Translesional Synthesis Past Acetylaminofluorene-Derived DNA Adducts Catalyzed by Human DNA Polymerase κ and *Escherichia coli* DNA Polymerase IV†

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ABSTRACT: Human DNA polymerase κ (pol κ) has a sequence significantly homologous with that of *Escherichia coli* DNA polymerase IV (pol IV). We used a truncated form of human pol κ (pol κΔC) and full-length pol IV to explore the miscoding properties of these enzymes. Oligodeoxynucleotides, modified site-specifically with *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-AF), were used as DNA templates in primer extension reactions that included all four dNTPs. Reactions catalyzed by pol κΔC were partially blocked one base prior to dG-AAF or dG-AF, and also opposite both lesions. At higher enzyme concentrations, a significant fraction of primer was extended. Analysis of the fully extended reaction product revealed incorporation of dTMP opposite dG-AAF, accompanied by much smaller amounts of dCMP, dAMP, and dGMP and some one- and two-base deletions. The product terminating 3′ to the adduct site contained AMP misincorporated opposite dC. On templates containing dG-AF, dAMP, dTMP, and dCMP were incorporated opposite the lesion in approximately equal amounts, together with some one-base and two-base deletions. Steady-state kinetics analysis confirmed the results obtained from primer extension reactions catalyzed by pol κ. In contract, primer extension reactions catalyzed by pol IV were blocked effectively by dG-AAF and dG-AF. At high concentrations of pol IV, full-length products were formed containing primarily one- or two-base deletions with dCMP, the correct base, incorporated opposite dG-AF. The miscoding properties of pol κ observed in this study are consistent with mutational spectra observed when plasmid vectors containing dG-AAF or dG-AF are introduced into simian kidney cells [Shibutani, S., et al. (2001) *Biochemistry 40,* 3717−3722], supporting a model in which pol κ plays a role in translesion synthesis past acetylaminofluorene-derived lesions in mammalian cells.

Acetylaminofluorene (2-AAF),[1] a prototypic aromatic amide and model chemical carcinogen (reviewed in ref *1*), is metabolically activated in cells and reacts with DNA to form primarily *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-AF) DNA adducts (structures in Figure 1) (*2*−*8*). Both adducts are mutagenic in mammalian cells, generating base substitutions and frameshift deletions (*1*, *9*−*13*). Site-specific mutagenesis techniques have been used to explore the miscoding properties of single acetylaminofluorene-derived DNA adducts in simian kidney (COS-7) cells (*14*−*16*). dG-AAF and dG-AF adducts incorporated into a single-strand shuttle vector promote G → T transversions and G → A transitions targeted to the site of the lesion (*15*). The mutation frequency varies significantly, depending on the 3′ and 5′ sequence flanking the lesion (*16*).

The miscoding properties of dG-AAF and dG-AF have been explored in vitro, using mammalian DNA polymerases and primer extension methods. In reactions catalyzed by pol α or pol δ, dG-AAF blocks primer extension while dG-AF is bypassed with dCMP, the "correct" base, incorporated opposite the lesion (*15*). In reactions catalyzed by pol α, dG-AAF promoted incorporation of small amounts of dAMP and dTMP, accompanied by two-base deletions.

A number of novel human DNA polymerases have recently been characterized (reviewed in ref *17*). These polymerases are unusual in that they efficiently catalyze DNA synthesis past various forms of damaged DNA. Among these "translesion" polymerases is human pol κ, a homologue of the *Escherichia coli* DinB polymerase (*17*−*20*). This enzyme belongs to the UmuC superfamily (*21*) that was recently named as the Y-family (*22*). The human *DINB1* gene is widely expressed in human tissues (*18*, *19*). *E. coli DINB* (pol IV) (*23*, *24*) and pol κ (*25*, *26*) lack 3′−5′ proofreading exonuclease activity and exhibit high error rates even on nondamaged DNA templates.

The ability of pol κ to bypass various DNA lesions has been tested in vitro. Pol κ catalyzes translesion synthesis past abasic sites (*27*, *28*) and 8-oxo-7,8-dihydrodeoxyguanosine (*28*), incorporating dAMP opposite both lesions. The enzyme catalyzes error-free translesion synthesis past (−)-*trans*-*anti*-benzo[*a*]pyrene-N^2-dG, readily extending the dC·dG−BPDE pair from the 3′ terminus (*28*). Pol κ is unable to bypass *cis*-*syn* and (6-4) thymine-thymine dimers (*27*, *28*) or

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[1] Abbreviations: AAF, acetylaminofluorene; dG-AAF, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; pol κ, human DNA polymerase κ; pol κΔC, truncated form of pol κ; pol IV, *E. coli* DNA polymerase IV; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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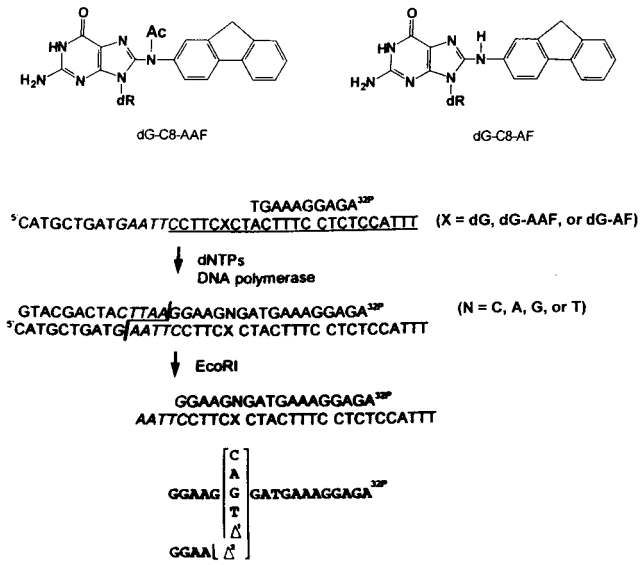

