Sequence Context Effects on Mutational Properties of cis-Opened Benzo[*c*]phenanthrene Diol Epoxide−Deoxyadenosine Adducts in Site-Specific Mutation Studies†

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ABSTRACT: Diastereomeric N^6-substituted dAdo adducts (cis B[*c*]PhDE-2/1*R* and cis B[*c*]PhDE-2/1*S*) that correspond to cis-opening at C-1 of the enantiomeric benzo[*c*]phenanthrene 3,4-diol 1,2-epoxides in which the epoxide oxygen and the benzylic hydroxyl group are trans (DE-2) were synthetically incorporated into oligonucleotide 16-mers. Each adduct was placed at the fourth nucleotide from the 5′-end of each of two different oligonucleotide sequences derived from the *E. coli supF* gene. Each adduct was also placed in two additional oligonucleotide sequences that were constructed by interchanging the adduct site and the immediately adjacent nucleotides between the two original sequences. These oligonucleotides were designed for use in site-specific mutation studies, with a single-stranded bacteriophage M13mp7L2 vector, to determine if the effects of sequence context on types and frequencies of base substitution mutations are attributable only to nucleotides immediately adjacent to these polycyclic aromatic hydrocarbon diol epoxide−dAdo adducts, or whether more distant nucleotide residues also affect the mutagenic response. In SOS-induced *Escherichia coli* SMH77, total base substitution mutation frequencies for the cis B[*c*]-PhDE-2/1*R*−dAdo adduct were relatively low (0.62−5.6%) compared with those for the cis B[*c*]PhDE-2/1*S*−dAdo adduct (11.9−56.5%). Depending on sequence context, cis B[*c*]PhDE-2/1*R*−dAdo gave predominantly A→T or a more equal distribution of A→T and A→G mutations whereas cis B[*c*]PhDE-2/1*S*−dAdo gave either predominantly A→T or predominantly A→G base substitutions. Our results clearly indicate that nucleotides that are distal as well as those that are proximal to the adduct site are capable of influencing both the mutation frequency and the distribution of base substitution mutations.

Polycyclic aromatic hydrocarbons (PAHs)[1] are products of incomplete combustion of fossil fuels, are present in cigarette smoke and in broiled meat, and are consequently widespread environmental pollutants [reviewed in (*1*−*3*)]. PAHs are carcinogenic after metabolic activation to bay-region or fjord-region diol epoxides (*4*−*7*) that are reactive toward DNA and are, therefore, mutagenic (*8*). Mutations in oncogenes or tumor suppressor genes, which are required for the carcinogenic process (*9*), could arise from reactions of these metabolites with cellular DNA.

Site-specific mutation studies using single- or double-stranded DNA vectors have been used to investigate both quantitative and qualitative aspects of mutations arising from individual PAH diol epoxide−deoxyribonucleoside adducts [reviewed in (*10, 11*)]. The PAH diol epoxide−deoxyribonucleoside adducts studied most extensively so far have been those derived from the diol epoxides of benzo[*a*]pyrene (*12*−*20*), though adducts derived from benz[*a*]anthracene (*21*) and dibenz[*a,j*]anthracene (*22*) have also been examined. As found for other carcinogen−DNA adducts (*23*−*25*), some of the benzo[*a*]pyrene diol epoxide adduct data indicate that the sequence in which an adduct is located influences both the types and frequencies of mutations that arise (*12, 14*−*18, 20*).

For example, in a system using a double-stranded vector, the trans-opened benzo[*a*]pyrene DE-2−dGuo adduct (with 10*S* configuration at the site of attachment to the nucleoside) either gave preferential G→T mutations in a 5′-C**T**G**C**AG context (*12*), preferential G→A mutations in a 5′-GACGC-**C**G**T**CATCC context (*18*), or a more even distribution of base substitutions in a 5′-G**C**G**G**CCAAAG context (*14*). Using a single-stranded vector and trans-opened adducts from

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[1] Abbreviations: PAHs, polycyclic aromatic hydrocarbons; B[*c*]Ph, benzo[*c*]phenanthrene; DE-1, diol epoxide where the benzylic hydroxyl group and the epoxide oxygen are cis or syn; DE-2, diol epoxide where the benzylic hydroxyl group and the epoxide oxygen are trans or anti; B[*c*]PhDE-2/1*R*, B[c]PhDE−dAdo adduct with 1*R* configuration at the adducted carbon; B[*c*]PhDE-2/1*S*, dAdo adduct with 1*S* configuration at the adducted carbon; DMT, dimethoxytrityl.

