Mutagenic Properties of 3-(Deoxyguanosin-N^2-yl)-2-acetylaminofluorene, a Persistent Acetylaminofluorene-Derived DNA Adduct in Mammalian Cells†

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ABSTRACT: The carcinogen 2-acetylaminofluorene is metabolically activated in cells and reacts with DNA to form *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF), *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF), and 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF) DNA adducts. The dG-N^2-AAF adduct is the least abundant of the three isomers, but it persists in the tissues of animals treated with this carcinogen. The miscoding and mutagenic properties of dG-C8-AAF and dG-C8-AF have been established; these adducts are readily excised by DNA repair enzymes engaged in nucleotide excision repair. In the present study, oligodeoxynucleotides modified site-specifically with dG-N^2-AAF were used as DNA templates in primer extension reactions catalyzed by mammalian DNA polymerases. Reactions catalyzed by pol α were strongly blocked at a position one base before dG-N^2-AAF and also opposite this lesion. In contrast, during translesion synthesis catalyzed by pol *η* or pol *κ* nucleotides were incorporated opposite the lesion. Both pol *η* and pol *κ* incorporated dCMP, the correct base, opposite dG-N^2-AAF. In reactions catalyzed by pol *η*, small amounts of dAMP misincorporation and one-base deletions were detected at the lesion site. With pol *κ*, significant dTMP misincorporation was observed opposite the lesion. Steady-state kinetic analysis confirmed the results obtained from primer extension studies. Single-stranded shuttle vectors containing 5′TCCTCCTCXCCTCTC (*X* = dG-N^2-AAF, dG-C8-AAF, or dG) were used to establish the frequency and specificity of dG-N^2-AAF-induced mutations in simian kidney (COS-7) cells. Both lesions promote G → T transversions overall, with dG-N^2-AAF being less mutagenic than dG-C8-AAF (3.4% vs 12.5%). We conclude from this study that dG-N^2-AAF, by virtue of its persistence in tissues, contributes significantly to the mutational spectra observed in AAF-induced mutagenesis and that pol *η*, but not pol *κ*, may play a role in this process.

2-Acetylaminofluorene (2-AAF),[1] a prototypic aromatic amide, is widely used as a model chemical carcinogen (reviewed in ref *1*). 2-AAF is metabolically activated in cells and reacts with DNA to form primarily *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) (*2*) and *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (Figure 1) (*3*−*5*). Approximately 5−15% of aminofluorene bound covalently to DNA in vitro or in vivo is recovered as the minor 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF) DNA adduct (Figure 1) (*4*, *6*−*12*). dG-N^2-AAF persists in tissues of animals treated with AAF (*4*, *6*) while dG-C8-AAF and dG-C8-AF are rapidly excised by nucleotide excision repair (*2*, *7*, *13*).

dG-C8-AAF and dG-C8-AF adducts are mutagenic in cultured mammalian cells, generating base substitutions and frameshift deletions (*1*, *14*−*18*). Site-specific mutagenesis techniques have been used to explore the miscoding properties of single AAF-derived DNA adducts in simian kidney (COS-7) cells (*19*−*21*). dG-C8-AAF and dG-C8-AF adducts inserted into a single-strand shuttle vector promote G → T transversions, along with fewer numbers of G → A transitions, targeted to the site of the lesion (*20*). The mutation frequency varies significantly depending on the 3′ and 5′ sequence flanking the lesion (*21*). The miscoding properties of dG-C8-AAF and dG-C8-AF also have been explored in vitro using mammalian DNA polymerases (pol) and primer extension methods (*20*). In reactions catalyzed by pol α or pol *δ*, dG-C8-AAF blocks primer extension while dG-C8-

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[1] Abbreviations: 2-AAF, 2-acetylaminofluorene; dG-N^2-AAF, 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene; dG-C8-AAF, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-C8-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; pol α, calf thymus DNA polymerase α; pol *η*, human DNA polymerase *η*; pol *κ*, DNA polymerase *κ*; pol *κ*ΔC, a truncated form of human DNA polymerase *κ*; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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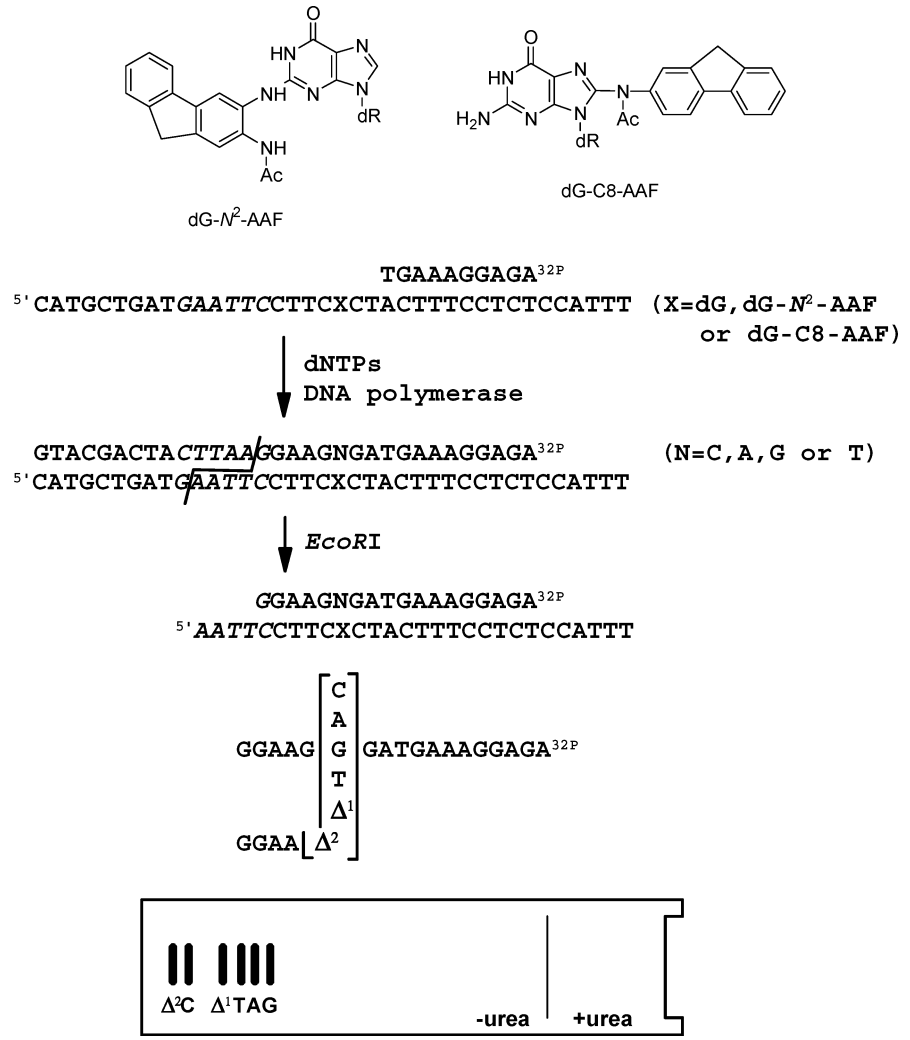

