bullock PA, Seo YS, Hurwitz J (1991) Initiation of simian virus 40 DNA synthesis in vitro. *Mol Cell Biol* 11:2350–2361.
10. Danna KJ, Nathans D (1972) Bidirectional replication of simian virus 40 DNA. *Proc Natl Acad Sci USA* 69:3097–3100.
11. Fareed GC, Garon CF, Salzman NP (1972) Origin and direction of simian virus 40 deoxyribonucleic acid replication. *J Virol* 10:484–491.
12. Li JJ, Kelly TJ (1985) Simian virus 40 DNA replication in vitro: Specificity of initiation and evidence for bidirectional replication. *Mol Cell Biol* 5:1238–1246.
13. Elbashir SM, Harboth J, Lendeckel W, Yalcin A, Weber K, Tuschi T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494–498.
14. Jakubczak JL, et al. (1996) Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage lambda transgene target. *Proc Natl Acad Sci USA* 93:9073–9078.
15. Besaratinia A, Pfeifer GP (2006) Investigating human cancer etiology by DNA lesion footprinting and mutagenicity analysis. *Carcinogenesis* 27:1526–1537.
16. Johnson RE, Washington MT, Prakash S, Prakash L (2000) Fidelity of human DNA polymerase η. *J Biol Chem* 275:7447–7450.
17. Matsuda T, Bebenek K, Masutani C, Hanaoka F, Kunkel TA (2000) Low fidelity DNA synthesis by human DNA polymerase η. *Nature* 404:1011–1013.
18. Haracska L, et al. (2001) Targeting of human DNA polymerase ι to the replication machinery *via* interaction with PCNA. *Proc Natl Acad Sci USA* 98:14256–14261.
19. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L (2000) Eukaryotic polymerases ι and ζ act sequentially to bypass DNA lesions. *Nature* 406:1015–1019.
20. Jain R, Nair DT, Johnson RE, Prakash L, Prakash S, Aggarwal AK (2009) Replication across template T/U by human DNA polymerase-ι. *Structure* 17:974–980.
21. Nair DT, Johnson RE, Prakash S, Prakash L, Aggarwal AK (2004) Replication by human DNA polymerase ι occurs via Hoogsteen base-pairing. *Nature* 430:377–380.
22. Nair DT, Johnson RE, Prakash S, Prakash L, Aggarwal AK (2006) An incoming nucleotide imposes an *anti* to *syn* conformational change on the templating purine in the human DNA polymerase-ι active site. *Structure* 14:749–755.
23. Washington MT, Johnson RE, Prakash L, Prakash S (2002) Human *DINB1*-encoded DNA polymerase κ is a promiscuous extender of mispaired primer termini. *Proc Natl Acad Sci USA* 99:1910–1914.
24. Haracska L, Prakash L, Prakash S (2002) Role of human DNA polymerase κ as an extender in translesion synthesis. *Proc Natl Acad Sci USA* 99:16000–16005.
25. Johnson RE, Prakash S, Prakash L (2000) The human *DINB1* gene encodes the DNA polymerase Polθ. *Proc Natl Acad Sci USA* 97:3838–3843.
26. Lone S, et al. (2007) Human DNA polymerase κ encircles DNA: Implications for mismatch extension and lesion bypass. *Mol Cell* 25:601–614.
27. Wang Y-C, Maher VM, Mitchell DL, McCormick JJ (1993) Evidence from mutation spectra that the UV hypermutability of xeroderma pigmentosum variant cells reflects abnormal, error-prone replication on a template containing photoproducts. *Mol Cell Biol* 13:4276–4283.
28. Waters HL, Seetharam S, Seidman MM, Kraemer KH (1993) Ultraviolet hypermutability of a shuttle vector propagated in xeroderma pigmentosum variant cells. *J Invest Dermatol* 101:744–748.
29. Johnson RE, Prakash L, Prakash S (2005) Distinct mechanisms of *cis-syn* thymine dimer bypass by Dpo4 and DNA polymerase η. *Proc Natl Acad Sci USA* 102:12359–12364.
30. Trincao J, Johnson RE, Escalante CR, Prakash S, Prakash L, Aggarwal AK (2001) Structure of the catalytic core of *S. cerevisiae* DNA polymerase η: Implications for translesion DNA synthesis. *Mol Cell* 8:417–426.
31. Washington MT, Prakash L, Prakash S (2003) Mechanism of nucleotide incorporation opposite a thymine-thymine dimer by yeast DNA polymerase η. *Proc Natl Acad Sci USA* 100:12093–12098.