A Single Site-specific trans-Open 7,8,9,10-
Tetrahydrobenzo[a]pyrene 7,8-Diol 9,10-Epoxide N²-Deoxyguanosine
Adduct Induces Mutations at Multiple Sites in DNA*§

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Site-specific mutagenicity of trans-opened adducts at the exocyclic N²-amino group of guanine by the (+)-(7R,8S,9S,10R)- and (−)-(7S,8R,9R,10S)-enantiomers of a benzo[a]pyrene 7,8-diol 9,10-epoxide (7-hydroxyl and epoxide oxygen are trans, BPDE-2) has been determined in Chinese hamster V79 cells and their repair-deficient counterpart, V-H1 cells. Four vectors containing single 10S-BPDE-dG or 10R-BPDE-dG adducts positioned at G₀ or G₁ in the analyzed 5'-ACTG₃G₃-AG sequence of the non-transcribed strand were separately transfected into the cells. Mutations at each of the seven nucleotides were analyzed by a novel primer extension assay using a mixture of one dNTP complementary to the mutated nucleotide and three other ddNTPs and were optimized to quantify levels of a mutation as low as 1%. Only G → T mutations were detected at the adducted sites; the 10S adduct derived from the highly carcinogenic (+)-diol epoxide was 40–50 and 75–140% more mutagenic than the 10R adduct in V79 and V-H1 cells, respectively. Importantly, the 10S adducts, but not the 10R adducts, induced separate non-targeted mutations at sites 5’ to the G₃ and G₀ lesions (G₀ → T and C → T, respectively) in both cell lines. Neither the T 5’ to G₀ nor sites 3’ to the lesions showed mutations. Non-targeted mutations may enhance overall mutagenicity of the 10S-BPDE-dG lesion and contribute to the much higher carcinogenicity and mutagenicity of (+)-BPDE-2 compared with its (−)-enantiomer. Our study reports a definitive demonstration of mutations distal to a site-specific polycyclic aromatic hydrocarbon adduct.

Mutagenic effects of benzo[a]pyrene, a carcinogenic environmental pollutant (1), are largely mediated through stereoisomers of its reactive diol epoxide (DE)† metabolite, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Early studies (2) suggested that BPDE was responsible for the major DNA adduct formed from benzo[a]pyrene. Two diastereomers, DE-1 and DE-2 (3) in which the benzylic 7-hydroxy group and epoxide oxygen are either cis or trans, respectively, are metabolically possible. Each of these exists as a pair of enantiomers (4). The predominantly formed (+)-(7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene ((+)-BPDE-2) (5) is the most mutagenic stereoisomer in cell cultures (6) and the strongest carcinogen in mice (7, 8). Its enantiomer, (−)-7S,8R-dihydroxy-9R,10S-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene ((−)-BPDE-2), has little or no carcinogenic activity (6). Treatment of cells with (+)-BPDE-2 or (−)-BPDE-2 causes DNA point mutations (mostly G → T transversions) and DNA frameshifts (9–11) that may lead to changes in expression or function of critical genes such as proto-oncogenes and tumor suppressors and eventually to carcinogenesis. (+)-BPDE-2 is ~5–6 times more active in forming DNA adducts than (−)-BPDE-2 (12). Although the (−)-BPDE-2 enantiomer gives substantial amounts of dA adducts (12, 13), both enantiomers react largely with the exocyclic N²-amino group of deoxyguanosine (dG) residues by trans opening of the epoxide ring at C-10 to produce 10S-BPDE-dG and 10R-BPDE-dG lesions (14) as shown in Fig. 1. Both of these lesions lie in the minor groove with the aromatic portion pointing toward the 5’- and 3’-ends of their respective adducted strands (15, 16).

