

Quantitative Analysis of Translesion DNA Synthesis across a Benzo[a]pyrene-Guanine Adduct in Mammalian Cells

THE ROLE OF DNA POLYMERASE κ *

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Replication across unrepaired DNA lesions in mammalian cells is effected primarily by specialized, low fidelity DNA polymerases. We studied translesion DNA synthesis (TLS) across a benzo[a]pyrene-guanine (BP-G) adduct, a major mutagenic DNA lesion generated by tobacco smoke. This was done using a quantitative assay that measures TLS indirectly, by measuring the recovery of gapped plasmids transfected into cultured mammalian cells. Analysis of *PolK*^{+/+} mouse embryo fibroblasts (MEFs) showed that TLS across the BP-G adduct occurred with an efficiency of $48 \pm 4\%$, which is an order of magnitude higher than in *Escherichia coli*. In *PolK*^{-/-} MEFs, bypass was $16 \pm 1\%$, suggesting that at least two-thirds of the BP-G adducts in MEFs were bypassed exclusively by polymerase κ (*pol* κ). In contrast, *pol* η was not required for bypass across BP-G in a human XP-V cell line. Analysis of misinsertion specificity across BP-G revealed that bypass was more error-prone in MEFs lacking *pol* κ . Expression of *pol* κ from a plasmid introduced into *PolK*^{-/-} MEFs restored both the extent and fidelity of bypass across BP-G. *Pol* κ was not required for bypass of a synthetic abasic site. *In vitro* analysis demonstrated efficient bypass across BP-G by both *pol* κ and *pol* η , suggesting that the biological role of *pol* κ in TLS across BP-G is due to regulation of TLS and not due to an exclusive ability to bypass this lesion. These results indicate that BP-G is bypassed in mammalian cells with relatively high efficiency and that *pol* κ bypasses BP-G *in vivo* with higher efficiency and higher accuracy than other DNA polymerases.

Genomic DNA is constantly subject to damage caused by both external agents, such as sunlight, and endogenous chemicals, such as reactive oxygen species. Most of this damage is eliminated by error-free DNA repair mechanisms, thereby restoring the DNA to its native sequence (1). However, a significant number of lesions escape repair and might therefore interfere with DNA replication and gene expression. Such in-

terference can be mitigated by DNA damage tolerance mechanisms, primarily translesion DNA synthesis (TLS¹; also termed translesion replication) (2–5) and postreplicative recombinational repair (1, 6–8). The key components in TLS are low fidelity DNA polymerases that specialize in lesion bypass (9–12). These proteins were conserved in evolution and are present in organisms ranging from *Escherichia coli* to humans (13). Humans contain at least four specialized DNA polymerases belonging to the Y superfamily (*pol* η , *pol* κ , *pol* λ , and REV1) as well as several from other polymerase families (e.g. *pol* ζ (14), *pol* μ (15, 16), and *pol* λ (16, 17)). Many of these polymerases have been implicated in TLS *in vitro* (18–24). However, there is a paucity of information about the efficiency and fidelity with which they support lesion bypass in living cells.

Pol η has a well established biological role in TLS, since it is mutated in all patients examined with the variant form of the hereditary disease xeroderma pigmentosum (10, 18). This disease is characterized by sensitivity to sunlight and a marked predisposition to skin cancer. Correspondingly, cells from xeroderma pigmentosum patients are sensitive to and hypermutable by UV light (1). Purified human *pol* η replicates across TT cyclobutyl pyrimidine dimers or normal TT sequences with similar efficiency and accuracy (19, 20). These observations suggest that in cells in which *pol* η is inactivated, the bypass of these lesions is effected by other DNA polymerases with reduced efficiency (resulting in increased UV sensitivity) and reduced fidelity (resulting in hypermutability and skin cancer). Evidence for a function in TLS *in vivo* has also been provided for *pol* ζ and hREV1. In these cases, decreased expression of the relevant genes with antisense RNA led to reduced UV mutability (14, 25).

In vivo experiments showed that fibroblasts in culture, derived from *PolK*^{-/-} mouse embryos, are sensitive to both killing and mutagenesis by BP, relative to otherwise isogenic cells from *PolK*^{+/+} mouse embryos, suggesting that *pol* κ is involved in bypass across BP-G adducts *in vivo* (26). *In vitro* experiments have shown that purified *pol* κ efficiently bypasses BP-G adducts, preferentially incorporating the correct nucleotide dCMP (21–23, 27). In this study, we used a quantitative indirect TLS assay to specifically analyze bypass across a site-specific BP-G adduct in mammalian cells. We found that 1) BP-G is bypassed in mammalian cells with a relatively high efficiency of 35–50%, an order of magnitude higher than in

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¹ The abbreviations used are: TLS, translesion DNA synthesis; BP, benzo[a]pyrene; BPDE, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide; BP-G, (+)-*trans*-benzo[a]pyrene-guanine adduct; MEF, mouse embryo fibroblasts; *pol*, DNA polymerase; MOPS, 4-morpholinepropanesulfonic acid.

E. coli, 2) polk is responsible for at least two-thirds of lesion bypass events, and 3) bypass by polk occurs with lower mutagenicity than bypass by other DNA polymerases.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride; minimum essential medium Eagle with Earle's salts and essential and nonessential amino acids; 100 mM glutamine; and fetal calf serum were from Sigma. Dulbecco's modified Eagle's medium and a mixture of penicillin and streptomycin for cell culture were from Invitrogen. JetPEI was from Polyplus-transfection (Illkirch, France).

Proteins—Restriction nucleases, *E. coli* polymerase I Klenow fragment, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs. Human DNA polymerases η , κ , and ι were from Enzymax (Lexington, KY).

