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Translesion replication of benzo[*a*]pyrene and benzo[*c*]phenanthrene diol epoxide adducts of deoxyadenosine and deoxyguanosine by human DNA polymerase ι

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ABSTRACT

Human DNA polymerase ι (polι) is a Y-family polymerase whose cellular function is presently unknown. Here, we report on the ability of polι to bypass various stereoisomers of benzo[*a*]pyrene (BaP) diol epoxide (DE) and benzo[*c*]phenanthrene (BcPh) DE adducts at deoxyadenosine (dA) or deoxyguanosine (dG) bases in four different template sequence contexts *in vitro*. We find that the BaP DE dG adducts pose a strong block to polι-dependent replication and result in a high frequency of base misincorporations. In contrast, misincorporations opposite BaP DE and BcPh DE dA adducts generally occurred with a frequency ranging between 2 × 10^{-3} and 6 × 10^{-4}. Although dTMP was inserted efficiently opposite all dA adducts, further extension was relatively poor, with one exception (a *cis* opened adduct derived from BcPh DE) where up to 58% extension past the lesion was observed. Interestingly, another human Y-family polymerase, polκ, was able to extend dTMP inserted opposite a BaP DE dA adduct. We suggest that polι might therefore participate in the error-free bypass of DE-adducted dA *in vivo* by predominantly incorporating dTMP opposite the damaged base. In many cases, elongation would, however, require the participation of another polymerase more specialized in extension, such as polκ.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are common environmental contaminants which are metabolized in mammals by a pathway involving cytochrome P-450 and epoxide hydrolase to give mutagenic and carcinogenic bay-region diol epoxides (DEs) (1,2). Each DE exists as a pair of diastereomers, one in which the benzylic hydroxyl group and the epoxide oxygen are *cis* and one in which these groups are *trans*. The latter diastereomer, whose DNA adducts are the subject of this study, consists of a pair of enantiomers with (*R*,*S*,*S*,*R*) and (*S*,*R*,*R*,*S*) absolute configurations at the carbon atoms of the tetrahydro benzo ring (Fig. 1). Notably, the (*R*,*S*,*S*,*R*) enantiomer is both the predominant isomer formed on metabolism of the hydrocarbon as well as the most carcinogenic (2,3). The PAH DEs generally form covalent DNA adducts at the exocyclic N^2 and N^6 amino groups of guanine and adenine, respectively, by *cis* or *trans* opening of the epoxide ring at the benzylic position (4). On reaction with DNA *in vitro*, DEs derived from benzo[*a*]pyrene (BaP) are largely selective for G adduct formation (5,6), whereas DEs derived from benzo[*c*]phenanthrene (BcPh) form significant proportions (up to 88%) of dA adducts (7). Site-specific mutagenesis studies in both bacterial (8–15) and mammalian (9,11) cell systems, as well as random mutagenesis studies with the DEs (16–18), indicate that the DE and their DNA adducts can induce a variety of mutations at both dG and dA sites.

Replicative DNA polymerases (pols), exemplified by *Escherichia coli* polIII and polδ from eukaryotes, are both highly accurate and processive when copying undamaged DNA templates (19–21). However, these enzymes are generally blocked by the presence of bulky or distortion-producing lesions in DNA, including PAH adducts, and either stall immediately before the lesion itself or after inserting a base opposite the lesion (22–24). The question then arises how such lesions can lead to heritable mutations rather than simply to blockage of replication. The answer lies in the discovery that many of the key participants in the mutagenic process are, in fact, DNA polymerases that can substitute for the replicative polymerase and facilitate lesion bypass (for recent reviews see 22,24–26 and references therein). Lesion bypass is also often

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referred to as translesion DNA synthesis (TLS) or translesion replication (TR) and is believed to occur in two mechanistically discrete steps (27,28). The first step is the incorporation of a base opposite the DNA adduct itself and this step can either be accurate or error-prone. The second step is extension from the (mis)incorporated base, so as to achieve complete lesion bypass. Many of the polymerases thought to be involved in translesion replication belong to the Y-family of DNA polymerases that are found in all kingdoms of life (29). Moreover, multiple Y-family orthologs are often present in one organism. *Escherichia coli*, for example, has two Y-family DNA polymerases, polIV and polV, while humans have four, polη, polι, polκ and Rev1 (29). Studies in which the ability of Y-family polymerases to bypass BaP DE adducts have been investigated reveal that the accuracy and efficiency of lesion bypass of any given stereoisomer varies depending upon the enzyme utilized and the local sequence context of the lesion. For example, *E.coli* polV can bypass BaP DE dA adducts with moderate accuracy, while polIV only misinserts a base opposite the lesion and is unable to facilitate extension beyond the lesion (30). Similar differences in the ability of polIV and polV to bypass BaP DE dG adducts have also been observed. Whereas polIV bypasses BaP DE dG adducts efficiently and accurately *in vitro* and *in vivo*, polV copies these lesions rather inefficiently and with low fidelity (30). Variations in the ability of human Y-family polymerases to bypass BaP DE lesions *in vitro* have also been reported (31–35). Polη, which normally bypasses a *cis-syn* thymine–thymine dimer efficiently and accurately (36,37), bypasses BaP DE dG adducts inefficiently and in the process usually misinserts dAMP or dGMP opposite the adducted dG (32). Polκ, on the other hand, bypasses the BaP DE dG adducts with the greatest efficiency and predominantly incorporates dCMP opposite the lesion (31,33–35).

