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Mutagenic properties of the 8-amino-2′-deoxyguanosine DNA adduct in mammalian cells

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ABSTRACT

The DNA adduct 8-amino-2′-deoxyguanosine (8-amino-dG) is found in liver DNA of rats treated with the hepatocarcinogen 2-nitropropane. Site-specifically modified oligodeoxynucleotides were used to explore the mutagenic potential of 8-amino-dG in simian kidney (COS-7) cells. Oligodeoxynucleotides (5′-TCCTCCT$\underline{X}_1$G$_2$CCTCTC and 5′-TCCTCCTG$_1\underline{X}_2$CCTCTC, $\underline{X}$ = dG or 8-amino-dG) with the lesion positioned at codon 60 or 61 of the non-coding strand of the human c-Ha-*ras1* gene were inserted into single-stranded phagemid vectors and transfected into COS-7 cells. The progeny plasmid obtained was used to transform *Escherichia coli* DH10B. Transformants were analyzed by oligodeoxynucleotide hybridization and DNA sequencing to establish the mutation frequency and spectrum produced by the modified base. The correct base, dCMP, was incorporated preferentially opposite 8-amino-dG at X_1 and X_2. When 8-amino-dG was at X_1, targeted $G^{NH2}\rightarrow T$ transversions were detected, along with smaller numbers of $G^{NH2}\rightarrow A$ transitions and $G^{NH2}\rightarrow C$ transversions. When the adduct was at X_2, only $G^{NH2}\rightarrow T$ transversions were observed. The frequencies of targeted mutation at X_1 and X_2 were 2.7 and 1.7%, respectively. Mutation frequency and mutagenic spectrum were sequence context dependent. In addition, non-targeted G→T transversions, accompanied by some G→A transitions, were detected 5′ to 8-amino-dG when the lesion was at X_2. We conclude that 8-amino-dG is a mutagenic lesion, generating G→T and G→C transversions and G→A transitions in mammalian cells.

INTRODUCTION

2-Nitropropane, an important industrial chemical (1) and a component of cigarette smoke (2), has been shown to be mutagenic in *Salmonella typhimurium* (3–5) and a potent hepatocarcinogen in rats when administered by inhalation (6) or by gavage (7).

8-Amino-2′-deoxyguanosine (8-amino-dG) and 8-oxo-7,8-dihydroxyguanine (8-oxo-dG) have been identified in liver DNA of rats treated with 2-nitropropane (8–10). 8-Oxo-dG, a well-known product of oxidative DNA damage, is mutagenic *in vitro* and *in vivo* (11–14). *Escherichia coli* and mammalian DNA polymerases incorporate dAMP and dCMP opposite this lesion: the dAMP/dCMP ratio depends on the DNA polymerase used (11). 8-Oxo-dG generates G→T transversions in *E.coli* and mammalian cells (12–14).

It has been hypothesized that 2-nitropropane is metabolized to hydroxylamine *O*-sulfonate or acetate, generating a reactive nitrenium ion, NH_2^+, that can react with DNA to form 8-aminoguanine (9). The aminating species is produced by aryl sulfotransferase (15). The incorporation of 8-amino-dGTP into DNA, catalyzed by several DNA polymerases, has been measured (16). Mammalian DNA polymerases α and β incorporate much higher amounts of 8-amino-dGTP opposite a template cytidine residue than of 8-oxo-dGTP (16). The contribution of a template 8-amino-dG to 2-nitropropane mutagenesis is not known.

In this paper, site-specifically modified oligodeoxynucleotides containing a single 8-amino-dG were inserted into a single-stranded shuttle vector that minimizes repair of DNA adducts (13). These vectors were used to establish the mutagenic specificity and frequency of 8-amino-dG in simian kidney (COS-7) cells. These studies reveal that 8-amino-dG has significant mutagenic potential, generating G→T transversions in mammalian cells.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. *Escherichia coli* DH10B was purchased from Gibco BRL. The simian kidney (COS-7) cell line was obtained from the tissue culture facility of SUNY at Stony Brook. T4 polynucleotide kinase and helper phage VCSM13 were purchased from Stratagene. A Waters 990 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.

