Translesion Synthesis by Human DNA Polymerase κ on a DNA Template Containing a Single Stereoisomer of dG-(+)- or dG-(−)-*anti*-N^2-BPDE (7,8-Dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene)†

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ABSTRACT: Several recently discovered human DNA polymerases are associated with translesion synthesis past DNA adducts. These include human DNA polymerase κ (pol κ), a homologue of *Escherichia coli* pol IV, which enhances the frequency of spontaneous mutation. Using a truncated form of pol κ (pol κΔC), translesion synthesis past dG-(+)- or dG-(−)-*anti*-N^2-BPDE (7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene) adducts was explored. Site-specifically-modified oligodeoxynucleotides containing a single stereoisomeric dG-N^2-BPDE lesion were used as DNA templates for primer extension reactions catalyzed by pol κΔC. Primer extension was retarded one base prior to the dG-N^2-BPDE lesion; when incubated for longer times or with higher concentration of enzyme, full primer extension was observed. Quantitative analysis of fully extended products showed preferential incorporation of dCMP, the correct base, opposite all four stereoisomeric dG-N^2-BPDE lesions. (+)-*trans*-dG-N^2-BPDE, a major BPDE−DNA adduct, promoted small amounts of dTMP, dAMP, and dGMP misincorporation opposite the lesion (total 2.7% of the starting primers) and deletions (1.1%). Although (+)-*cis*-dG-N^2-BPDE was most effective in blocking translesion synthesis, its miscoding properties were similar to other dG-N^2-BPDE isomers. Steady-state kinetic data indicate that dCMP is efficiently inserted opposite all dG-N^2-BPDE adducts and extended past these lesions. The relative frequency of translesion synthesis ($F_{ins} \times F_{ext}$) of dC·dG-N^2-BPDE pairs was 2−6 orders of magnitude higher than that of other mismatched pairs. Pol κ may play an important role in translesion synthesis by incorporating preferentially the correct base opposite dG-N^2-BPDE. Its relatively low contribution to mutagenicity suggests that other newly discovered DNA polymerase(s) may be involved in mutagenic events attributed to dG-N^2-BPDE adducts in human cells.

Human DNA polymerase κ (pol κ),[1] a homologue of *Escherichia coli* DinB (pol IV) (*1*, *2*), belongs to the Y-family of DNA polymerases (*3*). The fidelity of pol κ is low, increasing the frequency of spontaneous mutations (*4*, *5*). Pol κ catalyzes miscoding reactions at abasic sites (*6*), 8-oxo-7,8-dihydrodeoxyguanosine (*7*) and 1,N^6-ethenodeoxyadenosine (*8*), but does not catalyze bypass of thymine dimers (*6*, *7*) or cisplatin adducts (*6*, *9*). On templates containing *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF), pol κ incorporated primarily dTMP or dCMP opposite the lesion when the reaction mixture contained a single dNTP (*6*, *7*, *9*). Using steady-state kinetic studies and an in vitro experimental system that can quantify miscoding specificity in reactions containing four dNTPs (*10*, *11*), we observed that primer extension reactions catalyzed by pol κ were extended past dG-AAF by incorporating efficiently dTMP opposite the lesion (*12*). These results suggest that pol κ may be involved in translesion synthesis past damaged DNA adducts in human cells.

Benzo[*a*]pyrene (B[*a*]P) is a typical environmental carcinogen that belongs to the polycyclic aromatic hydrocarbon family. B[*a*]P is oxygenated in animal and human cells to form activated metabolites including (+)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-

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[1] Abbreviations: BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (+)-*anti*-BPDE, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7*R*,8*S*,9*S*,10*R* steric configuration); (−)-*anti*-BPDE, (−)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene (7*S*,8*R*,9*R*,10*S* steric configuration); (+)-*syn*-BPDE, (+)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7*R*,8*S*,9*S*,10*S* steric configuration); (−)-*syn*-BPDE, (−)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7*S*,8*R*,9*R*,10*R* steric configuration); AAF, acetylaminofluorene; dG-AAF, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG, 2′-deoxyguanosine; dNTP, 2′-deoxynucleotide triphosphate; pol κ, DNA polymerase κ; pol κΔC, a truncated form of pol κ; K_m, Michaelis constant; V_{max}, maximum rate of the reaction; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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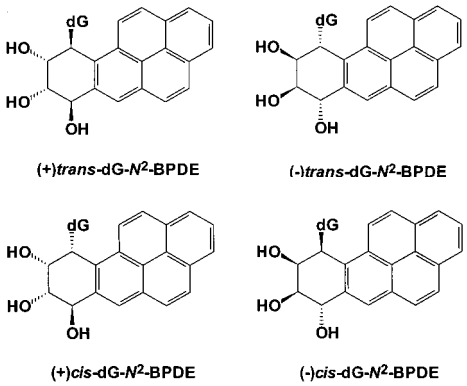

FIGURE 1: Structures of stereoisomers of dG-N^2-BPDE.