FIGURE 1: Structures of dG-AAF and dG-AF and a diagram of the method used to determine miscoding specificity. Structures of DNA adducts used in this study are shown. Unmodified or dG-AAF- or dG-AF-modified 38-mer templates are annealed to a ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed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

cisplatin adducts (*27*, *29*). Primer extension past dG-AAF is partially blocked at the adduct site. In studies conducted with a single dNTP, pol κ preferentially incorporates dTMP and dCMP opposite dG-AAF (*27*−*29*).

In this paper, base substitutions and deletions targeted to dG-AAF or dG-AF were observed during DNA synthesis in the presence of all four dNTPs in reactions catalyzed by pol κ or pol IV. Steady-state kinetic studies were performed with pol κ. The miscoding properties of this polymerase are compared with results of site-specific mutagenesis studies performed on dG-AAF and dG-AF adducts in simian kidney cells.

MATERIALS AND METHODS

General. [γ-^{32}P]ATP (specific activity of >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 1) were prepared by phosphoramidite chemistry using an automated DNA synthesizer (Applied Biosystems model 392) (*30*). To prepare dG-AAF-modified oligodeoxynucleotides, a 24-mer oligomer containing a single dG (5′CCTTCGCTACTTTCCTCTCCCTTT) was reacted with *N*-acetoxy-AAF (*31*, *32*). The dG-AF-modified oligomer was prepared by incubating the dG-AAF-modified oligomer under alkaline conditions in the presence of mercaptoethanol (*31*). Modified and unmodified oligodeoxynucleotides were purified on a Waters reverse-phase μBondapak C_{18} column (0.39 cm × 30 cm), using an isocratic condition of 0.05 M triethylammonium acetate (pH 7.0) containing 5% acetonitrile for 5 min and, subsequently, a linear gradient of 0.05 mM triethylammonium acetate (pH 7.0) containing 5 to 20% acetonitrile with an elution time of 55 min, at a flow rate of 1.0 mL/min. Modified 38-mer templates (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCCTTT, where X is dG-AAF or dG-AF) were prepared as described previously by ligating the dG-AAF- or dG-AF-modified 24-mer (5′CCTTCXCTACTTTCCTCTCCCTTT) to a 14-mer (5′CATGCTGATGAATT) (*15*). Briefly, 3 μg of dG-AAF- or dG-AF-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 (*33*), ligated to the 14-mer (3.6 μg), and then inbucated at 8 °C overnight using 3 μL of T4 DNA ligase (400 units/μL) and an 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 (*34*, *35*). The 38-mer product was isolated on a μBondapak C_{18} column (0.39 cm × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing containing 10 to 20% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min (*32*). The unmodified and modified 38-mers were purified by electrophoresis on a 20% nondenaturing polyacrylamide gel (35 cm × 42 cm × 0.04 cm). Bands were detected under ultraviolet light and extracted overnight with 2.0 mL of distilled water at 4 °C. Extracts were evaporated to dryness, and the 38-mers were isolated by HPLC. A fraction containing unmodified or dG-AAF- or dG-AF-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 (*33*) and subjected to 20% polyacrylamide gel electrophoresis (35 cm × 42 cm × 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 separation and purification of oligodeoxynucleotides. UV spectra and concentrations of oligomers were measured with a Hewlett-Packard 8452A diode array spectrophotometer.

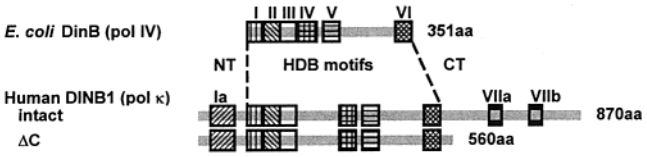

FIGURE 2: Schematic representation of *E. coli* DinB and human DINB1. Motifs I−VI are shared among UmuC/DinB family proteins, while motif Ia is conserved among mammalian and *Caenorhabditis elegans* DinB homologues (*19*). Motifs VIIa and VIIb denote zinc clusters (*18*). Pol $\kappa\Delta$C used in this study lacks motifs VIIa and VIIb. NT, N-terminal region; HDB, homology to DinB region; CT, C-terminal region.

Pol κ and Pol IV. A schematic representation of *E. coli* DinB (pol IV) and human DINB1 (pol κ) is shown in Figure 2. Pol $\kappa\Delta$C (560 amino acids) used in this study lacks motifs VIIa and VIIb that denote zinc clusters from the intact

DINB1 protein (870 amino acids) (*18*). Motifs Ia, VIIa, and VIIb are not conserved in pol IV (351 amino acids) (*19*).