Synthesis of oligodeoxynucleotides

An unmodified 15mer oligodeoxynucleotide (5′-TCCTCC-T$\underline{GG}$CCTCTC) was prepared by solid state synthesis, using an

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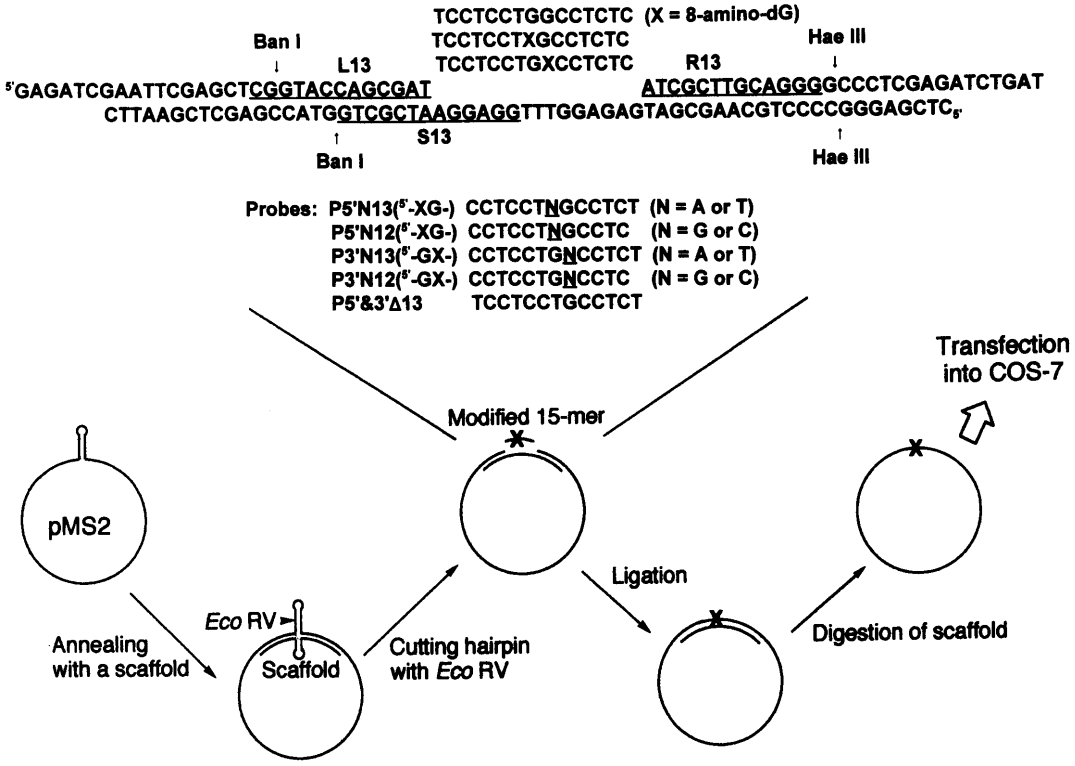

Figure 1. Construction of a single-strand vector containing a single 8-amino-dG. The upper strand is part of the single-strand pMS2 sequence; X represents 8-amino-dG. The underlined 13mer of the 61mer scaffold (bottom strand) was used to determine the concentration of the ssDNA construct. The underlined L13 and R13 probes were used to detect the correct insertion. The probes listed were used for oligodeoxynucleotide hybridization to determine mutation specificity of 8-amino-dG.

