Mutagenic Events in *Escherichia coli* and Mammalian Cells Generated in Response to Acetylaminofluorene-Derived DNA Adducts Positioned in the *Nar* I Restriction Enzyme Site†

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ABSTRACT: Comparative mutagenesis studies of *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) and *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene (dG-AF) adducts positioned in the *Nar* I restriction enzyme site were performed using *Escherichia coli* (*E. coli*) and simian kidney (COS-7) cells. Oligodeoxynucleotides (5′TCCTCG$_1$G$_2$CG$_3$CCTCTC) containing a recognition sequence for the *Nar* I restriction enzyme were modified site-specifically with dG-AAF or dG-AF. Modified and unmodified oligomers inserted into single-stranded phagemid shuttle vectors were used to transform *E. coli* or to transfect COS-7 cells. Following replication in host cells, progeny plasmids were recovered and analyzed for mutations. In SOS-induced *E. coli*, dG-AAF primarily induced one- and two-base deletions. The mutational frequency varied, depending on the position modified in the *Nar* I site; 91% two-base deletions were observed at G$_3$, while 8.4% and 2.8% deletions were detected at G$_2$ and G$_1$, respectively. In contrast, dG-AF at any position in the *Nar* I site failed to produce deletions, generating primarily G → T transversions (mutational frequency, 7.6−8.4%). In COS-7 cells, both dG-AAF and dG-AF primarily induced G → T transversions. Mutation frequencies for dG-AAF were 9.4−24%, the highest values being at G$_1$ and G$_3$. Mutation frequencies for dG-AF were 9.3-21%, the higher value at G$_2$. We conclude from this study that the mutation potential of dG-AAF and dG-AF depends on the structure of the adduct, the sequence context of the lesion, and the host cell used for the experiment.

2-Acetylaminofluorene (2-AAF[1]) is a strong rat liver carcinogen which, after metabolic activation, primarily binds DNA at the C-8 position of guanine to form *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) and its deacetylated form, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene (dG-AF) (reviewed ref *1*). The mutagenic properties of dG-AAF and dG-AF have been examined in *Escherichia coli (E. coli)* and various mammalian and human cells. When an SV40-based shuttle vector carrying the *supF* gene was treated with *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene (*2*) or *N*-acetoxy-AAF (*3*) and then transfected into human embryonic kidney cell lines, G → T transversions were generated at the lesion sites. Comparable mutational spectra were detected at the *dhfr* gene in Chinese hamster ovary cells treated with *N*-acetoxy-AAF (*4*) or *N*-hydroxy-2-AF (*5*). In support of these observations, G:C → T:A transversions were detected in the *lac*I reporter gene of B6C3F1 Big Blue transgenic mice treated with 2-AAF (*6*). In contrast, frameshift deletions predominated in a human *hprt* cDNA sequence integrated into chromosomal DNA of mouse VH-12 cells treated with *N*-acetoxy-AAF (*7*). In *E. coli*, dG-AAF induced primarily frameshift deletion mutations (*8*, *9*); a mutation hot spot was found in the recognition sequence for the restriction enzyme *Nar* I, where −2 frameshift mutations (5′-G$_1$G$_2$CG$_3$CC→G$_1$G$_2$CC) were observed at a high frequency (*10*, *11*). On the other hand, using pBR322 plasmids randomly modified with *N*-hydroxy-AF, dG-AF adducts induced mainly base substitutions with a high frequency of G → T transversions (*9*). The mutagenic potential of dG-AF positioned at the *Nar* I site has not been reported.

Site-specific mutagenesis studies have been performed in our laboratory using mammalian cells and single-stranded shuttle vectors containing a single dG-AAF or dG-AF adduct (*12*, *13*). Both dG-AAF and dG-AF mainly produce G → T transversions and lesser amounts of G → A transitions in COS-7 cells (*13*); mutational frequency and specificity are influenced significantly by the sequence context in which dG-AAF and dG-AF are positioned (*14*).

The single-stranded plasmid shuttle vector used for these studies replicates both in *E. coli* and mammalian cells (*15*). To clarify the contribution of sequence context and the host cell to the mutagenic properties of AAF-derived DNA adducts, oligodeoxynucleotides containing the mutation hotspot sequence, *Nar* I, modified by dG-AAF or dG-AF were inserted into the vector. Progeny plasmids recovered following transformation of *E. coli* and transfection of COS-7

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[1] Abbreviations: AAF, *N*-acetyl-2-aminofluorene; AF, *N*-2-aminofluorene; dG-AAF, *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-AF, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.

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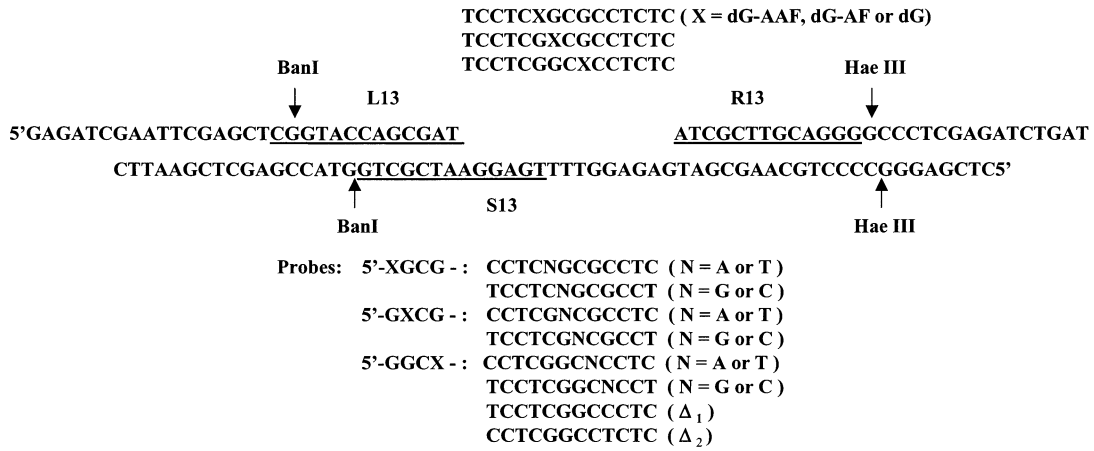