Mutagenicity of BPDE adducts depends upon efficiency of cellular repair systems to remove the adducts from DNA (mainly nucleotide excision repair (17, 18)) and fidelity of DNA polymerases replicating residual adducted sites. Although the normal replicative DNA polymerases polⅢ and polⅣ are blocked by bulky adducts, members of newly discovered Y superfamily of bypass DNA polymerases such as polⅢ, polⅣ, and polⅤ (19) were found to replicate past sites of various DNA lesions, albeit with low fidelity and low processivity. The frequency of nucleotide misincorporation of polⅢ (20), polⅣ (21), and polⅤ (22) replicating an undamaged template in vitro is in the range of 10⁻³–10⁻⁴. Assuming that the enzymes replicate not only the site of a lesion but possibly also a short stretch of DNA around the lesion with such a low fidelity suggests that besides incorporation of mismatched nucleotides opposite the adducted site, the bypass DNA polymerases could also introduce secondary mutations in the region flanking the adducted site, especially if its dihydroxy group and epoxide oxygen are either cis or trans, respectively; 10S-BPDE-dG and 10R-BPDE-dG, trans-opened N²-dG adducts derived from the (+)-(7R,8S,9S,10R)- and (−)-(7S,8R,9R,10S)-enantiomers of BPDE-2, respectively; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; DTT, dithiothreitol.
DNA structure is perturbed by the presence of the adduct. Multiple mutations in a shuttle vector treated with (+)-BPDE, believed to be generated by an error-prone polymerase, have been observed (23) in random mutagenesis experiments. In the present study, we have examined the mutagenicity of 10S-BPDE-dG and 10R-BPDE-dG lesions both at adducted sites and at unmodified flanking sites. We constructed double-stranded plasmid vectors bearing DNA of the HPRT gene of Chinese hamster V79 cells containing a single adduct positioned at each of the two adjacent sites in the non-transcribed strand of the gene and transfected them separately into V79 cells and also into their nucleotide excision repair-deficient derivative, V-H1 cells (24). These cells are defective in the xeroderma pigmentosum complementation group D/ERCC2 gene encoding for an ATP-dependent DNA helicase (25), an essential subunit of transcription and nucleotide excision repair complex TFIIH (26). They are 9-fold more sensitive to cytotoxic effects of (+)-BPDE-2 than V79 cells and have ~50% lower capacity for removal of (+)-BPDE-2-induced adducts from DNA compared with V79 cells (27). We qualitatively and quantitatively evaluated mutagenic effects of both 10R and 10S adducts in a sequence of seven nucleotides by a novel quantitative minisequencing method, and we compared their differences with respect to 10R/10S stereochemistry, position of the adducts at two adjacent sites, and also cellular DNA repair status. The most striking observation was that, in addition to mutations at the adduct sites, significant numbers of mutations were induced 1 or 2 bases 5’ to these sites by 10S but not 10R adducts.

**MATERIALS AND METHODS**

**Oligonucleotides—** All non-adducted oligonucleotides were prepared at IDT (Corvalal, IA). The 19-mer oligonucleotide and primers for quantitative minisequencing (Fig. 2) were gel-purified. Adducted oligonucleotide 18-mers (5’-AAACTGTCGAAAGCGACAAATGCTGGACTGGATGGCTGCGGGATCCCTCCTCACACCGCT/ 3’-AGGCTTTGTATTTGGCTTTCCCAGTTTCAGTAATGACACAAATATACTATTTGTGTCATTAGTG) and 37-mers—primers (5’-TGCGGGATCCCTCCTCACACCGCT/3’-AGGCTTTGTATTTGGCTTTCCCCAGTTTCAGTAATGACACAAATATACTATTTGTGTCATTAGTG) are 9-fold more sensitive to cytotoxic effects of (+)-BPDE-2 from DNA compared with V79 cells (27). We qualitatively and quantitatively evaluated mutagenic effects of both 10R and 10S adducts from DNA compared with V79 cells (27). We qualitatively and quantitatively evaluated mutagenic effects of both 10R and 10S adducts in a sequence of seven nucleotides by a novel quantitative minisequencing method, and we compared their differences with respect to 10R/10S stereochemistry, position of the adducts at two adjacent sites, and also cellular DNA repair status. The most striking observation was that, in addition to mutations at the adduct sites, significant numbers of mutations were induced 1 or 2 bases 5’ to these sites by 10S but not 10R adducts.

**Preparation of Plasmid Vectors Containing Site-specific 10S- or 10R-BPDE Adducts in the HPRT cDNA—** The HPRT cDNA was prepared by RT-PCR from total mRNA of V79 cells using primer A and primer D as described before (32). Primer A and primer D create BamHI and EcoRI sites, respectively. Double-stranded PCR product was digested with BamHI and EcoRI to create a 564-bp fragment for use as a template for single-stranded DNA synthesis. The DNA was purified by ethanol precipitation, and 50 pmol of single-stranded DNA was used as a template for PCR amplification using primers A and D. The PCR product was gel-purified and used as a template for site-directed mutagenesis using the ThermoSequenase Cycle Sequencing Kit (USB). The reactions were performed according to the manufacturer’s instructions. The resulting PCR products were digested with BamHI and EcoRI and cloned into the pCR3 vector (5.1 kb, Invitrogen) containing the HPRT cDNA. The construct with the antisense orientation (pCR3/HPRT antisense) was further verified by dideoxy sequencing using Thermosequenase Cycle Sequencing Kit (USB).