automated DNA synthesizer (17). Oligodeoxynucleotides containing a single 8-amino-dG (5′-TCCTCCT$\underline{X_1G_2}$CCTCTC or 5′-TCCTCCT$\underline{G_1X_2}$CCTCTC, X = 8-amino-dG) were synthesized by the phosphoramidite technique, as described previously (18). Modified and unmodified oligodeoxynucleotides were purified on a reverse phase μBondapak C_{18} column (0.39 × 30 cm; Waters), 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 (19). These oligomers were further purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea (35 × 42 × 0.04 cm) (19). Bands were detected under UV light and extracted overnight with 2.0 ml of distilled water at 4°C. Extracts were concentrated on a Centricon 3 filter (Amicon) by centrifugation at 5000 r.p.m. for 2 h then subjected to HPLC to remove urea (19). Oligonucleotides were labeled at the 5′-terminus by treatment with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (20) and subjected to acrylamide gel electrophoresis. The position and homogeneity of oligonucleotides following gel electrophoresis was determined by autoradiography using Kodak X-Omat XAR film.

Site-specific mutagenesis in COS cells

SV40-transformed simian kidney cell line COS-7 and a single-stranded shuttle vector, pMS2, which confers neomycin (Neo^R) and ampicillin (Amp^R) resistance (13), were used to establish mutagenic specificity. Construction of a circular single-stranded (ss)DNA containing a single DNA adduct followed procedures established previously in this laboratory (13). pMS2 DNA was annealed to a 61mer then digested with *Eco*RV to create a 15mer gap (Fig. 1). An unmodified or 8-amino-dG-modified 15mer was ligated to the gapped vector. A portion of the ligation mixture was used to establish ligation efficiency. The ligation mixture was incubated for 2 h with T4 DNA polymerase (1 U/pmol DNA) to digest the hybridized 61mer, then treated with *Eco*RV and *Sal*I to cleave residual single-stranded 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 single-stranded pMS2 were subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ssDNA. DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labeled S13 probe complementary to DNA containing the 15mer insert. The absolute amount of closed circular ssDNA was established by comparing the radioactivity in the sample with that in known amounts of ssDNA.

COS-7 cells were transfected with ssDNA (100 fmol) over 18 h using lipofectin (21), after which the cells were grown for 2 days in Dulbecco's modified Eagle's medium/10% fetal calf serum. Progeny plasmids were recovered by the method described by Hirt (22) and treated with S1 nuclease to digest input ssDNA. We confirmed using agarose gel electrophoresis that the sample of progeny plasmids contains only double-stranded vector. These plasmids were used to transform *E.coli* DH10B. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization (23,24). The oligodeoxynucleotide probes used to identify progeny phagemids are shown in Figure 1. Probes L13 and R13 were used to select phagemids containing the correct insert.

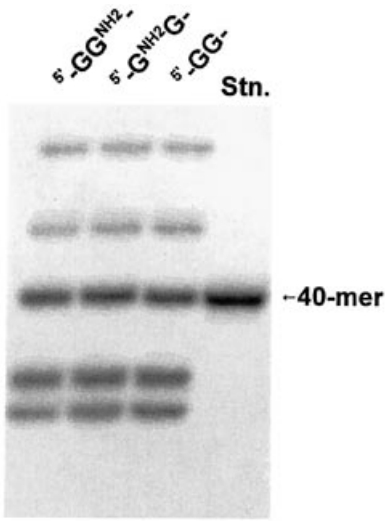

Figure 2. Analysis of products inserted into pMS2. A portion of the vector annealing with the 61mer scaffold was digested with *Ban*I and *Hae*III and subjected to 12% denaturing polyacrylamide gel as described in Materials and Methods.

Transformants that failed to react with L13 and R13 were omitted from the analysis. When L13/R13-positive transformants failed to hybridize to the probes designed to detect events targeted to the lesion site, double-stranded (ds)DNA was prepared and subjected to dideoxy-nucleotide sequencing analysis (25).