Characterization of polι *in vitro* reveals that its enzymatic properties can vary depending upon the local sequence context of template DNA or its structure (38–40). To determine how well polι responds in general to PAHs, we have measured the ability of the enzyme to misincorporate opposite four stereoisomeric BaP DE dA adducts and two BcPh DE dA adducts in two different local sequence contexts as well as two BaP DE dG adducts in two different local sequence contexts. We have also measured its ability to extend the primer beyond the adducted base. In general, BaP DE dG adducts posed a strong block to polι-dependent replication, and when bases were inserted opposite the lesion, misincorporations occurred with high frequency. In contrast, but in keeping with the ability of the enzyme to favor the correct incorporation of dTMP opposite unmodified dA templates (38), incorporation of dTMP opposite all six dA adducts was favored by factors of ~10^3–10^4 over the incorporation of incorrect nucleotides. In most cases, polι-dependent extension from the correctly paired, but structurally distorted, primer terminus was inefficient. However, we found that another Y-family DNA polymerase, polκ, was able to extend dTMP that had been inserted opposite a BaP DE dA adduct by polι. Based upon these observations, we suggest that polι (probably in combination with polκ) may help protect humans from the mutagenic consequences of exposure to PAHs by reducing the mutagenic potential of BaP DE dA and BcPh DE dA adducts.

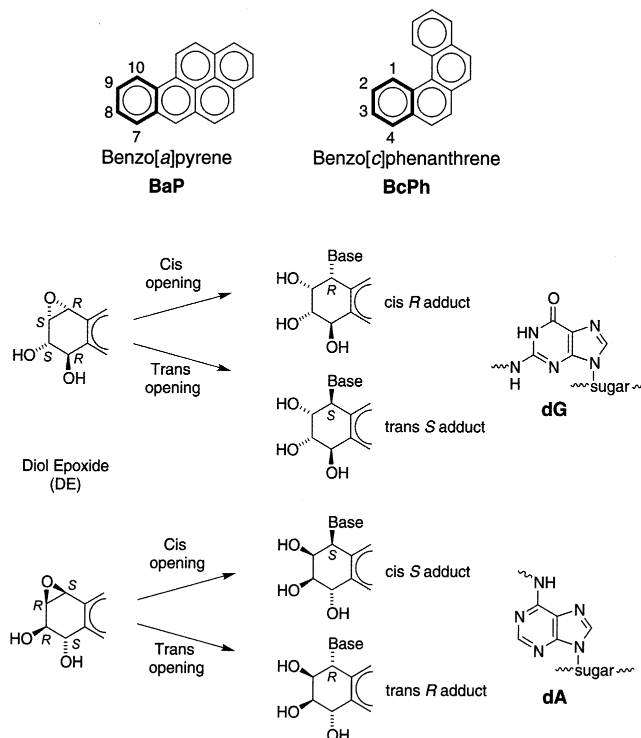

Figure 1. Structures of BaP and BcPh and their diol epoxide (DE) metabolites, with derived DNA adducts (Base = dG or dA residue with the point of attachment at N^2 of G or N^6 of dA as indicated). Only adducts derived from the DE shown (two enantiomers of the diastereomer [DE-2 or '*anti*' DE] in which the benzylic hydroxyl group and the epoxide oxygen are *trans*) were used in this study. The angular benzo ring of the hydrocarbon that is metabolized to the DE is shown in bold. Note that for *cis* adducts, the configuration at the benzylic C-10 or C-1 of the epoxide is retained, whereas for *trans* adducts it is inverted.

MATERIALS AND METHODS

PAH–DNA adduct nomenclature

In a number of previous studies, a system for naming purine–DE adducts has been utilized in which the configuration of the adducts is designated as (+) or (–), depending on the sign of the optical rotation of their parent DE. We find this system to be confusing, because it incorrectly implies known optical rotations for the adducts themselves and actually bears no direct relation to their structures. Consequently, in the present paper, as in previous studies (cf. 32), we use a convention that is based on the known absolute configuration (*R* or *S*) of the adducts at the point of attachment of the hydrocarbon to the exocyclic amino group of the purines, as well as the stereochemistry of epoxide ring opening (*cis* or *trans*) by this amino group. To facilitate comparisons with other work, we list here the dG and dA adducts used in this study (and whose structures are shown in Fig. 1), along with the alternative names in brackets as follows: BaP trans S [(+)-*trans-anti*]; BaP trans R [(–)-*trans-anti*]; BaP cis R [(+)-*cis-anti*]; BaP cis S [(–)-*cis-anti*]; BcPh cis S [(+)-*cis-anti*]; BcPh cis R [(–)-*cis-anti*]. Note, however, that there is no direct correspondence between *R*/*S* absolute configuration of the adducts and the signs used in the adduct names. As described in the Introduction, all the adducts in this study are derived

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from the same DE diastereomer, whose benzylic hydroxyl group and epoxide oxygen are *trans* (*anti* isomer), thus, for simplicity in the present study, we have not designated the diastereomer of the parent DE.