FIGURE 1: Structure of dG-N^2-AAF and diagram of the method used to determine miscoding specificity. Structures of DNA adducts used in this study are shown. Unmodified and dG-N^2-AAF- and dG-C8-AAF-modified 38-mer templates are annealed to a ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed by DNA pol η or pol κ 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 × 72 × 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.

AF accommodates translesion synthesis with dCMP, the "correct" base, incorporated opposite the lesion (*20*). In reactions catalyzed by pol α, dG-C8-AAF promotes incorporation of small amounts of dAMP and dTMP, accompanied by two-base deletions. A dG-N^2-AAF-modified oligodeoxynucleotide, prepared by postsynthetic methods, was used as a template for investigating the miscoding properties of the lesion in reactions catalyzed by the Klenow fragment of *Escherichia coli* DNA polymerase I (*22*). This study revealed that dG-N^2-AAF generates G → T transversions (*22*); however, the miscoding potential of dG-N^2-AAF lesions in reactions catalyzed by mammalian and human DNA polymerases has never been systematically explored.

In recent years, a number of novel human DNA polymerases have been characterized (reviewed in ref *23*). Among them, pol η (*24*) and pol κ (*25*, *26*), Y-family DNA polymerases (*27*), lack 3′ → 5′ proofreading exonuclease activity and exhibit high error rates even on nondamaged DNA templates (*28*−*30*). The ability of pol η (*31*−*39*) and pol κ (*31*, *32*, *40*−*43*) to catalyze translesion synthesis past various DNA lesions in vitro has been established.

Primer extension reactions catalyzed by pol η and pol κ have been investigated, using as templates oligomers modified with dG-C8-AAF or dG-C8-AF. In studies conducted with a single dNTP, pol κ preferentially incorporated dTMP and dCMP opposite dG-C8-AAF (*40*−*42*), while pol η preferentially incorporated dCMP opposite this lesion (*33*). In the presence of four dNTPs (*43*), pol κ catalyzed incorporation of dTMP opposite dG-C8-AAF, accompanied by lesser amounts of dCMP, dAMP, and dGMP incorporation and one- and two-base deletions. On templates containing dG-C8-AF, dAMP, dTMP, and dCMP were incorporated opposite the lesion in approximately equal amounts, with some deletions also occurring (*43*).

In the present study, we introduced a single lesion site-specifically into oligodeoxynucleotides to explore the miscoding potential of dG-N^2-AAF in vitro and to establish the mutational specificity and frequency of this adduct during extrachromosomal DNA replication in mammalian cells. The results of our study, coupled with the persistence of dG-N^2-AAF in animals treated with AAF (*4*, *6*), suggest that the contribution of this so-called "minor" adduct to overall

mutagenicity of the carcinogen may be disproportionate to the relative level of this adduct in mammalian cells.

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. Calf thymus DNA pol α (30000 units/mg) was purchased from Chimerx.

Synthesis of Oligodeoxynucleotides. Unmodified DNA templates (5′CCTTCGCTACTTTCCTCTCCATTT and 5′CATGCTGATGAATTCCTTCGCTACTTTCCTCTCCATTT), primers, and standard markers (Figure 1) were prepared using an automated DNA synthesizer (Applied Biosystems Model 392) (*44*). The dG-N^2-AAF-modified oligodeoxynucleotides were prepared by phosphoramidite chemistry (*45*). The dG-C8-AAF-modified oligodeoxynucleotides were prepared by postsynthetic methods as described previously (*20*). The unmodified and modified 38-mers were purified by electrophoresis on a 20% nondenaturing polyacrylamide gel (35 × 42 × 0.04 cm). Bands were detected under ultraviolet light, extracted overnight with 2.0 mL of distilled water at 4 °C, and then evaporated to dryness. The oligomers were 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 10% → 20% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min (*46*). Oligodeoxynucleotides were labeled at the 5′ terminus with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (*47*) 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. HPLC was performed using a 515 HPLC pump, 996 photodiode array detector, and pump control module. UV spectra and concentrations of oligomers were measured with a Hewlett-Packard 8452A diode array spectrophotometer.