Pol $\kappa\Delta$C with a six-His tag attached at the carboxyl terminus was prepared by overproduction using a baculovirus expression system, as described elsewhere (*27*). Pol IV was prepared from *E. coli* LMG194 (*36*) carrying pKE4, which is a derivative of pYP73 (*37*) encoding DinB with a six-His tag at the C-terminus. Frozen cells containing pKE4 (~20 g of wet weight) were thawed and resuspended in 100 mL of buffer A [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 10 mM imidazole, and 20% glycerol]. Lysozyme and phenylmethanesulfonyl fluoride (PMSF) were added to final concentrations of 0.1 mg/mL and 0.1 mM, respectively. After standing on ice for 30 min, the cells were disrupted by sonication. The cell lysates were centrifuged at 32 000 rpm for 30 min, and the supernatant was applied to a Hitrap chelating column (Amersham). The column was washed with a buffer and then eluted with a linear gradient of imidazole up to 500 mM. Pol IV protein was eluted around 350 mM imidazole. Fractions containing Pol IV were combined, dialyzed overnight against buffer B [20 mM sodium phosphate (pH 7.4), 100 mM NaCl, and 20% glycerol], and applied to a Hiload 16/10 Q Sepharose HP column (Amersham). The flow-through fraction was then applied to Hiload 16/10 SP Sepharose HP (Amersham). The column was washed with buffer B and then with a linear gradient of NaCl up to 1 M. The pol IV protein was eluted around 400 mM NaCl. Approximately 20 mg of purified pol IV was obtained.

Primer Extension Reactions. Using a 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 or pol IV 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 1). The reaction buffer for pol $\kappa\Delta$C and pol IV 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. The reaction was stopped by addition of formamide dye. The samples were subjected to 20% denaturing PAGE. The radioactivity levels of extended products were measured by β-phosphorimager (Molecular Dynamics).

Miscoding Specificity. Using dG-AAF- or dG-AF-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 unmodified template; 50 ng for dG-AAF- and dG-AF-modified templates) or pol IV (200 ng for unmodified template; 1000 ng for dG-AAF- and dG-AF-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 gel. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer and cleaved with *Eco*RI. To quantify base substitutions and deletions, the samples were subjected to two-phase PAGE (15 cm × 72 cm × 0.04 cm) (*38*, *39*) (Figure 1).

Steady-State Kinetic Studies. Kinetic parameters associated with nucleotide insertion opposite dG-AAF or dG-AF 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 a 24-mer template (150 fmol; 5′CCTTCXCTACTTTCCTCTCCCTTT, where X is dG, dG-AAF, or dG-AF) 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 assess chain extension. The reaction samples were subjected to 20% denaturating PAGE (35 cm × 42 cm × 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 $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dC\cdot dG]}$ (*40*, *41*).

FIGURE 3: Polyacrylamide gel electrophoresis of 38-mer oligodeoxynucleotides containing a single AAF-derived DNA adduct. The 38-mer oligodeoxynucleotides (5′CATGCTGATGAATTCCTTCXCTACTTTCCTCTCCCTTT) containing a single dG (lane 1), dG-AAF (lane 2), or dG-AF (lane 3) at the X position were prepared as described in Materials and Methods, labeled with [γ-^{32}P]ATP, and subjected to 20% polyacrylamide gel electrophoresis (35 cm × 42 cm × 0.04 cm). A 24-mer oligomer containing dG is shown in lane 4. The position and homogeneity of oligodeoxynucleotides following gel electrophoresis were determined by a β-phosphorimager.

RESULTS

Primer Extension Reactions Catalyzed by Pol $\kappa\Delta$C on dG-AAF- and dG-AF-Modified DNA Templates. dG-AAF- and dG-AF-modified 38-mer templates were prepared by ligation of the 24-mer containing a single dG-AAF or dG-AF with a 14-mer, and purified by HPLC and gel electrophoresis as described in Materials and Methods. An unmodified 38-mer was prepared by phosphoramidite chemical synthesis. The unmodified and modified 38-mers were analyzed by 20% polyacrylamide gel electrophoresis after labeling with ^{32}P at the 5′ terminus (Figure 3). The dG-AAF- and dG-AF-modified 38-mers (lanes 2 and 3, respectively) migrated slower than the unmodified oligomer (lane 1), as previously

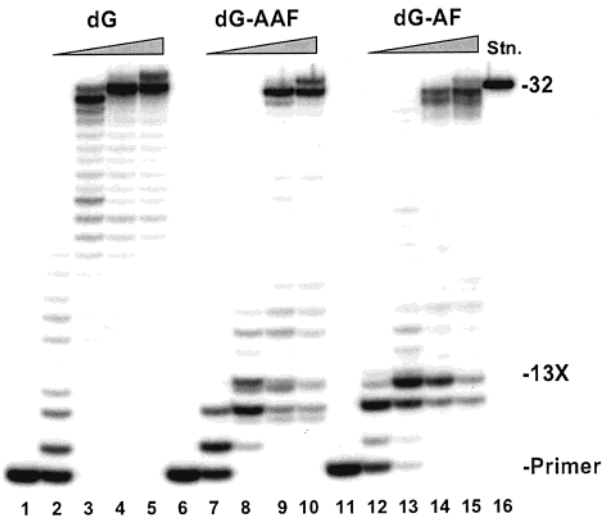

FIGURE 4: Primer extension reactions catalyzed by pol $\kappa\Delta$C on dG-AAF- and dG-AF-modified DNA templates. Using unmodified (lanes 1−5), dG-AAF-modified (lanes 6−10), or dG-AF-modified (lanes 11−15) 38-mer templates (150 fmol; 5′CATGCTGTTGAATTCCTTCXCTACTTTCCTCTCCCTTT, where X is dG, dG-AAF, or dG-AF) 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 in lanes 1, 6, and 11; 0.1 ng (1.6 fmol) for lanes 2, 7, and 12; 1.0 ng (16 fmol) for lanes 3, 8, and 13; 10 ng (160 fmol) for lanes 4, 9, and 14; and 50 ng (800 fmol) for lanes 5, 10, and 15], as described in Materials and Methods. One-third of the reaction mixture was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 cm × 42 cm × 0.04 cm). A 32-mer (5′AGAGGAAAGTAGCGAAGGAATTCAACAGCATG) was used as a marker of the fully extended product (lane 16). Radioactivity levels of extended products were measured by a β-phosphorimager. 13X represents the adducted position.