Primers and templates for replication assays

For most of our studies, the adducted templates contained a site-specific PAH lesion 4 bases from the end of a 16mer oligonucleotide template. The exception was a 29mer template in which a BaP DE trans S dA lesion was located 13 bases from the end of the template. Lesion-containing 16mer templates were prepared and purified as previously described (12–14,41). The 29mer template was synthesized on a 1.5 µmol scale by a semi-automated procedure essentially as described (42,43) except that a commercial 500 Å dG controlled pore glass (44 µmol/g) support was used. The *trans*-N^6-BaP DE–dA adducted phosphoramidite used in the manual coupling step consisted of a single diastereomer with known 10*S* configuration. Preparation and characterization of the diastereomerically pure 10*R* and 10*S* phosphoramidites will be described elsewhere. The 5′-dimethoxytrityl-protected oligonucleotide was subjected to HPLC at room temperature on a Higgins Analytical DNA Semi-Prep column (10 × 100 mm) (Thomson Instrument Co., Clear Brook, VA) eluted at 3 ml/min with a linear gradient that increased the percentage of solvent B in solvent A [0.1 M $(NH_4)_2CO_3$ buffer, pH 7.0] from 20 to 100% over 15 min, where solvent B (re-adjusted to pH 7.0–7.4) is a 1:1 mixture of acetonitrile and solvent A (t_r = 6.7 min). After standard cleavage of the dimethoxytrityl group, the deprotected oligonucleotide was purified by HPLC at 65°C on a Waters Xterra MS C_{18} column (2.5 µm, 4.6 × 50 mm) eluted at 1 ml/min with a linear gradient of solvent B in the above buffer that increased the composition of solvent B from 13 to 22% in 20 min (t_r = 8.0 min). Templates without lesions and oligonucleotide primers complementary to the 3′-end of each template were synthesized using standard techniques and gel purified by Lofstrand Laboratories (Gaithersburg, MD). Primers were 5′-labeled with [γ-^{32}P]ATP (5000 Ci/mmol) (Amersham Pharmacia Biotech, Piscataway, NJ), using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD). For the 12mer primers/16mer templates, four different template sequence contexts were used: 5′-TTTA*GAGTCTGCTCCC-3′ and 5′-CAGA*TTTAGAGTCTGC-3′ were used for BaP DE and BcPh DE adducts of dA; 5′-TTCG*AATCCTTCCCCC-3′ and 5′-GGGG*TTCCCGAGCGGC-3′ were used for the two BaP DE adducts of dG. For the 16mer primer/29mer template, only one sequence context was used and this was 5′-GCTCGT-CAGCAGA*TTTAGAGTCTGCAGTG-3′. In each case, the position of the adducted base is marked by an asterisk and the location of the primer is underlined.

Enzymes

Wild-type glutathione *S*-transferase (GST)-tagged human polι was purified from baculovirus-infected SF9 insect cells by glutathione–agarose (Pharmingen, San Diego, CA) chromatography and hydroxyapatite ion exchange chromatography, as described (38). A recombinant human polκΔC (human polκ residues 1–560) with a His tag at its C-terminus was purified from *E.coli* essentially as described for human polκΔC purified from baculovirus-infected insect cells (44). Rat DNA polymerase β was the generous gift of Dr S.H. Wilson (NIEHS, Research Triangle Park, NC).

Replication reactions

Radiolabeled primer–template DNAs were prepared by annealing the 5′-^{32}P-labeled primer to the unlabeled template DNA at a molar ratio of 1:1.5. The efficiency of annealing was examined by comparing the relative amounts of free primer and annealed primer–template that had been separated on a 12% native acrylamide gel. In all cases, we estimated that >95% of the radiolabeled primers were annealed to the corresponding template (data not shown). Standard 10 µl reactions contained 100 fmol annealed primer–template (expressed as primer termini), 30 fmol polι (3 nM final concentration), 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 250 µg/ml BSA, 60 mM KCl, 2.5% glycerol and 100 µM of each ultrapure dNTP (Amersham Pharmacia Biotech, NJ) and lasted for 30 min at 37°C. Where noted, KCl was omitted from the reactions and DTT was replaced with 10 mM β-mercaptoethanol. In reactions containing the longer 29mer template, both the length of the reaction and the concentrations of polκΔC and polι enzymes were varied as noted in the figure legends. Standard 10 µl reactions for rat polβ contained 100 fmol primer–template, 25 fmol enzyme, 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 20 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA, 2.5% glycerol and 100 µM of each ultrapure dNTP. After incubation at 37°C for 30 min, reactions were terminated by the addition of 10 µl of 95% formamide/10 mM EDTA and the reaction mixture was heated to 100°C for 5 min followed by immediate cooling at 0°C. Reaction mixtures (5 µl) were subjected to 20% polyacrylamide–7 M urea gel electrophoresis and replication products were visualized by autoradiography or PhosphorImager analysis (Molecular Dynamics, CA, or Fujifilm Software Inc., CA).

Kinetic analysis of replication products

Preliminary experiments were performed to identify DNA polymerase and dNTP concentrations and assay reaction times that would ensure 'single hit' conditions with no more than 20% of the radiolabeled primer utilized (45,46). As a consequence, the concentration of polι used in the steady-state assays was reduced to 2.5 nM and the concentration of dNTPs was varied from 0.1 to 10 µM for the correct incoming dNTP and between 1 and 100 µM for incorrect nucleotides. Two minute reactions were performed for the incorporation of correct nucleotides, whereas those measuring incorporation of incorrect nucleotides varied from 10 to 30 min. All the reactions were initiated by addition of the appropriate dNTP. Reaction products were separated in a 20% polyacrylamide gel containing 7 M urea and gels were dried prior to quantitative analysis using ImageQuant (Molecular Dynamics, CA) and Image Gauge V3.4 (Fujifilm, Sunnyvale, CA) software. Saturation plots of velocity as a function of dNTP concentration were determined by dividing the percentage product generated by the respective reaction time. The apparent V_{max} and K_m values were derived from non-linear least squares fits to a rectangular hyperbola using the SigmaPlot software (SPSS, Chicago, IL). Nucleotide misincorporation frequencies were calculated as previously described, and the data presented are the averages of three or four separate experiments (38,45,46).