DNA—DNA oligonucleotides without a lesion were supplied by Sigma-Genosys. Oligonucleotides containing synthetic abasic sites (dSpacer; Sterlin, VA) were synthesized by the Synthesis Unit of the Biological Services Department in our institute or were purchased from Metabion (Martinsried, Germany). Site-specifically modified 12-mer oligonucleotides containing (+)-*trans*-BPDE-*N*²-dG adduct were generated as previously described (28). The BPDE-modified 54-mer oligonucleotides used to construct the gap-lesion plasmids GP-BPG1 and GP-BPG2 were the products of ligating the BPG1 and BPG2 12-mer oligonucleotides to two 21-mer oligonucleotides, using a 34-mer as a scaffold (Fig. 1A). The resulting 54-mers were separated from the scaffold and excess 21-mers on a 12% denaturing polyacrylamide gel (containing 8 M urea). The construction of the gap-lesion plasmids GP21 (with a synthetic abasic site) and GP20 (without a lesion; Fig. 1B) was previously described (29, 30). The gap-lesion plasmids GP-BPG1 and GP-BPG2 were constructed similarly to GP21, except that the insert oligonucleotides contained the BPDE-modified 54-mer templates (Fig. 1B). The plasmid GP20-*cm* is a chloramphenicol-resistant derivative of GP20. This plasmid was prepared as GP20, except that the vector plasmid used was pSKSL-*cm*. Plasmid pSKSL-*cm* is the ligation product of the HindIII-XhoI fragment (2845 bp long) from plasmid pSKSL and a 773-bp fragment containing the chloramphenicol resistance gene from plasmid pACYC184 and carrying PCR-generated termini with XhoI and HindIII sites. The cDNA of the human *DINB1* gene, encoding polk, was obtained by reverse transcription-PCR from HeLa cell total mRNA as three overlapping fragments using the following primers: for the 5' portion of the *DINB1* gene, primer 361 (5'-CGGATAAGTTTAT-ACCATGGATAG-3') and primer 362 (5'-GGCAATGCCTGCACTGGC-TGTC-3'); for the middle portion of *DINB1*, primer 363 (5'-CTCAGTT-GTTTTTGGAAACATCAG-3') and primer 364 (5'-AGAACTCTTCTTA-TGAGACA-3'); for the 3' portion of *DINB1*, primer 365 (5'-CCATGA-GTGTACATTAGAGAA-3') and primer 366 (5'-TTAATGATAAAATGT-TCAATGTTTAC-3'). The three amplified fragments were cleaved with restriction nucleases PflMI, XbaI plus PflMI, and XbaI, respectively, and then ligated. Finally, the full cDNA was amplified from the ligation mixture using primers 361 and 366 and cloned into the EcoRV site of plasmid pACYC184 to yield plasmid pACYC184-polk. The sequence of the entire cloned *DINB1* gene was then determined. The polk expression vector, pC-polk, was generated by PCR using pACYC184-polk as a template, and the primers 5'-TTAGGATCCGGATAGCACAAAGGAGAA-GTGT-3' and 5'-GTTCAATGTTTACTTAACTCGAGATCAAGGGTA-TGTTTGGG-3'. The PCR product was digested with BamHI and XhoI and inserted into BamHI- and XhoI-cleaved pCDNA3 downstream to the cytomegalovirus immediate early promoter. The sequence of polk open reading frame was confirmed by DNA sequencing.

Cell Cultures—Cells from Polk-deficient and wild-type mouse embryonic fibroblasts were previously described (31). The SV40-transformed human fibroblasts, MRC5 (normal) and XP30RO (sv) (XPV; also designated GM3617 (32)) were gifts from A. R. Lehmann (University of Sussex, Brighton, UK). The human fibroblast cells were cultured in Eagle's minimum essential medium. The MEF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine. Each medium was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The cells were incubated at 37 °C in a 5% CO₂ atmosphere.

Mammalian TLS Assay in Cultured Cells—The quantitative indirect TLS assay is a modification of the assay previously described (33). The original assay involved transient transfection of mammalian cells with a gap-lesion plasmid (kan^R), along with a normalizing intact plasmid (cm^R) and a carrier plasmid (amp^R). A control transfection is conducted

in parallel with a gapped plasmid (kan^R) without a lesion (modified base), along with the same normalizing intact plasmid (cm^R) and carrier plasmid (amp^R). After incubation of the transfected cells to allow TLS, the plasmid contents are extracted, and gapped plasmids that were not completely filled and ligated are denatured by alkaline lysis. The plasmid mixture is then transformed into an *E. coli recA* strain, and transformants are selected in parallel on LB-kan plates (to select for plasmids that underwent TLS) and LB-cm plates (to select for the normalizing plasmid). The extent of bypass is calculated by dividing the ratio of kan^R/cm^R transformants obtained with the gap-lesion plasmid mixture by the ratio of kan^R/cm^R transformants obtained with the gapped plasmid (with no lesion) mixture (33). In the modified procedure, we replace the intact normalizing plasmid (cm^R) by a gapped plasmid without a lesion (cm^R), thereby simplifying the TLS assay. Mammalian cells are co-transfected with a plasmid mixture containing the gap-lesion plasmid (kan^R), a control gapped plasmid without a lesion (cm^R), and the carrier plasmid pUC18 (amp^R). After allowing time for gap filling and lesion bypass, the plasmids were extracted from the cells using alkali, such that only filled in plasmids remained intact. To assay the fraction of filled in plasmids, the plasmid mixture was transformed into an indicator *E. coli recA* strain and plated in parallel, as in the original method, on LB-kan plates (to select for plasmids that underwent TLS) and LB-cm plates (to select for the control filled in plasmid GP20-*cm*) (Fig. 2). TLS in this case was calculated by the ratio of kan^R/cm^R *E. coli* transformants. The two methods yielded similar results. Specifically, the cells were co-transfected with a DNA mixture containing 50 ng of a gap-lesion plasmid (GP21, GP-BPG1, or GP-BPG2; kan^R), 50 ng of a gapped plasmid without lesion (GP20-*cm*, cm^R), and 10 μ g of the carrier plasmid pUC18, using jetPEI/DNA complexes (34). The percentage of lesion bypass gap filling was calculated by dividing the number of GP21*, GP-BPG1*, or GP-BPG2* transformants (number of colonies on LB-kan plates) by the number of corresponding GP20 cm* transformants (number of colonies on LB-cm plates). When desired, plasmids were extracted from kan^R colonies, and the sequence opposite the lesion was determined by automated DNA sequencing analysis in the Biological Services Department in this Institute.

In Vivo Complementation Assay—For the polk complementation assay, the MEFs were co-transfected with a DNA mixture containing 1.5 μ g of gap-lesion plasmid (GP-BPG1, kan^R), 1.5 μ g of gapped plasmid without lesion (GP20-*cm*, cm^R), and 1 μ g of expression vector (pC-polk or the control pCDNA3). In this assay, the MEF cells were electroporated with Nucleofector™ (Amaxa GmbH, Köln, Germany) according to the manufacturer's protocol. When desired, plasmids were extracted from kan^R colonies, and the sequence opposite the lesion was determined by automated DNA sequencing analysis in the Biological Services Department in this institute.