FIGURE 1: Ligation of unmodified and modified 15-mer to *Eco*RV-digested ss pMS2 and probes used for analysis. The upper strand is part of ss pMS2 sequence. Inserted 15-mer containing recognition sequence for *Nar* I restriction enzyme (5′-GGCGCC). X represents dG, dG-AAF, and dG-AF. Restriction enzyme *Ban* I and *Hae* III were used to produce a 40-mer fragment if a 15-mer oligomer was successfully ligated. The underlined L13 and R13 probes were used to detect correct insertion. The underlined 13-mer sequence (S13 probe) of the 61-mer scaffold (bottom strand) was used to determine the concentration of ss DNA construct. Four base mismatches in the center (GGCG/TTTT) serve as a genetic marker to identify products of DNA replication. Probes listed below were used to detect mutations in progeny plasmids by differential oligodeoxynucleotide hybridization at each guanine site.

cells were analyzed for mutations. We find that dG-AAF primarily induces base substitutions in mammalian cells while deletions predominate in *E. coli*; in both hosts, mutational frequency and specificity vary, depending on the adducted position in the *Nar* I site. In contrast, dG-AF predominantly induced G → T transversions in both *E. coli* and COS-7 cells.

MATERIALS AND METHODS

Materials. [γ -^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. *E. coli* DH10B and M13KVO7 helper phage were purchased from Gibco/BRL. The simian kidney cells line (COS-7) was obtained from the tissue culture facility of SUNY at Stony Brook. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolab.

Preparation of Oligodeoxynucleotides. A 15-mer oligodeoxynucleotide (5′TCCTC$\underline{G_1G_2CG_3CC}$TCTC) was prepared by solid-state methods using an automated DNA synthesizer (*16*); the *Nar* I restriction enzyme site is underlined. dG-AAF-modified oligodeoxynucleotides were prepared by reacting a 15-mer oligomer (100 μg) at 37 °C for 10 min with *N*-acetoxy-AAF (10 μg/2 μL ethanol) as described previously (*17*, *18*). Modified oligonucleotides containing a single dG-AAF positioned at G_1, G_2, or G_3 in *Nar* I site were separated on a reverse phase μBondapak C_{18} column (0.80 × 30 cm, Waters), eluted over 60 min at a flow rate of 2.0 mL/min with a linear gradient of 0.05 M triethylammonium acetate (pH 7.0), containing 10−15% acetonitrile. The position of dG-AAF in the oligonucleotide was determined by digestion with venom phosphodiesterase I (*19*). In this method, unmodified or dG-AAF-modified 15-mer oligodeoxynucleotides were labeled at the 5′-terminus with ^{32}P (*20*). The ^{32}P-labeled oligomers (1.0 pmol) were digested with 1.0 × 10^{-5} unit of venom phosphodiesterase I in 100 mM Tris-HCl buffer, pH 8.0, for 3, 10, and 30 min at 25 °C and heated at 95 °C for 3 min. Samples were then subjected to electrophoresis on a denaturing 20% polyacrylamide gel (35 × 42 × 0.04 cm) for 6 h at 1500 V. dG-AF-modified oligodeoxynucleotides were prepared by deacetylation of dG-AAF-modified oligomers under alkaline condition (*18*). These oligodeoxynucleotides were purified further by electrophoresis on 20% denaturing polyacrylamide gels. Bands were extracted by soaking overnight with distilled water. Extracts were concentrated using a Centricon #3 molecular filter (Amicon) and precipitated by ethanol to remove urea. A Waters 900 HPLC instrument equipped with a photodiode array detector was used for separation and purification of oligodeoxynucleotides. UV spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer to determine amounts of purified oligomers.

Construction of Single-Stranded Plasmid Containing a Single dG-AAF and dG-AF Adduct. According to a published protocol (*15*), ss DNA bearing a single dG-AAF or dG-AF adduct was constructed (Figure 1). In short, ss pMS2 was annealed with a 61-mer scaffold at 9 °C overnight and digested with *Eco*RV to yield gapped ss DNA. An unmodified or modified 15-mer was phosphorylated at the 5′ end, hybridized to the gap, and then ligated into the vector at 4 °C for 2 days. The ligation mixture was washed with distilled water in Centricon #100 molecular filter (Amicon) to remove unligated 15-mer. A portion of the ligation mixture was used to confirm insertion of 15-mer into the ss vector. The remaining mixture was digested with exonuclease III and T4 DNA polymerase to remove the scaffold and cleavage residual vector. After extraction with phenol/chloroform, the ss vector was precipitated with ethanol and dissolved in 0.1×TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

To confirm ligation of the 15-mer insert, the ligation mixture was digested with *Ban* I and *Hae* III. The phosphates at 5′end of DNA fragment were replaced with γ-^{32}P using an exchange reaction. Following ethanol precipitation, ^{32}P-labeled DNA fragments were separated on 12% denaturing polyacrylamide gel. As shown in Figure 1, if a 15-mer oligodeoxynucleotide was ligated into a ss vector, a 40-mer band should appear.