BPDE)] and (−)-*anti*-BPDE; (+)-*anti*-BPDE is a predominant product (*13*−*17*). The (+)-*anti*-BPDE stereoisomer is tumorigenic in mouse skin (*18*) and the lung of newborn mice (*19*), while the mirror image (−)-*anti*-BPDE enantiomer is not. Both (+)- and (−)-*anti*-BPDE react with native DNA in vitro to form (+)-*trans*- and (−)-*cis*-dG-N^2-BPDE adducts and (−)-*trans*- and (+)-*cis*-dG-N^2-BPDE adducts, respectively, as shown in Figure 1 (*20*−*24*), (+)-*trans*-dG-N^2-BPDE being the predominant product (*25*).

Cytochrome P450 1A1 (CYP 1A1) is a major enzyme in the pathway that metabolizes B[*a*]P to the corresponding phenols and dihydrodiols. CYP 1A1 is induced in a ligand-dependent manner by the aryl hydrocabon receptor (AhR) and AhR nuclear translocator (Arnt) (*26*). The binding of aromatic compounds, such as B[*a*]P, to AhR followed by dissociation of Hsp90 from the receptor leads to translocation of the ligand attached-AhR from the cytosol to the nucleus. Arnt interacts with ligand-attached AhR in the nucleus to form a heteromeric DNA-binding complex that activates transcription of several genes, including that coding for CYP1A1, by binding to xenobiotic responsive element (XRE) (*27*). Recently, it was found that AhR activation in mouse cells increases expression of the gene coding for pol κ (*28*).

Random mutagenesis studies have been performed in mammalian cells using racemic mixtures of *anti*-BPDE (*29*−*32*) or optically pure (+)- or (−)-*anti*-BPDE (*33*, *34*). In these studies, G → T transversions predominated, followed by smaller fractions of G → A and G → C base substitutions. Site-specific mutagenesis studies were used to explore the mutagenic properties of (+)-*trans*- and (−)-*trans*-dG-N^2-BPDE adducts and (+)-*cis*- and (−)-*cis*-dG-N^2-BPDE adducts in mammalian cells (*35*, *36*). In COS-7 cells, dG-N^2-BPDE adducts inserted into a single-stranded shuttle vector promoted G → T transversions, accompanied by lesser amounts of G → A transitions and G → C transversions; the relative frequencies of these mutations varied depending on the stereochemistry of the dG-N^2-BPDE isomers and on the flanking sequence context (*35*−*42*).

Recently, primer extension reactions catalyzed by pol κ were shown to bypass (−)-*trans*-dG-N^2-BPDE, one of the minor BPDE-derived adducts, embedded site-specifically in an oligodeoxynucleotide template; dCMP, the correct base, was inserted opposite the lesion in reactions containing a single dNTP (*7*). Quantitative analyses of translesion synthesis were not performed in that study. We have confirmed and extended the observations of Zhang et al. (*7*), studying translesion synthesis past all four dG-N^2-BPDE stereoisomers, including the major BPDE adduct (+)-*trans*-dG-N^2-BPDE. Quantitative studies of dNTP incorporation opposite and extension from the 3′ terminus also were performed.

Using an in vitro experimental system developed in our laboratory (*10*, *11*), we find in this work that base substitutions and deletions occur opposite all four stereoisomeric dG-N^2-BPDE lesions. Steady-state kinetic experiments were performed to determine the frequencies of nucleotide insertion opposite dG-N^2-BPDE lesions and of primer extension from the 3′ terminus matched or mismatched dN·dG-N^2-BPDE (N = C, A, G, or T) base pairs. Taken together, we find that primer extension reactions catalyzed by pol κ were extended past dG-N^2-BPDE lesions by efficiently incorporating dCMP, the correct base, opposite each of the four lesions. dG-N^2-BPDE adducts promoted only small amounts of base substitutions and deletions, suggesting that the contribution of pol κ to BPDE mutagenicity in cells may be minimal.

MATERIALS AND METHODS

General. [γ-^{32}P]ATP (specific activity >6000 Ci/mmol) was obtained from Amersham Corp. dNTPs were from Pharmacia; T4 polynucleotide kinase was from Stratagene. *Eco*RI restriction endonuclease was purchased from New England BioLabs.

Synthesis of Oligodeoxynucleotides. Unmodified DNA templates (5′CCTTCGCTACTTTCCTCTCCCTTT and 5′CATGCTGATGAATTCCTTCGCTACTTTCCTCTCCCTTT), primers, and standard markers (Figure 2) were prepared using an automated DNA synthesizer (Applied Biosystems Model 392) (*43*). The 24-mer oligodeoxynucleotides, 5′CCTTCXCTACTTTCCTCTCCCTTT (X = modified position), containing single (+)-*trans*-dG-N^2-BPDE, (−)-*trans*-dG-N^2-BPDE, (+)-*cis*-dG-N^2-BPDE, or (−)-*cis*-dG-N^2-BPDE were prepared, isolated, and purified according to procedures previously outlined (*44*−*46*). Modified and unmodified oligodeoxynucleotides were purified on a Waters reverse-phase μBondapak C_{18} column (0.39 × 30 cm), using a linear gradient of 0.05 mM triethylammonium acetate (pH 7.0) containing 5−20% acetonitrile with an elution time of 60 min, at a flow rate of 1.0 mL/min.

