Sequence Context Profoundly Influences the Mutagenic Potency of Trans-Opened Benzo[*a*]pyrene 7,8-Diol 9,10-Epoxide−Purine Nucleoside Adducts in Site-Specific Mutation Studies†

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ABSTRACT: The postoligomerization method was used to prepare oligonucleotide 16-mers that contained dAdo or dGuo adducts, derived from trans opening of each enantiomer of the two diastereomeric benzo[*a*]pyrene 7,8-diol 9,10-epoxides, in two sequence contexts. These 16 oligonucleotides, along with the four corresponding oligonucleotides containing unsubstituted purines, were ligated into single-stranded DNA from bacteriophage M13mp7L2 and transfected into *Escherichia coli* SMH77. The mutagenic effects of replication past these adducts were then evaluated. The various adduct isomers induced point mutations at different frequencies and with different distributions of mutation types, as was anticipated. However, sequence context had the most substantial effects on mutation frequency. A high frequency of deletions of a single guanine was found in a context where the dGuo adduct was at the 3′-end of a run of five guanines, whereas no single base deletion was found in the other context studied, 5′-C<u>G</u>A-3′. Mutation frequencies in constructs containing dAdo adducts were much higher in a 5′-T<u>A</u>G-3′ context (37−58%, depending on the individual isomer) than in a 5′-G<u>A</u>T-3′ context (5−20%), and for a given adduct, mutation frequency was up to 10-fold higher in the former sequence than in the latter. These findings indicate that sequence context effects need more thorough evaluation if the goal of understanding the mechanism through which DNA adducts lead to mutation is to be achieved.

Following the demonstration that the transformed phenotype can be transferred to other cells by transferring only the transformed cell's DNA (*1*), it has become widely accepted that DNA is the key target of chemical carcinogens. Furthermore, extensive studies in many laboratories have narrowed the potential DNA targets to a series of genes that control cell growth and differentiation and have been termed protooncogenes and tumor suppressor genes (reviewed in ref *2*). Similar improvements in the definition of chemical carcinogens also have been made. For example, soot was recognized as a carcinogen in 1775 (*3*), but by the 1930s, it was clear that the polycyclic aromatic hydrocarbon components, such as benzo[*a*]pyrene, were the cancer-causing agents (*4*). Recognition of the important role played by metabolic activation in chemical carcinogenesis developed in the 1960s (*5*, *6*), and in the 1970s, it was shown that the activity of these carcinogenic hydrocarbon components is dependent on their conversion to chemically reactive diol epoxide metabolites (*7*). These reactive metabolites modify cellular DNA and primarily form purine deoxyribonucleoside adducts (reviewed in refs *8* and *9*). A question that is still open, however, concerns which of the various DNA adducts generated by a given chemical carcinogen are responsible for the mutations found in tumor cell DNA.

Methods for determining the mutagenic properties of individual carcinogen−DNA adducts were developed in the 1980s (reviewed in ref *10*). These involved the construction of oligonucleotides containing single adducts at specific sites, incorporation of these oligonucleotides into vectors, and evaluation of progeny resulting from the replication of these vectors in cellular systems. Related studies have involved the evaluation of products obtained from the replication of adducted templates with polymerases in vitro (reviewed in ref *11*). We have reported previously on the ability of various polymerases to replicate past benzo[*a*]pyrene 7,8-diol 9,10-epoxide (BPDE[1])−dAdo adducts in vitro (*12*). However, in these studies, different polymerases exhibited different properties, and it was not clear how well such systems would reflect the in vivo situation where the full replication machinery would be present. For this reason, we decided to shift our evaluation of the mutagenic potential of individual BPDE−deoxyribonucleoside adducts to a cellular system.

Several site-specific mutation studies with polycyclic hydrocarbon−deoxyribonucleoside adducts have been re-

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[1] Abbreviations: CPG, controlled pore glass; FdI, 2-fluorodeoxyinosine; DE, diol epoxide; BPDE, benzo[*a*]pyrene 7,8-diol 9,10-epoxide; CD, circular dichroism; THF, tetrahydrofuran; DMT, dimethoxytrityl.

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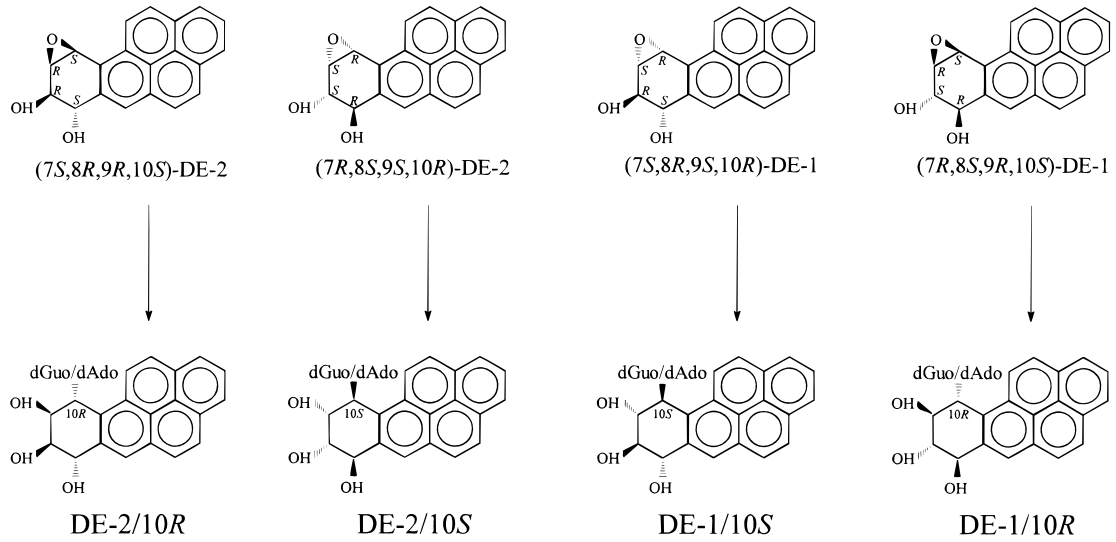

FIGURE 1: Structures of the four optically active isomers of BPDE and of the dGuo and dAdo adducts formed from each of these by trans opening of the epoxide ring by the amino group of the purine residues. Diol epoxides in which the epoxide oxygen and the benzylic 7-hydroxyl group are cis or trans are labeled DE-1 and DE-2, respectively (these are also frequently referred to as syn and anti diol epoxides, respectively, in the literature). The adducts are labeled with the diol epoxide from which they were derived (DE-1 or DE-2), and the configuration at C_{10} is appended to designate the diol epoxide enantiomer from which they are derived. Note that, in these trans adducts, the configuration at C_{10} in the adduct is always the inverse of that in the diol epoxide precursor.

ported (*13*−*20*). Of these, four studies from Loechler's laboratory have used a double-stranded vector in which the adduct is placed in one strand and the complementary strand has been UV-irradiated to prevent progeny formation from this strand when the vector subsequently replicates in an *Escherichia coli* host (*13*, *14*, *18*, *19*). These authors have pointed out that, because of various complexities with this system, including the possibility of repair of the adducted strand, mutation frequencies obtained are not necessarily good indicators of the intrinsic mutagenicity of a given adduct (*14*). Nevertheless, these studies have shown that the kinds of mutations obtained from the same BPDE−DNA adduct vary with the surrounding sequence context such that a trans dGuo adduct (DE-2/10*S* in Figure 1) preferentially gave G → T mutations (*13*) or G → A mutations (*19*) or gave G → T, G → A, and G → C mutations in a roughly 3:1:1 ratio (*14*). Other site-specific studies of hydrocarbon−deoxyribonucleoside adducts have used the single-stranded M13mp7L2 bacteriophage vector (or a derivative thereof) developed by Lawrence and his colleagues (*21*, *22*). With this system, dibenz[*a*,*j*]anthracene DE−dGuo and −dAdo adducts gave G → T and A → T mutations, respectively (*17*); BPDE−dGuo adducts gave G → T mutations predominantly (*16*, *20*), and BPDE−dAdo adducts gave only A → G mutations (*15*).