Primer Extension Reactions Catalyzed by DNA Polymerases. Pol η and a truncated form of pol κ (pol $\kappa\Delta$C) were prepared as described previously (*24*, *26*). Using a modified or unmodified 38-mer oligodeoxynucleotide (150 fmol) primed with a ^{32}P-labeled 10-mer (5′AGAGGAAAGT; 100 fmol), primer extension reactions catalyzed by pol α, pol η, or pol κ were conducted at 25 °C for the 10-mer primer in a buffer (10 μL) containing four dNTPs (100 μM each) (Figure 1). The reaction buffer for pol α, pol η, or pol κ 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. Extended reaction products were analyzed by denaturing 20% PAGE. The radioactivities of the extended products were measured with a β-phosphorimager (Molecular Dynamics).

Nucleotide Insertion Opposite the dG-N^2-AAF Lesion. Using a modified or unmodified 38-mer oligodeoxynucleotide (150 fmol) primed with a ^{32}P-labeled 12-mer (5′AGAGGAAAGTAG; 100 fmol), primer extension reactions catalyzed by pol α, pol η, or pol κ were conducted at 30 °C in a buffer (10 μL) containing a single dNTP (100 μM) (Figure 1). Primer extension reactions with a single dNTP were conducted on templates containing a dG-N^2-AAF-modified or unmodified 24-mer oligodeoxynucleotide (750 fmol) using pol α (25 fmol for the unmodified template; 250 fmol for the dG-N^2-AAF-modified template), pol η (100 fmol for the unmodified template; 500 fmol for the dG-N^2-AAF-modified template), or pol $\kappa\Delta$C (100 fmol for the unmodified template; 500 fmol for the dG-N^2-AAF-modified template), as described above.

Miscoding Specificity. Using dG-N^2-AAF- or dG-C8-AAF-modified or unmodified 38-mer oligodeoxynucleotides (750 fmol) primed with a ^{32}P-labeled 12-mer (5′AGAGGAAAGTAG; 500 fmol), primer extension reactions catalyzed by pol η (80 fmol for the unmodified template; 500 fmol for the dG-N^2-AAF- or dG-C8-AAF-modified templates) or pol $\kappa\Delta$C (80 fmol for the unmodified template; 500 fmol for the dG-N^2-AAF- and dG-C8-AAF-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 following 20% PAGE (35 × 42 × 0.04 cm). 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) (*48*, *49*) (Figure 1).

Steady-State Kinetic Studies. Kinetic parameters associated with nucleotide insertion opposite dG-N^2-AAF or dG-C8-AAF 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 η or pol $\kappa\Delta$C (0.8−80 fmol) 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′CCTTCXCTACTTTCCTCTCCATTT, where X is dG, dG-N^2-AAF, or dG-C8-AAF) 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 η or pol $\kappa\Delta$C (0.8−80 fmol) were used to measure chain extension. All samples were subjected to 20% denaturating 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 $F = (V_{max}/K_m)_{[\text{wrong pair}]}/(V_{max}/K_m)_{[\text{correct pair = dC·dG}]}$ (*50*, *51*).

Site-Specific Mutagenesis in Simian Kidney COS-7 Cells. A SV40-transformed simian kidney cell line COS-7 and a single strand (ss) shuttle vector, pMS2, that confers neomycin (Neo^R) and ampicillin (Amp^R) resistance (*52*), were used to establish mutagenic specificity. An oligodeoxynucleotide (5′TCCTCCTCXCCTCTC, where X is dG-N^2-AAF, dG-C8-AAF, or dG) containing a single dG-N^2-AAF or dG-C8-AAF was used for this experiment; the bases flanking dG-N^2-AAF and dG-C8-AAF (underlined) are the same as for the 38-mer used for in vitro studies.

A circular ss DNA containing a single DNA adduct was constructed according to procedures established previously in this laboratory (*52*). pMS2 DNA was annealed to a 61-mer and then digested with *Eco*RV to create a 15-mer gap (Figure 5). dG-N^2-AAF- or dG-C8-AAF-modified 15-mers were ligated to the gapped vector. To establish ligation efficiency, a portion of the vector annealed to the 61-mer

scaffold was digested with *Ban*I and *Hae*III, followed by exchange of the terminal phosphate residue using [γ-^{32}P]-ATP and T4 polynucleotide kinase, and subjected to 12% denaturing polyacrylamide gel electrophoresis (*52*). A second portion of the ligation mixture was incubated for 2 h with T4 DNA polymerase (1 unit/pmol of DNA) to digest the hybridized 61-mer and then treated with *Eco*RV and *Sal*I to cleave residual ss pMS2. The reaction mixture was extracted twice with phenol/chloroform, 1:1 (v/v), and twice with chloroform. Following ethanol precipitation, the DNA was dissolved in distilled water. A portion of the ligation mixture and known amounts of ss pMS2 were subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ss DNA. DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labeled S13 probe complementary to the DNA containing the 15-mer insert. The absolute amount of closed circular ss DNA was established by comparing the radioactivity in the sample with that in known amounts of ss DNA.