Modified 38-mer templates (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCCTTT, where X is dG-N^2-BPDE) were prepared as described previously by ligation of the dG-N^2-BPDE-modified 24-mer (5′CCTTCXCTACTTTCCTCTCCCTTT) to a 14-mer (5′CATGCTGATGAATT) (*47*). Briefly, 3 μg of dG-N^2-BPDE-modified 24-mer was phosphorylated at the 5′ terminus using 7.5 μL of T4 polynucleotide kinase (10 units/μL) and 3 μL of 10 mM ATP, ligated to the 14-mer (3.6 μg), and then incubated at 8 °C overnight using 3 μL of T4 DNA ligase (400 units/μL) and a 18-mer template (4.5 μg, 5′GTAGCGAAGGAATTCATC) in 100 μL of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 2.5 μg of BSA. The 38-mer product was isolated on a μBondapak C_{18} column (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing containing 10−20% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min (*48*). Unmodified and modified 38-mers were purified by electrophoresis on a 20% nondenaturing polyacrylamide gel (35 × 42 × 0.04 cm). Bands were extracted overnight with 2.0 mL of distilled water at 4 °C.

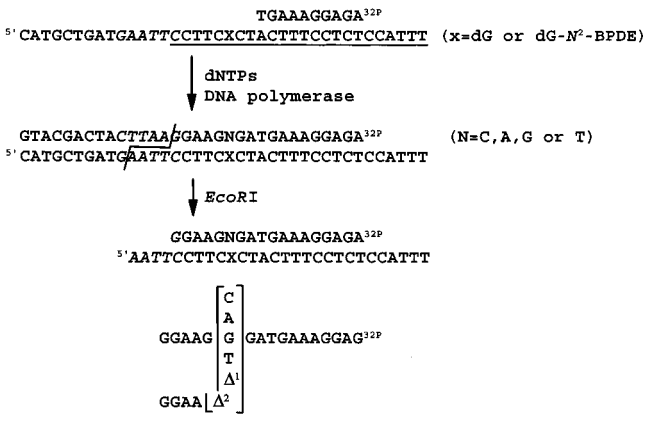

FIGURE 2: Diagram of the method used to determine miscoding specificities. Unmodified or dG-N^2-BPDE-modified 38-mer templates were annealed to a ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed by DNA pol $\kappa\Delta$C were conducted in the presence of four dNTPs. Fully extended products formed during DNA synthesis were recovered from the polyacrylamide gel, cleaved with *Eco*RI, and subjected to electrophoresis on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm), as described in Materials and Methods. To determine miscoding specificity, the mobility of the reaction products were compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one- (Δ^1) or two-base (Δ^2) deletions.

Extracts were evaporated to dryness, and the 38-mers were isolated by HPLC. A fraction containing unmodified, dG-N^2-BPDE-modified oligomers was evaporated to dryness and used for primer extension reactions and kinetic studies. Oligodeoxynucleotides were labeled at the 5′ terminus with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (*49*) and subjected to 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). The position and homogeneity of oligodeoxynucleotides following gel electrophoresis were determined using a Molecular Dynamics β-phosphorimager. A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of the oligodeoxynucleotides. Concentrations of oligodeoxynucleotides were calculated from their UV absorption spectra in solution, measured with a Hewlett-Packard 8452A diode array spectrophotometer.

Pol κ. Pol $\kappa\Delta$C (560 amino acids) used in this study lacks motifs VIIa and VIIb, which denote zinc clusters, from the intact DINB1 (pol κ) protein (870 amino acids) (*1*). Pol $\kappa\Delta$C with a 6× His tag attached at the carboxyl terminus was prepared by overproduction using a baculovirus expression system, as described elsewhere (6).

Primer Extension Reactions. Using dG-N^2-BPDE-modified or unmodified 38-mer oligodeoxynucleotide (150 fmol) primed with a ^{32}P-labeled 10-mer (5′AGAGGAAAGT; 100 fmol), 11-mer (5′AGAGGAAAGTA; 100 fmol), or 12-mer (5′AGAGGAAAGTAG; 100 fmol), primer extension reactions catalyzed by pol $\kappa\Delta$C were conducted at 25 °C for the 10-mer and 11-mer primers and at 30 °C for the 12-mer primer in a buffer (10 μL) containing four dNTPs (100 μM each) (Figure 2). The reaction buffer for pol $\kappa\Delta$C contains 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA, 60 mM KCl, and 2.5% glycerol. Reactions were stopped by addition of formamide dye. The samples were subjected to 20% denaturing PAGE. The radioactivity of extended products was measured by a β-phosphorimager (Molecular Dynamics).

Miscoding Specificity. Using dG-N^2-BPDE-modified or unmodified 38-mer oligodeoxynucleotide (750 fmol) primed with a ^{32}P-labeled 12-mer (5′AGAGGAAAGTAG; 500 fmol), primer extension reactions catalyzed by pol $\kappa\Delta$C (5 ng for the unmodified template; 50 ng for the dG-N^2-BPDE-modified templates) were conducted at 30 °C in a buffer (10 μL) containing four dNTPs (100 μM each). Extended reaction products (approximately 28−32 bases long) were extracted from the gels. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer and cleaved with *Eco*RI. To quantify base substitutions and deletions, samples were subjected to two-phase PAGE (15 × 72 × 0.04 cm) (*10*, *11*) (Figure 2).