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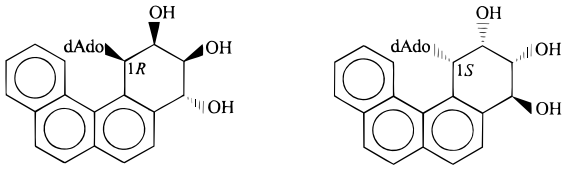

cis B[*c*]PhDE-2/1*R* **cis B[*c*]PhDE-2/1*S***

Context I(A): 5'-TT**T**A**G**AGTCTGCTCCC

Context II(A): 5'-CA**G**A**T**TTAGAGTCTGC

Context III(A): 5'-TT**G**A**T**AGTCTGCTCCC

Context IV(A): 5'-CA**T**A**G**TTAGAGTCTGC

FIGURE 1: Structures of the adducts formed from cis-opening of each enantiomer of benzo[*c*]phenanthrene diol epoxide-2 (B[*c*]-PhDE-2) by the exocyclic amino group of dAdo residues in DNA and the sequence contexts in which they were inserted.

each of the four isomeric benzo[*a*]pyrene diol epoxides, context-dependent variations in frequencies of individual base substitution mutations at adducted guanines were also noted, though G→T was the most prominent change (*20*). Although less data are available for hydrocarbon−dAdo adducts than for hydrocarbon−dGuo adducts, Latham et al. (*23*) have found that mutation frequencies for styrene oxide−dAdo adducts vary with sequence context, and Page et al. (*20*) also found context-dependent changes in frequencies, as well as in mutation types, for benzo[*a*]pyrene diol epoxide−dAdo adducts. Thus, sequence context clearly affects mutation frequencies and types of base substitutions found in site-specific mutation studies. However, little is known about the distance over which sequence changes can affect adduct mutagenesis. For example, are effects on mutagenesis restricted to the nucleotides immediately adjacent to the adduct, or can nucleotide changes more distant from the adduct also affect the mutational response?

The present experiments were designed to determine if sequence context effects on types of base substitution mutations and on base substitution mutation frequencies are limited to nucleotides immediately adjacent to PAH diol epoxide−dAdo adducts. In this report, the two dAdo adducts at the exocyclic N^6-amino group that would arise from cis-opening of each enantiomer of benzo[*c*]phenanthrene 3,4-diol 1,2-epoxide-2, in which the benzylic 4-hydroxyl group and epoxide oxygen are trans (referred to in this paper as cis B[*c*]PhDE-2/1*R* and cis B[*c*]PhDE-2/1*S*; see Figure 1 for structures), were incorporated as the fourth nucleotide from the 5′-end of each of four different sequence contexts. The Context I(A) and Context II(A) sequences were derived from the *E. coli supF* gene and have been used previously in studies of benzo[*a*]pyrene diol epoxide−dAdo adducts (*20, 26*). The other two sequences used were identical to the first two sequences except that the adduct and the immediately adjacent nucleotides on either side from Context II(A) replaced their counterparts in Context I(A) to yield Context III(A) and this triplet from Context I(A) was placed into Context II(A) to yield Context IV(A) (Figure 1). With this experimental design, it was possible to determine if the mutational properties of a given adduct were dependent only on the immediately adjacent nucleotides or if the sequence environment into which the adduct-containing triplet was placed substantially affected the mutational properties of the adduct. We found that both the immediately adjacent nucleotides and nucleotides beyond these immediate neighbors are important determinants of adduct mutagenicity.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzyme *Eco*RI, T4 DNA ligase, T4 polynucleotide kinase, Sequenase version 2.0 Sequencing Kit, and [γ-^{32}P]ATP or [α-^{32}P]dATP were purchased from Amersham Corp. Uracil−DNA glycosylase, 2 × Prehybridization/Hybridization solution, unmodified oligonucleotide 16-mers, and 56-mer scaffolds were from Gibco/BRL, and exonuclease III was from New England Biolabs, Inc. ABI-PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits were obtained from Perkin-Elmer. QIAprep spin M13 Kit and QIAprep 8 M13 Kit were from Qiagen Inc. *E. coli* SMH77 [F′ *lacZ*, Δ*M*15, *pro*$^+$, Δ(*pro-lac*), *leu*$^+$] and bacteriophage M13mp7L2 were kind gifts from C. W. Lawrence (University of Rochester, Rochester, NY).

