Mutagenic Potential of Benzo[*a*]pyrene-Derived DNA Adducts Positioned in Codon 273 of the Human *P53* Gene†

Huan Dong, Radha Rani Bonala, Naomi Suzuki, Francis Johnson, Arthur P. Grollman, and Shinya Shibutani*

Laboratory of Chemical Biology, Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651

Received August 17, 2004; Revised Manuscript Received October 2, 2004

ABSTRACT: Codon 273 (5′CGT) of the human *P53* gene is a mutational hot spot for the environmental carcinogen benzo[*a*]pyrene. We incorporated a single (+)- or (−)-*trans*-*anti*-benzo[*a*]pyrene diol epoxide (BPDE) DNA adduct at the second position of codon 273 of the human *P53* gene and explored the mutagenic potential of this lesion in mammalian cells. Oligodeoxyribonucleotides (5′GAGGTGCG^{BPDE}-TGTTTGT) modified with (+)- or (−)-*trans*-dG-N^2-BPDE were incorporated into single-stranded shuttle vectors and transfected into simian kidney cells. Progeny plasmids were then used to transform *Escherichia coli* DH10B. Transformants were analyzed by oligodeoxynucleotide hybridization and DNA sequence analysis to establish the mutation frequency and spectrum produced by the adducted base. We determined the mutational frequencies associated with (+)-*trans*-dG-N^2-BPDE and (−)-*trans*-dG-N^2-BPDE adduction to be 26.5% and 17.5%, respectively. The predominant mutations generated by both stereoisomers were G → T transversions, with some G → A transitions. When the cytosine 5′ to dG-N^2-BPDE was replaced by 5-methylcytosine, the mutational frequencies of (+)-*trans*-dG-N^2-BPDE and (−)-*trans*-dG-N^2-BPDE were reduced to 11.1% and 10.6%, respectively, while the mutational specificity remained unchanged. Thus, the mutational "hot spot" at codon 273 in P53 may reflect either sequence-specific reactivity of BPDE and/or inefficient repair of BPDE−DNA adducts positioned at this site.

Benzo[*a*]pyrene, one of several carcinogens present in cigarette smoke (*1*−*9*), has been implicated as a causative agent in lung cancer. Benzo[*a*]pyrene is a prototype of the ubiquitous class of environmental mutagens designated polycyclic aromatic hydrocarbons (PAHs), which are metabolized by cellular monooxygenases to form highly reactive epoxides and diol epoxides (*1*−*3*). Its biologically active metabolite, 7*β*,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(±)-*anti*-BPDE],[1] reacts covalently with the guanine residues of DNA to form covalent N^2-deoxyguanine-DNA adducts (*4*, *5*), adopting either trans or cis stereoisomeric configurations. The major BPDE−DNA adduct detected in mammalian cells is the trans adduct (*8*).

BPDE−DNA adducts generally are removed by nucleotide excision repair (NER), but if the lesion persists, translesion DNA synthesis catalyzed by one of several DNA polymerases (*6*, *7*) may result in a mutation(s). DNA adducts derived from BPDE are found in lung tissue of smokers (*9*). Approximately 60% of human lung cancers contain mutations in the *P53* tumor suppressor gene (*10*), distributed in a nonrandom fashion and characterized by mutational hot spots located at CpG sequences in codons 157, 248, and 273.

The dominant mutations found at the CpG sequence are G → T transversions (*10*, *11*), and are believed to be initiated by BPDE and/or other PAHs (*12*). Codon 273, located on exon 8, is one of the most frequently mutated codons, and contains a CpG sequence in which cytosine is preferentially methylated. The CpG sequence also is a mutational hot spot (*13*) found in certain human genetic diseases (*14*, *15*). To date, the mutagenic potential of a single (±)-*trans*-dG-N^2-BPDE (the structures shown in Figure 1) positioned at codon 273 of *P53* has not been investigated, and the effect of a flanking 5-methylcytosine (C^m) residue on the mutagenic properties of this adduct is not known.

Johnson et al. have recently developed a new and efficient method to synthesize benzo[*a*]pyrene adducted to 2′-deoxyguanosine (dG) at the N^2-position and to incorporate its phosphoramidite derivative into oligomeric DNA (*16*). In the present study, we used a single-strand shuttle vector to determine the specificity and frequency of mutations induced by (+)- or (−)-*trans*-dG-N^2-BPDE adducts positioned at the mutational hot spot codon 273 of the *P53* gene in mammalian cells. Oligonucleotides, modified site-specifically with (+)- or (−)-*trans*-dG-N^2-BPDE, were inserted into plasmid vectors and then transfected into simian kidney (COS-7) cells; progeny plasmids were analyzed for mutations. Oligodeoxynucleotides containing 5-methylcytosine at the CpG sequence of codon 273 were used to explore the contribution of a flanking C^m to the mutagenic potential and the specificity

† This research was supported by Grant ES04068 from the National Institutes of Environmental Health Sciences.

* To whom correspondence and requests for reprints should be addressed. Phone: 631-444-8018. Fax: 631-444-3218. E-mail: shinya@pharm.sunysb.edu.

[1] Abbreviations: BP, benzo[*a*]pyrene; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (+)-*anti*-BPDE, (+)-7*β*, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (−)-*anti*-BPDE, (−)-7*β*, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; dG, 2′-deoxyguanosine; G^+, (+)-*trans*-dG-N^2-BPDE; G^-, (−)-*trans*-dG-N^2-BPDE; C^m, 5-methylcytosine; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; ss DNA, single-stranded DNA.