Steady-State Kinetic Studies. Kinetic parameters associated with nucleotide insertion opposite dG-N^2-BPDE lesion and chain extension from the 3′ primer terminus were determined at 30 °C, using varying amounts of single dNTPs (0−500 μM). For insertion kinetics, reaction mixtures containing pol $\kappa\Delta$C (0.5−5 ng) and dNTP (0−500 μM) were incubated at 30 °C for 2 min in 10 μL of Tris-HCl buffer (pH 8.0) using 24-mer template (150 fmol; 5′CCTTCXCTACTTTCCTCTCCCTTT, where X is dG or dG-N^2-BPDE) primed with a ^{32}P-labeled 12-mer (100 fmol; 5′AGAGGAAAGTAG). Reaction mixtures containing a 24-mer template (150 fmol) primed with a ^{32}P-labeled 13-mer (100 fmol; 5′AGAGGAAAGTAGN, where N is C, A, G ,or T), varying amounts of dGTP (0−500 μM), and pol $\kappa\Delta$C (0.5−5 ng) were used to measure chain extension. The reaction samples were subjected to 20% denaturing PAGE (35 × 42 × 0.04 cm). The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Hanes−Woolf plots. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dC·dG base pair according to the equation $F = (V_{max}/K_m)_{wrong\ pair}/(V_{max}/K_m)_{correct\ pair=dC\cdot dG}$ (*50*, *51*).

RESULTS

Primer Extension Reactions Catalyzed by Pol $\kappa\Delta$*C on dG-*N^2*-BPDE-Modified DNA Templates.* A 38-mer dG-N^2-BPDE-modified template was prepared by ligation of the 24-mer containing a single stereoisomeric dG-N^2-BPDE adduct with a 14-mer and purified using HPLC and gel electrophoresis as described in Materials and Methods. An unmodified 38-mer was prepared by phosphoramidite automated DNA synthesis methods. The unmodified and modified 38-mers were analyzed by 20% polyacrylamide gel after labeling with ^{32}P at the 5′ terminus (Figure 3). The dG-N^2-BPDE-modified 38-mers migrated slower than the unmodified oligomer, as previously observed for shorter oligomers (*46*). Unmodified and modified 38-mers were purified to radiographic homogeneity using a combination of HPLC and gel electrophoresis.

Primer extension reactions were conducted on unmodified and dG-N^2-BPDE-modified 38-mer templates in the presence of four dNTPs and variable amounts of pol $\kappa\Delta$C (Figure 4). Primer extension readily occurred on the unmodified template to form extended products. Using 1.0 ng of pol $\kappa\Delta$C (molar ratio of enzyme:primer = 1:6.3), 93% of the starting primer was extended past the unmodified dG. In contrast, when dG-N^2-BPDE-modified templates were used, primer extension was retarded primarily one base prior to the lesions. Weak blockages were observed opposite the (+)-*trans*-dG-N^2-BPDE and (−)-*cis*-dG-N^2-BPDE lesions. Using 1.0 ng of

Table 1: Base Incorporation and Deletions opposite dG-N^2-BPDE in Reactions Catalyzed by Pol κ

DNA adduct	miscoding event (%)						total
	C[a]	A[a]	G[a]	T[a]	Δ^1 [a]	Δ^2 [a]	
dG	89.7 ± 2.9						0
(+)-*trans*-dG-N^2-BPDE	76.0 ± 3.6	0.47 ± 0.04	0.31 ± 0.03	1.9 ± 0.2	nd[b]	1.1 ± 0.1	3.78
(−)-*trans*-dG-N^2-BPDE	81.2 ± 2.2	0.48 ± 0.05	0.48 ± 0.02	2.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	6.36
(+)-*cis*-dG-N^2-BPDE	14.8 ± 1.4	0.79 ± 0.07	0.17 ± 0.02	0.92 ± 0.05	1.8 ± 0.1	0.60 ± 0.05	4.28
(−)-*cis*-dG-N^2-BPDE	62.3 ± 2.8	0.39 ± 0.03	0.28 ± 0.04	0.97 ± 0.04	0.74 ± 0.06	0.97 ± 0.1	3.35

[a] The percentage of nucleotide incorporation (C, A, G, and T) opposite the lesion and deletions (Δ^1 and Δ^2) were calculated on the basis of the amount of starting primer used for primer extension. Data are expressed as the mean ± SD obtained from three independent experiments. [b] Not detectable.

FIGURE 3: Polyacrylamide gel electrophoresis of 38-mer oligodeoxynucleotides containing a single stereoisomer of dG-N^2-BPDE. The 38-mer oligodeoxynucleotides (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCCTTT, where X is dG or dG-N^2-BPDE) were prepared as described in Materials and Methods, labeled with [γ-^{32}P]ATP, and subjected to 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). The position and homogeneity of oligodeoxynucleotides following gel electrophoresis were determined by a β-phosphorimager.

pol $\kappa\Delta$C, 1.8−25% of the starting primer was extended past the dG-N^2-BPDE lesions. Larger amounts of extended products were obtained by increasing the amount (10 and 50 ng) of pol $\kappa\Delta$C (Figure 4). Among the four diastereoisomers of dG-N^2-BPDE, primer extension past the (+)-*trans*- and (−)-*trans*-dG-N^2-BPDE lesions was more efficient than past the *cis*-dG-N^2-BPDE lesions. Primer extension past the (+)-*cis*-dG-N^2-BPDE lesion was especially poor; even when 10 and 50 ng of pol $\kappa\Delta$C was used, extended products were only 6.3% and 25%, respectively, of the starting primers.