**Preparation of Plasmid Vectors Containing Site-specific 10S- or 10R-BPDE Adducts in the HPRT cDNA—** Four plasmid vectors were constructed with the 10R-BPDE-dG adduct and the early eluting oligonucleotide (t_R 17.6 min) as containing the 10R adduct, on the basis of the CD spectrum (14, 30) of the known nucleoside adducts obtained upon enzymatic hydrolysis (12) of the late eluting adduct. The CD spectra of the 18-mer oligonucleotides themselves exhibited bands at 320–350 nm that were positive for the early eluting (10R) isomer and weakly negative for the late eluting (10S) isomer, consistent with previous observations (31) of other oligonucleotides containing trans-opened BPDE dG adducts of known absolute configuration as follows: 16-mer modified with the 10S or 10R adduct at the position G_5, 5’-AAACCTGGAAGCGACAAATGCTGGACTGGATGGCTGCGGGATCCCTCCTCACACCGCT/3’-AGGCTTTGTATTTGGCTTTCCCCAGTTTCAGTAATGACACAAATATACTATTTGTGTCATTAGTG; primer A, 5’-TGCGGGATCCCTCCTCACACCGCT/ 3’-AGGCTTTGTATTTGGCTTTCCCAGTTTCAGTAATGACACAAATATACTATTTGTGTCATTAGTG; primer B, 5’-CTCTTCTCCTGTCACAGCGG; primer C, 5’-AAATTTAACGACTCTACTATAGGG; primer D, 5’-GCAGATCCACTGGAATCTCTCCATCATC. Sequence of primers used for mutagenesis analysis is shown in Fig. 2.

**Scaffold-directed Extension of the Adducted 18-Mers to the Adducted 37-Mers—** The adducted oligonucleotides and the non-adducted control were extended on their 5’ termini with the 19-mer to the final 37-mers by scaffold-directed ligation. Adducted 18-mers or the corresponding non-adducted 18-mer (10 pmol), 5’-labeled with 32P, were incubated with the 19-mer and 5’-32P-labeled 59-mer scaffold in a molar ratio of 1:5. The reaction was incubated at 25 °C for 2 h at pH 7.5 and terminated by adding 15 µl of stop solution containing 90% formamide, 10 µM NaOH, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. Reaction products were separated on 10% PAGE and detected by autoradiography. The 37-mer bands were excised from the gel and briefly washed with water. The gel was repeatedly treated with water by thawing and cooling (65 °C for 5 min and 0 °C for 5 min) to elute the DNA, and the DNA was precipitated from the eluate with ethanol. The total yield of the ligation product was ~40% for the non-adducted oligonucleotide and ~25% for the adducted oligonucleotides.

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Multiple Mutations from a Single Benzo[α]pyrene DNA Adduct

Construction of plasmid vectors adducted with BPDE

The Chinese hamster V79 cells were obtained from the ATCC and propagated in minimum Eagle's medium containing 10% dialyzed and heat-inactivated fetal bovine serum in an incubator with a humidified atmosphere containing 5% CO2. Before transfection, the cells were seeded at a density 2 x 10⁶ per 60-mm dish. Following 24 h of incubation, the cells were transfected with 0.3 μg of one of the modified plasmids (105 or 10⁷ adduct at the G₀ or G₁ position) or the control plasmid using Effectene (Qiagen). The cells were incubated for 24 h, washed with phosphate-buffered saline, and supplied with fresh medium. After 48 h following the transfection, the cells were harvested by trypsinization and seeded in the same medium supplemented with 500 μg/ml G418 (Invitrogen) at a density 3 x 10⁶ per 60-mm dish to select permanently transfected cells. Ten dishes were prepared from each transfection. Cellular colonies developed after an 11-day incubation with G418 were harvested by trypsinization from each dish and collected by centrifugation. To ensure analysis of at least 100 independent cellular clones from each plasmid transfection, 10 plates containing at least 60 cellular colonies in a random mixture were harvested. Thus, each transfection experiment yielded 5 x 10 pooled samples, which were separately processed and analyzed.