TLS Assay in *E. coli*—Gapped plasmids carrying the BP-G adduct (GP-BPG1 and GP-BPG2; kan^R) and the control plasmid without the adduct (GP20; kan^R) were used in parallel to transform UV-irradiated *E. coli* cells as previously described (8, 29, 35). The cells were UV-irradiated at 20 Jm⁻², followed by a recovery period of 30 min at 37 °C, after which they were transformed in parallel with the gapped plasmids with and without the BP-G adduct using the Ca-MOPS method (8). Survival was calculated by dividing the number of transformants obtained with the gap-lesion plasmid by the number of transformants obtained with the gapped plasmid without the lesion. In this assay, results from two parallel transformations are compared. Although no internal control is included, the same stock of competent cells was used for the gapped plasmids with and without the lesion, and the reliability of the results was assured by performing multiple experiments for each pair of gapped plasmid constructs. The bacterial strains used in this study were *E. coli* AB1157 (*argE3*, *hisG4*, *leuB6*, *proA2*, *thr1*, *ara14*, *galK2*, *lacY1*, *mtl1*, *xyl5*, *thi1*, *tsx33*, *rpsL31*, *supE44*) and *E. coli* RW118 (*leuB+*, *araD139*, *sulA211*, *argE3*, *hisG4*, *leuB6*, *proA2*, *thr1*, *ara14*, *galK2*, *lacY1*, *mtl1*, *xyl5*, *thi1*, *tsx33*, *rpsL31*, *supE44*), both proficient in TLS.

In Vitro Lesion Bypass Assay—The DNA substrates used for this assay were prepared by annealing a 5'-³²P-end-labeled oligonucleotide primer to the BPDE-modified 54-mer templates, followed by purification on a BioSpin 30 gel filtration column (Bio-Rad). Primer 5'-CTG-GTTCAAGTAGCCAGGTAGGACG-3' was used for the BP-G1-modified 54-mer, whereas primer 5'-CTGGTTCAAGTAGCCAGGTAGGA-3' was used for the BP-G2 54-mer, both creating a substrate with the 3' terminus located opposite the template base preceding the lesion. Anal-

² The asterisks refer to the indicated plasmid after recovery from mammalian cells.

A

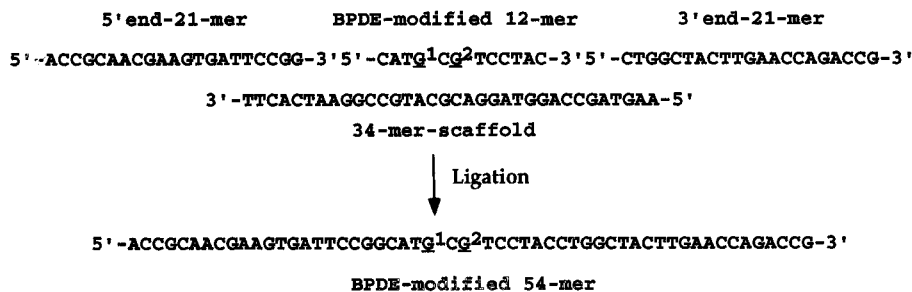
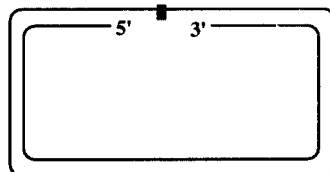


FIG. 1. Gap-lesion plasmids used to assay TLS in living cells. **A**, outline of the construction of 54-mer oligonucleotides carrying site-specific BP-G adducts that were used to build the gap-lesion plasmids. See "Experimental Procedures" for details. **B**, the structure of gapped plasmids used in this study. The *black rectangle* represents a damaged nucleotide. The DNA sequence at the gap region is shown. The *underlined G* represents the BP-G lesion. *G1* and *G2*, the two guanines that were modified with BP in plasmids GP-BPG1 and GP-BPG2, respectively (*underlined* in **A** and **B**); *X* (in GP21) represents an abasic site.

B



GP20

5'-CAACGAAAGTGATTCCCGTCGTGACTGGGAAACCCCTGGGCTACTTGAACCAG-3'
3'-GTTGCTTCACTAAGG-5' 3'-CCGATGAACTTGGTC-5'

GP21

5'-CAACGAAAGTGATTCCCGTCGTGACTGXGAAACCCCTGGGCTACTTGAACCAG-3'
3'-GTTGCTTCACTAAGG-5' 3'-CCGATGAACTTGGTC-5'

GP-BPG1

5'-CAACGAAAGTGATTCCCGCATGCGTCTACCTGGCTACTTGAACCAG-3'
3'-GTTGCTTCACTAAGG-5' 3'-CCGATGAACTTGGTC-5'

GP-BPG2

5'-CAACGAAAGTGATTCCCGCATGCGTCTACCTGGCTACTTGAACCAG-3'
3'-GTTGCTTCACTAAGG-5' 3'-CCGATGAACTTGGTC-5'

ysis by electrophoresis on native gels revealed that >95% of the primers were annealed to the template oligonucleotides. All primer extension reactions contained 5 mM dithiothreitol, 5 mM MgCl₂, 100 μM each of dATP, dCTP, dGTP, and dTTP, 50 nM primer/template, and 25 nM DNA polymerase. Primer extension reaction carried out by *E. coli* polymerase I Klenow fragment also contained 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 8 μg/ml bovine serum albumin, and 4% glycerol. Primer extension reaction carried out by human DNA polymerase η, human DNA polymerase κ, or human DNA polymerase ι also contained 25 mM potassium phosphate (pH 7.0), 100 μg/ml bovine serum albumin, and 10% glycerol. The primer extension reactions were carried out at 37 °C for 3–20 min. Reactions were stopped by adding an equal volume of a mixture of 99.5% formamide, 0.025% bromphenol blue, and 0.025% xylene cyanol. Samples were fractionated by electrophoresis on 15% polyacrylamide gels containing 8 M urea, after which they were dried, visualized, and quantified using a Fuji BAS 2500 phosphor imager. The extent of bypass was calculated by dividing the amount of bypass products by the amount of all extended primers.

RESULTS

TLS across a Benzo[a]pyrene-guanine Adduct Is More Efficient in Mammalian Cells than in E. coli—The lesion bypass assay system used is a modified version of a previously described assay based on transfection of cells in culture with a gapped DNA plasmid carrying a site-specific lesion at a predetermined site and carrying an intact *kan* gene. Plasmids are recovered from cells and are introduced into *E. coli recA* cells for survival and mutational analysis (Fig. 2). Plasmids in which gaps are repaired by TLS in the mammalian cells give rise to colonies in *E. coli* (33) (also see "Discussion"). To quantify the results, a normalizing control gapped plasmid without the lesion and carrying a different antibiotics marker (*cm*^R) was co-transfected along with the plasmid carrying the site-specific lesion. In addition, carrier DNA (pUC18, *amp*^R) is included in the plasmid mixture. The ratio of *kan*^R/*cm*^R transformants represents the extent of gap filling and lesion bypass (Fig. 2; for details see "Experimental Procedures"). Therefore, the assay

measures TLS indirectly via the recovery of gapped plasmids.