10.1021/bi0482194 CCC: $27.50
Published on Web 11/20/2004

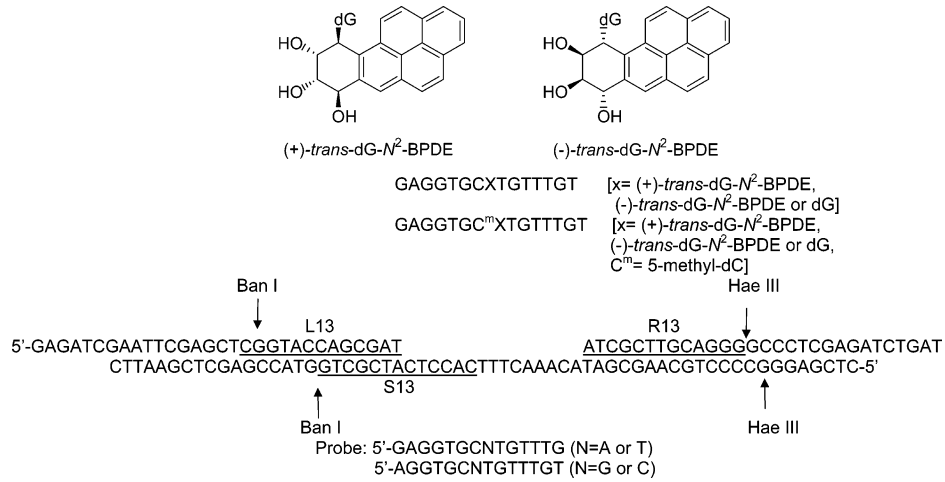

FIGURE 1: Ligation of unmodified and modified 15-mer to *Eco*RV-digested ss pMS2 vector and probes used for analysis. The upper strand is part of the ss pMS2 sequence. The inserted 15-mer contains codon 271−275 sequence for the human *P53* gene (5′-GAGGTGCGTGTTTGT; the underlined sequence represents codon 273). X represents dG and (±)-*trans*-BPDE-N^2-dG, while m represents 5-methylation of dC. Restriction enzyme *Ban*I and *Hae*III produce a 40-mer fragment if a 15-mer oligomer is 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. Probes listed below were used to detect mutations in progeny plasmids by differential oligodeoxynucleotide hybridization at each guanine site.

of BPDE-derived DNA adducts. We report here that (±)-*trans*-dG-N^2-BPDE has significant mutagenic potential in mammalian cells, generating primarily G → T transversions and some G → A transitions. Methylation at the 5′ flanking cytosine significantly reduces the mutational frequency for both enantiomers.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. (Piscataway, NJ). Lipofectamine, *E. coli* DH10B, and M13KO7 helper phage were purchased from Invitrogen (Carlsbad, CA). Simian kidney cells line (COS-7) was obtained from the tissue culture facility of SUNY at Stony Brook. T4 polynucleotide kinase, T4 DNA ligase, restriction endonucleases (*Eco*RI, *Ban*I, *Hae*III, and *Sal*I), T4 polymerase, and exonuclease III were purchased from New England Biolab (Beverly, MA). A phosphoramidite derivative of C^m was purchased from Glen Research (Sterling, VA).

Preparation of Oligodeoxynucleotides. The 59-mer scaffold and 15-mer unmodified oligodeoxynucleotides (5′GAGGTGCGTGTTTGT, codon 271−275), primers, and probes were prepared by solid-state methods using an automated DNA synthesizer (Applied Biosystems model 392) (*17*). Oligodeoxynucleotides containing (+)-*trans*-dG-N^2-BPDE, (−)-*trans*-dG-N^2-BPDE, and/or C^m were prepared by phosphoramidite chemical synthesis and purified according to procedures previously published (*16*). Modified and unmodified oligodeoxynucleotides were purified on a Waters reverse-phase μBondapak C_{18} column (0.39 × 30 cm, Waters), using a linear gradient of 0.05 mM triethylammonium acetate (pH 7.0) containing 5−20% acetonitrile with an elution time of 60 min, at a flow rate of 1.0 mL/min. These oligodeoxynucleotides were purified further by electrophoresis on 20% denaturing polyacrylamide gels. Bands were cut out and the DNA extracted by soaking with distilled water for at least 16 h. Extracts were concentrated using a Centricon-3 molecular filter (Amicon, Beverly, MA) and precipitated by ethanol to remove urea. The molecular weight of the modified oligomers was determined using a Voyager-DE STR matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer system. A Waters 900 HPLC instrument equipped with a photodiode array detector was used for purification of oligodeoxynucleotides. The amounts of purified oligomers were determined by an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Piscataway, NJ).

Construction of a Single-Strand Plasmid Containing (+)-trans-dG-N^2-BPDE, (−)-trans-dG-N^2-BPDE, and/or C^m Adducts. The procedure (Figure 1) followed an earlier protocol (*18*). Briefly, single-stranded (ss) pMS2 was annealed with a 61-mer scaffold at 10 °C overnight and digested with *Eco*RV to yield gapped ss DNA. The annealing mixture was washed with distilled water in a Centricon-100 molecular filter (Amicon) to remove small digestion products. An unmodified or modified 15-mer was phosphorylated at the 5′ end, hybridized to the gap, and then ligated into the vector at 10 °C for 16 h. Ligation mixtures were purified using a Qiagen PCR cleanup kit (Qiagen, Valencia, CA). A portion of the ligation mixture was used to confirm insertion of the 15-mer into the ss vector; the remaining mixture was digested with exonuclease III and T4 DNA polymerase to remove the scaffold and to cleave residual vector DNA and then extracted with phenol/chloroform. The ss vector constructs were precipitated with 70% ethanol and dissolved in 0.1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

To verify correct ligation of the 15-mer insert, the ligation mixture was digested with *Ban*I and *Hae*III (Figure 1). The phosphate group at the 5′-end of the DNA fragments was replaced with γ-^{32}P using an exchange reaction (*18*). Following ethanol precipitation, ^{32}P-labeled DNA fragments were separated on a 12% denaturing polyacrylamide gel. A 40-mer band is observed if the 15-mer oligodeoxynucleotide is ligated correctly (Figure 1).