The repair-efficient Chinese hamster V-H1 cells (kindly provided by Dr. M. Zdzienicka, Leiden University Medical Center, Leiden, The Netherlands) were grown and transfected in the same way with several changes. The cells were seeded at a density 2.6 x 10⁶ per a 60-mm dish, transfected with 0.5 μg of the plasmid, and seeded after the transfection at a density 1 x 10⁶ per 60-mm dish.

Preparation of DNA Samples for Mutation Analysis—Chromosomal DNA from harvested cells was isolated using High Pure PCR Template Preparation kit (Roche Molecular Biochemicals). DNA fragment (337 bp) encompassing the examined region was prepared by PCR amplification using primer B corresponding to region 437-457 of the HPRT gene (exon 6) (32) and primer C corresponding to the T7 promoter region of the plasmid construct; the endogenous cellular HPRT gene is not amplified using this set of primers. The reaction was carried out in a 50-μl mixture containing 200 ng of DNA, 200 nM primers, 200 μM dNTP, and 2.5 units/μl Taq DNA polymerase (0.025 unit/μl, Qiagen). Following 30 cycles (95°C for 30 s, 55°C for 10 s, and 72°C for 10 s) with the initial 3 min at 95°C and the final 7 min at 72°C, the residual dNTPs were removed by a 30-min alkaline phosphatase treatment (0.01 unit μl of the reaction). The product was purified using QiAquick PCR Purification kit (Qiagen). Concentration of the product was estimated based on absorbance at 260 nm, and the purity was checked on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Standard 337-bp fragments used as positive and negative controls for quantitative minisequencing were amplified from plasmids pCR3/HPRTcontrol containing all four nucleotides at each of the seven examined positions (Fig. 2). These plasmids were prepared by site-directed mutagenesis using a QuikChange Kit (Stratagene, La Jolla, CA).

Mutation Detection Assay Using Quantitative Minisequencing—The method is based on a single nucleotide primer extension assay (34, 35) modified to quantitate mutations in a known sequence context at levels lower than 10%. A primer annealed to the DNA template (337-bp fragments), one nucleotide before the analyzed site (Fig. 2), is extended with a mixture of one dNTP and three other ddNTPs. When annealed to a fragment carrying a specific analyzed nucleotide (mutation), the primer can be extended with the complementary dNTP before being terminated by ddNTP incorporation at non-mutated nucleotide positions, whereas elongation of the primer annealed to fragments containing the analyzed nucleotide is terminated immediately with ddNTP. The level of mutation is determined by calculating the ratio of differently extended primers following their separation on denaturing polyacrylamide gel and analysis with PhosphorImager (see Fig. 5). Primer extension was catalyzed by Thermosequenase DNA polymerase (0.4 units/μl, U. S. Biochemical Corp.) in a reaction mixture (10 μl) containing three ddNTPs (1 μM), one dNTP (10 μM) complementary to the analyzed nucleotide, 26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl₂, 50 mM ³²P-labeled primer, and 2.5 nM DNA template. Twenty-five cycles (95°C...
for 30 s, 50 °C for 10 s, and 72 °C for 10 s) with the initial 1 min at 95 °C were used. The reaction was stopped with 1 volume of 95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromphenol blue, and 0.05% xylene cyanol FF. Reaction products were separated on 15% PAGE in 1 x TBE buffer and visualized by autoradiography. To increase the throughput of the assay, up to four sets of samples were subsequently loaded into the same gel. For quantitative evaluation (Fig. 5), the signal from the gel was transferred onto a screen and scanned by Cyclone PhosphorImaging System (Packard Instrument Co.), and the amount of radioactivity associated with each spot was quantified using software provided with the system.