Miscoding Properties of dG-N^2-BPDE. Using 38-mer templates primed with ^{32}P-labeled 12-mer and 50 ng of pol $\kappa\Delta$C, primer extension reactions were conducted in the presence of four dNTPs and pol $\kappa\Delta$C. The fully extended products (approximately 28−32-mers) past dG or dG-N^2-BPDE were recovered as described in Materials and Methods and digested by *Eco*RI. Since the amounts of fully extended products for (+)-*cis*-dG-N^2-BPDE adduct were much less than for the other stereoisomeric adducts, fully extended products were recovered from the other four reaction samples. The products were subjected to a two-phase PAGE analysis for quantifying base substitutions and deletions formed opposite the lesion (Figure 5). A standard mixture of six ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, or dT opposite the lesion and products containing one- and two-base deletions could be resolved by this method. When the unmodified template was used, dCMP, the correct base, was inserted opposite dG (Figure 5 and Table 1); however, small amounts (3.9%) of unknown products also were observed. All *trans*- and *cis*-dG-N^2-BPDE promoted incorporation of dCMP (15−81%) as their primary product. (+)-*trans*-dG-N^2-BPDE, a major B[*a*]P adduct, promoted small amounts of incorporation of dTMP (1.9%), dAMP (0.5%), and dGMP (0.3%) opposite the lesion and two-base (1.1%) deletions. Similarly, (−)-*trans*-dG-N^2-BPDE promoted the incorporation of dTMP (2.5%), dAMP (0.5%), and dGMP (0.5%) opposite the lesion and one- (1.4%) and two- (1.5%) base deletions. (+)-*cis*-dG-N^2-BPDE was a relatively more blocking lesion as compared with other BPDE adducts; direct incorporation of dTMP (0.9%), dAMP (0.8%), and dGMP was observed at the lesion site, in addition to one-base (1.8%) and two-base (0.6%) deletions. The miscoding property of the (−)-*cis*-dG-N^2-BPDE adduct was similar to that of the (+)-*cis*-dG-N^2-BPDE. As shown by the arrows, small amounts of unknown products (4.8−7.8%) were also observed.

Kinetic Studies on dG-N^2-BPDE-Modified DNA Templates. Using steady-state kinetic methods, the frequency of dNTP incorporation (F_{ins}) was measured opposite a diastereoisomer of dG-N^2-BPDE within the linear range of the reaction (Table 2). For all dG-N^2-BPDE isomers, the F_{ins} values for dCTP, the correct base, were 2−4 orders of magnitudes higher than that for any of the other dNMPs. Interestingly, F_{ins} (2.52×10^{-4}) for dCTP opposite the (+)-*cis*-dG-N^2-BPDE was 9−13 times lower than that opposite any of the other isomers [$(2.25-3.29) \times 10^{-3}$] and was only 16, 32, and 41 times higher than that for dATP, dGTP, and dTTP, respectively. Chain extension reactions were carried out in the presence of dGTP. As a result, dC·dG-N^2-BPDE pairs were extended more efficiently in all cases than any of the other nucleotides paired with the BPDE-modified dGs (Table 2). F_{ext} for the dC·(+)-*trans*-dG-N^2-BPDE pair was 230 and 1100 greater than that of dA·(+)-*trans*-dG-N^2-BPDE and dG·(+)-*trans*-dG-N^2-BPDE, respectively, and 27 times greater than dT·(+)-*trans*-dG-N^2-BPDE. However, F_{ext} for the dC·(+)-*cis*-dG-N^2-BPDE pair was only 44 and 16 times greater than that of dA·(+)-*cis*-dG-N^2-BPDE and dT·(+)-*cis*-dG-N^2-BPDE, respectively. The relative frequency of translesion synthesis ($F_{ins} \times F_{ext}$) was estimated by multiplying F_{ins} by F_{ext} (Table 2). $F_{ins} \times F_{ext}$ for dC·dG-N^2-BPDE pairs were 2−6 orders of magnitudes higher than that of the other dN·dG-N^2-BPDE pairs. $F_{ins} \times F_{ext}$ for dC·(+)-*cis*-dG-N^2-BPDE was 650−680 times higher than that for dA·(+)-*cis*-dG-N^2-BPDE and dT·(+)-*cis*-dG-N^2-BPDE pairs.