Quantification of 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 DNAs were transferred to a nylon membrane and hybridized to a ^{32}P-labeled, S13 probe complementary only to DNA containing the 15-mer insert (Figure 1). The absolute amount of ss DNA was determined by comparing the radioactivity in the sample with that contained in known amounts of ss DNA.

Site-Specific Mutagenesis Studies in Simian Kidney Cells. Mutagenesis studies in COS-7 cells followed a previously used protocol with minor modifications (*19*). In brief, 3×10^5 cells were seeded on a 6 cm tissue culture plate and cultured in CO_2 incubator at 37 °C for 18 h. Using lipofectamine reagents, cells were transfected with 100 fmol of constructed vector for 24 h. Following transfection, cells were cultured for 48 h in DMEM medium containing 10% fetal calf serum. Progeny phagemids were recovered according to the method of Hirt (*20*), treated with nuclease S1/*Eco*RV to remove contaminating input ss DNA, and then used to transform competent *E. coli* DH10B cells by electroporation. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization, as described previously (*19*, *21*). 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. Transformants that failed to hybridize with L13 and R13 were omitted from the analysis. When L13/R13-positive transformants failed to hybridize to probes designed to detect events targeted to the lesion site, double-stranded (ds) DNA was prepared and subjected to dideoxynucleotide sequencing analysis (*22*). Mutational frequency is reported as the ratio of mutated colonies to total colonies analyzed.

RESULTS

Construction of Single Strand Plasmid DNA Containing a (+)-trans-dG-N^2-BPDE or (−)-trans-dG-N^2-BPDE Adduct with or without a 5′ Flanking C^m Residue at Codon 273 of P53 Gene. 15-mer oligodeoxynucleotodes containing dG-*N*^{2}-BPDE and/or C^m adduct were prepared using phosphoramidite chemical synthesis and purified using HPLC and gel electrophoresis. The modified oligomers were ligated into ss vectors, as shown in Figure 1. An unmodified 15-mer was used as a control. When the ligation mixture was cleaved with *Ban*I and *Hae*III and labeled with ^{32}P, a 40-mer product, indicated by an arrow, was detected by 12% denaturing PAGE (*23*), indicating that the unmodified and BPDE-modifed oligomers had been inserted correctly into the ss vector (Figure 2). Oligomers containing BPDE adducts migrated more slowly. No significant differences in ligation efficiency were observed among unmodified, C^m-, and dG-*N*^{2}-BPDE-modified oligodeoxynucleotides (data not shown). The final concentration of ss DNA vector was established by southern blot hybridization.

The S13 probe was hybridized to the ligation site of the ss vector (Figure 1). Using a β-phosphorimager, the amount of cc DNA in each construct was determined by comparing the product with pMS2 DNA standards. Concentrations of unmodified and modified ss vectors were 2.11 ng/μL (5′CGT), 7.15 ng/μL (5′C^{m}GT), 10.31 ng/μL (5′CG$^+$T), 10.04 ng/μL (5′CG$^-$T), 5.55 ng/μL (5′C^{m}G$^+$T), and 5.57 ng/μL (5′C^{m}G$^-$T), respectively. BPDE-modified pMS2 was used to transfect COS-7 cells; the number of transformants recovered were compared to those obtained from the unmodified control

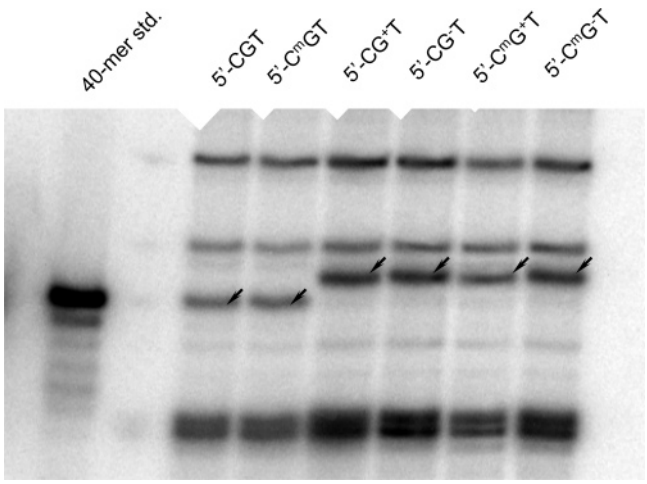

FIGURE 2: Confirmation of insertion of BPDE into the ss vector. A portion of the vector annealed to the 61-mer scaffold was digested with *Ban*I and *Hae*III followed by exchange of the terminal phosphate residue using [γ-^{32}P]ATP and T4 polynucleotide kinase and subjected to 12% denaturing polyacrylamide gel electrophoresis as described under Experimental Procedures. G$^+$, (+)-*trans*-dG-*N*^{2}-BPDE; G$^-$, (−)-*trans*-dG-*N*^{2}-BPDE; C^m, 5-methylcytosine.