The single-stranded M13 vector has a disadvantage in that it is perhaps not an ideal model for chromosomal DNA, which is double-stranded, but it has an advantage in that attempts to repair the vector are presumably lethal; therefore, a reasonable measure of the intrinsic mutagenic potential of a given adduct should be accessible. For this reason, we have chosen to use this system to investigate the relative mutagenic potentials of the eight adducts (derived from trans opening of each optically active BPDE by the exocyclic amino groups of dGuo and dAdo) shown in Figure 1. Since each of these adducts was studied in two different sequence contexts, the modulating effect of sequence context on each adduct was also evaluated. G → T and A → T were the major, but not exclusive, base substitution mutations found. Additionally, in a sequence where a dGuo adduct was placed in a run of dGuo residues, deletion of a single dGuo residue occurred at a high frequency. Although adduct structure affected mutagenic activity (up to ∼4-fold), larger effects (up to ∼10-fold) could be attributed to the differences in sequence context chosen for this investigation.

EXPERIMENTAL PROCEDURES

Materials. *Eco*RI restriction enzyme, T4 DNA ligase, T4 polynucleotide kinase, and [γ-^{32}P]ATP were obtained from Amersham Corp. Uracil DNA glycosylase, 2× Prehybridization/Hybridization solution, and some unmodified oligonucleotides and 56-mer scaffold oligonucleotides were from Gibco/BRL. Most unmodified oligonucleotides were provided by M. Powers (SAIC, NCI-FCRDC, Frederick, MD). For DNA sequencing, ABI-PRISM Dye Terminator Cycle Sequencing Ready Reaction kits were obtained from Perkin-Elmer. Qiagen QIAprep M13 kits were used for purification of the M13 DNA. *E. coli* SMH77 [*F*′lacZ, ΔM15, *pro*$^+$, Δ(*pro-lac)*, *leu*$^+$], a derivative of AB1157, and bacteriophage M13mp7L2 were generous gifts from C. W. Lawrence (University of Rochester, Rochester, NY).

Oligonucleotides. In a previous study (*12*), we described the preparation and properties of oligonucleotides containing the adducts derived from trans opening of the enantiomers of each diastereomeric BPDE by the exocyclic 6-amino group of dAdo. Each of the four dAdo adducts was placed in either of two sequence contexts labeled context I and context II. These same oligonucleotides were used in this work and are referred to here as context I(A) and context II(A), respectively, to distinguish them from the oligonucleotides containing dGuo adducts.

Oligonucleotides containing BPDE−dGuo adducts were prepared by postoligomerization modification (*23*) from the CPG-bound 16-mers 5′-TTC(2-FdI)AATCCTTCCCCC-3′ [context III(FdI)] and 5′-GGG(2-FdI)TTCCCGAGCGGC-

3′ [context IV(FdI)], in which 2-FdI represents a 2-fluoro-2′-deoxyinosine residue.

Synthetic Intermediates. BPDE-1 and -2 were prepared as described (*24*). 2-Fluoro-2′-deoxyinosine was prepared in quantitative yield from its O^6-benzyl derivative (*25*) (144 mg, 0.4 mmol) by catalytic hydrogenation (room temperature, 1 atm) in methanol (20 mL) in the presence of 96 mg of Pd (5%)/C. ^{1}H NMR [300 MHz, $(CD_3)_2CO$]: δ 8.27 (s, 1 H_8), 6.32 (t, 1 $H_{1'}$, J = 6.8 Hz), 4.55 (m, 1 $H_{3'}$), 3.99 (m, 1 $H_{4'}$), 3.71 (m, 2 $H_{5'}$), 2.67 (dt, 1 $H_{2'}$, J = ∼7, 13.2 Hz), 2.39 (ddd, 1 $H_{2'}$, J = 2.9, 6.1, 13.2 Hz).

2-Fluoro-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyinosine. 2-FdI (160 mg, 0.59 mmol) was allowed to react with a 2-fold molar excess of dimethoxytrityl tetrafluoroborate (added in three portions over the course of 2.5 h) in the presence of Li_2CO_3 (88 mg, 1.2 mmol) and 2,6-lutidine (1.7 mL). Fifteen minutes after the final addition of the tetrafluoroborate, the reaction mixture was diluted with ethyl acetate. Following standard workup, chromatography of the crude reaction product on a silica column eluted with CH_2Cl_2/MeOH/Et_3N (90:10:1) gave 2-fluoro-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyinosine as a white solid (183 mg, 54%). ^{1}H NMR [300 MHz, $(CD_3)_2CO$]: δ 7.84 (s, 1 H_8), 7.46, 7.43 (2 s, 2 H, aromatic), 7.32−7.20 (7 H, aromatic), 6.84−6.79 (4 H, aromatic), 6.31 (t, 1 $H_{1'}$, J = 6.6 Hz), 4.65 (m, 1 $H_{3'}$), 4.09 (app q, 1 $H_{4'}$, J = 4.5 Hz), 3.76 (s, 6 H, 2 OCH_3), 3.32 (m, 2 $H_{5'}$), 2.83 (app quint, 1 $H_{2'}$, J = 6.6 Hz), 2.40 (ddd, 1 $H_{2'}$, J = 4.1, 6.2, 13.2 Hz).

2-Fluoro-5′-O-(4,4′-dimethoxytrityl)-3′-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphinyl]-2′-deoxyinosine (2-FdI Phosphoramidite). To a mixture of 2-fluoro-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyinosine (181 mg, 0.32 mmol) and EtN(*i*-Pr)$_2$ (0.28 mL, 1.6 mmol) in 5.5 mL of CH_2Cl_2 being stirred at 0−5 °C under argon was added 2-(cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (0.17 mL, 0.78 mmol) in two equal portions over the course of 45 min. TLC indicated the reaction was complete after an additional 95 min. The crude reaction mixture was chromatographed on a silica column eluted with CH_2Cl_2/EtOAc/MeOH/Et_3N (80:15:5:1). The desired 2-FdI phosphoramidite was obtained as a white solid (177 mg, 75%). ^{31}P NMR (121.4 MHz in CD_2Cl_2 with phosphoric acid as an external standard): δ 148.69.