observed for shorter oligomers (*32*). There were no contaminants of the unmodified 38-mer and the starting modified 24-mer in the dG-AAF- or dG-AF-modified 38-mer. All unmodified and modified 38-mers were purified to homogeneity using a combination of HPLC and gel electrophoresis.

Using unmodified and dG-AAF- and dG-AF-modified 38-mer templates, primer extension reactions were conducted in the presence of four dNTPs using 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 (1:6.3 enzyme:primer molar ratio), 91% of the starting primer was extended past the unmodified dG. In contrast, when dG-AAF- or dG-AF-modified templates were used, primer extension was retarded one base prior to, and opposite, both lesions. Using 1.0 ng of pol $\kappa\Delta$C, 26.7 and 29.6% of the starting primer extended past the dG-AAF and dG-AF lesions, respectively. A large amount of extended products was obtained by increasing the amount (10 and 50 ng) of pol $\kappa\Delta$C (Figure 4). By comparison with the mobility of standard 32-mer (lane 16), primer extension reactions were shown to stop one or two nucleotides prior to the end of unmodified and modified templates. This phenomenon has been observed by us (E. Ohashi and H. Ohmori) (*27*) and by other laboratories (*20*, *29*). The failure to replicate precisely to the end of the DNA template may be due to the intrinsic property of pol κ (*29*).

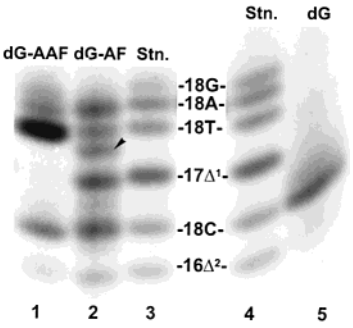

FIGURE 5: Miscoding specificities induced by dG-AAF and dG-AF DNA adducts. Using unmodified (lane 5), dG-AAF-modified (lane 1), and dG-AF-modified (lane 2) 38-mer templates (750 fmol) primed with a ^{32}P-labeled 12-mer (500 fmol; 5′AGAGGAAAGTAG), primer extension reactions were conducted at 30 °C for 30 min in a buffer containing four dNTPs (100 μM each) and pol $\kappa\Delta$C (5 ng for the unmodified template; 50 ng for dG-AAF- and dG-AF-modified templates), as described in Materials and Methods. The extended reaction products (approximately 28−32-mer) produced on the unmodified or dG-AAF- or dG-AF-modified templates were extracted following PAGE (35 cm × 42 cm × 0.04 cm). The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer and cleaved with the *Eco*RI restriction enzyme, as described in Materials and Methods. One-fifth of the reaction sample containing the unmodified template and the entire sample from the dG-AAF- or dG-AF-modified template were subjected to a two-phase 20% PAGE (15 cm × 72 cm × 0.04 cm). Mobilities of reaction products were 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 (lanes 3 and 4).

Miscoding Properties of dG-AAF and dG-AF. Because misincorporation occurred at the position prior to the dG-AAF lesion, a 12-mer primer, instead of a 10-mer, was used to assess incorporation of dNTP during translesional synthesis catalyzed by pol $\kappa\Delta$C. Following primer extension past dG-AAF or dG-AF in reaction mixtures containing four dNTPs, extended products (approximately 28−32-mer) were recovered and digested by *Eco*RI. Products were subjected to two-phase PAGE to quantify base substitutions and deletions formed opposite the lesion (Figure 1). A standard mixture of six ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions is resolved by this method (Figure 5, lanes 3 and 4). When the unmodified template was used, dCMP, the correct base, was inserted opposite dG (Figure 5, lane 5, and Table 1); however, small amounts (3.2%) of unknown products were also observed. dG-AAF directed misincorporation of dTMP (28.4%), along with smaller amounts of dCMP (7.7%), dAMP (5.3%), and dGMP (3.4%). A small amount of two-base deletions (1.7%) also was observed (lane 1). dG-AF promoted similar incorporation of dCMP (10.9%), dAMP (9.3%), and dTMP (6.6%) opposite the lesion (lane 2). One-base (10.5%) and two-base (2.7%) deletions also were detected. As shown by an arrow, an unknown product was detected. Miscoding frequencies of dG-AAF and dG-AF were 40 and 31%, respectively; the miscoding specificity of dG-AAF differed from that of dG-AF (Table 1). Although dG-AF is oxidized even in neutral buffers to form three products (*42*, *43*), this degradation can be prohibited by an antioxidant such as DTT (*43*). The reaction buffer used for primer extension reactions contains 10 mM DTT, and such degradation was not observed. Therefore, the miscoding specificity of the dG-AF adduct could not have been affected by oxidation products from dG-AF.