COS-7 cells were transfected over 18 h with ss DNA (100 fmol) using lipofection (*53*), after which the cells were grown for 2 days in Dulbecco's modified Eagle's medium/10% fetal calf serum. Progeny plasmids were recovered using the method described by Hirt (*54*), treated with S1 nuclease to digest input ss DNA, and used to transform *E. coli* DH10B. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization (*55*). Oligodeoxynucleotide probes used to identify progeny phagemids are shown in Figure 5. Probes L13 and R13 were used to confirm that the gapped plasmids were constructed correctly. Transformants that failed to react with L13 and R13 were omitted from the analysis. When L13/R13-positive transformants failed to detect events targeted to the lesion site, double-strand (ds) DNA was prepared and subjected to dideoxynucleotide sequencing analysis (*56*).

RESULTS

Primer Extension Reactions Catalyzed by DNA Polymerase on dG-N^2-AAF-Modified DNA Templates. Using unmodified and dG-*N*^{2}-AAF- and dG-C8-AAF-modified 38-mer templates, primer extension reactions were conducted in the presence of four dNTPs using variable amounts of pol α, pol η, or pol κ (Figure 2). Primer extension reactions catalyzed by pol α readily formed extended products on the unmodified template (Figure 2A). However, when a dG-*N*^{2}-AAF-modified template was used, primer extension was blocked one base prior to the lesion and opposite the lesion. Similar blockage was observed in experiments with dG-C8-AAF. Using 250 fmol of pol α, 2.0 ± 0.2% (mean ± SD from three independent experiments) and 4.8 ± 0.3% of the starting primer extended past the dG-*N*^{2}-AAF and dG-C8-AAF lesions, respectively. When pol η was used to catalyze the reaction, primer extension on dG-*N*^{2}-AAF- or dG-C8-AAF-modified templates resulted in fully extended products (Figure 2B). Blockage of primer extension was observed one base prior to dG-*N*^{2}-AAF while no blockage was detected at the dG-C8-AAF adduct site. Using 500 fmol of pol η, 64 ± 4%, 94 ± 3%, and 98 ± 5% of the starting primer extended past dG-*N*^{2}-AAF, dG-C8-AAF, and the unmodified dG, respectively. These results suggest that translesion synthesis past the dG-C8-AAF lesion is more efficient than with dG-*N*^{2}-AAF. With pol $\kappa\Delta$C, primer extension reactions readily occurred at the dG-*N*^{2}-AAF adduct site (Figure 2C). However, primer extension was slightly retarded one base before dG-C8-AAF and opposite the lesion. Using 500 fmol of pol $\kappa\Delta$C, 95 ± 3%, 88 ± 5%, and 99 ± 3% of the starting primer extended past the dG-*N*^{2}-AAF, dG-C8-AAF, and unmodified dG, respectively. The ability of pol κ to bypass the dG-*N*^{2}-AAF lesion is higher than that of pol η.