(±)-7β,8α,9β-Trihydroxy-10α-amino-7,8,9,10-tetrahydrobenzo[a]pyrene. (±)-7β,8α-Dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-1) (100 mg, 0.33 mmol) was heated under pressure in liquid NH_3 at 45−50 °C for 5 h. After evaporation of NH_3, the solid product was washed with MeOH/Et_2O (104 mg, 98%). ^{1}H NMR (500 MHz, Me_2SO-d_6/CD_3OD): δ 8.58 (d, 1 H_{11}, $J_{11,12}$ = 9.5 Hz), 8.42 (s, 1 H_6), 8.28 (d, 1 H_{12}), 8.26 and 8.28 (br d, 1 H_1 and 1 H_3, $J_{1,2}$ = $J_{2,3}$ = 7.7 Hz), 8.12 and 8.16 (2d, 1 H_4 and 1 H_5, $J_{4,5}$ = 9.0 Hz), 8.04 (t, 1 H_2, $J_{1,2}$ = $J_{2,3}$ = 7.7 Hz), 4.91 (d, 1 H_7, $J_{7,8}$ = 7.0 Hz), 4.87 (d, 1 H_{10}, $J_{9,10}$ = 4.0 Hz), 3.99 (q, 1 H_9), 3.62 (t, 1 H_8, $J_{8,9}$ = 7.0 Hz).

Fluorinated Oligonucleotides. These were prepared as described (*12*). The appropriate support-bound 11-mers were prepared by automated synthesis using 163 mg (15.5 μmol) of derivatized CPG (170 Å) loaded with N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-3′-succinic acid (95 μmol/g). Each support-bound oligomer was removed from the column and treated manually with 2-FdI phosphoramidite (46 mg, 59 μmol; 3.8-fold molar excess) for 16 h at room temperature in the presence of 0.25 mL of 0.5 M 1*H*-tetrazole in acetonitrile. Following this manual coupling step, end capping [pyridine/4-(dimethylamino)pyridine/acetic anhydride in THF] was performed manually, the beads were returned to the column, and the three final residues were added by the automated procedure to yield the CPG-bound fluorinated oligonucleotides, contexts III(FdI) and IV(FdI) (234 mg at 39 μmol/g and 252 mg at 45 μmol/g, respectively).

Adducted Oligonucleotides. Postoligomerization modification was carried out essentially as described (*12*). In a typical experiment, 2.2 μmol of support-bound context III(FdI) was heated for 5 days at 55−58 °C with 11.2 mg (35.1 μmol, 16-fold molar excess) of (±)-7β,8α,9β-trihydroxy-10α-amino-7,8,9,10-tetrahydrobenzo[*a*]pyrene (DE-1 aminotriol) or (±)-7β,8α,9α-trihydroxy-10β-amino-7,8,9,10-tetrahydrobenzo[*a*]pyrene (DE-2 aminotriol; *26*) and 10 μL of triethylamine in 336 μL of Me_2SO in the presence of 336 μL of hexamethyldisiloxane. The same procedure was used on a 4−6 μmol scale for postoligomerization modification of context IV(FdI). Workup of the glass beads and ammonia cleavage of the dimethoxytrityl (DMT) oligonucleotides were carried out as described (*12*). HPLC conditions for separation of adducted from nonadducted DMT oligonucleotides are given in the Supporting Information.

Since the amino triols used for postoligomerization modification were derived from trans opening of racemic diol epoxides, each modified oligonucleotide preparation contained two diastereomeric, adducted oligomers. These diastereomers were separated chromatographically after removal of the DMT group (80% acetic acid in H_2O, 30 min, room temperature). For HPLC conditions, see the Supporting Information. In our experience, the modified oligomers derived from the G-rich sequence [context IV(G)] consistently exhibited lower purity and poorer chromatographic characteristics than the context III(G) oligonucleotides and were obtained in much lower isolated yields (on the order of 1−2 A_{260} units of each diastereomer from 5 μmol of CPG-bound context IV, as opposed to 12−17 A_{260} units of each diastereomer from 2−3 μmol of CPG-bound context III). Retention times and absolute configurational assignments (see below) for the diastereomeric adducted oligonucleotides derived from the two enantiomers of each diol epoxide are given in Table 1.

Absolute configurations were assigned by enzymatic digestion to the nucleoside level (*12*, *27*) of the early eluting isomer in each pair of context III(G) adducts (from DE-1 and -2) and of both the early and late eluting isomers of the context IV(G) adducts. Comparison of the circular dichroism (CD) spectra of the resultant nucleoside adducts with those of the known optically pure dGuo and Guo adducts (*27*−*29*) established that the early eluting oligonucleotides were those with the 10*R* absolute configuration of the adducts in all cases (Table 1). The CD spectra of the adducted oligonucleotides of context III(G) exhibited bands in the pyrene chromophore region (320−350 nm) which had the same sign (positive for 10*R* and negative for 10*S*) as that observed for the corresponding nucleoside adducts. In contrast, CD spectra of the context IV(G) oligonucleotides were not indicative of adduct configuration, since their long-wavelength CD bands were weak, and the late eluting DE-2

Table 1: Retention Times and Absolute Configurations at C_{10} of Modified Oligonucleotides Containing Trans-Opened BPDE Adducts

	context III(G)[a]		context IV(G)[b]	
parent diol epoxide	retention time[c] (min)	absolute configuration	retention time[c] (min)	absolute configuration
(7*R*,8*S*,9*R*,10*S*)-DE-1	32.1	*R*	25.5	*R*
(7*S*,8*R*,9*S*,10*R*)-DE-1	34.0	*S*	29.0	*S*
(7*S*,8*R*,9*R*,10*S*)-DE-2	32.0	*R*	25.4	*R*
(7*R*,8*S*,9*S*,10*R*)-DE-2	35.9	*S*	28.2	*S*

[a] Context III(G), 5′-TTCGAATCCTTCCCCC-3′, assignments based on enzymatic hydrolysis of the early eluting oligonucleotides from DE-1 and -2 to monomeric dGuo adducts of known absolute configuration. [b] Context IV(G), 5′-GGGGTTCCCGAGCGGC-3′, assignments based on enzymatic hydrolysis of all four oligonucleotides to monomeric dGuo adducts. [c] On a Beckman Ultrasphere C_{18} column under the chromatographic conditions given in the Supporting Information.

adducted oligonucleotide (10*S* adduct) exhibited both positive and negative bands in this region.

Oligonucleotide Purification by Electrophoresis. All oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and examined by polyacrylamide gel electrophoresis. Since most oligonucleotides contained some traces of contaminating sequences, aliquots (~0.5 OD unit) were purified further by electrophoresis on a 20% denaturing polyacrylamide gel (0.3 cm). The oligonucleotides were detected under UV light; the corresponding area of gel was cut out, and the oligonucleotides were eluted overnight and then adsorbed to a reversed-phase Sep-Pak cartridge (Waters) and recovered by elution with 60% MeOH in H_2O (1 mL). After drying, the eluent was resuspended in H_2O (100 μL), and the purity was confirmed by further electrophoresis after ^{32}P-end labeling.