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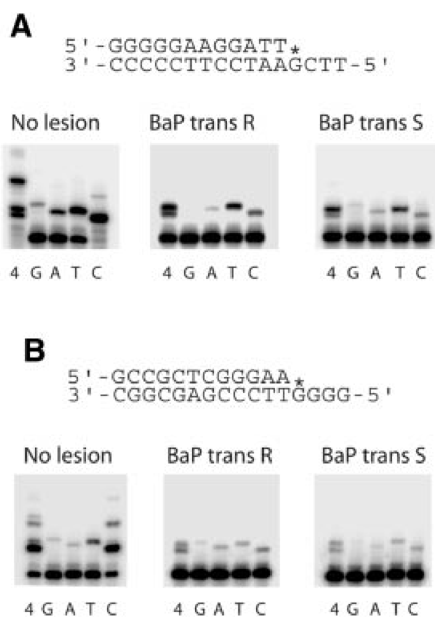

Figure 2. Ability of polι to replicate two stereoisomeric BaP DE dG adducts in two sequence contexts (**A** and **B**). For each panel, the sequence of the primer–template used in the reaction is shown above the reactions. Reactions were performed for 30 min at 37°C in the presence of the four dNTPs (4) at 100 µM each or individually, G, A, T and C (at 100 µM). The template either contained no lesion or a G adduct indicated by an asterisk in the template.

RESULTS

BaP DE and BcPh DE are potent carcinogens (2,3) whose biological activity most likely results from the formation of covalent DNA adducts which are incorrectly replicated. Notably, the spectra of mutations generated in the *supF* gene in shuttle vectors treated with the DE *in vitro* and then replicated in human cells suggest that mutations at adducted dA or dG residues are not evenly distributed. Instead, mutations occur with high frequency at mutagenic 'hot-spots', whereas the same bases in other regions mutate much less frequently and are therefore considered mutagenic 'cold-spots'. Our present study was aimed at analyzing the ability of polι to facilitate translesion replication of four stereoisomeric BaP DE and two BcPh DE adducts of adenosine that were incorporated into 16mer oligonucleotide templates in a sequence containing a hot-spot for BcPh DE-induced mutations (bases 137–122 of the template strand of the *supF* gene) and a cold-spot (bases 141–126 of *supF*) (47). Similarly, two BaP DE adducts of guanine were studied in sequences containing either a mutagenic hot-spot or cold-spot for BaP DE-induced mutations, corresponding to bases 161–176 and 102–117 of the coding strand of the *supF* gene, respectively (48).

Polι-dependent replication of two stereoisomeric BaP DE dG adducts in two template sequence contexts

We have previously demonstrated that at undamaged template guanines the misincorporation frequency in one sequence context is in the range ~10^{-1}–10^{-2} (38). Our subsequent studies revealed that the frequency of polι misincorporation and extension is, however, acutely sensitive to local sequence contexts and structures (39,40). Such sequence context variations can be seen in Figure 2, where misincorporation of dTMP or dAMP opposite the undamaged dG is clearly greater when the 5′ template bases are G*CTT-5′ compared to when they are G*GGG-5′ (cf. Fig. 2, upper and lower panels, No lesion). In both sequence contexts, the BaP trans R and BaP trans S dG adducts posed strong blocks to polι-dependent primer extension. Furthermore, the specificity of nucleotide misincorporation changed dramatically. Whereas the correct incorporation of dCMP is favored opposite the undamaged template G, in the G*CTT-5′ sequence context dTMP incorporation appears to be favored over the other three nucleotides when the lesion is present. In contrast, in the G*GGG-5′ sequence context, dAMP, dTMP or dCMP appear to be inserted with roughly equal efficiency. We have confirmed that the data reported in Figure 2, which were obtained under 'multiple hit' conditions, do in fact reflect a change in incorporation fidelity by performing kinetic analyses on the misincorporations in the G*CTT-5′ sequence context (Table 1). Under these conditions, we see that dTMP incorporation opposite BaP trans S and BaP trans R dG adducts is favored by a factor of 6- to nearly 10-fold over the correct base pair, while misincorporations of dAMP occurred with a relative frequency of 0.22 or 0.51, respectively. We were unable to reliably detect incorporation of dGMP opposite either the BaP trans S or BaP trans R dG adducts under these assay conditions (Table 1). We note, however, that when the BaP trans S dG adduct is located in a different sequence context, dGMP misincorporation is apparently favored over the (mis)incorporation of all other dNTPs (35).

Table 1. Kinetic analysis of polι-dependent incorporation opposite BaP trans R and BaP trans S dG adducts

Template	dNTP	BaP trans R		BaP trans S	
		V_{max}/K_m[a]	f_{inc}	V_{max}/K_m[a]	f_{inc}
3′-G*CTT-5′	G	N/A	N/A	N/A	N/A
	A	0.0031	0.22	0.0023	0.51
	T	0.087	6.2	0.043	9.6
	C	0.014	1	0.0045	1

[a]Units of V_{max}/K_m are the percentage primer extension product/min/µmol nucleotide. The V_{max}/K_m ratio was the average of three or more experiments with standard deviations not exceeding 20% of the V_{max}/K_m value.