Table 1: Base Incorporation and Deletions Occurred Opposite the dG-AAF or dG-AF Lesion in Reactions Catalyzed by Pol κ and Pol IV

		miscoding event[c] (%)					
DNA adduct	C[c]	A[c]	G[c]	T[c]	Δ^{1} [c]	Δ^{2} [c]	total
pol κ[a]							
dG	87.0 ± 2.6						**0**
dG-AAF	7.7 ± 0.6	5.3 ± 0.4	3.4 ± 0.3	28.4 ± 2.1	1.1 ± 0.2	1.7 ± 0.2	**39.9**
dG-AF	10.9 ± 1.2	9.3 ± 0.7	1.7 ± 0.1	6.6 ± 0.5	10.5 ± 0.8	2.7 ± 0.1	**30.8**
pol IV[b]							
dG	77.1 ± 3.1						**0**
dG-AAF	5.0 ± 0.4	0.36 ± 0.03	0.10 ± 0.01	1.6 ± 0.2	3.2 ± 0.2	5.6 ± 0.3	**10.9**
dG-AF	11.9 ± 0.8	0.97 ± 0.10	0.26 ± 0.01	nd[d]	1.3 ± 0.1	2.0 ± 0.1	**4.5**

[a] Data taken from Figure 5. [b] Data taken from Figure 6B. [c] C, A, G, T, Δ^1, and Δ^2 represent the amount of extended product containing dC, dA, dG, dT, and one- and two-base deletions at the lesion site, respectively. Data are expressed as means ± the standard deviation obtained from three independent experiments. [d] Not detectable.

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

Insertion dNTP ↓GATGAAAGGAGA^{32P} 5′CCTTCXCTACTTTCCTCTCCCTTT				Extension 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}xF_{ext}
X = dG							
C:G	0.83 ± 0.12[b]	89.1 ± 11.0[b]	**1.0**	0.24 ± 0.08[b]	98.3 ± 19.2[b]	**1.0**	**1.0**
X = dG-AAF							
C:X	122 ± 3.9	15.8 ± 0.35	**1.20 x 10^{-3}**	404 ± 30	12.0 ± 0.7	**7.24 x 10^{-5}**	**8.69 x 10^{-8}**
A:X	318 ± 38	7.84 ± 0.70	**2.32 x 10^{-4}**	335 ± 35	3.90 ± 0.63	**2.95 x 10^{-5}**	**6.84 x 10^{-9}**
G:X	381 ± 24	0.82 ± 0.03	**1.99 x 10^{-5}**	467 ± 21	3.94 ± 0.08	**2.06 x 10^{-5}**	**4.10 x 10^{-10}**
T:X	56.1 ± 2.9	17.4 ± 0.37	**2.88 x 10^{-3}**	263 ± 27	31.4 ± 1.3	**2.92 x 10^{-4}**	**8.41 x 10^{-7}**
X = dG-AF							
C:X	95.7 ± 2.1	15.9 ± 0.36	**1.54 x 10^{-3}**	141 ± 31	0.87 ± 0.09	**1.56 x 10^{-5}**	**2.40 x 10^{-8}**
A:X	303 ± 32	14.3 ± 1.5	**4.57 x 10^{-4}**	414 ± 14	8.25 ± 0.69	**4.86 x 10^{-5}**	**2.22 x 10^{-8}**
G:X	267 ± 4.8	9.22 ± 2.50	**3.21 x 10^{-4}**	466 ± 40	4.52 ± 0.92	**2.42 x 10^{-5}**	**7.77 x 10^{-9}**
T:X	290 ± 9.4	1.13 ± 0.24	**3.61 x 10^{-5}**	328 ± 30	1.91 ± 0.28	**1.37 x 10^{-5}**	**4.95 x 10^{-10}**

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dC\cdot dG]}$. X is a dG, dG-AAF, or dG-AF lesion. [b] Data expressed as means ± the standard deviation obtained from three independent experiments.

Kinetic Studies on dG-AAF and dG-AF-Modified DNA Templates. Using steady-state kinetic methods, the frequency of dNTP incorporation (F_{ins}) was measured opposite dG-AAF or dG-AF within the linear range of the reaction. Although the V_{max} for dTTP insertion opposite dG-AAF was similar to that for dCTP, the K_m for dTTP was 2.2 times lower than that for dCTP (Table 2). Therefore, F_{ins} for dTTP is 2.4 times higher than that for dCMP, the correct base. F_{ins} for dATP and dGTP was 12 and 150 times lower than that for dTTP, respectively. Chain extension reactions were carried out in the presence of dGTP. V_{max} for the dT·dG-AAF base pair was much higher, and K_m lower, than for other base pairs. As a result, the dT·dG-AAF pair was extended more efficiently than other pairs (Table 2). F_{ext} for the dT·dG-AAF base pair was 4.0, 10, and 14 times higher than those for the dC·dG-AAF, dA·dG-AAF, and dG·dG-AAF pairs, respectively.

For the dG-AF-modified template, V_{max} for dTTP insertion opposite dG-AF was 14 times lower than that for dCTP and K_m for dTTP was 3.0 times higher than that for dCTP. F_{ins} for dTTP was 43 times lower than that for dCMP, and F_{ins} for dCTP was 3.4 and 4.8 times higher than that for dATP and dGTP, respectively. F_{ext} for the dC·dG-AF pair was similar to that for the dT·dG-AF pair, and the F_{ext} of the dA·dG-AF pair was 2.0−3.5 times higher than that for other pairs (Table 2).