DISCUSSION

Some of the newly discovered DNA polymerases with low fidelity, especially those classified as Y-family polymerases, are associated with translesion synthesis past sites of DNA damage (reviewed in ref *52*). Such enzymes, in addition to the replicative DNA polymerases (pol α, δ, and ϵ), are likely to contribute to mutational events in mammalian and human cells (*47*). In fact, primer extension catalyzed by pol α is blocked at the site of dG-N^2-BPDE lesions (*53*). We also observed that primer extensions catalyzed by pol α and pol

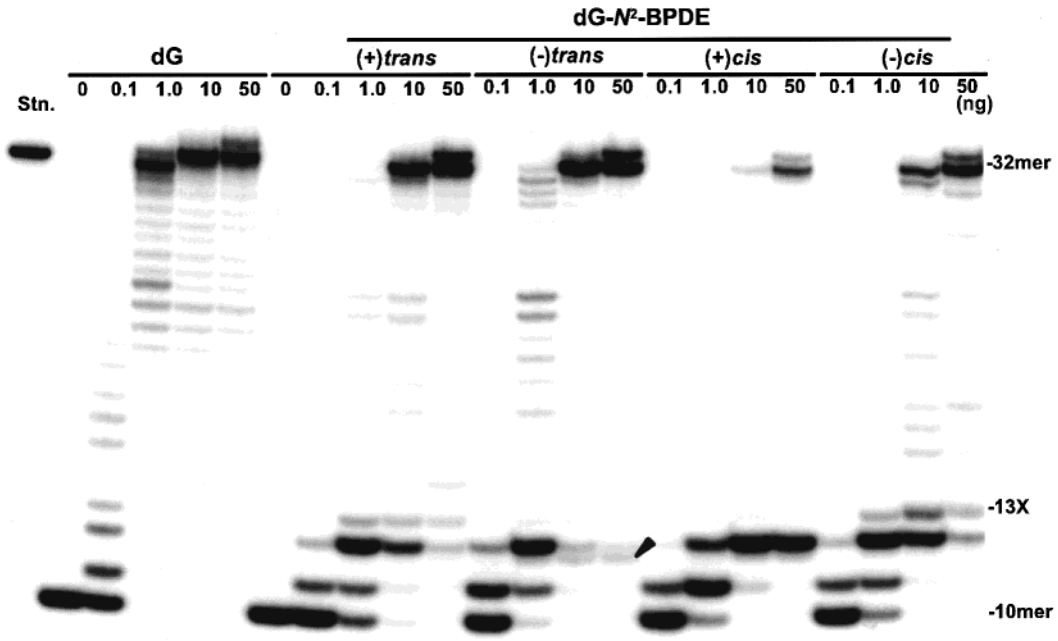

FIGURE 4: Primer extension reactions catalyzed by pol $\kappa\Delta$C on dG-N^2-BPDE-modified DNA templates. Using unmodified or dG-N^2-BPDE-modified 38-mer templates (150 fmol; 5′CATGCTGTTGAATTCCTTCXCTACTTTCCTCTCCCTTT, where X is dG or dG-N^2-BPDE) primed with a ^{32}P-labeled 10-mer (100 fmol; 5′AGAGGAAAGT), primer extension reactions were conducted at 25 °C for 30 min in a buffer containing four dNTPs (100 μM each) and variable amounts of pol $\kappa\Delta$C (no enzyme; 0.1 ng, 1.6 fmol; 1.0 ng, 16 fmol; 10 ng, 160 fmol; 50 ng, 800 fmol), as described in Materials and Methods. One-third of the reaction mixture was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). A 32-mer (5′AGAGGAAAGTAGCGAAGGAATTCAACAGCATG) was used as a marker of the fully extended product (Stn). The radioactivity of extended products was measured by a β-phosphorimager. 13X represents the adducted position.

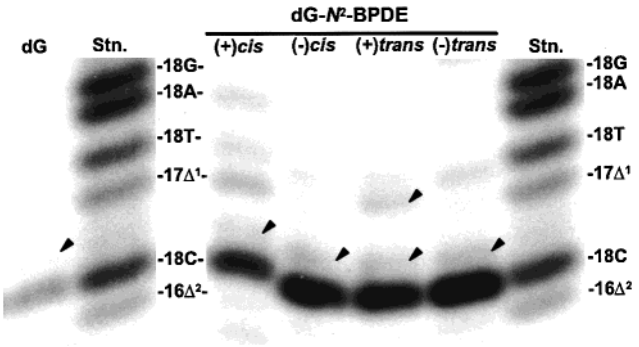

FIGURE 5: Miscoding specificities induced by dG-N^2-BPDE DNA adducts. Using unmodified or dG-N^2-BPDE-modified 38-mer templates (750 fmol) primed with a ^{32}P-labeled 12-mer (500 fmol; 5′AGAGGAAAGTAG), primer extension reactions were conducted at 30 °C for 30 min in a buffer containing four dNTPs (100 μM each) and pol $\kappa\Delta$C (5 ng for unmodified template; 50 ng for dG-N^2-BPDE-modified templates), as described in Materials and Methods. The extended reaction products (approximately 28−32-mer) produced on the unmodified, dG-N^2-BPDE-modified templates, except for (+)-*trans*-dG-N^2-BPDE, were extracted from one reaction sample following PAGE (35 × 42 × 0.04 cm). For the (+)-*trans*-dG-N^2-BPDE adduct, the extended reaction products were collected from four separated reaction samples. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer and cleaved with *Eco*RI, as described in Materials and Methods. One-fifth of the reaction sample containing the unmodified template and the entire sample from the dG-N^2-BPDE-modified template were subjected to two-phase 20% PAGE (15 × 72 × 0.04 cm). The mobility of reaction products was compared with those of 18-mer standards (Figure 1) containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions.