Polι-dependent replication of four stereoisomeric BaP DE dA adducts in two sequence contexts

Although polι is generally considered a low fidelity enzyme when copying template G, T or C, it is actually relatively accurate when replicating template A, with misincorporations in the range 1–2 × 10^{-4} (38,49,50). This is recapitulated with the undamaged templates within the sequence context ATTT-5′ or AGAC-5′, where all of the primer is extended in the presence of dTMP and only faint incorporations are observed in the presence of the other nucleoside triphosphates (Fig. 3, No lesion). When copying templates containing BaP trans S, BaP trans R, BaP cis S and BaP cis R lesions in both sequence contexts, dTMP incorporation opposite the lesion is clearly favored, as in many of the reactions all of the primer had been utilized (Fig. 3). Interestingly, while misincorporation of dG

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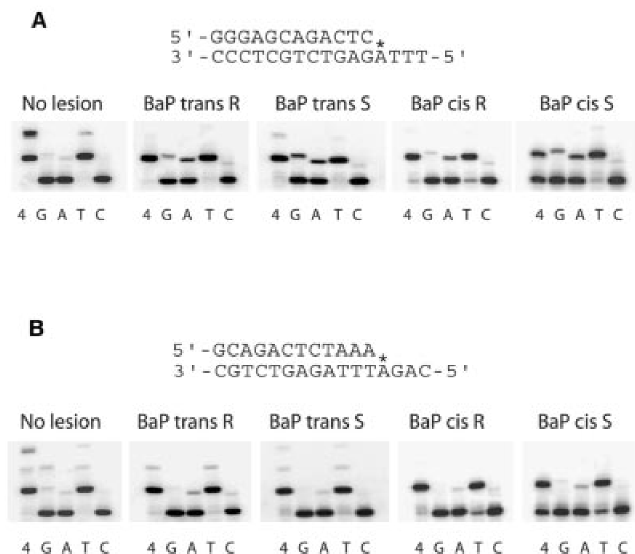

Figure 3. Ability of polι to replicate four stereoisomeric BaP DE dA adducts in two sequence contexts (**A** and **B**). For each panel, the sequence of the primer–template used in the reaction is shown above the reactions. Reactions were performed for 30 min at 37°C in the presence of the four dNTPs (4) at 100 µM each or individually, G, A, T and C (at 100 µM). The template either contained no lesion or an A adduct indicated by an asterisk in the template.

and dA is clearly seen in the context of A*TTT-5′, much lower levels are seen in the context of A*GAC-5′. Furthermore, within the A*TTT-5′ sequence context, the *cis* and *trans S* BaP DE adducts appear to be less accurately replicated than the corresponding *cis* and *trans R* adducts (Fig. 3). In all cases the incorporation of dCMP opposite the lesion was virtually undetectable (Fig. 3). While dTMP appears to be efficiently incorporated opposite the lesion, little to no extension beyond any of the lesions was observed.

To determine more accurately the fidelity of polι-dependent misinsertion when copying the BaP trans S and BaP trans R dA adducts, we measured the frequency of misincorporation opposite both lesions in two sequence contexts (Table 2). Although incorporation of dTMP opposite all four lesions was observed under 'multiple hit' conditions, the Michaelis–Menten analysis of initial rates revealed that in both sequence contexts, the efficiency of correctly incorporating dTMP opposite the *R* configuration of the BaP DE dA adducts is almost 10 times higher than for the *S* configuration (Table 2). Furthermore, the V_{max}/K_m for incorporation opposite the *R* isomer in the A*TTT-5′ context is only ~4-fold lower than the efficiency of incorporating dTMP opposite undamaged template A (38). In contrast, the efficiencies of misincorporating dGMP or dAMP opposite both the *R* and *S* isomers were roughly similar in both sequence contexts (Table 2). As a consequence, the relative frequencies of misincorporating dGMP or dAMP opposite the BaP trans S dA adduct are ~10-fold higher than the *R* isomer. Overall, the efficiency of incorporating bases (correct or incorrect) opposite both stereoisomers was slightly greater in the A*TTT-5′ context compared to the A*GAC-5′ context. Our data suggest, therefore, that for polι-dependent replication of the BaP DE dA adducts, the efficiency and accuracy of nucleotide incorporation opposite the adducted base is largely dependent upon the stereoisomer of the lesion and that, to a lesser extent, the local sequence context contributes to the overall efficiency of the reaction.

Table 2. Kinetic analysis of polι-dependent incorporation opposite BaP trans R and BaP trans S dA adducts in two DNA sequence contexts

Template	dNTP	BaP trans R		BaP trans S	
		V_{max}/K_m[a]	f_{inc}	V_{max}/K_m[a]	f_{inc}
3′-A*TTT-5′	G	0.021	1.03×10^{-3}	0.017	8.3×10^{-3}
	A	0.024	1.24×10^{-3}	0.03	1.47×10^{-2}
	T	19.3	1	2.04	1
	C	0.012	6.2×10^{-4}	0.0014	6.8×10^{-4}
3′-A*GAC-5′	G	0.002	1.5×10^{-4}	0.0012	9.9×10^{-4}
	A	0.025	1.9×10^{-3}	0.011	9.1×10^{-3}
	T	13.1	1	1.21	1
	C	0.01	7.4×10^{-4}	0.001	8.2×10^{-4}

[a]Units of V_{max}/K_m are the percentage primer extension product/min/µmol nucleotide. The V_{max}/K_m ratio was the average of three or more experiments with standard deviations not exceeding 20% of the V_{max}/K_m value.