Quantification of the Constructed Vectors by Southern Blotting. A portion of the ss vector with or without a lesion, together with known amounts of ss PMS2, was subjected to electrophoresis on 0.9% agarose gel to separate closed

circular (cc) and linear ss DNA. These DNA were transferred to a nylon membrane and hybridized to a ^{32}P-labeled S13 probe complementary only to DNA containing the 15-mer insert. The absolute amount of ss DNA was determined by comparing radioactivity in the sample with that in known amounts of ss DNA.

Site-Specific Mutagenesis Studies in Mammalian Cells and E. coli. Mutagenesis studies in COS-7 cells were conducted as previously described (*21*, *22*). In short, 5×10^5 cells were seeded in 6 cm plate, cultured overnight, and then transfected with 170 ng of ss vector for approximately 18 h with Lipofectin regents (Gibco/BRL) (*23*). After transfection, cells were cultured for 48 h in DMEM medium (Gibco/BRL) containing 10% fetal calf serum. Progeny phagemid were recovered by the method of Hirt (*24*), treated with nuclease S1/*EcoR* V to remove contaminating input ss DNA, and used to transform competent *E. coli* DH10B by electroporation. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization as described previously (*21*, *22*). When *E. coli* AB1157 was used for mutagenesis studies, SOS responses were induced by irradiation of exponentially growing bacteria suspensions with UV light (20 J/m^2) before they were made competent by the $CaCl_2$ method (*25*). Cells were transformed with unmodified and adduct containing vectors (50 ng ss DNA/100 μL competent cells) and immediately plated on ampicillin-containing (100 μg/mL) plates to ensure independent transformation. Transformants were analyzed by oligodeoxynucleotides hybridization as described above. The mutational frequency represented as a percentage by calculating the ratio of mutated colonies to total colonies analyzed.

RESULTS

Preparation of Oligodeoxynucleotides Containing a Single dG-AAF or dG-AF. A 15-mer oligomer (5′TCCTCG$_1$G$_2$CG$_3$-CCTCTC) containing three guanine residues was reacted with *N*-acetoxy-AAF in a buffered solution. HPLC was used to isolate the dG-AAF-modified 15-mers (Figure 2); eight products (peaks 2−8) containing one or more dG-AAF adducts and one unmodified oligomer (peak 1) were resolved. dG-AAF absorbs UV over 300 nm, and the absorbance is linearly related to the number of dG-AAF-modification; therefore, on the basis of the 320 nm/260 nm ratio, three products (peaks 2−4) are estimated to contain a single modification of dG-AAF, three additional products (peaks 5−7) contain two modifications of dG-AAF, and one product (peak 8) contains three modifications of dG-AAF. Following digestion of ^{32}P-labeled dG-AAF-modified oligomers by venom phosphodiesterase, the position modified is determined by blockage of enzyme digestion (*19*), as shown by arrows in Figure 3. Oligomers with a single dG-AAF adduct (peaks 2, 3, and 4) were modified at G$_1$ (5′G^{AAF}GCGCC), G$_3$ (5′GGCG^{AAF}CC), and G$_2$ (5′GG^{AAF}CGCC), respectively. No blockage was observed on the unmodified oligomer. Peaks 5, 6, and 7 represent oligomers doubly modified at G$_1$ and G$_3$ (5′G^{AAF}GCG^{AAF}CC), G$_1$ and G$_2$ (5′G^{AAF}G^{AAF}-CGCC), and G$_2$ and G$_3$ (5′GG^{AAF}CG^{AAF}CC), respectively (data not shown). Peak 8 represents an oligomer containing three modifications (5′G^{AAF}G^{AAF}CG^{AAF}CC). The acetyl group of the dG-AAF-modified 15-mer was released under alkaline condition to prepare the corresponding dG-AF-modified oligomer (*18*). As shown in Figure 3, dG-AAF-modified

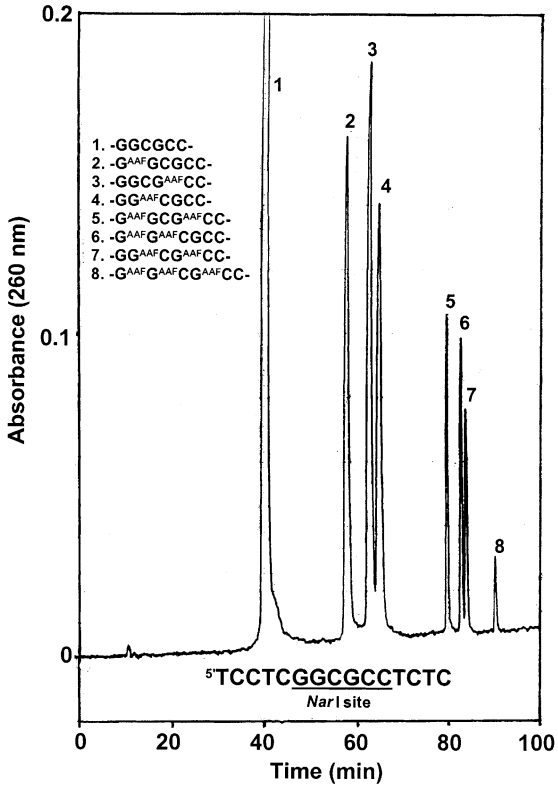

FIGURE 2: HPLC separation of 15-mer oligodeoxynucleotides containing dG-AAF and dG-AF. A 15-mer oligodeoxynucleotide (5′TCCTCG$_1$G$_2$CG$_3$CCTCTC, 100 μg) containing three dG was incubated with *N*-acetoxy-AAF (10 μg/2 μL ethanol), evaporated to dryness, and subjected to HPLC as described in Experimental Procedures. The dG-AAF-modified oligomers were isolated from unmodified oligomer on a reverse-phase μBondapak C$_{18}$ (0.80 × 30 cm, Waters) using a linear gradient of 0.05 M triethylammonium acetate, PH 7.0, containing 10−15% acetonitrile with an elution time of 60min and a flow rate of 2.0 mL/min.

oligomers migrate slower than the corresponding unmodified oligomer, as observed previously for shorter dG-AAF-modified oligomers (*18*). All dG-AAF-modified oligomers were homogeneous. Modified oligodeoxynucleotides were further purified by electrophoresis on denaturing 20% polyacrylamide gels and used for mutagenesis studies.