β were blocked at the lesion site.[2] However, primer extension reactions catalyzed by pol κ efficiently bypasses dG-N^2-BPDE adducts. Moreover, dCMP, the correct base, was predominantly incorporated opposite the lesions, resulting in only a low level of miscoding events. This observation was supported by steady-state kinetic studies. Pol κ may contribute to bypass bulky DNA adducts during DNA synthesis in cells.

On the basis of a ternary crystal structure obtained with an unmodified template/primer complexed with the Y-family polymerase Dpo4 (a DinB orthologue from *Sulfolobus solfataricus* P2), Ling et al. (*54*) showed that the active site is relatively open and less sterically hindered than in replicative polymerases. The authors speculated that such a structure might allow for local conformational flexibility that facilitates lesion bypass. Although there is considerable sequence homology between pol κ and pol Dpo4, the crystal structure of pol κ is not known. Multidimensional NMR studies reveal that the (+)-*trans*-dG-N^2-BPDE adduct adopts as anti conformation to pair with dC; the benzo[*a*]pyrene ring is positioned in the minor groove and directed toward the 5′ end of the modified strand (*55*). Therefore, when dCMP is inserted opposite (+)-*trans*-dG-N^2-BPDE, a Watson−Crick pair between dC and dG-N^2-BPDE may form during chain extension.

The behavior of pol κ on BPDE-derived adducts differs significantly from recent studies on AAF-derived DNA adducts in which a high level of miscoding occurs during DNA synthesis (*12*). When miscoding properties of dG-AAF was analyzed in the same sequence context using the same amount of pol $\kappa\Delta$C as for studies with dG-N^2-BPDE, the overall miscoding frequency for dG-AAF was as high as 39.9% (*12*). The frequency of base substitutions (37.1%) was approximately 14 times higher than that for (+)-*trans*-dG-N^2-BPDE (2.7%). The pattern of base substitutions associated with dG-AAF was different from that for (+)-*trans*-dG-N^2-

[2] S. Shibutani et al., unpublished data.

Table 2: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Pol κ[a]

	dNTP ↓GATGAAAGGAGA^{32P} 5′CCTTCXCTACTTTCCTCTCCCTTT			dGTP ↓NGATGAAAGGAGA^{32P} 5′CCTTCXCTACTTTCCTCTCCCTTT			
N·X	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	K_m (μM)	V_{max} (% min^{-1})	F_{ext}	$F_{ins} \times F_{ext}$
X = dG							
C·G	0.83 ± 0.12[b]	89.1 ± 11.0	1.0	0.24 ± 0.08	98.3 ± 19.2	1.0	1.0
X = (+)-*trans*-dG-N^2-BPDE							
C·X	14.1 ± 1.1	4.21 ± 0.27	2.78×10^{-3}	24.5 ± 2.5	39.2 ± 1.1	3.91×10^{-3}	1.09×10^{-5}
A·X	38.6 ± 8.8	(8.11 ± 1.66) × 10^{-2}	1.96×10^{-5}	152 ± 16	1.07 ± 0.01	1.72×10^{-5}	3.37×10^{-10}
G·X	186 ± 23	(4.75 ± 0.81) × 10^{-2}	2.36×10^{-6}	67.9 ± 15.9	(9.68 ± 0.47) × 10^{-2}	3.49×10^{-6}	8.24×10^{-12}
T·X	122 ± 6.7	(4.72 ± 0.50) × 10^{-2}	3.61×10^{-6}	101 ±1.6	5.97 ± 0.06	1.44×10^{-4}	5.20×10^{-10}
X = (−)-*trans*-dG-N^2-BPDE							
C·X	19.7 ± 1.6	6.93 ± 0.12	3.29×10^{-3}	17.0 ± 0.1	43.9 ± 0	6.30×10^{-3}	2.07×10^{-5}
A·X	136 ± 23	0.33 ± 0.01	2.30×10^{-5}	242 ± 46	7.62 ± 1.87	7.67×10^{-5}	1.76×10^{-9}
G·X	167 ± 32	0.13 ± 0.01	7.56×10^{-6}	238 ± 19	0.71 ± 0.07	7.35×10^{-6}	5.56×10^{-11}
T·X	133 ± 20	0.13 ± 0.01	9.19×10^{-6}	26.4 ± 2.1	11.1 ± 0.35	1.03×10^{-3}	9.47×10^{-9}
X = (+)-*cis*-dG-N^2-BPDE							
C·X	93.5 ± 22.0	2.49 ± 0.21	2.52×10^{-4}	14.7 ± 0.3	68.2 ± 1.2	1.13×10^{-2}	2.85×10^{-6}
A·X	85.1 ± 12.3	0.15 ± 0.01	1.61×10^{-5}	61.8 ± 11.6	6.56 ± 0.08	2.59×10^{-4}	4.17×10^{-9}
G·X	177 ± 15.0	0.15 ± 0.01	7.93×10^{-6}	808 ± 42	2.20 ± 0.28	6.64×10^{-6}	5.27×10^{-11}
T·X	65.0 ± 30.0	(3.86 ± 0.10) × 10^{-2}	6.22×10^{-6}	23.9 ± 0.8	6.95 ± 0.08	7.10×10^{-4}	4.42×10^{-9}
X = (−)-*cis*-dG-N^2-BPDE							
C·X	43.8 ± 4.5	10.6 ± 0.21	2.25×10^{-3}	36.7 ± 8.2	39.4 ± 5.7	2.61×10^{-3}	5.87×10^{-6}
A·X	41.9 ± 1.6	0.65 ± 0.02	1.45×10^{-4}	168 ± 2.5	1.65 ± 0.08	2.40×10^{-5}	3.48×10^{-9}
G·X	256 ± 18	0.28 ± 0.02	7.27×10^{-6}	43.3 ± 0.4	0.28 ± 0.01	1.58×10^{-5}	1.15×10^{-10}
T·X	155 ± 18	0.42 ± 0.01	2.53×10^{-5}	87.2 ± 7.1	3.62 ± 0.16	1.01×10^{-4}	2.56×10^{-9}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described under Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{wrong\ pair}/(V_{max}/K_m)_{correct\ pair}$. X = dG or dG-$N^2$-BPDE lesion. [b] Data are expressed as the mean ± SD.