We used gapped plasmids carrying site-specific BP-G adducts in order to study their bypass in mammalian cells. Two constructs were utilized, each containing the sequence GCGTCC derived from the *p53* gene, carrying the lung cancer mutational hotspot codon 157 (underlined) (36). Plasmid GP-BPG1 (*kan*^R), carries the sequence 5'-CATGCGTCCCTAC-3', whereas GP-BPG2 (*kan*^R) carries the sequence 5'-CATGCGTCTACTAC-3' (the G is modified with BP). As shown in Table I (top), when the plasmids were alkali-treated and introduced into the *E. coli* tester strain without prior passage through the murine cells, TLS was very low (0.05–0.07%), representing the background in this system. When plasmid GP-BPG1 (*kan*^R) was assayed in *PolK*^{+/+} cells, TLS was nearly 3 orders of magnitude above background, reaching an extent of 48 ± 4%. Similar results were obtained with plasmid GP-BPG2 (*kan*^R) (52 ± 4%; Table I (top)). This extent of bypass is much higher than values usually reported for bypass across BP-G adducts in *E. coli* (37–39). However, since the differences might stem from DNA sequence context effects, which are known to strongly affect bypass across BP-G adducts *in vitro* and in *E. coli in vivo* (40–43), we assayed TLS across the BP-G adduct in *E. coli* cells, using the same gapped plasmids used in the mammalian cells. The assay, performed as previously described (8, 29, 35), involved transformation of SOS-induced *E. coli* cells with plasmid GP-BPG1(*kan*^R) or GP-BPG2 (*kan*^R), followed by selection on LB-*kan* plates. As a control, the cells were transformed in parallel with the gapped plasmid without the lesion. TLS was calculated by the ratio of transformants obtained with the gap-lesion plasmids to the number of transformants obtained with the gapped plasmid without a lesion. As can be seen in Table I (bottom), bypass across the BP-G adduct was poor in *E. coli*, both with the GP-BPG1 (*kan*^R) and GP-BPG2 (*kan*^R) constructs, reaching 1.6% at most. SOS induction caused a

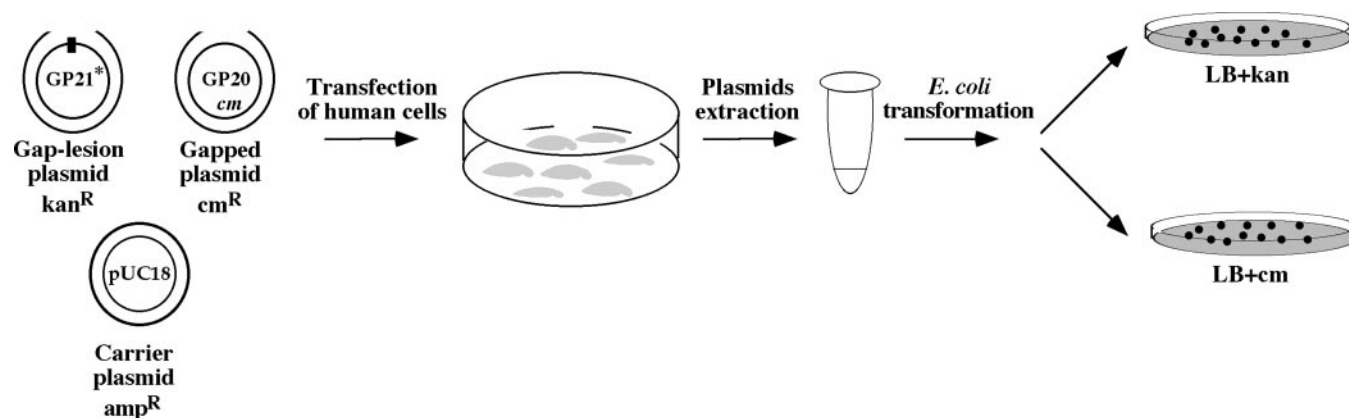


FIG. 2. **Outline of the quantitative TLS assay.** Cultured mammalian cells are transfected with a mixture of a gap-lesion plasmid (kan^R), a control gapped plasmid without a lesion (cm^R), and a carrier plasmid (amp^R). After gap filling by TLS in the mammalian cells, the plasmids were extracted and introduced into an indicator *E. coli* cell for analysis. Only gapped plasmids that were completely filled in the mammalian cells gave rise to bacterial colonies on selective media. TLS is calculated by the ratio of kan^R/cm^R colonies. See "Results" for details.

TABLE I
Extent of bypass across BP-G adducts in mouse embryofibroblasts and in E. coli cells

Plasmid mixtures containing the indicated gap-lesion plasmid (kan^R), the gapped plasmid GP20-*cm* (cm^R), and the carrier plasmid pUC18 (amp^R) were introduced into *PolK*^{+/+} mouse embryo fibroblasts by transfection, after which the DNA was extracted and introduced into the *E. coli* indicator strain. Top, bypass levels were calculated by dividing the number of kan^R colonies by the number of cm^R colonies. The table shows the average of at least six transfections. Bottom, *E. coli* cells, either unirradiated or UV-irradiated, were transformed in parallel with the indicated gap-lesion plasmid and with a control gapped plasmid without the lesion. Bypass levels were obtained by dividing the number of transformants obtained with the gap-lesion plasmid by the number of transformants obtained with the control gapped plasmid.

Mouse cells	Gap-lesion plasmid	<i>E. coli</i> transformants ^a		Lesion bypass
		Kan ^R	Cm ^R	
				%
MEF	GP-BPG1	325	700	48 ± 4
MEF	GP-BPG2	188	381	52 ± 4
None ^b	GP-BPG1	220	442000	0.05 ± 0.002
None ^b	GP-BPG2	123	176000	0.07 ± 0.0005
<i>E. coli</i> strain ^c	SOS induction	Gap-lesion plasmid	Kan ^R transformants ^d	Lesion bypass
				%
AB1157	–	GP-BPG1	442/29270	1.5 ± 0.6
	+	GP-BPG1	1129/64150	1.8 ± 0.7
RW118	–	GP-BPG1	807/50755	1.6 ± 0.4
	+	GP-BPG1	721/35410	2.0 ± 0.9
AB1157	–	GP-BPG2	1388/186060	0.7 ± 0.2
	+	GP-BPG2	4025/266550	1.5 ± 0.3
RW118	–	GP-BPG2	721/81650	0.9 ± 0.4
	+	GP-BPG2	609/40570	1.5 ± 0.4

^a The number of transformants obtained in a typical assay with 100 μl of the transformation mixture. For plates carrying over 300 transformants, appropriate dilutions were made in order to obtain an accurate colony count.