Table 1: Transformation Efficiency of *E. coli* DH10B with a COS-7 Cell-Derived Progeny Phagemid

ss DNA[a]	no. of transformants	transform efficiency (%)
5-CGT-3′	1.59×10^5	100
5-CG$^+$T-3′	3.80×10^4	23.9
5-CG$^-$T-3′	4.00×10^4	25.2
5-C^{m}GT-3′	1.19×10^5	74.8
5-C^{m}G$^+$T-3′	3.24×10^4	20.3
5-C^{m}G-T-3′	3.65×10^4	22.9

[a] Single-stranded DNA (100 fmol) was transfected into COS-7 cells. The progeny phagemid was used to transform *E. coli* DH10B for ampicillin resistance. G$^+$, (+)-*trans*-dG-*N*^{2}-BPDE; G$^-$, (−)-*trans*-dG-*N*^{2}-BPDE; C^m, 5-methylcytosine.

(Table 1). The presence of a (+)-*trans*-dG-*N*^{2}-BPDE or (−)-*trans*-dG-*N*^{2}-BPDE residue reduced transformation efficiency by more than 70%; this value was affected by the configuration of the adduct.

Mutagenicity of dG-N^2-BPDE Adducts in COS-7 Cells. Using COS-7 cells, the mutagenic properties of BPDE-*N*^{2}-dG adducts positioned in codon 273 of *P53* gene were analyzed (Table 2). Mutational frequencies of (+)-*trans*-dG-*N*^{2}-BPDE (26.5%) was higher than that of (−)-*trans*-dG-*N*^{2}-BPDE (17.5%) ($p = 0.09$, Student's *t*-test). G → T transversions (15.5% and 10.9%) predominated, accompanied by lesser amounts of G → A transitions (10.0% and 4.3%, respectively). Both enantiomers produced small numbers of G → C transversions (1.0% and 2.3%, respectively).

Similar mutational specificities were observed when dC 5′ to dG-*N*^{2}-BPDE adducts was methylated. In this case, G → T transversions also dominated the mutational spectrum [6.1% for (+)-*trans*-dG-*N*^{2}-BPDE and 9.1% for (−)-*trans*-dG-*N*^{2}-BPDE]. G → A transitions (5.0%) were associated with (+)-*trans*-dG-*N*^{2}-BPDE. For the (−)-*trans*-enantiomer, the frequency of G →A transitions decreased dramatically to 0.9%. Deletions were not observed with the modified sequences used in this study, and methylation alone was not associated with mutational events (Table 2). The presence of C^m 5′ to the adduct resulted in a significant decrease in

Table 2: Targeted Mutational Specificity of Hot Spot Sequence of GAGGTG CG^{BPDE}TGTTTGT (underlined codon 273) for Human *P53* Gene in COS-7 Cells[a]

plasmid	expt no.[b]	no. of analyzed colonies	targeted mutations (dG or dG^{BPDE} to dX)				
			G	A	T	C	others
5′-CGT-3′	1[b]	95	95	0	0	0	0
	2	128	128	0	0	0	0
	3	140	140	0	0	0	0
	total	363	363 (100)	0	0	0	0
5′-CG^{+}T-3′	1	106	67	18	21	0	0
	2	97	76	7	14	0	0
	3	107	85	6	13	3	0
	total	310	228 (73.5)	31 (10.0)	48 (15.5)	3 (1.0)	0
5′-CG^{-}T-3′	1	94	76	6	11	1	1
	2	103	84	3	13	3	1
	3	105	89	4	9	3	0
	total	302	249 (82.5)	13 (4.3)	33 (10.9)	7 (2.3)	2[d]
5′-C^{m}GT-3′	1	131	131	0	0	0	1[c]
	2	125	125	0	0	0	0
	3	130	130	0	0	0	0
	total	386	386 (100)	0 (0)	0 (0)	0 (0)	1
5′-C^{m}G^{+}T-3′	1	87	74	6	7	0	3
	2	89	78	6	5	0	0
	3	85	80	1	4	0	0
	total	261	232 (88.9)	13 (5.0)	16 (6.1)	0 (0)	3[e]
5′-C^{m}G^{-}T-3′	1	103	93	2	7	1	0
	2	97	82	1	13	1	0
	3	119	110	0	9	0	0
	total	319	285 (89.4)	3 (0.9)	29 (9.1)	2 (0.6)	0[e]

[a] G^{+}, (+)-*trans*-dG-N^2-BPDE; G^{-}, (−)-*trans*-dG-N^2-BPDE; C^{m}, 5-methylcytosine. Numbers in parentheses are percents. Adducted ss DNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was used to transform *E. coli* DH10B for analysis of mutation. [b] Experiments 1−3 represent independent experiments. [c] Nontargeted mutation associated with a targeted event: 5′-GAGGTG(G)CGTGTTTGT-3′ one G insertion. [d] Nontargeted mutation associated with a targeted event: 5′-GAGGT(G)CGTGTTTGT-3′ one G deletion and 5′-GAGGTG(T)CGTGTTTGT-3′ one G → T. [e] Nontargeted mutation associated with a targeted event: 5′-GAG(A)TGCGTGTTTGT-3′ two G → A and 5′-GAGGTG(T)CGTGTTTGT-3′ one G → T.