Ligation of Oligonucleotides into M13mp7L2 Bacteriophage DNA. Bacteriophage M13mp7L2 was grown in *E. coli* SMH 77. M13mp7L2 is a modification of M13mp7 with two nucleotides replaced by four nucleotides (*30*) to stabilize the duplex hairpin region containing the *Eco*RI site in the single-stranded molecule. Following protocols established by Lawrence and colleagues (*30*) with some minor variations, M13 DNA (200 μg) was cut with *Eco*RI [0.5 unit/μg of DNA for 2.5 h at 37 °C (for experiment I) or 4 units/μg of DNA for 2.5 h at 30 °C (for experiment II)] to linearize the DNA. After a phenol extraction, DNA (2 μg) was converted to a gapped circular species by annealing with an equimolar amount of a 56-mer uracil-containing scaffold. The latter was designed to be complementary to 20 nucleotides at each end of the M13 molecule and included a central 16-mer complementary to the oligonucleotides that were to be ligated into the viral DNA. Thereafter, the appropriate 5′-phosphorylated 16-mer oligonucleotides (either adducted or unadducted) were added in a 10-fold molar excess [the DE-2/10*S*-containing context III(G) was used in equimolar amounts because of limited availability] and, after annealing (37 °C for 15 min followed by at least 2 h at room temperature), was ligated overnight at 16 °C with T4 DNA ligase (0.15 unit/μL). In the case of the context I(A) oligonucleotides, construction of the modified M13 vector was most successful when the oligonucleotides were first annealed with the 56-mer scaffold (25:1 molar ratio; 50 °C for 5 min and then slowly cooled to room temperature) and then annealed with the linear M13 DNA (0.5 molar ratio). All ligation reaction mixtures were exposed to uracil-DNA glycosylase (1 unit/200 μL) at 37 °C for 40 min to remove the uracil residues in the scaffold and create abasic sites in their place that would lead to degradation of the scaffold by AP endonuclease and exonucleases immediately after transfection into the bacterial cells.

Ligation Efficiencies. Estimates of ligation efficiencies were obtained by separating ligation mixtures (100 ng) on a 1.4% agarose gel, transferring the products to a nitrocellulose membrane by Southern blotting, and probing with radioactive probes complementary to M13 sequences or complementary to M13 and adjacent oligonucleotide insert sequences. A phosphorimager (Molecular Dynamics) was then used to determine the relative amounts of closed circular DNA and linear DNA in each sample.

Transfection of E. coli with M13 DNA. E. coli SMH 77 cells were SOS-induced (UV irradiation at 40 J/m^2) immediately before they were made competent for transfection ($CaCl_2$) or were made competent without SOS induction (*30*). An aliquot of the ligation mixture, corresponding to 15 ng of total DNA, was transfected into 100 μL of a competent cell suspension. After transfection, the M13-containing bacteria were mixed with top agar, poured onto agar plates, and grown overnight at 37 °C.

Analysis of Progeny Bacteriophage. Progeny bacteriophage were analyzed by two different methods. In studies referred to here as experiment I, the protocols used by Banerjee et al. (*30*), with some modifications to distinguish background events from the desired events (*17*), were followed. Briefly, 100 plaques were transferred to master plates, and the resultant bacterial colonies were blotted with a nitrocellulose membrane (Schleicher and Schuell). This membrane was probed to determine which of the original plaques contained M13 with the normal base at the former adduct site (i.e., nonmutated progeny) (all probes used in these studies are summarized in Table 2). Before the M13 bacteriophage were sequenced, the remaining plaques were transferred to a new plate and screened, as above, but using a probe that would detect progeny that had rejoined the M13 ends without incorporating the 16-mer oligonucleotide. This step eliminated background plaques with no insert. The membrane was then stripped (*31*) and probed again with a probe that overlapped the 3′-end of the oligonucleotide insert and some of the M13 sequence. The M13 DNAs from the plaques that were positive in this last screen were then sequenced using a dideoxynucleotide protocol and an automated sequencer (ABI).

In studies referred to as experiment II, analysis using oligonucleotide probes was fashioned after the procedures described by Chary et al. (*15*) and Moriya et al. (*16*). M13 DNA was transferred from each agar plate to four nitrocellulose filters. After the filters were baked for 2 h at 80 °C in a vacuum oven, filters were washed with 3× SSC containing 0.1% SDS for 2 h and then prehybridized to eliminate nonspecific binding with 2× Prehybridization/Hybridization solution (Gibco/BRL) for 1.5 h at 37 °C. One of four radiolabeled probes (1 ng/mL final concentration), designed to be complementary to the sequence on either side of the adduct site, but containing either A, C, G, or T opposite the adduct site (Table 2), was then added to each filter at 37 °C, and the solutions were cooled slowly to room temperature

Table 2: Various Oligonucleotides Used in Vector Construction and Progeny Analysis

M13 construction[a]		progeny analysis	
constructed 16-mer[b]	scaffold[c]	experiment I probe[d]	experiment II probe[e]
context I(A)	*M13*-GGGAGCAGACUCUAAA-*M13*	wild-type CAGACTCTAAACAC	CAGACTC*B*AAACAC
TTTXGAGTCTGCTCCC		insert probe GTGAATTGGGAGCA	
context II(A)	*M13*-GCAGACUCUAAAUCUG-*M13*	wild-type TCTAAATCTGCAC	TCTAAA*B*CTGCAC
CAGXTTTAGAGTCTGC		insert probe GTGAATTGCAGACT	
context III(G)	*M13*-GGGGGAAGGAUUCGAA-*M13*	wild-type AAGGATTCGAACAC	AAGGATT*B*GAACAC
TTCXAATCCTTCCCCC		insert probe GTGAATTGGGGGAA	
context IV(G)	*M13*-GCCGCUCGGGAACCCC-*M13*	wild-type CTCGGGAACCCCCAC	CTCGGGAA*B*CCCCAC
GGGXTTCCCGAGCGGC		insert probe GTGAATTGCCGCTC	

[a] All sequences are listed in the 5′−3′ direction. [b] X represents one of the four trans adducts of dAdo or dGuo shown in Figure 1. [c] *M13* indicates sequences of 20 nucleotides at each end of the scaffold that are complementary to the M13 ends. These are the same in each of the four scaffolds and are the sequence 5′-AAAACGACGGCCAGUGAAUU...CACUGAAUCAUGGUCAUAGC-3′. [d] The probe for detecting progeny that had not incorporated the insert was the same for all contexts since only M13 sequences are present in these background events; i.e., the no insert probe was GTGAATTCACTGAA. [e] The four probes used to analyze progeny from a given oligonucleotide context all have the same sequence except that the base labeled *B* in the table was either A, C, G, or T. The probes used to determine ligation efficiency were the insert probes shown above and the probe GGCGAAAGGGGGATGTGC, which is complementary only to M13 sequences. This latter oligonucleotide was also used as the primer in sequencing the progeny.

and left overnight under constant agitation. The filters were then washed (4 × 30 min) with 6× SSC at room temperature followed by 30 min at the appropriate stringent temperature [context I(A), 38 °C; context II(A), 35.5 °C; context III(G), 38 °C; and context IV(G), 38.5 °C]. The filters were subsequently exposed to X-ray film with an intensifying screen at −70 °C overnight.

RESULTS

Each of the four dAdo and dGuo adducts illustrated in Figure 1 [these are identified by the diol epoxide diastereomer from which they were derived, DE-1 or DE-2, and the absolute configuration at C_{10}, i.e., 10*S* or 10*R* (*32*)] was introduced into two oligonucleotide sequences, context I(A) and context II(A) (*12*) for the dAdo adducts and context III-(G) and IV(G) for the dGuo adducts (see Table 2 for sequences). The sequences were selected from the *supF* gene that has been used extensively as a target gene in shuttle vector-based mutation studies with polycyclic aromatic hydrocarbon diol epoxides (*33*−*38*). In context III(G), BPDE−dGuo adducts were placed at a site in the *supF* sequence known to be amenable to mutation by racemic BPDE-2 (*33*) and by each enantiomer of benzo[*c*]phenanthrene DE-1 and DE-2 (*35*) (position 164), whereas in context IV(G), the adducts were placed at a site where mutation by these agents was very rare (position 105) (*33*, *35*). BPDE−dAdo adducts were placed at a site in the *supF* sequence that benzo[*c*]phenanthrene diol epoxides mutate fairly frequently [position 134, context I(A)] (*34*, *35*) and at a site that they do not mutate [position 138, context II(A)]. Unlike benzo[*a*]pyrene diol epoxides, benzo[*c*]phenanthrene diol epoxides generate extensive adducts with adenine residues in DNA (*39*, *40*) and produce substantial mutation frequencies at A·T pairs.