^b A DNA mixture containing the indicated gap-lesion plasmid was introduced into *E. coli* JM109*recA*, without prior passage through the mouse cells.

^c The *E. coli* strains used in this study are proficient in TLS. Genotypes are given under "Experimental Procedures."

^d Number of colonies obtained with the gap-lesion plasmid (number on the left) and the control gapped plasmid (number on the right) in a typical assay with 100 μl of the transformation mixture. In order to obtain an accurate colony count, appropriate dilutions were made to get 100–300 colonies/plate.

slight increase in bypass, but even under these conditions bypass did not exceed 2%. Thus, bypass across the BP-G adduct was at least an order of magnitude more efficient in mice embryo fibroblasts than in *E. coli*.

***PolK*^{–/–} Mouse Embryo Fibroblasts Are Deficient in TLS across a Benzo[a]pyrene-guanine Adduct**—We examined the role of *polk* in bypass across BP-G adducts by performing the TLS assay in parallel in *PolK*^{+/+} and *PolK*^{–/–} cells. As can be seen in Fig. 3A and Table II, bypass across the BP-G adduct in gapped plasmid GP-BPG1 (kan^R) in *PolK*^{–/–} MEFs was 16 ± 1%, 3-fold lower than in *PolK*^{+/+} MEFs. Similar results were obtained with plasmid GP-BPG2 (kan^R). In this case, TLS was 52 ± 4 and 20 ± 3% in *PolK*^{+/+} and *PolK*^{–/–} cells, respectively (Table II).

In order to examine whether the decreased TLS in *PolK*^{–/–} is indeed due to the lack of *polk*, we used a plasmid expressing

human *polk* from the cytomegalovirus immediate early promoter to complement the deficiency in *PolK*^{–/–} cells. Fig. 3A and Table II show the results of such complementation experiments performed with plasmid GP-BPG1 (kan^R). TLS in the control *PolK*^{–/–} cells that were transfected with the empty expression vector was 20 ± 2.5%. However, when co-transfected with the plasmid expressing human *polk*, bypass increased to 49 ± 6%, similar to the levels observed in *PolK*^{+/+} cells (Table II; Fig. 3A). Expression of *polk* by transient transfection of a cytomegalovirus immediate early promoter-driven construct may have caused an overexpression of *polk*. However, at this point, it is not clear to what extent this might have affected the results, since the extent of bypass under complementation conditions was similar to the bypass observed in *PolK*^{+/+} cells. In conclusion, *polk* has a major role in the bypass of the BP-G adduct and is responsible for the bypass of at least

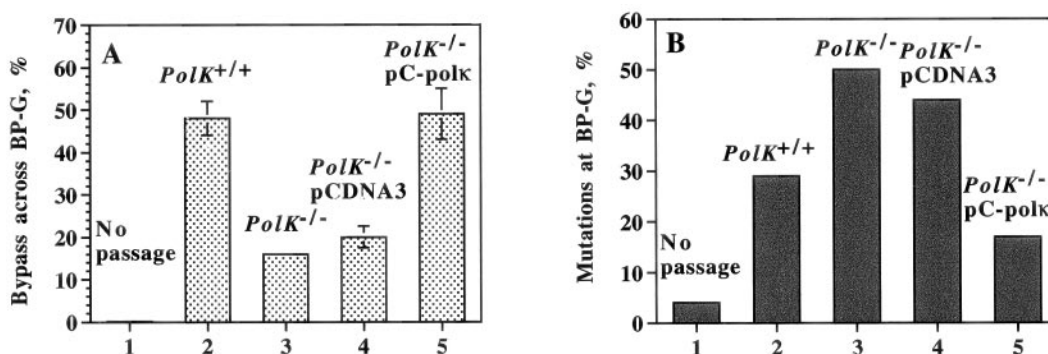


FIG. 3. Extent and mutagenicity of TLS across a BP-G adduct in $PolK^{+/+}$ and $PolK^{-/-}$ mouse embryo fibroblasts. A, the extent of bypass. B, the mutagenicity of bypass, namely the percentage of nucleotides other than C that were inserted opposite the lesion. Columns 4 and 5 in each panel represent complementation experiments with an empty vector (pCDNA3) and with a vector expressing polκ (pC-polκ), respectively. The results, obtained with gap-lesion plasmid GP-BPG1, were taken from Tables II and III.

TABLE II

Extent of bypass across a BP-G adduct in $PolK^{+/+}$ and $PolK^{-/-}$ MEFs in the presence or absence of polκ expressed from a plasmid

The plasmid mixtures contained the indicated gap-lesion plasmid (kan^R) and the control gapped plasmid GP20-cm (cm^R) and, when indicated, also the polκ expression vector pC-polκ, or the empty vector pCDNA3. The plasmids were transfected into $PolK^{+/+}$ or $PolK^{-/-}$ MEFs, after which the DNA was extracted and introduced into the *E. coli* indicator strain. Bypass levels were calculated by dividing the kan^R/cm^R transformants obtained. The table shows the average bypass results of at least three transfections.

Mouse cells (MEFs)	Gap-lesion plasmid	Expression vector	<i>E. coli</i> transformants ^a		Lesion bypass %
			Kan ^R	Cm ^R	
$PolK^{+/+}$	GP-BPG1		325	700	48 ± 4
$PolK^{-/-}$	GP-BPG1		122	785	16 ± 1
$PolK^{-/-}$	GP-BPG1	pCDNA3	95	460	20 ± 3
$PolK^{-/-}$	GP-BPG1	pC-polκ	179	380	49 ± 6
$PolK^{+/+}$	GP-BPG2		188	381	52 ± 4
$PolK^{-/-}$	GP-BPG2		110	479	20 ± 3

^a The number of transformants obtained in a typical assay with 100 μl of transformation mixture except for the complementation experiments, where 500 μl transformation mixture was used to compensate for a lower efficiency of the electroporation transfection method. This did not affect the results of lesion bypass, which are calculated by the ratio of Kan^R/cm^R colonies.

two-thirds of the BP-G adducts in our system.