Construction of ss Plasmid DNA Containing a Single dG-AAF or dG-AF Adduct at Nar I Site. Fifteen-mer oligomers containing a single modification of dG-AAF and dG-AF were ligated into ss vectors, as shown in Figure 1. The unmodified 15-mer was used as a control. When part of the ligation mixture was cleaved with *Ban* I and *Hae* III and labeled with ^{32}P, a 40-mer product was detected on 12% denaturing PAGE, indicating that the oligomer was inserted into the ss vector (data not shown), as shown in a previous study (*13*). Unmodified oligomers migrate with the 40-mer standard; dG-AAF or dG-AF modifications result in a slower migration. No significant differences in ligation efficiency were observed among unmodified, dG-AAF- and dG-AF-modified oligodeoxynucleotides (data not shown). The final concentration of ss DNA vector was quantified by southern blot hybridization in order to accurately transfect and transform COS-7 cells and *E. coli*. The S13 probe was hybridized to the ligation site of the ss vector (Figure 1). Using a β-phosphorimager, the net production of cc DNA of each construct was estimated by comparison with pMS2 DNA standards. Concentrations of unmodified and modified cc

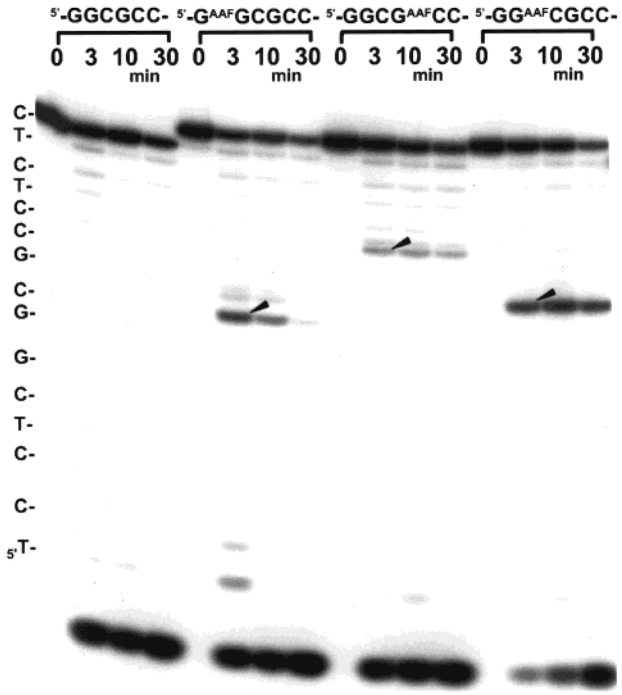

FIGURE 3: Polyacrylamide gel electrophoresis of enzymatically digested unmodified and dG-AAF-modified oligodeoxynucleotides. Unmodified or dG-AAF-modified 15-mer oligodeoxynucleotides (5′TCCTCG$_1$G$_2$CG$_3$CCTCTC, where G$_1$, G$_2$, or G$_3$ is dG-AAF) were labeled at the 5′-terminus with ^{32}P as described in Material and Method. The ^{32}P-labeled oligomers (1.0 pmol) were digested with 1.0×10^{-5} unit of venom phosphodiesterase I in 100 mM Tris-HCl buffer, pH 8.0, for 3, 10, and 30 min at 25 °C, heated at 95 °C for 3 min, and then subjected to electrophoresis on 20% polyacrylamide gel ($35 \times 42 \times 0.04$ cm) for 6 h at 1500 V.

vector samples were 23.5 ng/μL (5′GGCGCC), 14.4 ng/μL (5′G^{AAF}GCGCC), 26.0 ng/μL (5′GG^{AAF}CGCC), 20.7 ng/μL (5′GGCG^{AAF}CC), 6.64 ng/μL (5′G^{AF}GCGCC), 14.3 ng/μL (5′GG^{AF}CGCC), and 5.62 ng/μL (5′GGCG^{AF}CC), respectively.

Mutagenicity of dG-AAF and dG-AF Adducts in E. coli. Using *E. coli* AB1157 with SOS functions induced by UV radiation, the mutagenic frequency and specificity of dG-AAF at various positions in the *Nar* I site were analyzed (Table 1). One- and two-base deletions were predominantly induced. When dG-AAF was at G$_1$, only one-base deletions (Δ^1) were observed: the mutation frequency was 2.8%. When G$_2$ was modified with dG-AAF, one-base (5.0%) or two-base (3.4%) deletions were detected. The mutation frequency increased dramatically to 91.3% when dG-AAF was positioned at G$_3$ in *Nar* I. Among these mutations, 90.2% were two-base deletions; only 1.1% G → T transversions were detected. These results are consistent with those reported for studies in bacteria by Fuchs et al. (*8*). In contrast, mutation frequencies of dG-AF positioned at G$_1$, G$_2$, and G$_3$ in *Nar* I were 7.6%, 8.4%, and 8.2%, respectively. G → T transversions predominated. G → C transitions also were observed when dG-AF was positioned at G$_1$ and G$_3$. No deletions were detected. No mutations were observed at unmodified G$_1$, G$_2$, and G$_3$.