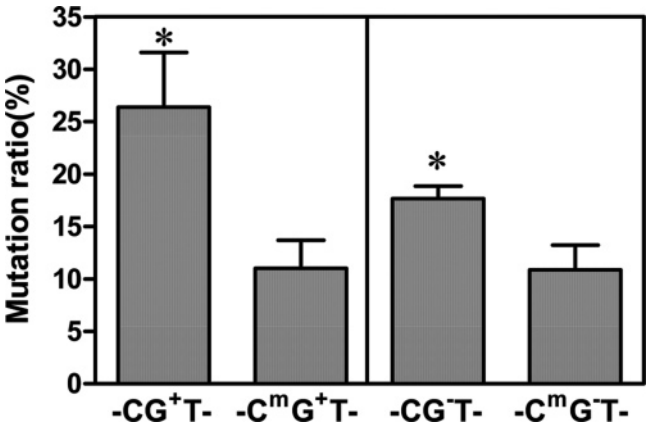

FIGURE 3: Effect of 5-methylation of dC on mutational frequencies of (+)-*trans*-dG-N^2-BPDE and (+)-*trans*-dG-N^2-BPDE. Data were taken from Tables 1 and 2. G^{+}, (+)-*trans*-dG-N^2-BPDE; G^{-}, (−)-*trans*-dG-N^2-BPDE; C^{m}, 5-methylcytosine. *, Student's *t*-test, $n = 3$, $p = 0.03$.

mutational frequencies (by 16.4% for (+)-trans- dG-N^2-BPDE and by 7.0% for (−)-*trans*-dG-N^2-BPDE) (Table 2 and Figure 3). The Student's *t*-test analysis showed a *p* value < 0.03. No mutations were observed at the C and C^{m} flanking the dG-N^2-BPDE adducts.

DISCUSSION

Mutations in the *P53* gene are associated with a number of human cancers (*24*−*28*). In lung cancer, *P53* mutations have been related to smoke exposure (*10*) and occur frequently at preferred sites of modification by polycyclic hydrocarbons, including benzo[*a*]pyrene. Codon 273 is one such target site in this gene (*29*).

In the current study, we used ss plasmid vector to establish the mutagenic specificity of the two major BPDE−DNA adducts, (+)- and (−)-*trans*-dG-N^2-BPDE, in COS-7 cells. A ss vector is converted to its ds form in COS-7 cells (*30*, *31*). Levine et al. has shown, using human cells and a different DNA adduct, that the mutagenic event observed with ss vector was similar to that for ds vector (*32*). Therefore, the ss vector can be used to study mutagenic events in mammalian cells. When (+)- and (−)-*trans*-dG-N^2-BPDE adducts were positioned at the second base of codon 273, targeted G → T transversions were detected, with somewhat fewer G → A transitions (Table 2). These results are similar to those recorded in the *P53* mutation database where, in codon 273, a disproportionate number of G:C → T:A mutations are found in lung cancer tissues of smokers (*33*). The (+)-*trans*-dG-N^2-BPDE adducts were more mutagenic (26.5%) than were the (−)-*trans*-enantiomers (17.5%), consistent with reports that the configuration of BPDE adducts plays a role in mutagenesis (*34*, *35*).

Moriya et al. (*35*) investigated the mutagenic potential of (+)- and (−)-*trans*-dG-N^2-BPDE at codons 60 and 61 in the human c-Ha-*ras1* protooncogene (*35*). They reported that the stereochemistry of the *trans*-*anti*-dG-N^2-BPDE adducts and the nucleotide sequence context in which these lesions are positioned strongly influence translesional synthesis, thereby altering the relative frequency (13−45%) of bases incorporated opposite the adduct; G → T transversions predominated in this study. We observed a 26.5% mutation

frequency at codon 273 of *P53*, which contains a CpG context. As the same experimental system was used for both studies, it is clear that sequence context can dramatically influence mutational frequency. However, the mutational specificity of the BPDE adducts in both studies was similar, suggesting that adduct structure, including stereochemistry, is the dominant factor in determining mutational spectra.

Primer-extension studies catalyzed by different human DNA polymerases (pols) have been used to explore mechanisms of BPDE bypass during translesional synthesis. In reactions catalyzed by pol κ, the correct base, dCMP, generally was incorporated opposite (+)-*trans*- and the (−)-*trans*-dG-N^2-BPDE adducts. Small amounts of dTMP and even smaller amounts of dAMP were misincorporated opposite these lesions (*5*, *36*). Wang et al. reported that pol η preferentially incorporates dAMP opposite the (+)- and (−)-*trans*-dG-N^2-BPDE DNA adducts and, additionally, frequently misinserts dGMP and dTMP (*37*). Pol μ produced frameshift deletions predominantly, thereby bypassing (+)- and (−)-*trans*-dG-N^2-BPDE (*38*). Pol ι is error-prone and preferentially incorporates dGMP opposite the (+)-*trans*-adduct, whereas both dAMP and dGMP are incorporated only weakly opposite (−)-*trans*-dG-N^2-BPDE (*36*). Therefore, fidelity of insertion is dependent on adduct stereochemistry and the polymerase(s) involved in translesion synthesis. It is likely that several DNA polymerases work together to catalyze translesional synthesis past BPDE−DNA adducts in cells, as proposed for other DNA adducts (*39*).