RESULTS

Construction of Site- and Stereospecifically Adducted Plasmid Vectors—BPDE-adducted plasmid vectors were constructed by the in vitro enzymatic extension of 37-mer oligonucleotides after their annealing to a single-stranded plasmid template as shown in Fig. 3 and described under “Materials and Methods.” The plasmid contains the HPRT gene inserted in the antisense orientation toward the plasmid promoter. The 37-mers were prepared from 18-mer oligonucleotides modified with 10S or 10R adducts at the G₀ or G₋₁ sites (see “Materials and Methods” and Fig. 4A). The sequence of the 18-mers corresponds to region 629–646 of the non-transcribed strand of the HPRT gene in Chinese hamster cells. The use of 37-mers instead of 18-mers in the reaction substantially increases the yield of the covalently closed circular reaction products (data not shown). After the reaction, covalently closed circular DNA was digested with restriction enzymes EcoRI and BamHI and re-ligated yielding a vector with the HPRT gene in the sense orientation and a single BPDE adduct in the non-transcribed strand of the gene (Fig. 3). Purity of the final products was assessed both the type and the level of mutations at the region as described under “Materials and Methods” and Fig. 5.
To validate accuracy and sensitivity of the assay model, binary mixtures of 337-bp fragments differing in a single nucleotide at each site of the region (pseudo-pools) were analyzed. These fragments were prepared by PCR amplification of 337-bp region from the HPRT cDNA manipulated in plasmid vectors by site-directed mutagenesis. The relative content of one fragment in the other was 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19, and 0%. Composition of these binary mixtures represented all potential single nucleotide mutations in the analyzed region (G0, C0, and T0). The ratio between a level of the fragment detected by the assay (f measured) and a predicted level of the fragment in the mixture (f predicted) represents a correction factor (k). The means ± S.D. of the correction factor were calculated at each analyzed point from at least three independent experiments and plotted. The relationship between k and the level of the analyzed fragment was found linear between 0.78–50%. Fig. 8 is included as Supplemental Material and shows the results from analyses of model binary fragment mixtures differing by a nucleotide in positions found mutated in the study (C0, G0, and G−1). The average k values...
(0.85–1.05) calculated for each mutation in the region from model binary mixtures (the rest of the results not shown) were used to re-calculate levels of mutations found in unknown samples. The results demonstrate accuracy and linearity of the assay in tested binary mixtures containing more than 1% of the analyzed fragment. Accuracy of the assay decreases sharply when levels of the analyzed fragment are lower than 1% and limits sensitivity of the assay to 0.3% (see Supplemental Material Fig. 8). Thus, mutagenic changes at levels higher than 1% can be qualitatively detected and at levels higher than 1% can be accurately quantified by the assay.

Mutagenicity of BPDE Adducts in Repair-proficient and Repair-deficient Cells—Mutagenicity of 10S-BPDE-dG and 10R-BPDE-dG lesions in the sequence 5′-A,G,C,T,G-3′ to 5′-C,G,A,T,G-3′ was examined at the adducted sites (G₀ and G₁) and also at their 5′- and 3′-flanking sites (from +3 to −3, Fig. 2 and also Fig. 6). G₀ corresponds to the hotspot (G-634) of the non-transcribed strand of the gene found in random mutagenesis studies of (+)-BPDE-2 in V79 cells (10, 32).

In repair-proficient V79 cells, the guanine adduct derived from (+)-BPDE-2, 10S-BPDE-dG, induced 2.5 ± 1.3 and 2.8 ± 1.1% of G → T mutations of the adducted nucleotides at the G₀ and G₁ sites, respectively. The guanine adduct derived from (-)-BPDE-2, 10R-BPDE-dG, induced 1.8 ± 1.0 and 1.9 ± 0.7% of G → T mutations of the adducted nucleotides at the G₀ and G₁ sites, respectively (Fig. 7A). Analysis of secondary mutations of nucleotides at sites flanking the lesion on the 5′-end revealed that the 10S adduct, but not the 10R adduct, induced C → T mutations (0.7 ± 0.4%) at the (+2) position when the G₀ site was adducted and G → T mutation (1.3 ± 0.5%) at the G₀ position when the G₁ site was adducted. Interestingly, adducts at the G₀ or G₁ site did not induce mutations of the T nucleotide located at the (+1) position. Also, no mutations were found in the 3′-vicinity of the adducted sites (up to 3 nucleotides) or further upstream in the 5′-direction (3 and 4 positions analyzed overall from the adducted G₀ and G₁ sites, respectively).