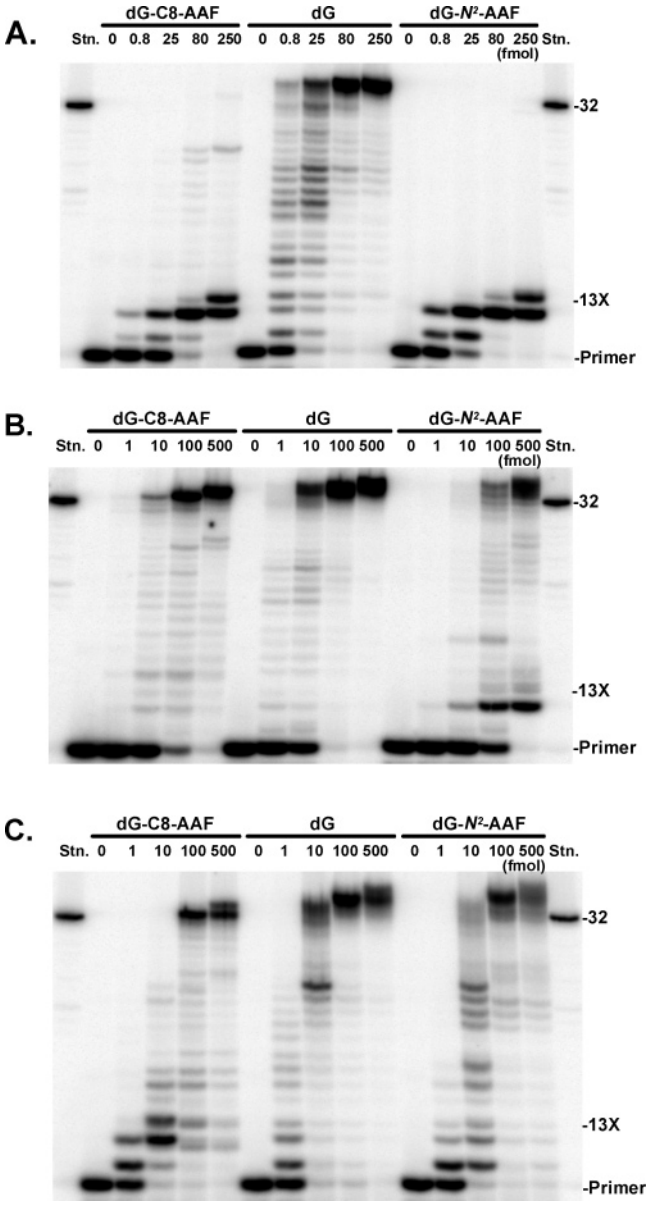

FIGURE 2: Primer extension reactions catalyzed by pol α, pol η, or pol κ on dG-*N*^{2}-AAF- and dG-C8-AAF-modified DNA templates. Using unmodified and dG-C8-AAF- or dG-*N*^{2}-AAF-modified 38-mer templates (150 fmol; 5′CATGCTGTTGAATTCCTTCXC-TACTTTCCTCTCCATTT, where X is dG, dG-C8-AAF, or dG-*N*^{2}-AAF) primed with a ^{32}P-labeled 10-mer (100 fmol; 5′AGAG-GAAAGT), primer extension reactions were conducted at 25 °C for 30 min in a buffer containing four dNTPs (100 μM each) and variable amounts of (A) pol α (0, 8.0, 25, 80, and 250 fmol), (B) pol η (0, 1.0, 10, 100, and 500 fmol), or (C) pol $\kappa\Delta$C (0, 1.0, 10, 100, and 500 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. Radioactivities of extended products were measured by a β-phosphorimager. 13X represents the adducted position.

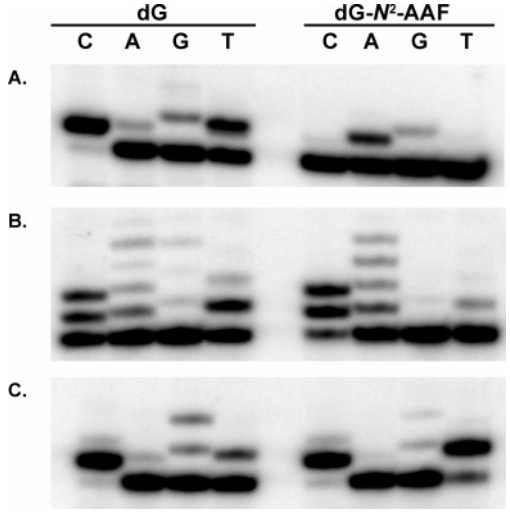

FIGURE 3: Nucleotide incorporation opposite the dG-N^2-AAF adduct in reactions catalyzed by DNA polymerases. Using a dG-N^2-AAF-modified 38-mer template (150 fmol) primed with a ^{32}P-labeled 12-mer (100 fmol; 5′AGAGGAAAGTAG), nucleotide incorporation was performed at 30 °C for 30 min in a buffer containing a single dNTP (100 μM) and (A) pol α (25 fmol for unmodified dG and 250 fmol for dG-N^2-AAF), (B) pol η (100 fmol for unmodified dG and 500 fmol for dG-N^2-AAF), or (C) pol $\kappa\Delta$C (100 fmol for unmodified dG and 500 fmol for dG-N^2-AAF). One-third of the reaction mixture was subjected to the denaturing 20% PAGE (35 × 42 × 0.04 cm), as described in the legend of Figure 2.

Nucleotide Insertion Opposite the dG-N^2-AAF Adduct. Nucleotide insertion opposite dG-N^2-AAF was determined in the presence of a single dNTP using 38-mer templates primed with a ^{32}P-labeled 12-mer. With pol α, dAMP was primarily inserted opposite the lesion (Figure 3A). With pol η, dCMP and dAMP were preferentially inserted opposite dG-N^2-AAF, along with a small amount of dTMP (Figure 3B). Using pol $\kappa\Delta$C, preferential incorporation of dCMP and dTMP was observed opposite dG-N^2-AAF; insertion of dAMP was not observed (Figure 3C). We conclude that pol α and pol η tend to misinsert dAMP opposite dG-N^2-AAF while pol κ tends to misinsert dTMP opposite the adduct site.

Miscoding Properties of dG-N^2-AAF. To determine the miscoding frequency and specificity of dG-N^2-AAF, primer extension catalyzed by either pol η or pol $\kappa\Delta$C was performed in reactions containing four dNTPs (Figure 1). Base substitutions and deletions observed at the targeted lesion were analyzed using two-phase PAGE. A 12-mer primer, instead of a 10-mer, was used for this analysis, because misincorporation had been observed previously at the position 3′ prior to dG-C8-AAF (*34*). Following primer extension past dG-N^2-AAF or dG-C8-AAF, 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. A standard mixture of six ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions can be resolved by this method (Figure 4, lanes 2 and 6). When the unmodified template was used, dCMP, the correct base, was inserted opposite dG (Figure 4, lanes 3 and 7). When pol η was used with a template containing dG-N^2-AAF, dCMP (53.9 ± 2.7% of the starting primers) was incorporated, along