Bypass across the BP-G Adduct Is More Mutagenic in the Absence of Polκ—Next we analyzed the specificity of nucleotide insertion opposite BP-G adducts. As shown in Table III, correct incorporation of C opposite BP-G adducts was more frequent than the incorrect incorporation of other nucleotides in both $PolK^{+/+}$ and $PolK^{-/-}$ cells. However, incorporation of the incorrect nucleotides (A, G, or T) was significantly greater in $PolK^{-/-}$ than in $PolK^{+/+}$ cells (Table III). With plasmid GP-BPG1 (kan^R), 29% of the bypass events in $PolK^{+/+}$ cells resulted in mutations, compared with 50% in $PolK^{-/-}$ cells (Fig. 3B, Table III). With plasmid GP-BPG2 (kan^R), bypass was only slightly more mutagenic in $PolK^{-/-}$ cells (71%) compared with $PolK^{+/+}$ (53%). With both plasmids, the main mutagenic event was misinsertion of A opposite the BP-G adduct. Complementation experiments revealed that while in the $PolK^{-/-}$ cells transfected with the empty vector, 44% of the bypass events were mutagenic, in such cells co-transfected with the polκ-expressing plasmid, only 17% of the bypass events were mutagenic (Fig. 3B, Table III). Thus, expressing polκ in $PolK^{-/-}$ cells renders TLS across the BP-G adduct less mutagenic than in cells lacking polκ.

Polκ Is Not Required for TLS across a Synthetic Abasic Site in Mouse Embryo Fibroblasts—To examine the substrate specificity of TLS by polκ, we performed experiments with gapped plasmids carrying a site-specific synthetic abasic site. Bypass across the abasic site reached levels of 36 ± 4% and 38 ± 4% in $PolK^{+/+}$ and $PolK^{-/-}$ cells, respectively (Table IV, top). Analysis of the specificity of base insertion opposite the abasic site revealed that purines, primarily A, were preferentially inserted opposite the abasic site (Table IV, bottom), consistent with our results in human cells in culture (33). Similar spectra of base

insertions were observed in $PolK^{+/+}$ and $PolK^{-/-}$ cells (Table IV, bottom). These results indicate that polκ is not required for *in vivo* bypass across an abasic site.

Polη Is Not Required for Bypass across the BP-G Adduct—To explore the polymerase specificity for TLS, we examined the ability of polη encoded by the human XPV gene to bypass BP-G lesions in DNA. As shown in Table V (top), bypass in the human cell line MRC5 (XPV^{+/+}) was 35 ± 4 and 39 ± 4% for plasmids GP-BPG1 (kan^R) and GP-BPG2 (kan^R), respectively. Similarly, bypass in the human XPV-defective cell line XP30R was 40 ± 3 and 44 ± 5% for plasmids GP-BPG1 (kan^R) and GP-BPG2 (kan^R), respectively (Table V, top). We also examined the specificity of nucleotide insertion opposite the BP-G adduct and found that in both cell lines bypass was largely accurate: 90% C insertion in XPV^{+/+} cells and >95% C insertion in XPV^{-/-} cells. This accuracy of bypass is higher than in the $PolK^{+/+}$ MEFs (71%; Table III). The reason for the difference in accuracy is not clear. It may stem from differences between human and mice cells and/or transformed cell lines *versus* embryo cells. In conclusion, polη is not required for bypass across the BP-G adduct in living cells.

In Vitro Bypass across the BP-G Adduct by Specialized DNA Polymerases—We examined the possibility that the primary role of polκ in TLS across the BP-G adduct stems from the failure of other DNA polymerases to bypass this lesion. It was previously shown that polη can bypass BP-G adducts (44–46). However, since TLS across BP-G is strongly influenced by DNA sequence context (40–43), we determined the ability of purified human polκ, polη, and polι to bypass the BP-G adduct in the same DNA sequence context that was used in our *in vivo* studies. Fig. 4 shows the kinetics of TLS across the BP-G lesion by purified recombinant human DNA polymerases κ, η, and ι.

TABLE III
Insertion specificity opposite a BP-G adduct during TLS in $PolK^{+/+}$ and $PolK^{-/-}$ MEFs in the presence or absence of $polk$ expressed from a plasmid

The plasmid mixtures contained the indicated gap-lesion plasmid (kan^R) and the control gapped plasmid GP20-*cm* (cm^R) and, when indicated, also the $polk$ expression vector pC- $polk$ or the empty vector pCDNA3. The plasmids were transfected into $PolK^{+/+}$ or $PolK^{-/-}$ MEFs, after which the DNA was extracted and introduced into the *E. coli* indicator strain. Plasmids were extracted from kan^R colonies and subjected to DNA sequence analysis. The table shows the DNA sequence opposite the lesion obtained for individual clones.

Mutation type	Gapped plasmid GP-BPG1				Gapped plasmid GP-BPG2	
	$PolK^{+/+}$	$PolK^{-/-}$	$PolK^{-/-}$ + pCDNA3	$PolK^{-/-}$ + pC- $PolK$	$PolK^{+/+}$	$PolK^{-/-}$
A	7 (25%)	11 (37%)	9 (30%)	4 (13%)	11 (29%)	20 (59%)
C	20 (71%)	15 (50%)	17 (56%)	25 (83%)	18 (47%)	10 (29%)
G	1 (4%)	2 (7%)	2 (7%)	2 (7%)	2 (5%)	2 (6%)
T	—	2 (7%)	2 (7%)	1 (3%)	7 (18%)	2 (6%)
Total mutations	8 (29%)	15 (50%)	13 (44%)	5 (17%)	20 (53%)	24 (71%)
Total isolates	28	30	30	30	38	34

TABLE IV
Extent and specificity of bypass across an abasic site in $PolK^{+/+}$ and $PolK^{-/-}$ MEFs

The plasmid mixtures containing the indicated gapped plasmid (GP21; kan^R or GP20-*cm*; cm^R) and the carrier plasmid pUC18 were introduced into the indicated mammalian cells, after which the DNA was extracted and introduced into the *E. coli* indicator strain. Top, bypass levels were calculated by dividing the number of kan^R colonies obtained for GP21 (containing the abasic site) by that obtained for GP20-*cm* (with no lesion; cm^R). The average of three experiments is presented. Bottom, plasmids were extracted from kan^R colonies containing GP21 descendants obtained in the top of the table and subjected to DNA sequence analysis. The table shows the base opposite the lesion, obtained for individual clones.