BPDE lesion, dTMP (28.4%) being preferentially incorporated opposite the lesion, accompanied by lesser amounts of dAMP (5.3%) and dGMP (3.4%). The frequency of deletions observed for the dG-AAF (2.8%) was slightly higher than for (+)-*trans*-dG-N^2-BPDE (1.1%). Thus, the miscoding property varies depending on the nature of the adduct. In addition to differences in structure, the adducts are positioned at the N^2 and C8 position of the respective base, which may influence miscoding frequency and specificity.

The (+)-*trans*-dG-N^2-BPDE adduct is the dominant lesion among the four stereoisomeric dG-N^2-BPDE adducts (*46*). The (+)-*trans*-dG-N^2-BPDE promoted only a low level of base substitutions and deletions (Table 1). This result is inconsistent with observations of a high level of base substitutions in mammalian cells (*35*, *36*). We conclude that pol κ does not contribute significantly to mutations generated by dG-N^2-BPDE. Other newly discovered DNA polymerase-(s) may play this role in mammalian cells.

Interestingly, two bands were observed at position 12 prior to the (−)-*trans*-dG-N^2-BPDE lesion. The upper band was affected by the amount of pol $\kappa\Delta$C present (Figure 4), while the lower band (3.1−2.5%) remained essentially unchanged. By comparing the mobility of these products to 12-mers containing dG, dA, dC, or dT at the 3′ terminus, the upper band appears to represent incorporation of dGMP, the correct base, while the lower band appears to represent incorporation of dAMP at this position (data not shown). Such semitargeted incorporation of dAMP opposite dC prior to dG-N^2-BPDE was not observed in mammalian cells (*36*). As shown by arrows in Figure 5, unknown products were formed on dG-N^2-BPDE-modified templates during translesion synthesis catalyzed by pol κ. Small amounts of unknown products also were observed on unmodified templates. The amounts of these faint bands were not sufficient to analyze the sequence by Maxam−Gilbert methods. These products may be generated by inaccurate translesion synthesis by pol κ, as observed in other laboratories (*4*, *7*). The low frequency of misincorporation may occur elsewhere than the adducted site.

We have used purified *E. coli* pol IV carrying a His tag at the C-terminus (*12*) to catalyze bypass of dG-N^2-BPDE with the same DNA templates used in our studies with pol κ. Pol IV bypassed all of the four stereoisomeric dG-N^2-BPDE adducts efficiently, incorporating dCMP predominantly opposite the adducts (data not shown). Miscoding properties of dG-N^2-BPDE adducts observed with pol IV were similar to those observed with pol κ and in good agreement with results recently reported for (+)-*trans*- and (−)-*trans*-dG-N^2-BPDE and pol IV (*56*). Although only 30% of the amino acids of pol IV are identical to those of pol κ at the HDB region (*1*), the ability to bypass dG-N^2-BPDE efficiently and accurately appears to be conserved between *E. coli* pol IV and human pol κ.

The human *POLK* gene is localized to chromosome 5q13 and widely expressed in human tissues (*1*, *2*). Pol κ was overexpressed in tumor tissue derived from 21 of 29 patients with lung cancer, as compared with their nontumor counterparts, suggesting that pol κ overexpression may be correlated with the development of lung cancer (*57*). Although tobacco smoking is a major risk factor for lung cancer and B[*a*]P is a potent carcinogen found in tobacco smoke (*19*), no correlation was observed between pol κ overexpression and smoking history of the patients examined (*57*). Pol κ can bypass BPDE−DNA adducts efficiently and promotes only a low level of miscoding during DNA synthesis. Thus, other newly discovered DNA polymerase-(s) may be primarily associated with mutagenic events generated by BPDE.

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NOTE ADDED AFTER ASAP POSTING

This article was inadvertenty released ASAP on 04/20/02 before final corrections had been made. The units for the $K_m$values in Table 2 are now μM. The correct version was posted 05/07/02.

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