Following purification, all oligonucleotide constructs were end labeled with radioactive phosphate and their purity was

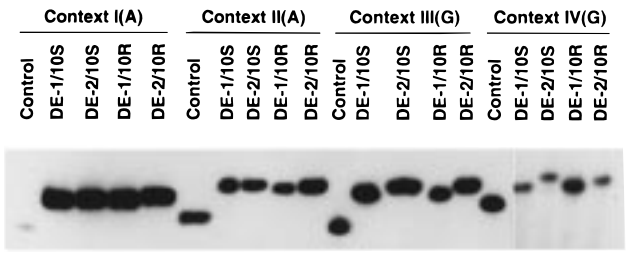

FIGURE 2: Control 16-mer oligonucleotides (see Table 2 for sequences) containing a normal dGuo (or dAdo) and oligonucleotides containing the indicated BPDE−deoxyribonucleoside adducts (see Figure 1 for structures) were purified by HPLC and by gel electrophoresis and then end labeled and examined by polyacrylamide gel electrophoresis.

demonstrated by denaturing polyacrylamide gel electrophoresis (Figure 2). Different BPDE adducts in the same oligonucleotide sequence led to some minor variations in migration through the gel, and the hydrocarbon residue retarded the constructs in comparison to the unsubstituted oligonucleotide. The methods used for synthesis allow only the desired trans adducts to be formed.

Data were gathered from two different methods of progeny analysis (Figure 3). In experiment I, where both SOS-induced and uninduced *E. coli* cells were used, progeny were analyzed by a combination of oligonucleotide hybridization and sequencing, following the approaches of the laboratories of Lawrence (*30*) and DiGiovanni (*17*). In experiment II, where only SOS-induced cells were used, a differential hybridization method of progeny analysis, based on that previously used by the laboratories of Lloyd and Moriya (*15*, *16*, *41*), was employed so that a larger number of progeny could be screened (Figure 3). Estimates of ligation efficiency varied from experiment to experiment (see the Supporting Information) but were substantially lower for context I(A) in experiment I and experiment IIa, in which the scaffold and cut M13 were annealed first and the adducted (or unadducted) 16-mer was added subsequently, than for the

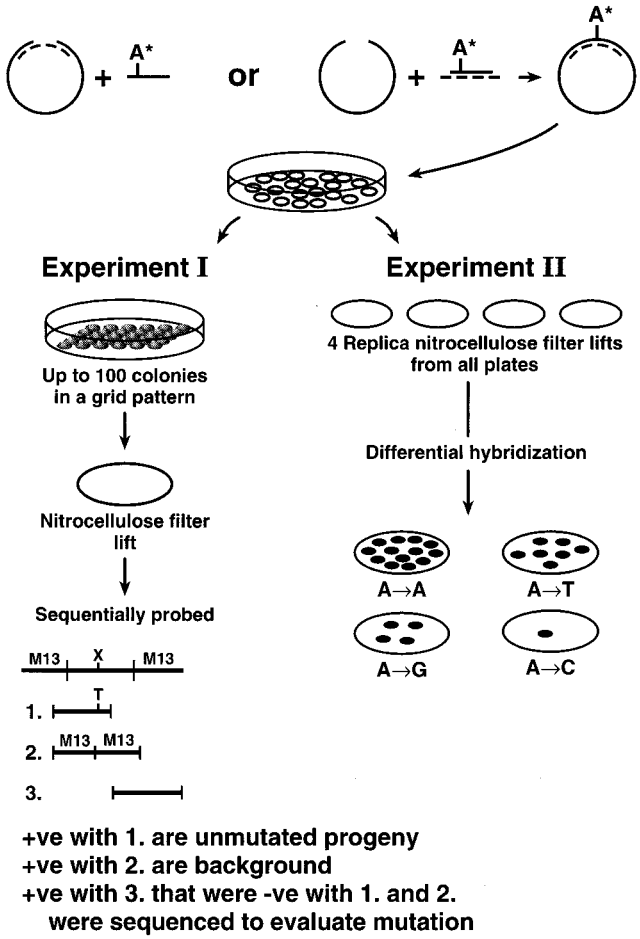

FIGURE 3: Schematic representation of experimental approaches used in M13 vector construction and progeny analysis illustrated for a dAdo adduct-containing construct. The dashed line represents the scaffold, and the short solid line with the A* represents the adducted context I or II in this case. The probes are as follows: (1) the wild-type probe that detects progeny in which the adduct is replaced by the normal A, (2) the no-insert probe that detects progeny that have arisen from ligation of the two ends of the cut M13 without the oligonucleotide having been incorporated, and (3) the insert probe that is complementary to the M13 sequence and to the adjoining oligonucleotide insert sequence and therefore detects progeny that contain an inserted oligonucleotide. The sequences of all the probes are listed in Table 2 (note the no-insert probe is given in the footnote). The sequences of all oligonucleotide contexts are also summarized in Table 2. In experiment I, progeny that are positive (+ve) with probe 1 are unmutated progeny. Those that are positive with probe 2 are background events, and of the remainder, those positive with probe 3 are evaluated by sequencing. The probes are placed in Figure 3 under the regions of the construct to which they are complementary. See the text for further explanation.

other sequence contexts. However, with context I(A), as with the other three, ligation efficiencies were similar for the adducted and unadducted oligonucleotides. In experiment IIb, 16-mer and scaffold were annealed together and the cut M13 was added thereafter (*15*) (Figure 3), resulting in greatly improved ligation efficiencies for the context I(A) constructs (see the Supporting Information).

The sum of mutant and nonmutant progeny arising from an adducted vector depended on the efficiency of each ligation and on the efficiency of transfection into the bacteria, and not surprisingly, therefore, substantial variation was obtained here, as in other studies (*20*). However, when comparisons were made between the uninduced and SOS-induced cells in experiment I, the progeny yield in uninduced cells averaged over all 16 adducted sequences (~21/plate containing 15 ng of M13 DNA) was usually less than that in SOS-induced cells (~35/plate). Additionally, more mutants were recovered from the SOS-induced cells [56 base substitutions and 133 single-base deletions at the adduct site [all but one of the deletions arose from the context IV(G) oligonucleotides]] than from the uninduced cells [9 base substitutions and 10 deletions, with all the latter arising from the context IV(G) oligonucleotides].

The low yield of mutants in the absence of SOS induction was not sufficient for comprehensive analysis of mutation frequencies, and further studies labeled experiment II were carried out in only SOS-induced cells. Furthermore, the laborious progeny analysis used in experiment I was replaced by a differential hybridization method of screening progeny. Altogether, experiment II led to the accumulation of a total 857 base substitution mutations. Almost 600 of these came from experiment IIa. In experiment IIb, mutations were studied only for the context I(A) and some adducted context II(A) oligonucleotides because very few mutations had been accumulated from context I(A), in particular, to this point. In experiment IIa, ligation efficiencies for adducted oligonucleotides were lower than in experiment I [~2% for context I(A) and in the range of ~3 to 9% for the others]. Nevertheless, because all plaques obtained were screened (rather than a randomly selected sample of 100 plaques in experiment I), it was comparatively easy to identify a large number of mutations from the contexts other than context I(A). Several samplings of plaques were sequenced to ensure that the method was correctly identifying both mutant and nonmutated progeny.