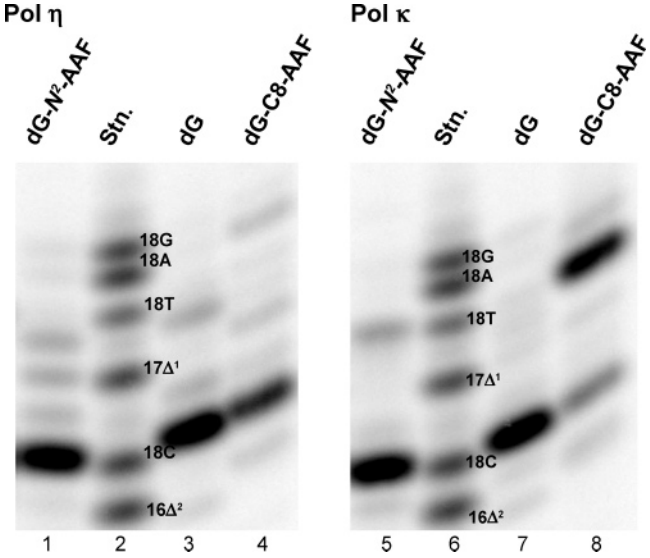

FIGURE 4: Miscoding specificities in primer extension reactions catalyzed by pol η and pol κ. Using unmodified (lanes 3 and 7) and dG-N^2-AAF- (lanes 1 and 5) and dG-C8-AAF- (lanes 4 and 8) modified 38-mer templates (750 fmol) primed with a ^{32}P-labeled 12-mer (500 fmol; 5′AGAGGAAAGTAG), primer extension reactions were conducted at 30 °C for 30 min in a buffer containing four dNTPs (100 μM each) and pol η (80 fmol for the unmodified template; 500 fmol for dG-C8-AAF- and dG-N^2-AAF-modified templates) or pol $\kappa\Delta$C (80 fmol for the unmodified template; 500 fmol for dG-C8-AAF- and dG-N^2-AAF-modified templates). The extended reaction products (approximately 28−32-mer) produced on the unmodified and dG-N^2-AAF- or dG-C8-AAF-modified templates were extracted following PAGE (35 × 42 × 0.04 cm). The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer and cleaved with *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-N^2-AAF- or dG-C8-AAF-modified template were subjected to a two-phase 20% PAGE (15 × 72 × 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 2 and 6).

with lesser amounts of dAMP (0.73 ± 0.05%) and dGMP (0.70 ± 0.03%). A small number of one-base (3.7 ± 0.3%) deletions also was observed (lane 1). Similarly, dG-C8-AAF promoted incorporation of dCMP (66.6 ± 4.3%) opposite the lesion (lane 4), accompanied by smaller amounts of dAMP (4.5 ± 0.3%), dGMP (0.9 ± 0.1%), and dTMP (1.1 ± 0.2%). One-base (4.5 ± 0.3%) and two-base (3.5 ± 0.1%) deletions also were detected. In the presence of pol $\kappa\Delta$C, dG-N^2-AAF promoted incorporation of dCMP (77.5 ± 4.7%) and lesser amounts of dTMP (6.6 ± 0.4%) (lane 5). No deletions were detected. In contrast, dG-C8-AAF promoted preferential incorporation of dTMP (61.3 ± 3.0%) opposite the lesion, accompanied by dCMP (15.7 ± 1.7%) and dAMP (4.2 ± 0.3%) (lane 8). One-base (1.0 ± 0.1%) and two-base (3.9 ± 0.2%) deletions also were observed. Pol η promoted G → T and G → C transversions and/or G → A transitions opposite both dG-N^2-AAF and dG-C8-AAF. On the other hand, pol κ promoted primarily G → A transitions opposite the both lesions. The miscoding frequencies of dG-N^2-AAF were 5.1 ± 0.4% for pol η and 6.6 ± 0.4% for pol $\kappa\Delta$C, respectively; these are lower than observed at dG-C8-AAF using pol η (14.5 ± 1.2%) and pol $\kappa\Delta$C (70.4 ± 5.3%).

Kinetic Studies on the dG-N^2-AAF-Modified DNA Template. Steady-state kinetic studies were performed using pol

Table 1: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Pol η and Pol κ[a]

	Insertion dNTP ↓GATGAAAGGAGA^{32P} 5'CCTTCXCTACTTTCCTCTCCATTT			Extension dGTP ↓NGATGAAAGGAGA^{32P} 5'CCTTCXCTACTTTCCTCTCCATTT			
N:X	K_m (μM)	V_{max}(%min^{-1})	F_{ins}	K_m (μM)	V_{max}(%min^{-1})	F_{ext}	F_{ins}xF_{ext}
Pol η							
X = dG							
C:G	0.26 ± 0.04[b]	55.3 ± 1.9[b]	**1.0**	0.51 ± 0.01[b]	89.9 ± 8.3[b]	**1.0**	**1.0**
X = dG-N^2-AAF							
C:X	8.23 ± 1.03	23.2 ± 0.57	**1.33 x 10^{-2}**	2.07 ± 0.44	39.1 ± 1.6	**0.115**	**1.53 x 10^{-3}**
A:X	31.4 ± 4.7	4.92 ± 0.11	**7.30 x 10^{-4}**	92.7 ± 6.6	1.99 ± 0.19	**1.28 x 10^{-3}**	**9.34 x 10^{-7}**
G:X	44.3 ± 6.9	0.038 ± 0.01	**3.90 x 10^{-6}**	91.2 ± 11.2	1.26 ± 0.03	**8.32 x 10^{-5}**	**3.24 x 10^{-10}**
T:X	148 ± 24	2.06 ± 0.20	**6.44 x 10^{-5}**	18.4 ± 3.2	8.00 ± 0.29	**2.64 x 10^{-3}**	**1.70 x 10^{-7}**
Pol κ							
X = dG							
C:G	0.42 ± 0.10[b]	44.2 ± 5.0[b]	**1.0**	0.26 ± 0.09[b]	78.9 ± 6.3[b]	**1.0**	**1.0**
X = dG-N^2-AAF							
C:X	28.8 ± 1.6	37.4 ± 0.45	**1.23 x 10^{-2}**	2.27 ± 0.05	58.6 ± 1.9	**8.51 x 10^{-2}**	**1.05 x 10^{-3}**
A:X	124 ± 1.5	0.22 ± 0.01	**1.69 x 10^{-5}**	5.75 ± 0.48	0.76 ± 0.03	**4.35 x 10^{-4}**	**7.35 x 10^{-9}**
G:X	326 ± 12	1.46 ± 0.05	**4.26 x 10^{-5}**	9.45 ± 4.60	0.72 ± 0.10	**2.51 x 10^{-4}**	**1.07 x 10^{-8}**
T:X	200 ± 10	19.6 ± 0.30	**9.32 x 10^{-4}**	2.97 ± 0.07	4.74 ± 0.11	**5.26 x 10^{-3}**	**4.90 x 10^{-6}**

[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 = dG or dG-$N^2$-AAF lesion. [b] Data expressed as the mean ± SD obtained from three independent experiments.