Oligonucleotide Synthesis. Recently we described an aminohydroxylation procedure in which racemic *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene was converted in one step to a diastereomeric pair of deoxyadenosine adducts corresponding to cis-opening of benzo[*a*]pyrene diol epoxide-2 (*27, 28*). We have now applied this approach to the conversion of (±)-*trans*-3,4-dihydroxy-3,4-dihydrobenzo-[*c*]phenanthrene (*29, 30*) with 3′,5′-di-*O*-*tert*-butyldimethylsilyl-dAdo to produce a diastereomeric mixture of N^6-[1*S*-(2*S*,3*R*,4*S*-trihydroxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthryl)]-3′,5′-bis-*O*-(*tert*-butyldimethysilyl)-2′-deoxyadenosine and N^6-[1*R*-(2*R*,3*S*,4*R*-trihydroxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthryl)]-3′,5′-bis-*O*-(*tert*-butyldimethysilyl)-2′-deoxyadenosine. Acetylation of the hydrocarbon hydroxyl groups with pyridine/acetic anhydride, removal of the silyl protecting groups on the sugar with pyridine/HF, and conversion into 5′-dimethoxytrityl-3′-diisopropylcyanoethylphosphoramidites as previously described (*31*) provided the desired mixture of diastereomers ready for incorporation into oligonucleotides. Details of the synthesis will be published separately (Yagi et al., in preparation).

Oligonucleotides were prepared on a 2 or 2.5 μmol scale using 170 Å controlled-pore glass (CPG) loaded with N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-3′-succinic acid (80−95 μmol/g). Support-bound oligonucleotides containing the appropriate 12-base sequence 3′ to the adduct were synthesized on an automated DNA synthesizer by standard methodology with modifications as described (*32*), including the use of a nonaqueous oxidant. The CPG beads bearing the oligonucleotide were removed from the column, and the modified dAdo residue was introduced manually by treatment with 6 μmol of the above mixture of diastereomeric phosphoramidites and 50 μL of 0.5 M 1*H*-tetrazole in acetonitrile for 16 h. End-capping was omitted following the manual step. After returning the support-bound oligonucleotide to the column, the remaining three residues were added by reaction on the synthesizer, the oligonucleotide was cleaved from the support by the standard procedure, and protective groups were removed by ammonolysis (16 h, 58 °C). Typical yields on manual coupling of the modified

Table 1: HPLC Retention Times and Configurational Assignments at C-1 of the Adduct for the Modified Oligonucleotides

context	Rt (min)[a]	configuration at C-1	parent DE[b]
I(A)	16.2	*R*	(−)-(*RSSR*)
	17.4	*S*	(+)-(*SRRS*)
II(A)	13.0	*S*	(+)-(*SRRS*)
	14.3	*R*	(−)-(*RSSR*)
III(A)	38.1[c]	*R*	(−)-(*RSSR*)
	39.4[c]	*S*	(+)-(*SRRS*)
IV(A)	17.0	*R*	(−)-(*RSSR*)
	18.8	*S*	(+)-(*SRRS*)