Mouse cells	Gap-lesion plasmid	<i>E. coli</i> transformants ^a		Lesion bypass
		Kan^R	Cm^R	
$PolK^{+/+}$	GP21 (kan^R)	140	401	36 ± 4
$PolK^{-/-}$	GP21 (kan^R)	310	853	38 ± 4

Mutation type	Cell type	
	$PolK^{+/+}$	$PolK^{-/-}$
Base substitution		
A	17 (90%)	16 (84%)
G	1 (5%)	1 (5%)
T	—	—
C	—	—
Deletion		
-1	1 (5%)	2 (11%)
Total isolates	19	19

^a The number of transformants obtained in a typical assay, with 100 μ l of transformation mixture.

Bypass was assayed using primed oligonucleotides, whose DNA sequence is the same as in the gap lesion plasmids used in the *in vivo* experiments. $Polk$ bypassed the BP-G adduct in either of the two sequence contexts examined with similarly high efficiency, reaching ~40% in 10 min (Fig. 4). In contrast, $pol\eta$ exhibited different bypass extents at the two sequence contexts. On template G1, bypass was slower than by $polk$. However, on template G2, $pol\eta$ and $polk$ showed similar bypass extents, although the product distribution was different. $PolI$ was unable to bypass the adducts under these conditions. Thus, consistent with previous results (44–46), at least one other DNA polymerase, $pol\eta$, is capable of bypassing a BP-G adduct *in vitro*, although it is not essential for this bypass reaction *in vivo*.

DISCUSSION

The multiplicity of DNA polymerases, particularly from the Y superfamily, suggests that lesion bypass is an important housekeeping process in protecting mammalian cells from genotoxic agents. The study of the intricate network of TLS polymerases and their mode of action in the cell requires quantitative tools. We have used a plasmid-based quantitative TLS

TABLE V
Extent and specificity of bypass across BP-G adducts in human MRC5 and XP3ORO cells

Plasmid mixtures containing the indicated gap-lesion plasmid (kan^R), the gapped plasmid GP20-*cm* (cm^R), and the carrier plasmid pUC18 were introduced into the indicated human cell lines by transfection, after which the DNA was extracted and introduced into the *E. coli* indicator strain. Top, bypass levels were calculated by dividing the number of kan^R colonies by the number of cm^R colonies. The table shows the average of at least three transfections. Bottom, plasmids were extracted from kan^R colonies containing GP-BPG1 descendants obtained in the top of the table and subjected to DNA sequence analysis. The table shows the base opposite the lesion, obtained for individual clones.

Cell line	Gap-lesion plasmid	Transformants ^a		Bypass levels
		Kan^R	Cm^R	
MRC5	GP-BPG1	135	375	35 ± 4
XP3ORO (XP-V)	GP-BPG1	122	301	40 ± 3
MRC5	GP-BPG2	354	863	39 ± 4
XP3ORO (XP-V)	GP-BPG2	290	690	44 ± 5

Mutation type	Cell lines	
	MRC5 (normal)	XP3ORO (XP-V)
Base substitution		
A	—	2 (10%)
C	20 (100%)	18 (90%)
G	—	—
T	—	—
Total isolates	20	20

^a The number of transformants obtained in a typical assay, with 100 μ l of transformation mixture.

assay, developed in our laboratory, to study the involvement of $polk$ in TLS in cultured cells. The major advantages of this system are its specificity for TLS and its quantitative results, which are essential for the in depth understanding of *in vivo* TLS. The main disadvantages of this system are that it is indirect (based on plasmid recovery) and that it utilizes a nonreplicating episomal substrate rather than a chromosomal substrate. Nonetheless, the system can be viewed as a model for gap filling during lagging strand DNA replication. Indeed, the system is responsive to the type of DNA damage and to the cellular composition of DNA polymerases, as shown in this and in previous studies (33, 47). The specificity of this assay for TLS is dictated by the structure of the plasmid used, namely a gap-lesion plasmid. Indeed, nucleotide excision repair is known not to act on single-stranded DNA regions (1), and in the absence of an intact complementary strand, recombinational repair cannot act either, as demonstrated previously (33). Base excision repair, on the other hand, can potentially interfere with the assay, since some DNA damage-specific DNA glycosylases, which initiate this pathway, can act on single-stranded DNA, leading to the formation of abasic sites (1). This means that if a DNA glycosylase were to excise the BP-G adduct, the