Polι-dependent replication of two stereoisomeric BcPh DE dA adducts in two sequence contexts

We have also investigated the ability of polι to copy two stereoisomeric BcPh DE dA adducts in the same two sequence contexts. Overall, the pattern of misincorporation opposite BcPh DE dA adducts was similar to that observed with BaP DE dA. Based upon the amount of primer utilization, incorporation of dTMP opposite BcPh cis S and BcPh cis R is clearly favored in both sequence contexts (Fig. 4). However, although the correct base is inserted opposite the BcPh DE dA adduct, further elongation was essentially abolished. The striking exception was the BcPh cis S lesion when located in the A*-GAC-5′ context, where significant extension was observed in the presence of all four dNTPs (Fig. 4).

Comparison of polι and polβ when replicating BaP DE and BcPh DE dA lesions in two sequence contexts

Our primer extension assays suggest that for the four stereoisomers of BaP DE dA and the two stereoisomers of BcPh DE dA adducts studied, polι can efficiently incorporate the correct base, dTMP, opposite the lesion, but in general is unable to elongate the correctly paired, yet presumably distorted, primer terminus. We were interested in determining how these properties might compare to another low fidelity DNA polymerase, polβ. Previous studies have indicted that BaP DE adducts pose a strong block to polβ-dependent replication (8). We therefore analyzed the ability of both enzymes to incorporate and bypass the four BaP DE and two BcPh DE adducts in the presence of all four nucleotides (Fig. 5). In agreement with the previous experiments (Figs 3 and 4), polι efficiently incorporated a base opposite all six lesions in both sequence contexts, whereas further extension was generally limited. As noted above, the exception was the BcPh cis S dA adduct located in the A*-GAC-5′ context (Fig. 5, lower panel, polι, lane 8). We quantitated the extent of lesion bypass and found that under these conditions, ~58% of the primers were elongated past the BcPh cis S adduct. In contrast, the second best bypass event was with the BaP trans S

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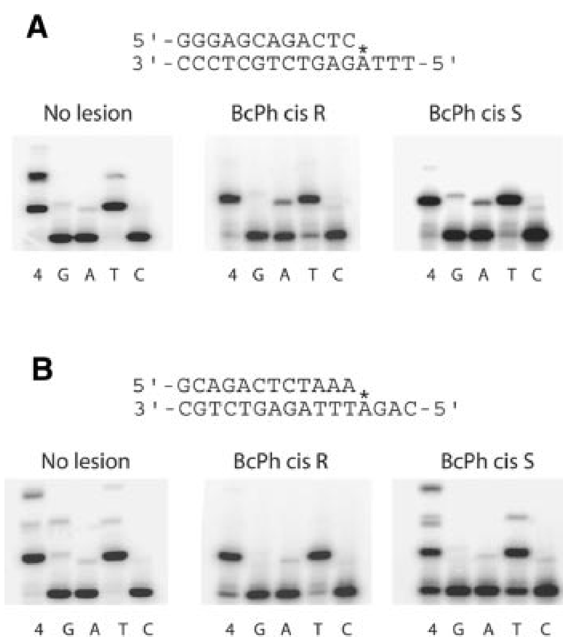

Figure 4. Ability of polι to replicate two stereoisomeric BcPh DE dA adducts in two sequence contexts (**A** and **B**). For each panel, the sequence of the primer–template used in the reaction is shown above the reactions. Reactions were performed for 30 min at 37°C in the presence of the four dNTPs (4) at 100 µM each or individually, G, A, T and C (at 100 µM). The template either contained no lesion or a dA adduct indicated by an asterisk in the template.

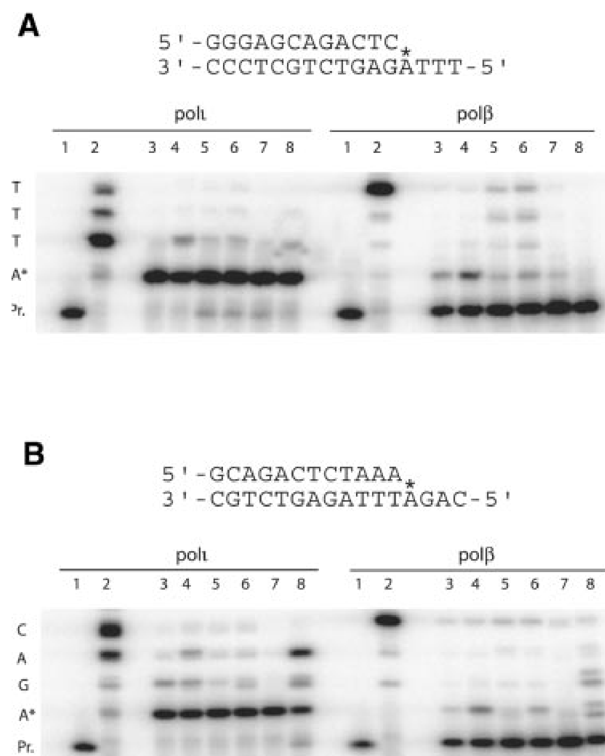

Figure 5. Comparison of the ability of human polι and rat polβ to bypass four stereoisomeric BaP DE dA adducts or two stereoisomeric BcPh DE dA adducts in two sequence contexts (**A** and **B**). The sequence of the primer–template used in the reactions is shown above their respective panels. Reactions were performed for 30 min at 37°C in the presence of the four dNTPs at 100 µM each; in these assays, KCl was omitted from the reactions and DTT was replaced with 10 mM β-mercaptoethanol. Lane 1, radiolabeled primer (no template); lane 2, no lesion; lane 3, BaP trans R; lane 4, BaP trans S; lane 5, BaP cis R; lane 6, BaP cis S; lane 7, BcPh cis R; lane 8, BcPh cis S.

adduct in the A*TTT-5′ context, which gave ~9% lesion bypass. As noted above, whereas all of the primers were extended on the undamaged DNA template, the presence of the BaP DE or BcPh DE adducts largely blocked polβ-dependent primer extension. Greatest incorporation was seen with the BaP trans S adduct in the A*TTT-5′ context (Fig. 5, upper panel, polβ, lane 4), where ~5% of the primers were extended by 1 bp and synthesis terminated opposite the lesion. However, we do note that limited bypass (2–5% of primers) occurred with some of the BaP DE and BcPh DE adducts in both sequence contexts (Fig. 5).