[a] Retention times on a Hamilton PRP-1 column, 7 × 305 mm, eluted at 3 mL/min with a linear gradient of acetonitrile in 0.1 M ammonium carbonate buffer, pH ∼7.5, that increased the acetonitrile concentration from 0 to 17.5% over 20 min, unless otherwise noted. [b] Configurational designations for the tetrahydro benzo-ring carbons, proceeding around the ring from C-4 (benzylic hydroxyl substituent) to C-1 (benzylic epoxide). [c] On a Beckman Ultrasphere C_{18} column (5 μ, 4.6 × 250 mm) eluted at 1.2 mL/min with a linear gradient of acetonitrile in 0.1 M ammonium carbonate buffer, pH 7−7.5, that increased the acetonitrile concentration from 5 to 11% over 40 min.

phosphoramidite were ∼50%, as estimated from the release of dimethoxytrityl (DMT) alcohol on detritylation after this step. In our experience, the diastereomeric oligonucleotides derived from the enantiomeric diol epoxides were consistently separated better by HPLC after removal of the 5′-DMT protecting group. Thus, this group was generally removed either on the synthesizer or by treatment (80% acetic acid in water) of the product mixture obtained after cleavage from the support and ammonolysis of the protected oligonucleotide. In one case [Context II(A)], the detritylated, crude oligonucleotide mixture contained an impurity that eluted close to the early-eluting adducted oligonucleotide, and therefore preliminary HPLC purification of the diastereomeric 5′-DMT oligonucleotides was required (Hamilton PRP-1 column, 7 × 305 mm, eluted at 3 mL/min with a gradient that increased the percent of acetonitrile in 0.1 M ammonium carbonate buffer, pH 7.5, from 15 to 32.5% over 35 min). The desired pair of 5′-DMT-substituted, adducted oligonucleotides, which coeluted at ∼16 min, was isolated, detritylated, and rechromatographed. HPLC conditions and retention times for the adducted oligonucleotides with a free 5′-hydroxyl group are given in Table 1. Typically, 20−30 A_{260} units of each isomer were obtained after HPLC purification.

Aliquots of adducted and unadducted oligonucleotides (∼0.5 A_{260} unit) were purified further by electrophoresis on a denaturing 20% polyacrylamide gel (*20*). The oligonucleotides were detected under UV light, and the corresponding band was cut out. The oligonucleotides were eluted overnight with 0.5 M ammonium acetate and 10 mM magnesium acetate and then adsorbed to reversed-phase Sep-Pak cartridges and recovered by elution with 60% MeOH in H_2O (1 mL). After drying, the eluate was resuspended in H_2O (150 μL). The purity of these products was then determined by polyacrylamide gel electrophoresis of ^{32}P-end-labeled aliquots.

Configurations of the adducts at the point of attachment of the hydrocarbon to N^6 of dAdo in these oligonucleotides were determined after enzymatic hydrolysis (*33*) to nucleosides (see below). The digests from Contexts I(A) and II(A) were subjected to HPLC [DuPont Zorbax ODS column (4.6 × 250 mm) eluted at 1.2 mL/min isocratically with 5% methanol in 50 mM sodium phosphate buffer, pH 7.0 (solvent A), for 8 min, followed by a linear gradient of solvent B (10% water in MeOH) that increased the percent of solvent B to 10% over the next 7 min and a second gradient that increased the proportion of solvent B to 100% over the following 15 min]. The nucleoside adducts (28−30 min) were isolated, and their absolute configurations were determined by comparison of their CD spectra with those of the known cis dAdo adducts from B[*c*]PhDE-2, which exhibit a positive band at ∼250 nm and a negative band at ∼280 nm for the 1*S* adduct and bands of the opposite signs for the 1*R* adduct (*34*).