The relative frequency of translesion synthesis ($F_{ins}F_{ext}$) was estimated by multiplying F_{ins} by F_{ext} (Table 2). $F_{ins}F_{ext}$ for the dT·dG-AAF pair was 10 times higher than that for the dC·dG-AAF pair and 120−2100 times higher than those for other pairs. In contrast, $F_{ins}F_{ext}$ for the dT·dG-AF pair was much lower than those for other pairs; for example, $F_{ins}F_{ext}$ was 48 times lower than that for the dC·dG-AF pair. $F_{ins}F_{ext}$ for the dC·dG-AF pair was similar to that for the dA·dG-AF pair and slightly higher than that for the dG·dG-AF pair.

Primer Extension Reactions Catalyzed by Pol IV. Primer extension catalyzed by pol IV readily occurred on an unmodified DNA template to form fully extended products (Figure 6A). However, primer extension reactions were blocked both one base prior to dG-AAF or dG-AF and opposite the lesions (Figure 6A). Using 200 ng of pol IV (a 50-fold molar excess over the primer), 6.5 and 10.2% of the starting primer was extended past dG-AAF and dG-AF, respectively.

At a higher temperature or when the mixture was incubated for longer times, the amount of fully extended products increased; these were recovered from the gel, digested by *Eco*RI, and subjected to two-phase PAGE to quantify miscoding (Figure 6B). When the unmodified template was used, dCMP, the correct base, was inserted primarily opposite dG (Figure 6B, lane 1, and Table 1); however, small amounts (1.3%) of unknown products were also observed. The presence of dG-AAF was associated with one-base (3.2%) and two-base (5.6%) deletions (Figure 6B, lane 3, and Table 1). Targeted incorporation of dCMP (5.0%), the correct base, and dTMP (1.6%) were observed, along with smaller amounts of dAMP (0.36%) and dGMP (0.10%). In contrast,

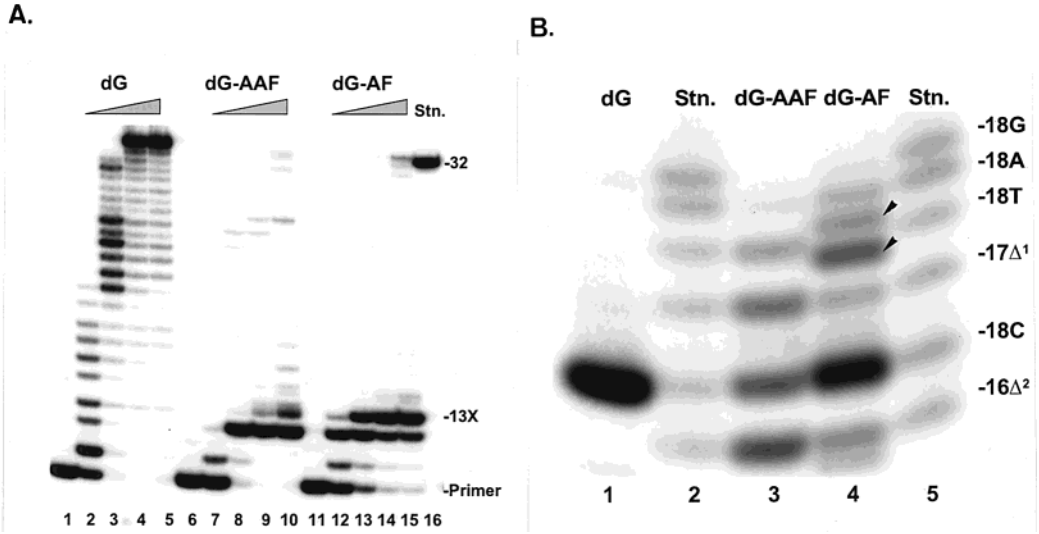

FIGURE 6: Primer extension reactions catalyzed by pol IV on dG-AAF- and dG-AF-modified DNA templates and a quantitative miscoding analysis. (A) Using unmodified (lanes 1−5), dG-AAF-modified (lanes 6−10), or dG-AF-modified (lanes 11−15) 38-mer templates (150 fmol) primed with a ^{32}P-labeled 10-mer (100 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 IV [no enzyme in lanes 1, 6, and 11; 0.4 ng (10 fmol) for lanes 2, 7, and 12; 4.0 ng (100 fmol) for lanes 3, 8, and 13; 40 ng (1 pmol) for lanes 4, 9, and 14; and 200 ng (5 pmol) for lanes 5, 10, and 15], as described in Materials and Methods. One-third of the reaction mixture was subjected to the denaturing 20% PAGE (35 cm × 42 cm × 0.04 cm). A 32-mer (5′AGAGGAAAGTAGCGAAGGAATTCAACAGCATG) was used as a marker of the fully extended product (lane 16). The radioactivity levels of extended products were measured by a β-phosphorimager. (B) Using unmodified (lane 1), dG-AAF-modified (lane 3), and dG-AF-modified (lane 4) 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 1.5 h in a buffer containing four dNTPs (100 μM each) and pol IV (200 ng for the unmodified template; 1000 ng for the dG-AAF- and dG-AF-modified templates), as described in Materials and Methods. The fully extended reaction products recovered from PAGE (35 cm × 42 cm × 0.04 cm) were used for miscoding analysis using two-phase 20% polyacrylamide gel electrophoresis (15 cm × 72 cm × 0.04 cm) as described in the legend of Figure 5.

dG-AF promoted preferential incorporation of dCMP (11.9%) opposite the lesion, accompanied by small amounts of dAMP (0.97%) and dGMP (0.26%) (lane 4). One-base (1.3%) and two-base (2.0%) deletions also were observed (lane 4). As shown by the arrows, several unknown products were detected.