Our data demonstrate, therefore, the unique capacity of polι to incorporate bases accurately and efficiently opposite BaP DE and BcPh DE dA adducts. In at least one sequence context, polι can extend the primer beyond the adduct site so as to achieve unassisted lesion bypass.

Ability of polκ to extend bases inserted opposite BaP DE trans S dA by polι

It is clear, however, that despite efficiently incorporating the correct base opposite both BaP DE dA and BcPh DE dA adducts, in a variety of sequence contexts, further polι-dependent extension is generally limited. Given that the prevailing model for TR posits that it might occur in two steps, and could conceivably be facilitated by two different polymerases, we were interested in determining if the polι-dependent incorporation might be extended by another polymerase. Candidate enzymes for such a reaction appear to be polζ and polκ. Unfortunately, human polζ has yet to be purified and characterized at the biochemical level. While *Saccharomyces cerevisiae* polζ is available, we chose not to investigate the combined actions of *S.cerevisiae* polζ with human polι, as *S.cerevisiae* does not possess a polι ortholog and our assays would only serve as a cross-phyla hypothetical model. Instead, we chose to assay the ability of human polκ, which has extension properties similar to *S.cerevisiae* polζ (51), to extend polι-dependent incorporations incurred opposite a BaP trans S dA adduct (Fig. 6). As controls for the reactions, we also assayed the ability of polκ to extend an undamaged template as well as insert bases opposite the BaP trans S dA lesion. Consistent with earlier studies with both full-length and truncated polκ (34,44,52,53), the polκΔC assayed in these studies extended the undamaged template efficiently, and the products were 1–2 bases shorter than one might expect based upon the length of the template, indicating that the enzyme stops synthesis 1 or 2 nt before the end of the template (Fig. 6). When replicating the BaP DE trans S lesion, polκ appeared to favor the misincorporation of dAMP. However, the misincorporated dAMP was poorly extended, as was evident by the amount of primer extension in the presence of all four dNTPs (in the presence of various concentrations of polκΔC) (Fig. 6). In contrast to the weak misincoporation of dAMP opposite the BaP DE trans S lesion by polκ, polι efficiently incorporated the correct base, dTMP (cf. Fig. 3 and Table 1), but was largely unable to extend the correct base pair, even after prolonged reaction times.

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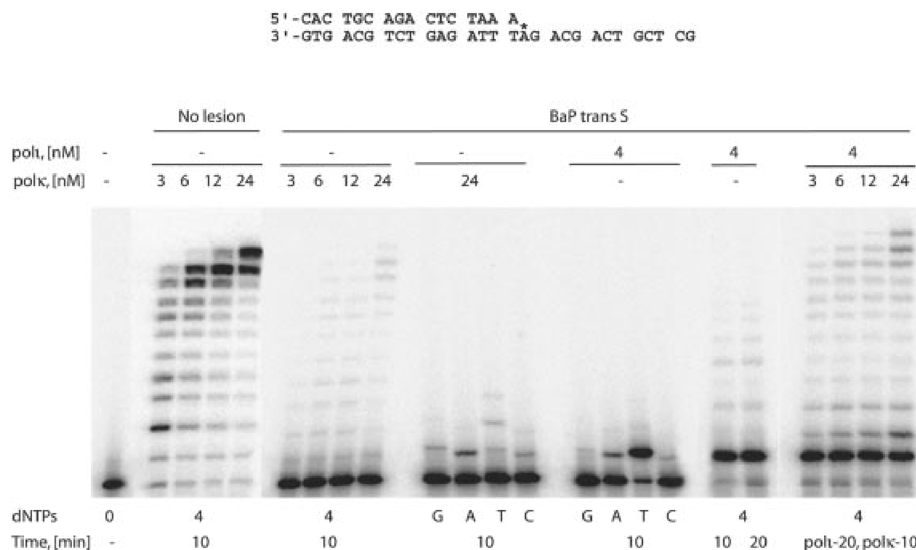

Figure 6. Ability of human polκ to misincorporate bases opposite a BaP DE trans S dA lesion and extend polι-dependent incorporation of dTMP opposite BaP DE trans S dA. The sequence of the 16mer primer/29mer BaP DE dA template used in the reactions is shown above the replication assays. The location of the adduct is indicated by an asterisk. In these assays, KCl was omitted from the reactions and DTT was replaced with 10 mM β-mercaptoethanol. Reactions were performed for the times noted at 37°C in the presence of the four dNTPs (4) at 100 μM each or individually, G, A, T and C (at 100 μM). The concentration of polι was kept constant at 4 nM, while the concentration of polκΔC was varied as noted above each reaction. In the case of the polι plus polκ reactions, 4 nM polι was allowed to extend the 16mer primer annealed to the damaged template in the presence of all four dNTPs (100 μM each) for 10 min before the subsequent addition of various concentrations of polκΔC for an additional 10 min. As a consequence, the total reaction time indicated on the figure for polι is 20 min.