Mutagenicity of dG-C8-AAF and dG-C8-AF Adducts in COS-7 Cells. Using COS-7 cells, the mutagenic properties of dG-AAF and dG-AF positioned in *Nar* I site were analyzed. As shown in Table 2, mutation frequencies of dG-AAF at G$_1$ (23.6 %) and G$_3$ (18.7%) were higher than that at G$_2$ (9.4%). When G$_1$ was modified with dG-AAF, G → T transversions (17.0%) predominated, accompanied by lesser amounts of G → A transitions (5.5%). Similar results were obtained from G$_2$-AAF although the number of G → T transversions observed in exp. 1 (3.1%) and exp. 2 (13.5%) varied significantly. When dG-AAF was positioned at G$_3$, G → C transversions were induced (11.5%), along with lesser numbers of G → T transversions (5.4%) and G → A transitions (1.8%). On the other hand, dG-AF adducted at the same position in the *Nar* I promoted primarily G → T transversions. A higher mutational frequency was observed at G$_2$-AF (20.9%). Significant numbers of deletions were not observed when dG-AAF and dG-AF mutations were detected at G$_1$, G$_2$, and G$_3$ in the *Nar* I site.

DISCUSSION

The *Nar* I site is known to be a mutational hot spot in bacteria. Using site-specifically modified shuttle vectors containing a single dG-AAF or dG-AF positioned within this sequence, the mutagenic specificity and frequency associated with these lesions in *E. coli* and mammalian cells were compared.

dG-AAF Induced Mutations in E. coli. In *E. coli* AB1157, following SOS induction, dG-AAF positioned at G$_3$ within the *Nar* I site (5′G$_1$G$_2$CG$_3$CC) promoted 90% two-base deletions (Figure 4 and Table 1). This result is consistent with that observed by Fuchs and his colleagues (*8*, *26*) using a gapped-duplex vector or ss vector system. To explain the formation of two-base deletions by AAF adducted at G$_3$, Koffel-Schwartz and Fuchs proposed a mechanism involving template-primer misalignment with a two-nucleotide slipped mutagenic intermediate (*27*). Since slippage of either the G^{AAF}C (5′GGCG^{AAF}CC) or CG^{AAF} (5′GGCG^{AAF}CC) dinucleotide in *Nar* I forms the same two-base deletions (5′GGCC), in vivo experiments, including our results, cannot distinguish between the adducted species. On the basis of primer extension reactions and steady-state kinetic studies using the Klenow fragment of *E. coli* DNA pol I (*17*), the ability of a damaged nucleotide to generate one- and two-base deletions has been shown to be determined by the following parameters: (a) the nature of the base inserted opposite the adduct, (b) the sequence context of the lesion, and (c) the overall rate of translesional DNA synthesis. dCTP was preferentially incorporated opposite dG-AAF, accompanied by lesser amounts of dGMP incorporation (*17*). In reactions catalyzed by Klenow fragment (*17*) on templates containing the sequence 5′GCG^{AAF}C, two-base deletions were formed when C (underlined) was 3′ to the lesion, but not when it was replaced by A or T. Our mechanism predicts that a newly inserted dCMP opposite the lesion and a 5′G flanking base at the primer terminus could pair with complementary GC bases 5′ to the lesion, resulting in formation of two-base deletions via a G^{AAF}C bulge (Figure 5A).

Although Fuchs and his colleagues did not observe significant numbers of mutations when dG-AAF was positioned at G$_1$ and G$_2$ in the *Nar* I site (*8*), we detected significant numbers of one-base deletions (2.8%) at dG$_1$-AAF and one-base (5.0%) and two-base (3.4%) deletions at dG$_2$-AAF. Mutation frequencies varied strikingly, depending on the adducted position, as noted by Fuchs et al. (*8*, *26*).

Table 1: Mutational Specificity of dG-AAF and dG-AF Adduct Embedded in the *Nar* I Site in *Escherichia coli* under SOS Induction[a]

plasmids (pMS2)		no. of targeted mutants on G^{AAF} and G^{AF} site						mutational frequency
		G	T	A	C	Δ^1	Δ^2	
5′GGCGCC	exp1	86	0	0	0	0	0	
	exp2	87	0	0	0	0	0	
	total	173 (100%)	0 (<0.6%)	0 (0.6%)	0 (<0.6%)	0 (<0.6%)	0 (<0.6%)	<0.5%
5′G^{AAF}GCGCC	exp1	87	0	0	0	1	0	
	exp2	88	0	0	0	4	0	
	total	175 (97.2%)	0 (<0.6%)	0 (<0.6%)	0 (<0.6%)	5 (2.8%)	0 (<0.6%)	2.8%
5′GG^{AAF}CGCC	exp1	82	0	0	0	3	2	
	exp2	80	0	0	0	6	4	
	total	162 (91.6%)	0 (0.6%)	0 (<0.6%)	0 (<0.6%)	9 (5.0%)	6 (3.4%)	8.4%
5′GGCG^{AAF}CC	exp1	8	0	0	0	0	85	
	exp2	8	2	0	0	0	81	
	total	16 (8.7%)	2 (1.1%)	0 (<0.5%)	0 (<0.5%)	0 (<0.5%)	166 (90.2%)	91.3%
5′G^{AF}GCGCC	exp1	78	5	0	1	0	0	
	exp2	81	7	0	0	0	0	
	total	159 (92.4%)	12 (7.0%)	0 (<0.6%)	1 (0.6%)	0 (<0.6%)	0 (<0.6%)	7.6%
5′GG^{AF}CGCC	exp1	81	3	0	0	0	0	
	exp2	72	11	0	0	0	0	
	total	153 (91.6%)	14 (8.4%)	0 (<0.6%)	0 (<0.6%)	0 (<0.6%)	0 (<0.5%)	8.4%
5′GGCG^{AF}CC	exp1	79	3	0	4	0	0	
	exp2	77	4	0	3	0	0	
	total	156 (91.8%)	7 (4.1%)	0 (<0.6%)	7 (4.1%)	0 (<0.6%)	0 (<0.6%)	8.2%