In the *P53* gene, cytosine, especially at CpG sites, is frequently methylated, and a large number of mutations are located at codon 273 in exon 8 (*11*, *15*). Several carcinogens, including benzo[*a*]pyrene diol epoxide, benzo[*g*]chrysene diol epoxide, aflatoxin B1 8,9-epoxide, and *N*-acetoxy-2-acetylaminofluorene, preferentially bind to dG at methylated CpG sequences in mutational hot spots. Thus, mutational hot spots at methylated CpG sequences may be a consequence of preferential carcinogen binding at these sites (*40*−*43*). To determine the effects of methylation of flanking cytosine on mutagenic frequency and specificity associated with (+)-trans- and (−)-*trans*-dG-N^2-BPDE, we incorporated C^m immediately 5′ to the modified guanine. A similiarly methylated oligomer with an unmodified dG was used as a control (Table 2). Unexpectedly, when the dG was modified by BPDE, methylation of the 5′-flanking cytosine resulted in a decrease of mutational frequency for both enantiomers (Figure 3), while mutational specificity was not significantly altered (Table 2). With both adducts, G → T transversions predominated over G → A transitions. The results of our methylation studies are inconsistent with certain other studies that used different DNA adducts. Tommasi et al. (*44*) reported that cyclobutane pyrimidine dimers (CPDs) containing 5-methylcytosine play an important role in formation of sunlight-induced skin tumors; methylation of CpG sequences appeared to create preferential targets for environmental mutagens and carcinogens (*44*, *45*). In contrast, our experiments support the report of Tornaletti et al. (*15*), who concluded that tissue-specific methylation does not contribute to mutation patterns observed in tumors. In that study, the occurrence of mutational hot spots in specific CpG sequences was not related to selective methylation at these sites (*15*). Hu et al. (*46*) found that, following methylation of cytosine, a CpG site in codon 282 was a strong binding site for *trans*-4-hydroxy-2-nonenal, while CpG sites in codons 175, 170, 245, and 273 were not (*46*). Thus, binding of a carcinogen to the C^mpG sequence appears to depend primarily on its intrinsic structure and stereochemistry.

When C^m is deaminated to form thymine during the course of the experiments, C → T transition mutations are promoted (*47*). Such mutations could be removed by mismatch repair (*48*). In our study, two C → T and one C → A mutations were detected opposite the C^m in experiment 3 (but, not in experiments 1 and 2) in the sequence context 5′-C^mG^+T-3′ (data not shown). No mutations were detected in any experiments for other C^m-modified oligomers. Therefore, the three mutations observed were not significant, suggesting that the conversion from C^m to thymine is minimal.

In our study, mutational frequencies observed opposite the dG-N^2-BPDE at C^mpG^{BPDE} sites were lower than those for CpG^{BPDE} sites. A methylated site may be repaired more easily by nucleotide excision or mismatch repair. Recent studies by Geacintov et al. support this hypothesis (*49*, *50*). These investigators found that excision of the predominant (+)-*trans*-anti-BPDE-dG adduct situated in a mutational hot spot sequence (codon 273 of the *p53* gene) was stimulated by CpG methylation. Our ss vector experimental system minimizes the contribution of DNA repair (*18*). However, once the ss vector containing dG-N^2-BPDE and/or C^m is replicated in cells, the duplex molecules could be exposed to repair. Therefore, in vivo repair is not completely eliminated. Excision activity may increase when a single C^m residue flanks the adduct in the damaged strand.

Jost (*51*) reported that nuclear extracts of chicken embryos have demethylation activity of DNA and that demethylation of 5′-C^m occurs through nucleotide excision repair in hemimethylated DNA (i.e., methylated on one strand only). Hanawalt et al. also found that demethylation enhances removal of pyrimidine dimers from the overall genome and from specific DNA sequences in Chinese hamster ovary cells (*52*). Such demethylation mechanisms also may contribute to the lower mutational frequency observed at the C^mpG^{BPDE} sites.

In summary, (+)-*trans*-dG-N^2-BPDE and (−)-*trans*-dG-N^2-BPDE DNA adducts positioned at codon 273 of the *P53* gene are highly mutagenic in mammalian cells, generating G → T transversions and G → A transitions, accompanied by a small number of G → C transversions. When cytosine 5′ to the adduct was replaced by 5-methylcytosine, the mutational specificity remained the same, but the mutational frequency significantly decreased, suggesting that 5-methylation of cytosine reduces the mutagenicity of *trans*-anti-BPDE-DNA adducts in mammalian cells.

ACKNOWLEDGMENT

We thank Ms. Cecelia Torres for preparing the modified and unmodified oligodeoxynucleotides.

REFERENCES

1. Conney, A. H., Chang, R. L., Jerina, D. M., and Wei, S. J. (1994) Studies on the metabolism of benzo[*a*]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite, *Drug Metab. Rev. 26*, 125−163.
2. Graslund, A., and Jernstrom, B. (1989) DNA−carcinogen interaction: Covalent DNA-adducts of benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxides studied by biochemical and biophysical techniques, *Q. Rev. Biophys. 22*, 1−37.