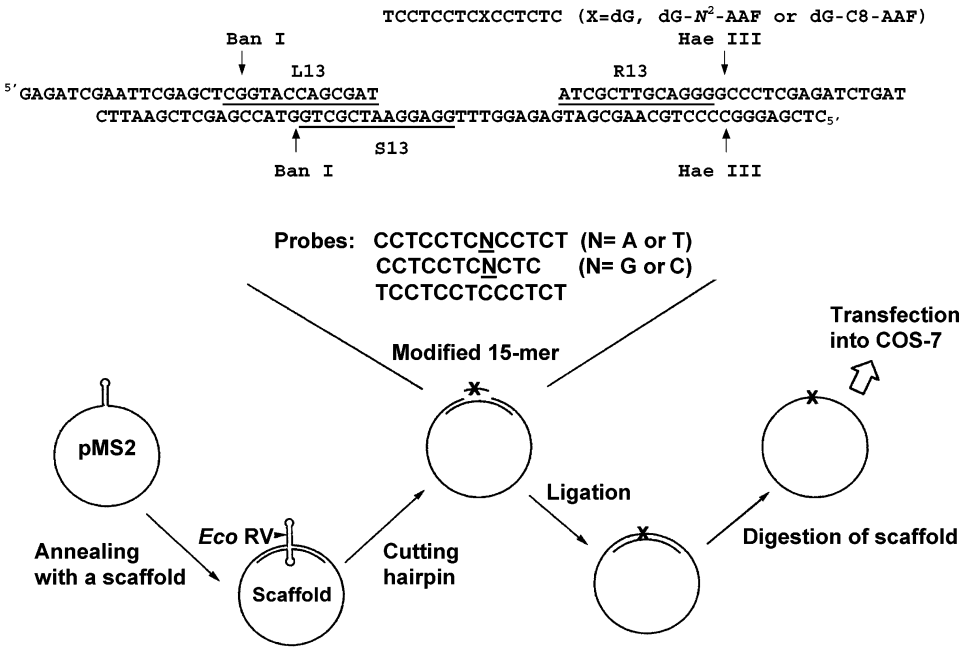

FIGURE 5: Construction of a single strand vector containing a single dG-N^2-AAF or dG-C8-AAF.

η and pol $\kappa\Delta$C to determine the frequency of dNTP incorporation (F_{ins}) opposite the dG-N^2-AAF lesion and chain extension (F_{ext}) from the primer terminus. Using pol η, the F_{ins} for dCTP, the correct base, was much higher than that for other dNTPs (Table 1). However, F_{ins} for dATP was only 18 times lower than that for dCTP, suggesting that dATP can be inserted opposite the lesion. The F_{ext} for the dC·dG-N^2-AAF pair was also higher than that for other dNMPs paired with dG-N^2-AAF. As a result, the relative frequency estimated for translesion synthesis ($F_{ins} \times F_{ext}$) past dC·dG-N^2-AAF was at least 3 orders of magnitudes higher than past other pairs. Similarly, with pol $\kappa\Delta$C, $F_{ins} \times F_{ext}$ past dC·dG-N^2-AAF was much higher than for other base pairs. F_{ins} and F_{ext} for dTTP were 13 times and 16 times, respectively, lower than that for dCTP. F_{ins} and F_{ext} of dATP were considerably lower that that for dTTP. Thus, pol η tends to misincorporate dAMP opposite the dG-N^2-AAF lesion while pol $\kappa\Delta$C tends to misincorporate dTMP during translesion synthesis.

Mutational Specificity in Vivo. Unmodified, dG-N^2-AAF-modified, and dG-C8-AAF-modified 15-mer oligodeoxynucleotides, having the same neighboring bases as those used for in vitro studies, were inserted into a gapped single-strand vector (Figure 5). pMS2 modified with dG-N^2-AAF or dG-C8-AAF was used to transfect COS-7 cells; the number of transformants recovered was compared to those obtained with

Table 2: Mutational Specificity of dG-N^2-AAF and dG-C8-AAF in COS Cells[a]

plasmid		targeted mutations (dG, dG-N^2-AAF, or dG-C8-AAF → X)					
		G	A	T	C	Δ^1	others
5′-CGC-	expt 1[b]	126	0	0	0	0	0
	expt 2	158	0	0	0	0	0
	total	284 (100%)	0 (<0.4%)	0 (<0.4%)	0 (<0.4%)	0 (<0.4%)	0
5′-CG$^{N^2-AAF}$C-	expt 1	132	0	4	1	0	0
	expt 2	122	0	2	2	0	1[c]
	total	254 (96.6%)	0 (<0.4%)	6 (2.3%)	3 (1.1%)	0 (<0.4%)	1
5′-CG^{C8-AAF}C-	expt 1	119	5	9	5	0	0
	expt 2	105	4	7	2	0	1[d]
	total	224 (87.5%)	9 (3.5%)	16 (6.3%)	7 (2.7%)	0 (<0.4%)	1