RESULTS

Mutational specificity *in vivo*

Unmodified and 8-amino-dG-modified 15mer oligodeoxynucleotides were inserted into a gapped single-stranded vector as described in Materials and Methods. The construct was cleaved with *Ban*I and *Hae*III and subjected to 12% denaturing polyacrylamide gel electrophoresis (Fig. 2). The digestion product containing the unmodified or 8-amino-dG-modified 15mer migrated identically to the 40mer standard marker. This result indicates that unmodified and modified oligomers were inserted. No significant difference in ligation efficiency was observed.

The final concentration of ssDNA vector was quantified by Southern blot hybridization (data not shown). The S13 probe hybridized to the ligation site of the single-stranded vector (Fig. 1). Using a β-phosphorimager, the net product of the closed circular (cc)DNA of each construct was estimated by comparison to pMS2 DNA standards. The concentration of the closed circular vector sample was 5.1 ng/μl for the unmodified oligomer (5′-GG-) and 5.0 (5′-G^{NH2}G-) and 3.0 ng/μl (5′-GG^{NH2}-) for the 8-amino-dG-modified oligomers, respectively.

pMS2 modified with 8-amino-dG was used to transfect COS7 cells; the number of transformants recovered were compared to those in the unmodified control (Table 1). The presence of a single 8-amino-dG residue reduced transformation efficiency by 65–87%, depending on the position of the adduct.

Table 1. Transformation efficiency of *E.coli* DH10B with a COS-7 cell-derived progeny phagemid

ssDNA[a]	Number of transformants
pMS2 (5′-GG)	2385 (100%)
pMS2 (5′-G^{NH2}G)	2090 (87%)
pMS2 (5′-GG^{NH2})	1557 (65%)

[a]100 fmol of ssDNA was transfected into COS-7 cells. The progeny phagemid was used to transform *E.coli* DH10B for ampicillin resistance.

Sequence context effects

When 8-amino-dG was at X_1, preferential incorporation of dCMP (95.6%), the correct base, was observed opposite the lesion (Table 2). Ten targeted G^{NH2}→T transversions were detected, along with four G^{NH2}→A transitions and four G^{NH2}→C transversions. The targeted mutation frequency was 2.7%. Some non-targeted mutations were also observed, as shown in Figure 3. When 8-amino-dG was at X_2, preferential incorporation of dCMP (95.6%) was observed opposite the lesion (Table 2). Nine targeted G^{NH2}→T transversions were detected, along with one G^{NH2}→A transition; no G^{NH2}→C transversions were observed. The frequency of targeted mutations at X_2 was 1.7%; the overall mutation frequency was 1.6 times less than that observed at the X_1 site. In addition, 13 non-targeted mutants showing G→T transversions were detected opposite the dG positioned 5′ to 8-amino-dG at the X_2 site, accompanied by three G→A transitions. The frequency of these non-targeted mutations was 3.1%.

DISCUSSION

Mutagenic specificity in mammalian cells

A single-stranded plasmid vector was used to establish the mutagenic specificity of 8-amino-dG in COS-7 cells. Single-stranded SV40-based shuttle vectors have been shown to be converted to its double-stranded form in several cultured cells (26,27). Crude extracts of *Xenopus* eggs (28) or COS cells (13) have an ability to convert ssDNA to dsDNA. DNA polymerase α/primase has been shown to be involved in replication on non-primed ssDNA templates (29). Thus, ssDNA could be used to study mutagenic events in mammalian cells.

When 8-amino-dG was positioned opposite the third base of codon 60 in the non-coding strand of the human c-Ha-*ras1* gene (5′-TGG^{NH2}C-), targeted mutations representing G→T transversions were detected (Table 2). When 8-amino-dG was opposite the first base of codon 61 in the non-coding strand (5′-TG^{NH2}GC-), G→A transitions and G→C transversions were also observed. Thus the mutational frequency and spectrum of 8-amino-dG varies according to the sequence context of the lesion.