DISCUSSION

The discovery of prokaryotic and eukaryotic DNA polymerases that efficiently catalyze translesion synthesis past sites of DNA damage (reviewed in ref *17*) supports the hypothesis that enzymes in addition to DNA polymerases α, δ, and ϵ play important roles in determining mutational specificity (*15*). In this paper, we report studies of DNA synthesis past dG-AAF and dG-AF in reactions catalyzed by human pol κ and *E. coli* pol IV. Although the sequences of the two enzymes are significantly homologous, their intrinsic miscoding properties and propensity to generate deletions differ significantly as summarized in Table 1.

Studies with Human Pol κ. Reactions involved in translesion synthesis (nucleotide insertion, proofreading, and chain extension from the 3′ terminus) are conveniently dissected by performing primer extension reactions on DNA templates containing a single adducted base. Translesion synthesis catalyzed by pol κ was partially blocked at the lesion site and at the 3′ flanking position. Significant translesion synthesis occurred when the enzyme:template molar ratio was 0.16; fully extended products appeared when this ratio was increased 10-fold. These observations reflect the processivity of pol κ, which is intermediate between that of pol η and pol β and consistent with the several roles postulated for this enzyme in cellular mutagenesis (*25*). In our study, base substitutions and deletions generated by pol κ were identified in the presence of all four NTPs (100 μM each). In parallel, under steady-state conditions, F_{ins} and F_{ext} were established for each base−DNA adduct pair. The highest K_m value that was observed was 470 μM. Although high concentrations of dNTPs may affect translesion synthesis catalyzed by pol κ, we chose not to examine it under highly unphysiological conditions. Taken together, these assays can be used, in part, to predict the spectrum of mutations associated with a given DNA adduct in mammalian cells, as determined by site-specific mutagenesis techniques (*15*, *16*).

dTTP was preferentially incorporated opposite dG-AAF with dAMP, dCMP, and dGMP incorporated in smaller amounts. These observations are in good agreement with studies in which the relative level of incorporation of a single dNTP in reactions catalyzed by pol κ was measured in several sequence contexts (*27*−*29*). Interestingly, fidelity assays on unmodified templates reveal that the highest error rate for pol κ (truncated enzyme and GST fusion product) is associated with the T−dCMP mispair (*25*). Our in vitro miscoding studies predict G → A and G → T mutations and a smaller number of G → C base substitutions targeted to the lesion site in cells. This mutation spectrum was observed when dG-AAF was introduced into a single-strand DNA vector and replicated in simian kidney (COS) cells (*15*). In that study, G → A transversions predominated; however, we have shown that the relative frequency of G → T and G → A mutations in this experimental system is strongly affected by the nature of the bases flanking the lesion (*16*).

At least two bands were observed at position 12 prior to dG-AAF, but not prior to dG-AF. The magnitude of the upper

band decreased depending on the amount of pol $\kappa\Delta$C present (Figure 4, lanes 7−10), while the lower band (3.1−3.7%) remained essentially unchanged. By comparison of 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, and the lower band appears to represent incorporation of dAMP at this position (data not shown). Semitargeted incorporation of dAMP opposite dC prior to the dG-AAF lesion was observed in vitro and occasionally in COS cells (*15*, *16*).

Translesion synthesis catalyzed by pol κ in vitro is dominated by the targeted misincorporation of dTMP opposite dG-AAF. However, the overall mutation frequency of dG-AAF embedded in a similar sequence in COS cells is only 11% (*15*). Various factors may operate to minimize mutagenicity in mammalian cells. For example, pol δ and ϵ have powerful 3′ → 5′ exonucleases that can excise misincorporated dTMP from blocked replication forks. Additionally, pol η efficiently catalyzes error-free synthesis past dG-AAF adducts (*44*, *45*) and may contribute to translesion synthesis in vivo.

One- and two-base deletions, targeted to the site of the lesion, are invariably present in our primer extension experiments. On the basis of our previous studies using the Klenow fragment of *E. coli* DNA pol I and dG-AAF-modified oligodeoxynucleotide templates with different sequence contexts (*31*), the propensity to form misaligned intermediates that lead to such deletions was found to be determined by two parameters: the rate of translesion synthesis, as determined by steady-state kinetics, and the nature of the base incorporated opposite the lesion relative to the sequence context in which the lesion is embedded. This model was used successfully to predict deletions generated by a variety of DNA lesions, such as 3,N^4-etheno-2′-deoxycytidine (*39*) and abasic sites (*46*), in reactions catalyzed by DNA pol α and β. On the basis of the sequence context and frequencies of insertion of dGMP and dAMP opposite dG-AAF, the one- and two-base deletions observed in reactions catalyzed by pol κ are those predicted by our model. Thus, F_{ins} for dAMP opposite dG-AAF was 5.2 and 12.4 times lower, respectively, than for dCMP and dTMP, and F_{ins} for dAMP opposite dG-AF was 3.4 times lower than for dCMP. A small amount of targeted incorporation of dAMP was observed opposite dG-AAF and dG-AF. The newly inserted dAMP and G 5′ flanking base is expected to pair with TC 5′ to the lesion, promoting two-base deletions (Figure 7B). Since F_{ins} for dGMP opposite dG-AAF was much lower than for other dNMPs, dGMP is unlikely to be incorporated. On the other hand, F_{ins} for dGMP opposite dG-AF was similar to that for dAMP and incorporation of dGMP was observed during DNA synthesis. Thus, dGMP inserted opposite the dG-AF could pair with C 5′ to the lesion, forming one-base deletions (Figure 7A). The mechanism described previously (*31*) also may account for the one- and two-base shortened reaction products observed in some studies of pol κ with dG-AAF and dG-AF (*18*−*20*).