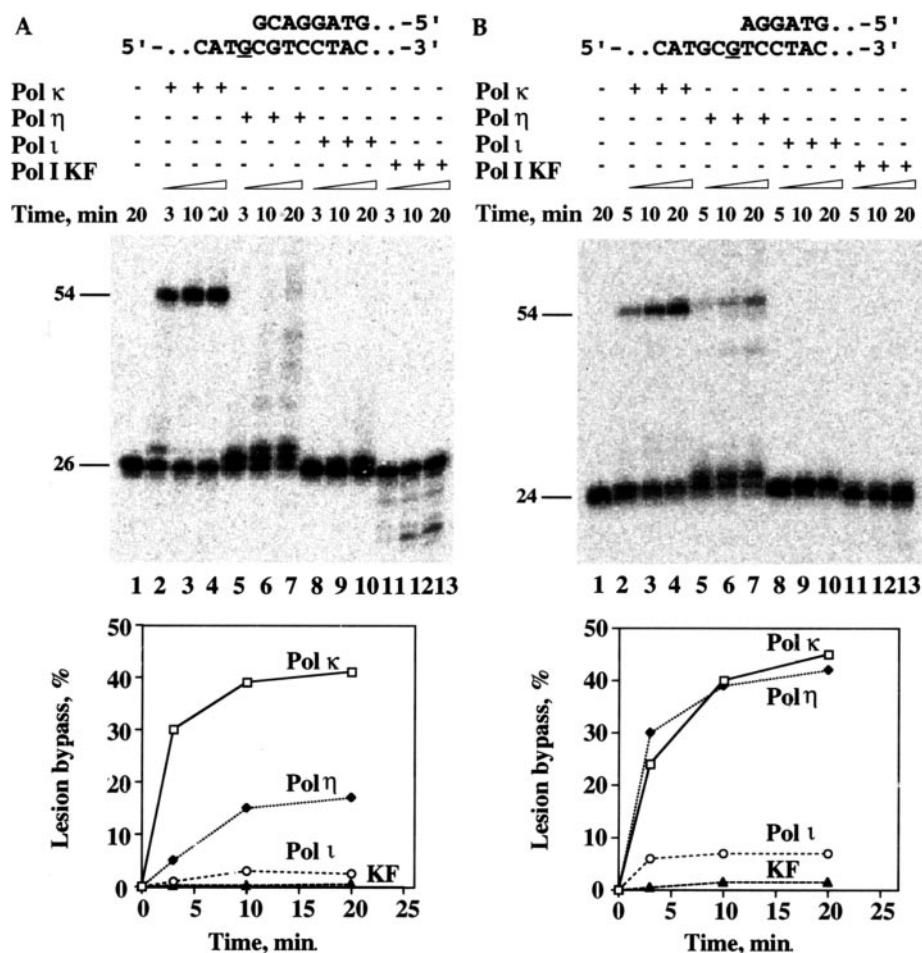


FIG. 4. *In vitro* bypass across BP-G by purified Y family human DNA polymerases. Primer extension assays were performed with the indicated purified recombinant human DNA polymerases, using primed oligonucleotides carrying a site-specific BP-G adduct in the template strand, as described under "Experimental Procedures." A and B, experiments performed with BP-G at two different sequence contexts, which are shown at the top of the gel images (the modified G is underlined). The upper panels show phosphor images of reaction products, whereas the lower panels show the quantification of lesion bypass based on these images. Lane 1 in each panel shows a control incubation of the reaction mixture without any DNA polymerase. Other lanes contain the products of reactions performed with the following DNA polymerases: human polκ (lanes 2–4); human polη (lanes 5–7); human polι (lanes 8–10); *E. coli* polymerase I (Klenow fragment) (lanes 11–13). For each DNA polymerase, three time points of the reaction are shown. The bands of oligonucleotides shorter than 26 nucleotides (A, lanes 11–13) represent excision products of the 3'–5' proofreading activity of polymerase I KF.

observed effects would have been due to abasic sites rather than the BP-G adduct. This possibility is ruled out by two arguments: (a) there was no difference in bypass across an abasic site in *PolK*^{+/+} and *PolK*^{-/-} cells, whereas there were clear differences in the bypass across BP-G in these two cell types, as described above; (b) there is no known DNA glycosylase that acts on BP-G. Thus, the results obtained with the gapped plasmid carrying the BP-G adduct in the single-stranded DNA region can be attributed solely to TLS in the mammalian cells.

Our results indicate that the BP-G adduct is bypassed in mammalian cells with considerable efficiency, amounting to 35–50%. This is more than 20-fold higher than the extent of bypass in SOS-induced *E. coli* across the same BP-G lesion, using the same gapped plasmid constructs. These results, together with previous results demonstrating much higher bypass across an abasic site in mammalian cells compared with *E. coli* (33), indicate that TLS in general is much more efficient in mammalian cells than in *E. coli* and is therefore likely to be a more significant repair function in mammals than in *E. coli*.

Two lines of evidence implicate polκ in cellular responses to BP; *PolK*^{-/-} mouse embryo fibroblasts were found to be sensitive to killing and mutagenesis by BP (26), and the *PolK* gene is subject to arylhydrocarbon receptor-dependent inducible

transcription (48). These results clearly indicate that polκ plays an important role in protecting mouse cells from the killing and mutagenic effects of BP, consistent with a role of polκ in TLS *in vivo*. However, the same results can be explained by the involvement of polκ in responses to DNA damage other than TLS, such as error-free repair and checkpoint activation. Indeed, a link between polκ and checkpoint activation, including the physical interaction between DinB and Hus1 and Rad1, was recently reported in *Schizosaccharomyces pombe* (49). Similarly, pole was reported to be a checkpoint protein (50). The experiments presented in this study provide evidence for the direct involvement of polκ in TLS across BP-G adducts in living cells, since cells lacking polκ exhibited a 3-fold reduction in TLS across this lesion, and expression of human polκ in these cells resulted in complementation of the bypass defect. Based on these results, polκ is responsible for the bypass of at least two-thirds of the BP-G adducts. In fact, since in the absence of polκ another polymerase may carry out bypass across BP-G to some extent, it is possible that in *PolK*^{+/+} cells polκ is responsible for the bypass of more than two-thirds of the BP-G lesions. This is quite remarkable, given the multiplicity of DNA polymerases present in mammalian cells (at least 14 additional DNA polymerases) (5, 51), underscoring the principle of DNA damage specificity in the bypass activity of at least certain TLS DNA polymerases.

The important role of polk in bypass across the BP-G adduct does not stem from an inherent inability of other DNA polymerases to bypass this lesion, since purified pol η was found to bypass the BP-G adduct in one of the constructs tested with an efficiency similar to that of polk. This is consistent with the idea that regulation of TLS DNA polymerases plays an important role in the final TLS outcome. It is clear, however, that polk is not the only DNA polymerase that can bypass BP-G *in vivo*, since bypass extents of 16–20% were obtained in cells lacking polk. At this point, the identity of these polymerases is still unknown; however, a possible candidate is pol η , based on its ability to bypass the BP-G adduct *in vitro*, as shown in this (Fig. 4) and in previous studies (44–46). It is currently unknown whether polk functions in the bypass of DNA lesions other than BP-G. However, it is likely that additional modified bases, perhaps adducts with other aromatic compounds, will be bypassed by polk.

It was previously reported that purified polk is a promiscuous extender of mispaired termini *in vitro* (52, 53). At this stage, we do not know whether polk performs both the misinsertion and extension steps *in vivo* or whether an additional polymerase is involved in the bypass reaction. Analysis of the specificity of nucleotide incorporation opposite the BP-G site indicates that the major mutagenic event involves the misinsertion of A, consistent with previous results (54, 55). Interestingly, bypass was more mutagenic in the absence of polk, resulting in an ~2-fold higher frequency of incorporation of incorrect nucleotides. This is not a big effect on mutation frequency, but it may translate to a much bigger effect on the mutagenic outcome of BP adducts in carcinogenesis, since the cumulative mutational outcome of DNA lesions grows exponentially with the number of hits. Overall, the results presented here, together with previous results, indicate that polk bypasses BP-G adducts with higher efficiency and higher accuracy than other DNA polymerases. This resembles the action of pol η on cyclobutyl pyrimidine dimers, making polk the second demonstrated case of a TLS polymerase with the ability to bypass a specific lesion with higher accuracy than other DNA polymerases. Collectively, these results suggest that at least some DNA polymerases function to bypass particular DNA lesions with higher efficiency and higher accuracy than others. In this sense, pol η and polk represent DNA damage tolerance enzymes, which are dedicated to minimizing the hazards of certain unrepaired lesions in DNA by restoring DNA replication in the face of arrest, with a reduced probability of mutations compared with other polymerases.

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