[a] Adducted ss DNA (50 ng) was used to transform SOS induced *E. coli* AB1157 (by UV 20J/m^2); progeny phagemids obtained were subject to oligonucleotide hybridization and sequence analysis to detect mutational specificity. exp1 and exp2 represent independent experiments. Nontargeted mutations associated with a targeted event are listed below:

5′TCCTCGGCG^{AAF}CCTCTC
----------Δ---------- 1 G→ Δ^1

5′TCCTCG^{AF}GCGCCTCTC
----------C---------- 1 G→ C

At dG_1-AAF, the newly inserted dGMP opposite the lesion, can pair with the 5′ flanking base, C, forming a one-base deletion (Figure 5B). With dG_2-AAF, the newly inserted dCMP may pair with G 5′ to the lesion, forming one-base deletions (Figure 5C). Formation of small numbers of two-base deletions induced by dG_2-AAF is ruled out by our mechanism (*17*); however, dGMP inserted opposite dG_2-AAF could pair with C two bases 5′ to the lesion, forming two-base deletions.

Recently, three SOS-inducible DNA polymerases, pol II, pol IV, and pol V, were shown to be involved in translesion synthesis on damaged DNA (*28*). On the basis of studies of pol II (*polB*), pol IV (*dinB*), and pol V (*umuDC*) mutants transformed vectors containing a single dG-AAF (*28*), pol II is required for forming two-base deletions at dG_3-AAF within the *Nar* I site and pol V is required for error-free translesion synthesis (*28*, *29*). Pol V is an essential polymerase that promotes one-base deletions and error-free elongation on a sequence containing contiguous dG residues (5′GGG^{AAF}-) (*28*). The contribution of pol IV to the bypass of dG-AAF lesions positioned in such specific sequences may be minimal (*28*), although we recently found that pol IV promotes primarily one- and two-base deletions opposite dG-AAF positioned in sites other than *Nar* I and contiguous dG sequences during in vitro DNA synthesis (*30*). Pol IV has been shown to catalyze replication past benzo[*a*]pyrene adducts (*28*). Therefore, bacteria may use several translesional DNA polymerases to bypass certain damaged DNA lesions; the polymerase(s) involved may vary depending on the nature of the adduct (*28*).

dG-AF Induced Mutations in E. coli. Interestingly, dG-AF adducts located within the *Nar* I site promote only base substitutions; G → T transversions predominate (Table 1). No deletions were observed. Results obtained from our site-specific approach are consistent with earlier observations using plasmids modified randomly with dG-AF (*9*). Unlike dG-AAF, mutational frequencies (7.6−8.4%) for dG-AF at G_1, G_2, and G_3 in *Nar* I site were similar (Figure 4). Sequence context effects for dG-AF-induced mutagenesis were not significant.

For dG-AF, base substitutions predominated, while dG-AAF is associated primarily with deletions. This difference may be partly explained by structural studies showing that the dG-AF adduct produces less distortion than dG-AAF in the DNA helix (*31*, *32*). Multidimensional NMR studies reveal that the guanine bearing the C8-AAF adduct in double strand DNA rotates from anti to syn conformation with the fluorene ring inserted into the helix (*33*). In contrast, dG-AF adopts two interchangeable conformations. In this case, part of the fluorene ring remains outside the helix, whereas in the other, the fluorine ring is partially intrahelical (*32*, *34*). The ratio of these conformations depend on the sequence context in which the adduct is embedded (*35*, *36*). Thus, structural distortion by dG-AAF tends to promote deletions, while the more flexible dG-AF adduct is primarily associated with base substitutions.

We have observed in in vitro primer-extension experiments that pol IV extends past dG-AF by primarily incorporating dCMP, the correct base, opposite the lesion, together with lesser amounts of dAMP and one- and two-base deletions (*30*). In the present study, misincorporation of dAMP also was observed; however, deletions were not observed in vivo (Table 1). Primer extension reactions catalyzed by the α subunit of pol III were blocked one base prior to dG-AAF, but extended readily past dG-AF by incorporating dCMP, the correct base, opposite the lesion.[2] SOS-inducible DNA

[2] Shibutani, S. and Maki, H., unpublished data.