The effect of repair deficiency in the V-H1 cells on the observed frequency of mutations is relatively small (a factor of 2 or less) and is only significant for the constructs containing 10S adducts at G₀ (Fig. 7B). For example, in the VH-1 cells, the 10S adduct induced 4.6 ± 1.5 and 2.1 ± 0.6% of G → T mutations of the adducted nucleotides at G₀ and G₁ sites, as compared with 2.5 and 2.8%, respectively, in the V79 cells (see above). The 10R adduct induced 1.9 ± 0.8 and 1.2 ± 0.6% of G → T mutations at the adducted nucleotides when at the G₀ and G₁ sites, respectively. Analysis of secondary mutations of nucleotides at sites flanking the lesion on the 5′-end showed the same pattern of mutations found in V79 cells; the 10S adduct, but not the 10R adduct, induced C → T mutations (1.7 ± 0.6%) at the (+2) position when the G₀ site was adducted and G → T mutations (1.5 ± 0.7%) at the G₀ position when G₁ was the adducted site. As with the V79 cells, no other mutation type or other mutated nucleotide was found in the analyzed region. The formation of secondary mutations depends on the presence of the 10S adduct but probably not on the formation of primary mutations. If so, tandem mutations (two mutations on the same analyzed DNA fragment) would be detected. In the primer extension assay tandem T mutations would give rise to multiple A incorporations in the primers used. No such multiple extensions of the primer were observed. Although we cannot exclude the possibility of formation of tandem mutations in levels below the detection limit of the assay (0.3%), random formation of secondary mutations independent of the primary mutations is a more probable scenario. Large standard deviations of the means of the presented data originate in large differences in the individual data from each of the 10 samples containing pools of cellular colonies, not from irreproducibility of the assay. Repeated analyses of the same samples showed remarkable reproducibility (maximum scatter did not exceed 10% of the calculated values, data not shown).
Multiple Mutations from a Single Benzo[a]pyrene DNA Adduct

DISCUSSION

At the target sites (G₀ and G⁻1), the 10S adduct and the 10R adduct induced only G → T mutations. This mutation was found to be dominant in previous studies examining mutagenicity of site-specific adducts both in prokaryotic (31, 36, 37) and eukaryotic cells (37), although G → C and G → A mutations have also been observed in some sequences. The sequence context of our adducted G₀ site is identical to the TGG sequence examined in simian kidney cells (37). There was no significant difference between mutagenicity at the adducted site for the 10S-BPDE-dG lesion relative to the 10R-BPDE-dG lesion, and a preponderance of G → T mutations was observed, as in the present study. However, the level of mutation induced by both adducts was substantially higher than in our study (13 versus 1.8–2.5%), and low levels (<1%) of G → C and G → A mutations were also detected. These differences may stem mainly from differences in the experimental systems; the single-stranded vector system used by Page et al. (31) and Moriya et al. (37) eliminates the involvement of DNA repair, whereas the double-stranded vector system used in our study is sensitive to DNA repair (both in V79 and in V-H1 cells, which have ~50% remaining capacity to remove adducts derived from the (+)-7R,8S,9S,10R-enantiomer of BPDE from DNA compared with V79 cells (27)). Consequently, the results of our study show substantially lower mutagenicity of the examined adducts and reflect both DNA repair of the adducts and fidelity of their bypass.

Results from DNA repair-proficient (V79) and DNA repair-deficient (V-H1) cells show only minor differences. It is possible that the defect of V-H1 cells in the xeroderma pigmentosum complementation group D/ERCC2 gene (25) influences mainly transcriptional coupled repair (18) and causes a decreased ability of the cells to preferentially repair BPDE adducts from the transcribed strand of active genes (27), whereas the efficiency of global genomic repair (18), which is responsible for the repair of the non-transcribed strand (location of the adducts in this study), may be affected only marginally. Both lesions in both adducted G sites generated the same kind of mutation (G → T), and the 10S adduct induced identical secondary mutations in these two cell lines. Quantitatively, no significant difference in mutagenicity of the 10R adduct was observed in corresponding sites in V79 and V-H1 cells. However, mutagenicity of the 10S adduct was significantly higher at the G₀ site in the V-H1 cells than in the V79 cells (p < 0.006), although similar at the G⁻₁ site in both cell lines (p > 0.1). The data suggest that repair of the 10S adduct is more efficient than the 10R adduct in the non-transcribed strand but also that repair efficiency of the 10S adduct may differ from site to site. These results are in accordance with a study by Custer et al. (29) who demonstrated a remarkable resistance of the 10R adduct to DNA repair in vitro compared with 10S adduct using a whole cell extract. However, in a different sequence context, no difference in repair efficiency of these two adducts in vitro was observed (38).