Primer extension reactions catalyzed by DNA polymerases α and δ proceeded readily on an 8-amino-dG-modified template (Shibutani *et al.*, unpublished data). Both of these replicative enzymes incorporate primarily dCMP, the correct base, opposite 8-amino-dG. In addition, polymerases α and δ misincorporate some dAMP and dGMP, respectively, opposite the lesion (Shibutani *et al.*, unpublished data). Based on these results, 8-amino-dG was predicted to generate G→T and G→C transversions in mammalian cells. Thus, the miscoding specificity and

Table 2. Mutational specificity of the 8-amino-dG adduct in mammalian cells[a]

Plasmid		No. of colonies analyzed	Number of Targeted Mutations (dG or 8-amino-dG → X)					
			G	T	A	C	Δ	others[b]
5'-TGG-	Exp.1[c]	172	171	0	0	0	0	1
	Exp.2	150	150	0	0	0	0	0
	Exp.3	280	277	0	1	0	0	2
	Total	602	598 (99.3%)	0 (<0.2%)	1 (0.2%)	0 (<0.2%)	0 (<0.2%)	3 (0.5%)
5'-TG^{NH2}G-	Exp.1[c]	235	226	3	1	0	0	5
	Exp.2	159	152	4	2	0	0	1
	Exp.3	265	252	3	1	4	0	5
	Total	659	630 (95.6%)	10 (1.5%)	4 (0.6%)	4 (0.6%)	0 (<0.2%)	11(1.7%)
5'-TGG-	Exp.1[c]	172	171	0	0	0	0	1
	Exp.2	150	150	0	0	0	0	0
	Exp.3	280	278	0	0	0	0	2
	Total	602	599 (99.5%)	0 (<0.2%)	0 (<0.2%)	0 (<0.2%)	0 (<0.2%)	3 (0.5%)
5'-TGG^{NH2}-	Exp.1[c]	190	180	2	0	0	0	8
	Exp.2	141	133	3	0	0	0	5
	Exp.3	286	277	4	1	0	0	6
	Total	619	590 (95.3%)	9 (1.5%)	1 (0.2%)	0 (<0.2%)	0 (<0.2%)	19 (3.1%)

[a]Adducted ssDNA (250 and 170 ng) was used to transfect COS-7 cells. The progeny phagemid was recovered and used to transform *E.coli* DH10B for mutation analysis.
[b]Non-targeted mutations are listed in Figure 3.
[c]Data of experiments 1–3 were obtained using independently prepared progeny phagemids.

Figure 3. Non-targeted mutations induced by 8-amino-dG lesions. The number and frequency of non-targeted mutations induced by 8-amino-dG or unmodified dG are described in Table 2.

frequency obtained *in vitro* are similar to those observed in COS-7 cells.

Using experimental conditions similar to those reported here, we have determined the mutagenic properties of 8-oxo-dG (Shibutani *et al.*, unpublished data). Preferential incorporation of dCMP, the correct base, was observed opposite 8-oxo-dG, as noted with 8-amino-dG. When 8-oxo-dG was opposite the third base of codon 60, G→T transversions and a lesser amount of G→A transitions were detected. When 8-oxo-dG was opposite the first base of codon 61, only G→T transversions were detected. Except for a few G→C transversions, the mutational specificity of 8-amino-dG was quite similar to that of 8-oxo-dG (11).

We conclude from this study that 8-amino-dG has significant mutational potential, generating primarily G→T transversions in mammalian cells. Frequencies of targeted mutations induced by 8-amino-dG (1.7–2.7%) were 1.9–4.0 times lower than the mutational frequency of 8-oxo-dG (5.2–6.8%) under comparable conditions. However, the level of 8-amino-dG adducts in the liver DNA of rats treated with 2-nitropropane was 5–6 times higher than the level of 8-oxo-dG (10). Thus, the contribution of 8-amino-dG to mutagenesis induced by 2-nitropropane may be greater than that of 8-oxo-dG.

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