Table 2: Mutational Specificity of dG-AAF and dG-AF Adducts Embedded in the *Nar* I Site in COS-7 Cells[a]

plasmids (pMS2)		no. of targeted mutants on G^{AAF} and G^{AF} site						mutational frequency
		G	T	A	C	Δ^1	Δ^2	
5′GGCGCC	exp1	112	0	0	0	0	0	
	exp2	149	0	0	0	0	0	
	total	261 (100%)	0 (<0.4%)	0 (0.4%)	0 (<0.4%)	0 (<0.4%)	0 (<0.4%)	<0.4%
5′G^{AAF}GCGCC	exp1	65	21	5	1	0	0	
	exp2	74	10	5	1	0	0	
	total	139 (76.4%)	31 (17.0%)	10 (5.5%)	2 (1.1%)	0 (<0.5%)	0 (<0.5%)	23.6%
5′GG^{AAF}CGCC	exp1	91	3	3	0	0	0	
	exp2	64	10	0	0	0	0	
	total	155 (90.6%)	13 (7.6%)	3 (1.8%)	0 (<0.6%)	0 (<0.6%)	0 (<0.6%)	9.4%
5′GGCG^{AAF}CC	exp1	89	4	2	12	0	0	
	exp2	46	5	1	7	0	0	
	total	135 (81.3%)	9 (5.4%)	3 (1.8%)	19 (11.5%)	0 (<0.6%)	0 (<0.6%)	18.7%
5′G^{AF}GCGCC	exp1	95	10	1	1	0	0	
	exp2	100	8	0	0	0	0	
	total	195 (90.7%)	18 (8.4%)	1 (0.5%)	1 (0.5%)	0 (<0.5%)	0 (<0.5%)	9.3%
5′GG^{AF}CGCC	exp1	71	18	0	0	1	0	
	exp2	83	23	0	0	0	0	
	total	154 (78.6%)	41 (20.9%)	0 (<0.5%)	0 (<0.6%)	1 (0.6%)[b]	0 (<0.5%)	21.4%
5′GGCG^{AF}CC	exp1	95	18	2	0	1	0	
	exp2	113	6	1	1	0	0	
	total	208 (87.8%)	24 (10.1%)	3 (1.3%)	1 (0.4%)	1 (0.4%)	0 (<0.4%)	12.2%

[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. exp1 and exp2 represent independent experiments. [b] Three base deletions (5′-TCCTCGG^{AF}CGCCTCTC → 5′- TCCTCGΔ^3CCTCTC). Nontargeted mutations associate with a targeted event are listed below:

5′TCCTCGGCG^{AAF}CCTCTC

----------T----------	7	(C→ T)
----------T----------	1	(C→ T)

5′TCCTCGG^{AF}CGCCTCTC

---------C-----G----------	2	(G→ C, C→ G)
---G----------	2	(C→ G)
---A----------	2	(C→ A)

5′TCCTCGGCG^{AF}CCTCTC

---------C----------	1	(G→ C)
----------TA------	1	(C→ T, T→ A)

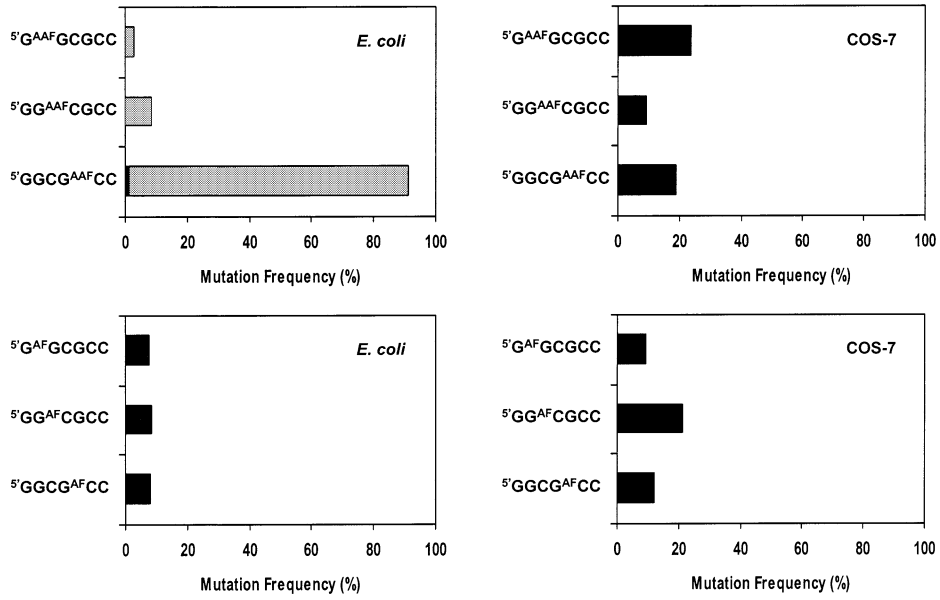

FIGURE 4: Comparison of mutational frequencies of dG-AAF and dG-AF positioned in the *Nar* I sequence in *E. coli* and COS-7 cells. Data for *E. coli* and COS-7 cells were taken from Table 1 and Table 2, respectively. The gray and black columns represent the frequencies of deletions and base substitutions, respectively.

polymerases may associate with pol III, assisting in catalyzing bypass of dG-AAF and dG-AF lesions. In *E. coli*, this results in deletions and base substitutions during DNA synthesis. Similarly, the proofreading function of Pol III may minimize deletions.

Mutagenesis of AAF-Derived DNA Adducts in Mammalian Cells. In COS-7 cells, dG-AAF promoted base substitutions opposite the lesion. At dG_1-AAF and dG_2-AAF, G → T transversions were preferentially formed within the *Nar* I site, accompanied by lesser numbers of G → A transitions.

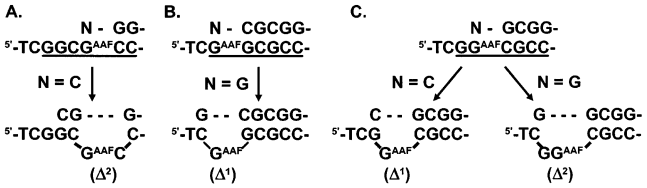

FIGURE 5: Proposed mechanisms for one-base and two-base deletions.