Mutations remote from a specifically modified base in DNA were first described by Lambert et al. (39) for frameshifts induced by an acetylaminofluorene adduct. The present study reports a definitive demonstration of substitution mutations remote from the target site induced by a single, site-specific polycyclic aromatic hydrocarbon DE adduct. These non-targeted mutations were observed only when the adduct has the 10S configuration and were found within two nucleotides on the 5'-side of the adducted base, namely at G₂ when G₀ was modified and at G₇ when G⁻₁ was modified (for sequence see Fig. 2). Notably, the absence of non-targeted mutations with 10R adducts at either position provides strong internal evidence that these mutations with the 10S adducts do not result from any artifact of the oligonucleotide synthesis, since for each sequence the 10R and 10S adducted oligonucleotides were prepared together from the mixed 10R/10S diastereomers of the phosphoramidite (see "Materials and Methods"), and thus the two diastereomeric oligonucleotides underwent identical treatment prior to final chromatographic purification. The non-targeted mutations at both sites represented mutagenic changes of the original nucleotides to T, suggesting their bypass with a mismatched A. Furthermore, the T that is immediately 5' to the G₀ lesion or separated by one base from the G⁻₁ lesion was not found to be mutated in the presence of either lesion. An attractive explanation for these data is that translesion synthesis results in A being inserted (either correctly or incorrectly) as the preferential nucleotide at the adducted site and at the two additional 5'-flanking nucleotides. In vitro studies with isolated bypass DNA polymerases (pol ζ, η, 1, and θ) on templates containing a single 10S-BPDE-dG or 10R-BPDE-dG lesion identified pol θ as involved in low fidelity insertions at these lesions in vitro (40, 41). The enzyme incorporates mostly A and G opposite the lesion site (40, 41) and thus is likely to be responsible at least in part for mutations induced by BPDE-dG lesions in mammalian cells (mostly G → T with minor G → C and G → A). Although the enzyme extends the primer beyond the lesion site rather inefficiently, its preference to extend mispaired primers containing a purine opposite the adduct and its high misincorporation frequency on non-damaged templates (10⁻⁵–10⁻⁶) (41) are features that may contribute to the formation of non-targeted mutations and would be worthy of further exploration by studies in vitro and in cells lacking functional pol θ.

The 10S BPDE adducts at the G₀ and G⁻₁ sites induced identical non-targeted mutations in both repair-proficient (V79) and repair-deficient (V-H1) cell lines, and the frequency of the non-targeted mutations correlated with the frequency of targeted mutations. The increased level of the targeted mutation at the G₀ site (G → T) in V-H1 cells was accompanied by a similar increase in the level of the non-targeted mutations (C → T) in these cells compared with V79 cells, whereas no difference in the frequency of non-targeted mutation (G → T at the G₀ site) between V-H1 and V79 cells was observed when the levels of the targeted mutation (G → T at the G⁻₁ site) were similar in both cell lines. Assuming that the differences in the targeted mutation frequency between these two cell lines reflect only the efficiency of the 10S adduct removal from a particular site, it seems likely that the adduct level determines the frequency of not only targeted but also non-targeted mutations.

The present observation that mutations are induced 1 or more bases away from the target site by a polycyclic aromatic hydrocarbon DE lesion is consistent with results of our recent study (42) of mutations in an E. coli-M13 system induced by several cis-opened adducts derived from both BPDE-2 (the diastereomer used in the present study, whose benzylic hydroxyl group and epoxide oxygen are trans) and BPDE-1 (benzylic hydroxyl group and epoxide oxygen cis). More limited data in E. coli by Jelinsky et al. (36) using a single trans-opened 10S adduct of BPDE-2 had also led to the tentative suggestion of non-targeted mutations at a base immediately adjacent to this adduct. In our previous study (42) using the sequence -G₀C₃G₄G₅G₆G₇G₀ with cis-opened adducts at G₀, non-targeted substitution mutations 4 and 6 bases remote from the target site were described, but their significance was not fully recognized. No significant non-targeted substitutions had been detected in this DNA sequence containing trans-opened BPDE-1 or BPDE-2 dG adducts in the same experimental system (31). The most prevalent non-targeted mutations (fre-
quence 2–4%) induced by the cis-opened BPDE-1 and BPDE-2 dG adducts were T substitutions at G$_6$ and G$_4$, and they occurred both in the absence of mutations at the target site (G$_0$) and in combination with G$_0$→T mutations at this site. These non-targeted mutations were not observed with the control (non-adducted) sequence, and most significantly they were observed only when the adducts at G$_6$ had 10f concentration, in analogy to our present observations. Because no significant non-targeted mutations were observed in a different (-G$_6$C$_6$G$_7$T$_7$G$_8$C$_8$) sequence containing BPDE-1 or -2 adducts at G$_6$ (42), the above mutations were most likely related to the run of 5 guanines in the (-G$_6$C$_6$G$_7$G$_8$G$_9$C$_9$-sequence. Despite the specific sequence effect, as well as the differences in experimental systems, the similarity between these results in E. coli and the present observations in mammalian cells is intriguing and suggests that mutations remote from the lesion site induced by polyaromatic hydrocarbon DE adducts may constitute a not uncommon mechanism of polyaromatic hydrocarbon mutagenesis whose significance has not been appreciated previously.