Optimal conditions for annealing context I(A) oligonucleotides with the corresponding scaffold oligonucleotides for experiment IIb were established by examining the ability of DNA polymerase to extend the 16-mer after annealing. Ligation efficiency estimates using annealing conditions selected in this fashion were quite high [~20 and 24% for the context I(A) and II(A) oligonucleotides, respectively]. Mutants from the context I(A) oligonucleotides were then relatively easily obtained. The mutation frequencies for all four adducts in this context were >35%. Estimates of survival of the adducted M13 constructs (plaques derived from adducted constructs compared with plaques derived from nonadducted constructs) for experiments IIa and IIb were quite low. For all adducted constructs, survival was less than 10%, and in many cases, it was less than 5% (see the Supporting Information).

Mutation Frequencies. Because of the method used to analyze progeny, only targeted base substitution mutations were detected in experiment II. However, it was clear from experiment I that deletion of a single guanine residue was a frequent occurrence when guanine adducts were placed at the 3′-end of a run of guanines in context IV(G) (see Table 2) (since the terminal nucleotide on the M13 is a guanine residue, the adduct is at the 3′-end of a run of five guanines in the M13 construct). With the four adducted context IV(G) constructs, the frequency of deletion was 13, 27, 25, and 30% for the DE-2/10*R*, DE-2/10*S*, DE-1/10*S*, and DE-1/10*R* adducts (Figure 1), respectively. These frequencies are based on the collection of 26, 48, 48, and 10 deletions in each case, in the order listed above. The adduct sequence

Table 3: Summary of the Number of Each Type of Substitution Mutation Recovered from SOS-Induced Cells and the Percentage of Total Progeny Found To Be Base Substitution Mutations (MF)[a]

	context I(A)				context II(A)				context III(G)				context IV(G)			
	mutation no.			MF	mutation no.			MF	mutation no.			MF	mutation no.			MF
adduct	A→T	A→G	A→C	(%)	A→T	A→G	A→C	(%)	G→T	G→A	G→C	(%)	G→T	G→A	G→C	(%)
DE-2/10*R*	56	1	12	58	60	17	13	20	37	10	8	3	27	13	14	15
DE-2/10*S*	50	2	8	51	18	12	5	5	30	5	15	11	38	13	14	11
DE-1/10*S*	29	3	7	44	21	20	8	11	38	19	14	5	25	24	14	5
DE-1/10*R*	46	1	4	37	26	13	13	8	35	14	10	3	32	16	8	4

[a] In all experiments combined, no mutations were detected at the site at which adducts were introduced in any of the four control sequence contexts even though 1454, 16050, 22070, and 17398 plaques were screened for the context I(A), II(A), III(G), and IV(G) controls, respectively. Thus, spontaneous mutation frequencies in these experiments were all <0.07%. Since most of the data were obtained in experiment II, the pooled mutation frequencies given here are very similar to those obtained in experiment II. Only in the cases of the DE-2/10*R* construct of context I(A) (73% in experiment II and 58% in the combined data) and the DE-1/10*S* construct of context I(A) (49% in experiment II and 44% in the combined data) is there a substantial difference. In all other cases, the values for the combined data and those for experiment II are the same or differ by no more than 2%.

context is clearly a major factor determining the frequency of deletion since the same adducts in a different sequence context, i.e., context III(G), did not yield a single base deletion. Similarly, no deletion mutations were detected in the 182 progeny recovered from the unsubstituted guanine-containing context IV(G) construct in SOS-induced cells in experiment I (frequency < 0.5%), showing that the deletions do require the presence of BPDE−dGuo adducts. Engineering errors cannot account for the deletions because, if the terminal guanine had been inadvertently lost from the cut M13, both the adducted and nonadducted constructs would yield deletions. Additionally, the frequency of deletion was much greater in SOS-induced cells than in noninduced cells, and this would not be the case if there had been loss of a guanine from the 16-mer.

The sequencing approach used in experiment I revealed that the adduct, together with 4−18 adjacent nucleotides, was deleted in some progeny. Thirteen such deletions were found in the SOS induction studies and three in the uninduced studies. Nine of this total were produced from context II(A) constructs, but with the overall numbers of such mutations being small for any particular construct, it is difficult to assign a specific frequency to these events. A few other base substitutions and deletions that did not involve the adduct site were also detected in experiment I. Of these, it was notable that deletion of the G that is the 3′-neighbor of the DE-1/10*S* adduct in context II(A) was found five times in the absence of SOS induction and twice in the presence of such induction, suggesting that this may be attributable to the adduct. No other deletion mutations were found at a frequency that suggested biological significance.

In addition to these deletions, a large number of base substitution mutations were recovered in these experiments. About 95% of the total base substitution mutations obtained were derived from the SOS-induced cells and differential hybridization screening used in experiment II. However, in Table 3, all of the base substitution mutations obtained from the SOS-induced cells (in both experiments I and II) are summarized together. Both mutation frequencies (mutant progeny as a percentage of total progeny, i.e., mutant plus nonmutant progeny) and the number of each kind of base substitution obtained are given.

It is important to note that no base substitution mutations were found in the constructs made with unadducted purines even though many thousands of plaques were screened. Thus, the spontaneous base substitution mutation frequency was very low (<0.07%) in these studies. The mutation frequencies for the adducted constructs are all based on a substantial number of mutants. In only two cases were less than 50 mutants collected [DE-1/10*S* in context I(A) and DE-2/10*S* in context II(A)]. The frequencies should be relatively reliable numbers, therefore, and the dAdo constructs of context I(A) clearly exhibited notably higher base substitution mutation frequencies than did the other constructs. Overall, the context III(G) constructs seemed to generate the least number of base substitutions as a group.

As expected, the different isomeric adducts examined (Figure 1) gave different mutation frequencies (Table 3). In context I(A), the mutation frequencies for adduct isomers differed by less than a factor of 2, whereas in other sequence contexts, variations of up to 4-fold were seen. In the case of the dAdo adducts, the sequence context had major effects on mutation frequency for each adduct isomer. Thus, frequencies were approximately 3-, 10-, 4-, and 5-fold higher in context I(A) versus II(A) for DE-2/10*R*, DE-2/10*S*, DE-1/10*S*, and DE-1/10*R*, respectively. It is somewhat surprising that the same adduct could have such different mutagenic properties depending on the sequence context, but in yet another sequence context for dAdo adducts, DE-2/10*S* was found to have a mutation frequency of ∼1% (J. E. Page et al., unpublished), indicating that sequence context effects can result inasmuch as 50-fold differences in frequency. The sequence contexts used in these studies for the dGuo adducts did not have such substantial effects on base substitution mutation frequency, except in the DE-2/10*R* case. However, since we have only examined two sequence contexts, it cannot be concluded that other contexts would not have major effects on base substitution mutation frequencies of the dGuo adducts also.