Although the overall miscoding frequencies for dG-AF and dG-AAF are approximately the same (30.8 vs 39.9%), the pattern of base incorporation opposite these lesions was quite different. dCMP and dAMP are preferentially incorporated opposite dG-AF, and a significant number of targeted one-base deletions are formed. These observations are based on

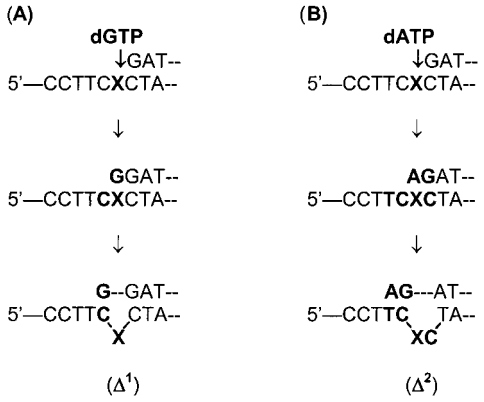

FIGURE 7: Proposed mechanism of one-base and two-base deletions. (A) When dGMP is inserted opposite the adducted lesion, the newly inserted nucleotide pairs with the 5′ flanking base (C) to form a one-base deletion. (B) When dAMP is inserted opposite the adducted lesion, the newly inserted nucleotide and the 5′ flanking base (G) can pair with TC 5′ to the adducted base to form a two-base deletion.

sequence analysis of full-length reaction products formed in the presence of four dNTPs and are consistent with results of steady-state kinetics analyses. Thus, $F_{ins}F_{ext}$ values for dC·dG-AF and dA·dG-AF pairs are approximately the same, 50-fold higher than that for the dT·dG-AF pair. These comparisons were made in vitro on DNA templates in which dG-AF was embedded in the sequence context TTCXCTA; in cells, mutagenic frequency and specificity of dG-AF vary significantly, depending in part on the nature of the base(s) flanking the lesion (*16*). Kinetic parameters for a bulky adduct may reflect several factors, including the nature of the adduct, the intrinsic properties of pol κ, and imperfect hybridization of the primer and adducted template.

Studies with E. coli Pol IV. The sequence of *E. coli* pol IV is considerably homologous with that of pol κ, but *E. coli* pol IV lacks the processivity associated with the latter enzyme. It is instructive to compare the miscoding properties of these enzymes on templates containing dG-AF or dG-AAF. In experiments with pol IV, translesion synthesis is observed only at high enzyme:primer ratios. The overall level of miscoding for both adducts with pol IV is much lower than for pol κ. Our results are consistent with the conclusion of Fuchs and his colleagues that pol IV is unlikely to be involved in translesion synthesis past dG-AAF (*47*). The majority (8.8%) of miscoding events for pol IV are one- and two-base deletions. The latter is consistent with the proposed mechanism for frameshift mutagenesis in vitro (*31*) since both lesions block progression of pol IV, promoting formation of misaligned intermediates. Although we were unable to obtain kinetic parameters for pol IV under steady-state conditions due to the high degree of blockage of translesion synthesis at the adducted sites, the preferential incorporation of dGMP and dAMP opposite dG-AAF or dG-AF observed in reactions catalyzed by large amounts of enzyme (data not shown) predicts formation of one- and two-base deletions, as proposed in panels A and B of Figure 7. According to this mechanism (*31*), formation of deletions depends on the sequence context of the oligodeoxynucleotide templates that were used.

As shown by the arrows in Figures 5 and 6B, unknown products formed on dG-AF-modified templates during translesional synthesis by pol κ and pol IV. Small amounts of

unknown products also were observed on unmodified templates (Figure 5, lane 5, and Figure 6B, lane 1). These products may be generated by inaccurate translesional synthesis of pol κ and pol IV, as observed in other laboratories (*23*−*26*). Alternatively, their production may be due to a low frequency of misincorporation at a site other than the adducted site.

Fuchs et al. recently found that the β clamp allows pol IV to form a stable initiation complex that increases the processivity of pol IV from 1 to 300−400 nucleotides (*48*). Although several of us (E. Ohashi and H. Ohmori) confirmed this phenomenon using the M13 gap-filling assay, the error frequency was reduced only by half.[2] Therefore, the fidelity of pol IV may reflect an intrinsic property of the enzyme, rather than the processivity factor.

Although human pol κ is a homologue of *E. coli* pol IV, only 30% of the amino acids of pol κ are identical to those of pol IV in the HDB region (Figure 2) (*18*), possibly accounting for the different properties of the two polymerases.

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