3. Rubin, H. (2001) Synergistic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: A bio-historical perspective with updates, *Carcinogenesis 22*, 1903−1930.
4. Grollman, A. P., and Shibutani, S. (1994) Mutagenic specificity of chemical carcinogens as determined by studies with single DNA adducts. In *DNA adducts: carcinogens and mutagenic agents: chemistry, identification, and biological significance* (Hemminki, K., Dipple, A., Shuker, D., Kadlubar, F. F., Segerback, D., and Bartsch, H., Eds.) No. 125, pp 385−397, IARC Scientific Publications, Lyon, France.
5. Suzuki, N., Ohashi, E., Kolbanovskiy, A., Geacintov, N. E., Grollman, A. P., Ohmori, H., and Shibutani, S. (2002) Translesion synthesis by human DNA polymerase κ on a DNA template containing a single stereoisomer of dG-(+)- or dG-(−)-anti-N^2-BPDE (7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene), *Biochemistry 41*, 6100−6106.
6. Zou, Y., Ma, H., Minko, I. G., Shell, S. M., Yang, Z., Qu, Y., Xu, Y., Geacintov, N. E., and Lloyd, R. S. (2004) DNA Damage Recognition of Mutated Forms of UvrB Proteins in Nucleotide Excision Repair, *Biochemistry 43*, 4196−4205.
7. Braithwaite, E., Wu, X., and Wang, Z. (1998) Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: Involvement of two excision repair mechanisms in vitro, *Carcinogenesis 19*, 1239−1246.
8. Geacintov, N. E., Cosman, M., Hingerty, B. E., Amin, S., Broyde, S., and Patel, D. J. (1997) NMR solution structures of stereoisometric covalent polycyclic aromatic carcinogen-DNA adduct: Principles, patterns, and diversity, *Chem. Res. Toxicol. 10*, 111−146.
9. Gyorffy, E., Anna, L., Gyori, Z., Segesdi, J., Minarovits, J., Soltesz, I., Kostic, S., Csekeo, A., Poirier, M. C., and Schoket, B. (2004) DNA adducts in tumour, normal peripheral lung and bronchus, and peripheral blood lymphocytes from smoking and nonsmoking lung cancer patients: Correlations between tissues and detection by ^{32}P-postlabelling and immunoassay, *Carcinogenesis 25*, 1201−1209.
10. Toyooka, S., Tsuda, T., and Gazdar, A. F. (2003) The TP53 gene, tobacco exposure, and lung cancer, *Hum. Mutat. 21*, 229−239.
11. Pfeifer, G. P., Tang, M., and Denissenko, M. F. (2000) Mutation hotspots and DNA methylation, *Curr. Top. Microbiol. Immunol. 249*, 1−19.
12. Campling, B. G., and el Deiry, W. S. (2003) Clinical implications of p53 mutations in lung cancer, *Methods Mol. Med. 75*, 53−77.
13. Fix, D. F., and Glickman, B. W. (1986) Differential enhancement of spontaneous transition mutations in the lacI gene of an Ung-strain of *Escherichia coli*, *Mutat. Res. 175*, 41−45.
14. Lee, D. H., O'Connor, T. R., and Pfeifer, G. P. (2002) Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG → TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells, *Nucleic Acids Res. 30*, 3566−3573.
15. Tornaletti, S., and Pfeifer, G. P. (1995) Complete and tissue-independent methylation of CpG sites in the p53 gene: Implications for mutations in human cancers, *Oncogene 10*, 1493−1499.
16. Johnson, F., Bonala, R., Tawde, D., Torres, M. C., and Iden, C. R. (2002) Efficient synthesis of the benzo[*a*]pyrene metabolic adducts of 2′-deoxyguanosine and 2′-deoxyadenosine and their direct incorporation into DNA, *Chem. Res. Toxicol. 15*, 1489−1494.
17. Takeshita, M., Chang, C. N., Johnson, F., Will, S., and Grollman, A. P. (1987) Oligodeoxynucleotides containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/apyrimidinic endonucleases, *J. Biol. Chem. 262*, 10171−10179.
18. Moriya, M. (1993) Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-Oxoguanine in DNA induces targeted G. C→T. A transversions in simian kidney cells, *Proc. Natl. Acad. Sci. U.S.A. 90*, 1122−1126.
19. Tan, X., Suzuki, N., Grollman, A. P., and Shibutani, S. (2002) Mutagenic events in *Escherichia coli* and mammalian cells in response to acetylaminofluorene-derived DNA adducts positioned in the *Nar* I restriction enzyme site, *Biochemistry 41*, 14255−14262.
20. Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures, *J. Mol. Biol. 26*, 365−369.
21. Shibutani, S., Suzuki, N., Tan, X., Johnson, F., and Grollman, A. P. (2001) Influence of flanking sequence context on the mutagenicity of acetylaminofluorene-derived DNA adducts in mammalian cells, *Biochemistry 40*, 3717−3722.
22. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
23. Shibutani, S., Fernandes, A., Suzuki, N., Zhou, L., Johnson, F., and Grollman, A. P. (1999) Site-specific mutagenesis of the *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridin e DNA adduct in mammalian cells, *J. Biol. Chem. 274*, 27433−27438.
24. Berner, A., Geitvik, G., Karlsen, F., Fossa, S. D., Nesland, J. M., and Borresen, A. L. (1995) TP53 mutations in prostatic cancer. Analysis of pre- and posttreatment archival formalin-fixed tumour tissue, *J. Pathol. 176*, 299−308.
25. Cooper, M. J., Haluschak, J. J., Johnson, D., Schwartz, S., Morrison, L. J., Lippa, M., Hatzivassiliou, G., and Tan, J. (1994) p53 mutations in bladder carcinoma cell lines, *Oncol. Res. 6*, 569−579.
26. Fenoglio-Preiser, C. M., Wang, J., Stemmermann, G. N., and Noffsinger, A. (2003) TP53 and gastric carcinoma: A review, *Hum. Mutat. 21*, 258−270.
27. Giglia-Mari, G., and Sarasin, A. (2003) TP53 mutations in human skin cancers, *Hum. Mutat. 21*, 217−228.
28. Miller, C. W., Simon, K., Aslo, A., Kok, K., Yokota, J., Buys, C. H., Terada, M., and Koeffler, H. P. (1992) p53 mutations in human lung tumors, *Cancer Res. 52*, 1695−1698.
29. Krawczak, M., and Cooper, D. N. (1998) p53 mutations, benzo-[*a*]pyrene and lung cancer, *Mutagenesis 13*, 319−320.
30. Menck, C. F. M., Madzak, C., Renault, G., Margot, A., and Sarasin, A. (1989) SV40-based shuttle viruses, *Mutat. Res. 220*, 101−106.
31. Madzak, C., and Sarasin, A. (1991) Mutation spectrum following transfection of ultraviolet-irradiated single-stranded or double-stranded shuttle vector DNA into monkey cells, *J. Mol. Biol. 218*, 667−673.
32. Levine, R. L., Yang, I. Y., Hossain, M., Pandya, G. A., Grollman, A. P., and Moriya, M. (2000) Mutagenesis induced by a single 1,N^6-ethenodeoxyadenosine adduct in human cells, *Cancer Res. 60*, 4098−4104.
33. Pfeifer, G. P. (2000) p53 mutational spectra and the role of methylated CpG sequences, *Mutat. Res. 450*, 155−166.
34. Fernandes, A., Liu, T., Amin, S., Geacintov, N. E., Grollman, A. P., and Moriya, M. (1998) Mutagenic potential of stereoisomeric bay region (+)- and (−)-*cis*-anti-benzo[*a*]pyrene diol epoxide-N^2-2′-deoxyguanosine adducts in *Escherichia coli* and simian kidney cells, *Biochemistry 37*, 10164−10172.
35. Moriya, M., Spiegel, S., Fernandes, A., Amin, S., Liu, T., Geacintov, N., and Grollman, A. P. (1996) Fidelity of translesional synthesis past benzo[*a*]pyrene diol epoxide-2′-deoxyguanosine DNA adducts: Marked effects of host cell, sequence context, and chirality, *Biochemistry 35*, 16646−16651.
36. Rechkoblit, O., Zhang, Y., Guo, D., Wang, Z., Amin, S., Krzeminsky, J., Louneva, N., and Geacintov, N. E. (2002) trans-Lesion synthesis past bulky benzo[*a*]pyrene diol epoxide N2-dG and N6-dA lesions catalyzed by DNA bypass polymerases, *J. Biol. Chem. 277*, 30488−30494.
37. Zhang, Y., Wu, X., Guo, D., Rechkoblit, O., Geacintov, N. E., and Wang, Z. (2002) Two-step error-prone bypass of the (+)- and (−)-*trans*-anti-BPDE-N^2-dG adducts by human DNA polymerases η and κ, *Mutat. Res. 510*, 23−35.
38. Zhang, Y., Wu, X., Guo, D., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. (2002) Lesion bypass activities of human DNA polymerase mu, *J. Biol. Chem. 277*, 44582−44587.
39. Plosky, B. S., and Woodgate, R. (2004) Switching from high-fidelity replicases to low- fidelity lesion-bypass polymerases, *Curr. Opin. Genet. Dev. 14*, 113−119.
40. Chen, J. X., Zheng, Y., West, M., and Tang, M. S. (1998) Carcinogens preferentially bind at methylated CpG in the p53 mutational hot spots, *Cancer Res. 58*, 2070−2075.
41. Maenhaut-Michel, G., Janel-Bintz, R., and Fuchs, R. P. (1992) A umuDC-independent SOS pathway for frameshift mutagenesis, *Mol. Gen. Genet. 235*, 373−380.
42. Bintz, R., and Fuchs, R. P. (1990) Induction of −2 frameshift mutations within alternating GC sequences by carcinogens that bind to the C8 position of guanine residues: Development of a specific mutation assay, *Mol. Gen. Genet. 221*, 331−338.
43. Fuchs, R. P., Schwartz, N., and Daune, M. P. (1981) Hot spots of frameshift mutations induced by the ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene, *Nature 294*, 657−659.