At G_3-AAF, G → C transversions predominated, accompanied by G → T transversions (Table 2). Deletions were not observed at any of the dG-AAF adducts positioned within the *Nar* I site, as observed previously using dG-AAF-modified oligomers embedded in a different sequence contexts (*14*). Although dG_2-AAF in $^{5'}$TCGG^{AAF}CGC- and G_3-AAF in $^{5'}$GGCG^{AAF}CCT- have 3′ and 5′ flanking bases similar to those in $^{5'}$CTGG^{AAF}CCT- and $^{5'}$CTCG^{AAF}CCT- (*14*), the mutational frequencies of dG_2-AAF (9.8 ± 5.2%) and dG_3-AAF (19.4 ± 3.7%) were 2.3 times lower and 1.8 times higher, respectively, than those observed with the corresponding sequences (22.7 ± 2.2% and 10.6 ± 3.2%). Notably, the mutagenic specificity of dG_3-AAF, showing preferential G → C transversions, differed from the preferential G → T transversions of dG-AAF, as observed in the $^{5'}$CTCG^{AAF}CCT- sequence. Like dG-AAF, dG-AF positioned within the *Nar* I sequence promoted preferentially G → T transversions, producing no deletions (Table 2). As reported previously (*14*), significant differences of mutagenic specificity and frequency were observed when dG-AAF- and dG-AF-modified oligomers embedded in the same sequence were transfected into COS-7 cells. Therefore, the similar mutagenic properties observed for dG-AAF and dG-AF adducts in this experiment cannot be due to intracellular deacetylation of dG-AAF. The mutagenic frequency of dG_2-AF (21.4 ± 0.4%) was 2.3 and 1.8 times higher than that of dG_1-AF (9.3 ± 2.7%) and dG_3-AF (12.3 ± 8.1%), respectively. We have reported previously that dG-AF positioned in the sequence $^{5'}$CTGG^{AF}CCT− promoted 70% base substitutions representing primarily G → T transversions (*14*). Although the bases flanking dG_2-AF ($^{5'}$TCGG^{AF}CGC−) were similar to those in the sequence used in earlier experiments, the mutational frequency at dG_2-AF was 3.3 times lower. We conclude that mutational frequency and specificity are affected by the 3′ and 5′ sequence context, not only by the bases flanking the lesion.

The mutational properties of dG-AAF observed in mammalian cells were strikingly different from those observed in *E. coli*. This difference may reflect different patterns of DNA replication in prokaryotes and eukaryotes (*37*, *38*). The miscoding properties of dG-AAF and dG-AF have been explored in vitro, using mammalian DNA polymerases and primer-extension techniques (*13*). In reactions catalyzed by pol α and pol δ, dG-AAF blocks primer extension, while dG-AF is bypassed with dCMP, the "correct" base, incorporated opposite the lesion (*13*). When pol α (template: enzyme = 1.2:1.0) was used, dG-AAF and dG-AF promoted small amounts of misincorporation of dAMP and dTMP (0.4% for dG-AAF and 2.9% for dG-AF) (*13*). This low frequency of misincorporation does not account for the high frequency of mutations observed in mammalian cells (*13*).

A number of novel human DNA polymerases have recently been found (reviewed in ref 39). 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 *E. coli* DinB polymerase (pol IV) (*39*−*42*). The ability of pol κ to bypass various DNA lesions in vitro, including 8-oxo-7,8-dihydro-deoxyguanosine (*43*), (−)-trans-*anti*-benzo[*a*]-pyrene-N^2-dG (*43*), and abasic sites (*43*, *44*) has been reported. In studies conducted with a single dNTP, pol κ preferentially incorporates dTMP and dCMP opposite dG-AAF (*43*−*45*). We have recently observed that primer-extension reactions catalyzed by pol κ are partially retarded at dG-AAF and dG-AF then extended past these lesions (*30*). dG-AAF promoted incorporation of dTMP opposite the lesion, accompanied by smaller amounts of dCMP, dAMP, and dGMP and some deletions. On templates containing dG-AF, dAMP, dTMP, and dCMP were incorporated opposite the lesion in approximately equal amounts, together with some deletions (*30*). These in vitro miscoding studies using pol κ predict G → A and G → T mutations and a lesser number of G → C base substitutions targeted to the lesion site in cells. This mutational spectrum is consistent with that observed when dG-AAF and dG-AF adducts replicate in COS-7 cells, although the relative frequencies of G → T and G → A mutations are affected by the sequence context to the lesions (*14*). Additionally, another DNA polymerase, pol η, efficiently catalyzes error-free synthesis past dG-AAF adducts (*46*, *47*). These newly found DNA polymerase(s) may participate in translesion synthesis in the presence of pol α and pol δ when extending past AAF-derived DNA adducts. In addition, dG-AAF tends to promote deletions in *E. coli* and base substitutions in COS-7 cells. To clarify the different molecular mechanisms of mutagenesis for AAF-derived adducts in *E. coli* and mammalian cells, the DNA polymerase(s) and/or other cellular factors involved must be identified.

In summary, in *E. coli*, dG-AAF positioned within *Nar* I site promotes frameshift mutations, whereas dG-AF in the same sequence promotes base substitutions mutations. In mammalian cells, both dG-AAF and dG-AF produce base substitutions mutations. The mutational specificity and frequency vary depending on the sequence context in which the lesion is embedded. We conclude that mutagenic properties of AAF-derived DNA adducts are significantly influenced by the chemical structure of the adduct, the sequence context in which the lesion is placed, and the nature of the host cell.

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