[a] Adducted ss DNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was used to transform *E. coli* DH10B and analyzed for mutations. [b] Expt 1 and expt 2 represent independent experiments. [c] Nontargeted mutation associated with a targeted event is 5′T(C→A)CTCCTCXCCTCTC. [d] Nontargeted mutation associated with a targeted event is 5′TC(C→T)TCCTCXCCTCTC.

an unmodified vector. The presence of a single dG-N^2-AAF and dG-C8-AAF adduct reduced transformation efficiency to 67 ± 10% and 81 ± 9% of control values, respectively. No significant difference in the transformation efficiencies between dG-N^2-AAF and dG-C8-AAF was observed. Vectors modified with dG-N^2-AAF preferentially incorporated dCMP (96.6%) opposite the lesion (Table 2). Targeted G → T transversion mutations (2.3%) were detected, along with lesser amounts of G → C transversions (1.1%). No deletions were detected. In contrast, dG-C8-AAF promoted targeted G → T transversions (6.3%), G → A transitions (3.5%), and G → C transversions (2.7%). The overall mutation frequency of dG-N^2-AAF was 3.7 times lower than that of dG-C8-AAF. Some nontargeted mutations were observed using dG-N^2-AAF- and dG-C8-AAF-modified plasmids. No mutations were observed with the unmodified vector.

DISCUSSION

Mutagenic Specificity in Mammalian Cells. A single-strand shuttle plasmid vector was used to compare the mutagenic specificity of the dG-N^2-AAF adduct in simian kidney cells with that of dG-C8-AAF. As observed previously (*20*), dG-C8-AAF promoted targeted G → T transversions, accompanied by fewer G → A transitions and G → C transversions. Therefore, the mutational frequency and specificity as determined by the site-specific mutagenesis approach were highly reproducible. The mutational spectrum of dG-N^2-AAF was characterized by G → T and G → C transversions. Although the overall mutational frequency of the N^2 adduct (3.4%) was 3.7 times lower than that of dG-C8-AAF (12.5%) (Table 2), the persistence of this adduct in mammalian cells suggests that it may contribute significantly to the overall mutagenicity of AAF. In view of the striking sequence context effects observed in mutagenesis studies of AAF-derived dG-C8 adducts (*21*), it is clear that more detailed analyses will be required to fully describe the mutagenic potential of dG-N^2-AAF.

Miscoding Properties during Translesion Synthesis Catalyzed by DNA Polymerases. Several recently discovered human DNA polymerases, including pol η and pol κ, have been associated with translesion synthesis past bulky DNA adducts (*23*). In fact, while primer extension reactions catalyzed by pol α and pol δ were blocked (data not shown) at the dG-N^2-AAF lesion, primer extension reactions catalyzed by pol η and pol κ readily extended past the lesion. Both enzymes bypass the lesion by incorporating primarily dCMP, the correct base, opposite dG-N^2-AAF in reactions containing four dNTPs. The majority of misincorporation events detected at the lesion site was incorporation of dAMP for pol η and dTMP for pol κ (Figures 3 and 4). Our results obtained by two-phase PAGE assay were supported by steady-state kinetic studies. Therefore, the miscoding spectrum and mutation frequency observed in reactions with pol η were consistent with mutagenic events of dG-N^2-AAF in single-strand vectors replicated in COS-7 cells, suggesting that pol η, rather than pol κ, may be primarily involved in translesion synthesis past the dG-N^2-AAF adduct. In addition, pol α inserted dATP, not dCTP, opposite the dG-N^2-AAF, but these intermediates were only weakly to form fully extended product (Figure 3A). As proposed as a switching translesion mechanism (*57*), pol η and/or pol κ may extend the dA•dG-N^2-AAF pair formed by pol α to form the fully extended products.

The relative "bypass" frequency past the dC•dG-N^2-AAF pair (1.05×10^{-3}) in reactions catalyzed by pol κ was at least 4 orders of magnitude higher than that observed previously for dC•dG-C8-AAF (8.69×10^{-8}) or dC•dG-C8-AF (2.40×10^{-8}) (*34*). Similarly, efficient bypass was observed with pol κ for N^2 guanine adducts, such as (+)-*trans*-dG-N^2-BPDE (*58*) and dG-N^2-estrogen (*59*). Primer extension reactions catalyzed by pol η (1.53×10^{-3}) also efficiently bypassed the dG-N^2-AAF lesion, incorporating dCMP opposite the lesion. These differences may be due to adducts at the N^2 and C8 position of the respective base, which may influence miscoding frequency and specificity.

Conformation of dG-N^2-AAF in Duplex DNA. Molecular modeling studies of the dG-N^2-AAF adduct embedded in duplex DNA (*60*) suggest that Watson−Crick base pairing is retained with the fluorenyl moiety lying in the minor groove. The precise orientation of the fluorenyl group may vary according to the neighboring sequence (*60*). In this conformation, dG-N^2-AAF adopts an anti orientation and pairs with dC (anti). When dAMP is placed opposite dG-N^2-AAF, the mispaired adenine adopts a syn orientation with Hoogsteen pairing to the modified guanine (anti) (*60*). The relative resistance of the dG-N^2-AAF adduct to excision by DNA repair enzymes may reflect the lack of structural distortion when it lies in the B-DNA minor groove. Thus, dG-N^2-AAF is a persistent lesion, and its relative contribution to AAF carcinogenesis may be disproportionate to its level and mutagenic potential in mammalian cells.

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