The individual base substitution mutations obtained are also shown in Table 3. Overall, the base substitutions obtained most frequently for the dAdo adducts were A → T transversions and for the dGuo adducts were G → T transversions. The frequencies at which each individual base substitution arose from the adducted dAdo substrates are summarized in Figure 4. The variations in A → T mutation frequency with sequence context [compare contexts I(A) and II(A) for each individual adduct] were quite dramatic. For example, the A → T mutation frequency for the DE-2/10*S* constructs was 42% for context I(A) and only 3% for context

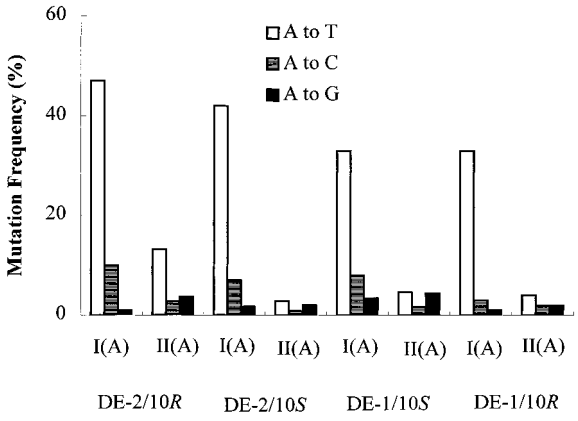

FIGURE 4: Mutation frequencies for base substitution mutations obtained with sequence contexts I(A) and II(A) each containing one of the four dAdo adducts shown in Figure 1.

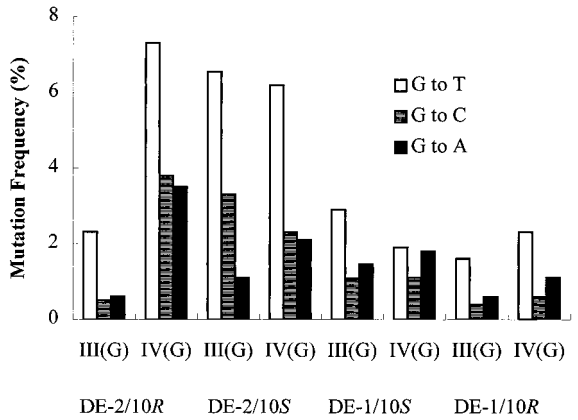

FIGURE 5: Mutation frequencies for base substitution mutations obtained with sequence contexts III(G) and IV(G) each containing one of the four dGuo adducts shown in Figure 1.

II(A), i.e., a 14-fold difference. The frequency of the other transversion mutation, A → C, also varied substantially, up to 7-fold, with sequence context, but in contrast, A → G transition mutations were fairly similar in each context for most adducts. Another aspect revealed in Figure 4 is that, in the context I(A) constructs, A → T mutations dominated the mutations found for all adducts examined, whereas in the context II(A) series, all three base substitutions were more evenly represented.

A summary of the frequencies for individual base substitution mutations in BPDE−dGuo adduct-containing constructs is presented in Figure 5. The scale is expanded in this plot compared with that for the dAdo adduct-containing constructs, and most of the frequencies in Figure 5 are comparable to those found for the context II(A) constructs in the previous figure. Except for the DE-2/10*R* context III(G) and IV(G) constructs, sequence context had lesser effects on base substitution mutations than was the case for dAdo adducts. Adduct structure also played a significant role in determining mutation frequencies. For example, the DE-2/10*S* adduct-containing construct (this adduct is the major adduct formed from benzo[*a*]pyrene in vivo) gave a substantial mutation frequency in both sequence contexts and was far more active than the DE-1-derived constructs.

DISCUSSION

Determination of mutation frequencies in site-specific mutation studies is complicated by strand bias and repair with double-stranded vectors (*14*, *42*) and by the possibility that scaffolding oligonucleotides used in single-stranded vector construction might be extended to copy the vector genome, without necessitating trans lesion synthesis. To avoid this latter problem, uracil residues were incorporated into the scaffolds used in this study, and prior to transfection, ligation mixtures were treated with uracil-DNA glycosylase to generate abasic sites that should ensure rapid destruction of the scaffold by cellular apurinic endonucleases and by exonucleases [uracil-containing scaffolds have been used also by Bailey et al. (*43*)]. In the first study of experiment II, the mutation frequency for the DE-2/10*R* version of context I(A) was 73%, a value close to 75% (expected if each of the four nucleoside triphosphates could be inserted opposite the adduct with equal ease, i.e., if the adduct was completely noninformational). Thus, the methods used are capable of providing a good approximation of the mutation frequencies during trans lesion synthesis, and since reasonably large numbers of progeny have been analyzed for each construct [these can be determined from Table 3 and range from 88 for the DE-1/10*S* construct of context I(A) to ∼2000 for the DE-1/10*R* and DE-2/10*R* constructs of context III(G)], the mutation frequencies obtained in these studies should be fairly reliable.

A simple pattern of factors determining base substitution mutation is not obvious from our data. Earlier work on replication past BPDE−dAdo adducts by single polymerases in vitro (*12*) and on site-specific mutation with BPDE−dGuo adducts (*18*) had indicated that the configuration of the linkage between the nucleoside and hydrocarbon (*R* or *S*) was a more important determinant of biological effect than the configuration of the hydroxyl groups on the adduct residue. However, examination of Figure 4 shows that data for adducts with the same configuration at C_{10} but derived from DE-1 or DE-2 can be quite different [compare the 10*R* adducts in context II(A) in Figure 4] or relatively similar [compare the 10*S* adducts in context II(A)]. Similar observations can be made from the data in Figure 5 also, and it seems clear that all aspects of structure can have effects on the mutagenic properties of individual adducts, as might be expected. The most dramatic overall effect seen in these data is the profound effect of sequence context on mutation frequency, but a much greater sampling of possible sequences will be required to determine general associations between sequence context and base substitution mutations.

Substantial numbers of deletion mutants were collected for the adducted context IV(G) constructs where the adduct was at the underlined G in a 5′-GGGGT-3′ sequence. All four of the dGuo adducts examined led to deletions at one of the guanines in this sequence, but the frequency for the DE-2/10*R* adduct was only about half of that for the other three adducts. In other site-specific studies, the DE-2/10*S* adduct has been studied in a 5′-GCGGC-3′ sequence (*14*, *20*) or a 5′-GCGGC-3′ sequence (*20*), but no single guanine deletions at the adduct site or at its 3′-neighbor were reported. Moriya et al. (*16*) placed this adduct at either underlined G in a 5′-CCTGGC-3′ sequence and found no deletions, suggesting that more than two adjacent guanines are required for a high frequency of single-base deletions. However, two deletions were recovered when the DE-2/10*R* adduct was placed at the 5′-G site, but this only represented a frequency of 0.6% (*16*).

The higher frequency of single-base deletions found in our study is consistent with findings on acetylaminofluorene−DNA adducts reported by Lambert et al. (*44*). These workers found that the frequency of single-base deletions increased with increasing numbers of guanines on the 5′-side of the adducted guanine. They argued that it is difficult to extend the nascent DNA strand after it has extended opposite the adduct. However, if the adducted guanine subsequently loops out, the terminal C on the growing strand can slip and pair with the guanine neighboring the adduct to yield an intermediate that is more readily extended; the number of looped-out intermediates (and, therefore, mutation frequency) increases with the number of guanines on the 5′-side of the adduct (*45*). On the basis of this model, it is possible that the lower frequency of deletion found with the DE-2/10*R* construct of context IV(G) can be partially attributed to the greater base substitution mutation frequency found for this construct because deletion depends on the presence of cytosine opposite the adduct (*45, 46*). Cosman et al. (*47*) have studied an oligonucleotide duplex with a deletion opposite the adduct. In this structure, the benzo[*a*]pyrene residue is intercalated between base pairs neighboring the deletion, and hydrophobic base-stacking interactions help stabilize such structures.