44. Tommasi, S., Denissenko, M. F., and Pfeifer, G. P. (1997) Sunlight induces pyrimidine dimers preferentially at 5-methylcytosine bases, *Cancer Res. 57*, 4727−4730.
45. Yoon, J. H., Smith, L. E., Feng, Z., Tang, M., Lee, C. S., and Pfeifer, G. P. (2001) Methylated CpG dinucleotides are the preferential targets for G-to-T transversion mutations induced by benzo[*a*]pyrene diol epoxide in mammalian cells: Similarities with the p53 mutation spectrum in smoking-associated lung cancers, *Cancer Res. 61*, 7110−7117.
46. Hu, W., Feng, Z., Eveleigh, J., Iyer, G., Pan, J., Amin, S., Chung, F. L., and Tang, M. S. (2002) The major lipid peroxidation product, *trans*-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma, *Carcinogenesis 23*, 1781−1789.
47. Rideout, W. M., Coetzee, G. A., Olumi, A. F., and Jones, P. A. (1990) 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes, *Science 249*, 1288−1290.
48. Schofield, M. J., and Hsieh, P. (2003) DNA mismatch repair: Molecular mechanism and biological function, *Annu. Rev. Microbiol. 57*, 579−608.
49. Muheim, R., Buterin, T., Colgate, K. C., Kolbanovsij, A., Geacintov, N. E., and Naegeli, H. (2003) Modulation of human nucleotide excision repair by 5-methylcytosines, *Biochemistry 42*, 3247−3254.
50. Huang, X., Colgate, K. C., Kolbanovskiy, A., Amin, S., and Geacintov, N. E. (2002) Conformational changes of a benzo[*a*]-pyrene diol epoxide-N(2)-dG adduct induced by a 5′-flanking 5-methyl-substituted cytosine in a (Me)CG double-stranded oligonucleotide sequence context, *Chem. Res. Toxicol. 15*, 438−444.
51. Jost, J. P. (1993) Nuclear extracts of chicken embryos promote an active demethylation of DNA by excision repair of 5-methyldeoxycytidine, *Proc. Natl. Acad. Sci. U.S.A 90*, 4684−4688.
52. Ho, L., Bohr, V. A., and Hanawalt, P. C. (1989) Demethylation enhances removal of pyrimidine dimers from the overall genome and from specific DNA sequences in Chinese hamster ovary cells, *Mol. Cell Biol. 9*, 1594−1603.

BI0482194