However, when polκΔC was added to reactions following polι, the extent of lesion bypass increased considerably from ~3–9% in the presence of polκ, or polι, respectively, to ~30% in the presence of both enzymes. We conclude from these *in vitro* studies that while polκ has some ability to insert an incorrect base opposite the BaP trans S dA adduct, it is unable to extend the mispair. It can nevertheless extend a base that is correctly incorporated opposite the adduct by another DNA polymerase, such as polι.

DISCUSSION

It is becoming increasingly clear that members of the Y-family of lesion bypass polymerases exhibit unique misincorporation and bypass properties. A good example is the relative accuracy and efficiency of *cis-syn* thymine–thymine dimer and BaP DE adduct bypass performed by human polη, polι and polκ. Polη is able to bypass a *cis-syn* cyclobutane thymine–thymine dimer accurately and efficiently while polι does so inefficiently and inaccurately (36,37,54). Polκ is unable to insert bases opposite the thymine–thymine cyclobutane pyrimidine dimer (31,44,52). In contrast, polκ can bypass BaP DE adducted dG residues efficiently and accurately (31,33–35). Polη bypasses the same lesion inefficiently and in doing so misinserts dAMP or dGMP opposite the lesion (32). We have shown here that BaP DE dG is also a significant block to polι replication.

The spectra of mutations recovered from human cells (55) or mice (56) exposed to BaP or mammalian cells exposed to BaP DE (9,11,57,58) suggest that insertion of dAMP is favored at the adducted guanine. Under our assay conditions, we find that the BaP DE dG adducts pose strong blocks to polι-dependent replication, and polι predominantly inserts dTMP rather than dAMP when incorrect incorporations occur (Table 1). We conclude, therefore, that polι is unlikely to play a major role in the bypass of dG adducted BaP DE lesions *in vivo*. Based upon *in vitro* data, that role appears to be performed in an error-prone manner by polη (32,35) and an error-free manner by polζ (59) or polκ (31,33–35). Indeed, recent *in vivo* studies with mouse cells carrying a homozygous knockout of murine polκ exhibited both an increased sensitivity to BaP and an increase in BaP-induced mutagenesis (60), suggesting that polκ plays a major role in the error-free bypass of PAH adducts *in vivo*.

In keeping with the ability of the enzyme to favor the correct incorporation of dTMP opposite unmodified dA templates (38), we find that polι can accurately and efficiently insert dTMP opposite both BaP DE and BcPh DE dA adducts. Misincorporation frequencies were ~10-fold higher at the BaP DE dA adducts compared to an undamaged base and were generally in the range 10^{-3}–10^{-4}. The exception was at the BaP trans S lesion where misincorporation of dAMP occurred with a frequency of 1.47×10^{-2} (Table 2). These misincorporation frequencies are in the same range as that observed for polη at *cis-syn* thymine–thymine dimers (37) and for polκ at BaP DE dG adducts (33,34), and both enzymes are considered as being error-free when copying these lesions. Thus, it is possible that one cellular role of polι is to reduce the mutagenic potential of BaP DE and BcPh DE dA adducts through the correct incorporation of dTMP opposite the lesion *in vivo*.

Despite the efficient insertion of the correct base opposite the various PAH lesions, with the exception of the BcPh cis S lesion in one sequence context, further primer extension by polι is largely inhibited. Current models of lesion bypass suggest that it may occur in a two-step process; (mis)incorporation followed by extension (27,28). In the case of *cis-syn* thymine–thymine dimers, polη can perform both steps efficiently (36,37). We have previously hypothesized

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that efficient polι-dependent bypass of a cyclobutane pyrimidine dimer might require the participation of another polymerase, such as polζ (54). The possibility that polι might work in such a two-step process has indeed been demonstrated in a model system where *S.cerevisiae* polζ was mixed with human polι so as to achieve bypass of a synthetic abasic site and a 6–4 thymine–thymine dimer (49,61). It does not seem unreasonable, therefore, that a similar two-step process might also occur at BaP DE and BcPh DE dA adducts. Recent studies have suggested that, like *S.cerevisiae* polζ, human polκ can elongate mispairs and certain (mis)incorporations inserted opposite certain damaged bases (33,51). For example, although polκ cannot insert a base opposite a *cis-syn* thymine–thymine dimer (31,44,52), it can efficiently elongate dG and dA (but not dC or dT) bases that have been inserted opposite the 3′-thymine of the dimer (51). In a similar scenario, we found that polκ has only a weak ability to incorporate a base opposite a BaP DE trans S dA adduct and, when it did, dAMP was the preferred incorporation (Fig. 6). In contrast, however, when human polκ was added to replication reactions after polι had apparently successfully incorporated dTMP opposite the lesion, significant extension and complete lesion bypass was observed (Fig. 6). Thus, the combined actions of both polι and polκ lead to complete bypass of a BaP DE trans S dA adduct *in vitr*o. Our observations therefore parallel those of Zhang *et al*. (62), who found that polκ was able to extend dCMP that was inserted by human Rev1 opposite BaP DE trans R or trans S dG lesions. Thus, based upon a considerable amount of data generated from *in vitro* studies, as well as recent *in vivo* studies (60), polκ appears to play a major role in the bypass of PAHs. It can either bypass BaP DE adducted dG unassisted or it could play a role in TR by extending bases correctly inserted opposite BaP DE dG or dA adducts by Rev1 or polι, respectively.

While we hope that our *in vitro* studies with polι begin to provide clues as to its possible role(s) in lesion bypass *in vivo*, its true biological function will probably only be determined with the characterization of mice or cell lines lacking polι, and experiments toward generating such mice/cell lines are currently in progress.

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