In studies of the mutation spectra for several hydrocarbon diol epoxides in the *supF* gene, deletions in the run of guanines contained in context IV(G) have been very rare (*37*, *38*, *48*, *49*), although 7-bromomethylbenz[*a*]anthracenes induced many deletions in a different run of guanines in the forward mutation *supF* assay (*50*). The reasons for the differences in the site-specific studies and the forward mutation shuttle vector assay may be due to the fact that mutations arise during replication in a human cell in the latter assay (*51*), whereas replication occurs in *E. coli* in the former one. Also, Fuchs and colleagues (*44*) have demonstrated a dependence on SOS induction for the site-specific studies in *E. coli*, and this could well be another important difference in the two types of assays. A limited amount of single-base deletion was found when DNA polymerase in vitro replicated past trans DE-2/10*R* and -10*S* dGuo adducts in oligonucleotide templates (*52*).

Base substitution mutations for some polycyclic aromatic hydrocarbon−deoxyribonucleoside adducts in site-specific mutation studies have been reported earlier. The DE-2/10*S* dGuo adduct has been investigated most extensively, but in some studies, the mutagenic effect of this adduct was compared with that of the DE-2/10*R* dGuo adduct (*16*, *18*). In a 5′-TG̲G-3′ sequence context, Moriya et al. (*16*) found that the DE-2/10*S* adduct gave a considerably higher (∼10-fold in SOS-induced cells) mutation frequency than did the DE-2/10*R* adduct, and though G → T transversions were predominant in the DE-2/10*S* case, the recovery of mutants was too low for the DE-2/10*R* construct to allow comparison of the distribution of mutation types. In the work of Shukla et al. (*18*), a slightly higher mutation frequency was reported for the DE-2/10*S* adduct constructs, and distinct differences in mutation distributions were recorded for the constructs containing each stereoisomer. Thus, G → T, G → A, and G → C substitutions were 10, 82, and 3% of the total mutations for the DE-2/10*S* constructs and 25, 34, and 41% for the DE-2/10*R* construct in the 5′-CG̲T-3′ sequence context used. In this work, these two adducts were compared in two sequence contexts, 5′-CG̲A-3′ [context III(G)] and 5′-GG̲T-3′ [context IV(G)]. Although the DE-2/10*S* construct exhibited the higher mutation frequency in the III(G) context in concert with the literature reports above, this was not the case in the IV(G) context (Table 3), indicating that even the rank order of mutagenic frequency for two adducts can change with sequence context. Mutation distributions for G → T, G → A, and G → C were 67, 18, and 15% and 50, 24, and 26% for the DE-2/10*R* construct in contexts III-(G) and IV(G), respectively and 60, 10, and 30% and 58, 20, and 22% for DE-2/10*S* constructs in these same sequence contexts (Table 3). These distributions are very different from those reported by Shukla et al., even though each context used here differs in one case only at the 5′-position and in the other only at the 3′-position from that used in this recent work. However, the strains of *E. coli* used in the Shukla study and this study were different, and the bacterial strain can have profound effects in these assays (*43*).

Comparisons of constructs containing all four of the dAdo adducts examined in this work have also been reported previously by Lloyd's laboratory (*15*). Mutation frequencies reported for DE-2/10*R*, DE-2/10*S*, DE-1/10*S*, and DE-1/10*R* all in a 5′-CA̲A-3′ sequence context were 0.48, 0.26, 0.7, and 1.0%, respectively, and the only mutations reported were A → G transitions. These mutation frequencies are much lower than those reported here for these same adducts in different sequence contexts, but we have also found comparably low frequencies when the two DE-2 adducts were examined in the 5′-CA̲A-3′ sequence context (J. E. Page et al., unpublished). We have not found exclusive A → G mutations, however, and preliminary experiments in collaboration with Lloyd's laboratory suggest that the use of different bacterial strains in our two laboratories may be responsible for these differences.

Few mutations were generated by BPDE at A·T pairs in forward mutation shuttle vector studies (*33*), but the context I(A) sequence that was mutated more extensively by benzo[*c*]phenanthrene diol epoxides in shuttle vector studies than context II(A) (*35*) also gave more mutations in this work. Context II(A) constructs did give rise to mutations (5−20% frequencies) in these studies, however, so the inactivity of the adduct site in context II(A) in the shuttle vector is not simply a function of local sequence context. Other factors, such as the extent of reaction at this site or the rate of repair at this site (*53*), must presumably limit mutation in the shuttle vector assay. Similarly, consideration of the dGuo construct data also indicates that sequence context alone does not determine mutations in the shuttle vector assay. This follows because, with equal adduct formation and the absence of repair, like in the present investigations, the adduct site that was inactive in the shuttle vector assay [context IV(G)] was equally or more active in base substitution mutation and vastly more active in single-base deletions than context III-(G) in these site-specific studies.

Overall, differences were found for mutation frequencies for different adduct isomers in the same sequence context. In some cases, these differences were substantial (up to ∼3−4-fold), but in others, the differences were relatively small (Table 3). Studies of the conformation of adducts in double-stranded oligonucleotides (reviewed in refs *54* and *55*) have shown that conformation is affected by neighboring bases and by the nature of the bonding (cis or trans) of the

hydrocarbon. For example, the hydrocarbon residues in adducts with an *S* configuration at the site of attachment to the purine lie in the opposite direction compared to that in adducts with an *R* configuration. Such large conformational differences might effect substantial differences in mutation frequencies in all such cases. However, the template DNA must be single-stranded at the point of replication, and therefore, conformations seen in double-stranded constructs may not be directly relevant to those present during replication. For this reason, some studies of oligonucleotides that are partially single-stranded and resemble primer−template junctions have also been undertaken. For the DE-2/10*S* adduct, a structure was obtained wherein the benzo[*a*]pyrene residue was stacked over the base pair at the junction but the resolution was not sufficient to allow a structure for the DE-2/10*R* adduct to be obtained (*55*).

It has been clear from earlier work that sequence context plays an important role in determining mutational consequences, and the present findings clearly emphasize this point. Mutation frequencies in context I(A) versus II(A) all differ substantially, but it is apparent that the effects of sequence context on the frequency of A → T transversion mutations, in particular, are far more substantial than on other mutations (Figure 4). Similarly, the two sequence contexts used for the dGuo adducts gave profoundly different frequencies of single-base deletions, zero for context III(G) and 13−30% for context IV(G), yet except for the DE-2/10*R* construct, base substitution mutation frequencies were not very different. Unfortunately, the sequence context question is complex because it is not restricted to the immediate neighbors of the adduct in the case of single-base deletions (*44*), and it remains to be seen whether base substitutions will be similarly affected by nucleotides that are not immediately adjacent to the adduct. Though difficult, the question of the effect of neighboring sequence on mutagenic potential will have to be resolved if a complete understanding of the mechanism through which carcinogen−DNA adducts lead to mutation is to be achieved.

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SUPPORTING INFORMATION AVAILABLE

Descriptions of the details of the chromatographic purification of adducted oligonucleotides and two tables listing ligation efficiencies and two tables listing the survival of adducted M13 constructs in the various experiments (6 pages). Ordering information is given on any current masthead page.

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