A limited number of site-specific mutagenesis studies with other types of bulky DNA adducts has demonstrated induction of non-targeted substitution mutations at non-adducted sites in the vicinity of the lesion. They include studies of the N7-guanyl adduct of aflatoxin B$_1$, 8,9-epoxide and the adduct’s ring opened formamidopyrimidine form in SOS-induced E. coli (43, 44) as well as N-deoxyguanosin-8-yl)-2-acetylaminofluorene and N-deoxyguanosin-8-yl)-2-aminofluorene in COS-7 cells (45). Although it is difficult to make any generalization about the mechanism of these non-targeted mutations by comparing the data of these studies to those presented here (different experimental models, structure of the adducts, and sequence context), it is clear at this point that the formation of various non-targeted mutations is likely related to the bulky character of the adducts and that the orientation of the adducts may play a significant role. The hydrocarbon of the 10f BPDE-dG adduct orients toward the 5′-side of the modified base in the minor groove of duplex DNA in the sequence 5′-CCG (15) as well as 5′-TGG (46) and could thus cause structural perturbations 5′ to the lesion in our 5′-TGG and 5′-GGG, resulting in the observed non-targeted mutations 5′ to the adduct. In contrast, the 10f BPDE-dG adduct, which orients in the opposite direction toward the 3′-end of the modified strand in duplex DNA (16), was not found to cause any mutations at non-target bases on either side of the adduct. Interestingly, about 13% of the total mutations in SOS-induced E. coli caused by a dG adduct of aflatoxin B$_1$, 8,9-epoxide (43), which intercalate into the helix on the 5′-side of the modified G base (47, 48), were primarily C→T mutations at the dC immediately 5′ to the lesion (G) in a 5′-CQA- sequence. In contrast, a N-deoxyguanosin-8-yl)-2-acetylaminofluorene lesion, whose hydrocarbon moiety displaces the modified G and intercalates in its place opposite the complementary C (49), gave rise to base misincorporation on the 3′-side of the lesion site (50).

The dependence of non-targeted mutations on adduct configuration is of particular interest in light of the marked differences in mutagenicity and carcinogenicity between (+)- and (-)-BPDE-2 enantiomers. Higher tumorigenic activity of (+)-BPDE-2 compared with (-)-BPDE-2 has been demonstrated. (+)-BPDE-2 induced lung tumors and skin tumors when injected intraperitoneally into newborn mice and applied topically to the skin of adult mice, respectively; but (-)-BPDE had little or no carcinogenic activity (7, 8). Although the present mutagenesis study showed little difference in the mutagenicity of (+)- and (-)-BPDE-2 dG adducts at specific sites, (+)-BPDE-2 was 11 times more mutagenic than (-)-BPDE-2 in the HPRT gene of V79 cells on a per dose basis in a random mutagenesis study (11). Many factors can contribute to these different results, including more efficient adduct formation from (+)- to (-)-BPDE-2 (12), differences in relative proportions of dG and dA adducts formed from the two enantiomers (12, 13), as well as sequence effects on adduct formation, repair (51), and bypass (41). Furthermore, the higher mutagenicity of (+)-BPDE-2 adducts observed in random mutagenesis studies may result in part from their mutagenic effects on the whole region (i.e. non-targeted mutations in the vicinity of the adduct) rather than only at a particular adduct site. Because not all DNA mutations lead to amino acid substitutions, and some substitutions may be silent in terms of function, it is obvious that induction of a mutation at more than one site by a single adduct increases the probability of an amino acid change that could generate a protein with a compromised function. Consequently, the mutagenic and tumorigenic potential of a BPDE adduct leading to “multiposition” mutations would be significantly higher. In summary, results of this study contradict the intuitive notion that point mutations arise exclusively by erroneous replication of the modified base and suggest that the higher mutagenic and carcinogenic activity of (+)-BPDE-2 compared with (-)-BPDE-2 may partly stem from the capability of its major dG adduct to induce DNA mutations at multiple sites.
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A Single Site-specific trans-Opened 7,8,9,10-Tetrahydrobenzo[a]pyrene 7,8-Diol 9,10-Epoxide N²-Deoxyguanosine Adduct Induces Mutations at Multiple Sites in DNA

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