Typically, enzymatic hydrolysis of oligonucleotides was as follows. Approximately 2 A_{260} units of oligonucleotide were treated for 3 h at 37 °C with bovine pancreatic DNase I (0.15 mg) in 270 μL of Tris-HCl buffer, pH 7.0, containing 10 mM $MgCl_2$. The pH was then adjusted to ∼9.0 by addition of 25 μL of 1 M Tris base, snake venom phosphodiesterase (∼0.05 unit) was added, and digestion was allowed to proceed for 2 days. *E. coli* alkaline phosphatase (3 units) was then added, and the incubation was continued for 3 h. Under these conditions, the early-eluting (1*S*) diastereomer of Context II(A) was hydrolyzed essentially completely to the nucleoside adduct (29.0 min) with 1*S* configuration based on its CD spectrum (*34*). In contrast, under the same conditions, the major adduct-containing species obtained from the early-eluting (1*R*) diastereomer of Context I(A) was a product of incomplete digestion that eluted at 25.9 min, earlier than expected for a monomeric adduct; concomitantly, recovery of thymidine from the early-eluting Context I(A) diastereomer was approximately 50% of that expected. The isolated adduct-containing peak was resistant to further digestion by snake venom phosphodiesterase, but treatment (*35*) with ∼0.8 unit of bovine spleen phosphodiesterase (0.2 M sodium succinate, pH 6.0, 4 h, 37 °C), followed by alkaline phosphatase as above, resulted in its complete hydrolysis to thymidine and the expected monomeric adduct (29.2 min) whose CD spectrum indicated 1*R* configuration (*34*). Enzymatic digestion of the late-eluting diastereomers of Contexts I(A) and II(A) with 3 times the quantity (0.15 unit) of snake venom phosphodiesterase led to a similar result. The Context II(A) diastereomer with 1*R* configuration was incompletely hydrolyzed, whereas the Context I(A) diastereomer with 1*S* configuration was fully hydrolyzed.

Subsequently, the early-eluting (1*R*) diastereomers from Contexts III(A) and IV(A) were hydrolyzed for 3 days with a total of 0.2 unit of snake venom phosphodiesterase added in two portions at time zero and after 1 day. These hydrolysis mixtures were chromatographed on a Beckman Ultrasphere C_{18} column (4.6 × 250 mm) eluted at 1.2 mL/min with a linear gradient of methanol in water that increased the percent of methanol from 25 to 65% over 5 min, followed by a second linear gradient that increased the percent of methanol to 95% over the next 7.5 min; only the latest-eluting peak from the digest (8.9 min, monomeric nucleoside adduct) was isolated for CD measurement. No attempt was made to assess the extent of hydrolysis.

Ligation of Oligonucleotides into M13 Vector DNA. The vector DNA, M13mp7L2, was linearized with *Eco*RI (2 units/μg of DNA at 30 °C for 2.5 h) and then purified by

phenol extraction. Ligation of the oligonucleotide insert sequences into the cut M13 was in principle as described by Lawrence and co-workers (*36*) but used a uracil-containing scaffold (*20*). The 20 bases on each end of the scaffold were complementary to the M13 terminal sequences, and the middle 16 bases were complementary to the given insert sequence. Some ligation reactions involved annealing the 56-mer uracil-containing oligonucleotide scaffold (2 pmol) with the cut M13 (1 pmol) in 25 mM NaCl at 37 °C for 15 min followed by cooling to room temperature over at least 2 h. Phosphorylated unmodified or adducted oligonucleotide insert (100 pmol) was added, the solution was kept at room-temperature overnight, and T4 DNA ligase (30 units) was then added. Ligation reactions were left at 16 °C overnight after which uracil−DNA glycosylase (UDG, 1 unit) was added and the solution was incubated at 37 °C for 1 h. UDG generates abasic sites in the scaffold and makes it susceptible to cleavage by cellular endonucleases. Most ligation reactions followed an alternative protocol of annealing phosphorylated oligonucleotide insert 16-mers (50 pmol) with scaffolds (2 pmol) in 25 mM NaCl at 50 °C for 5 min followed by slow cooling overnight. The linearized M13 DNA (1 pmol) was then added, and the protocol above was followed thereafter.

Ligation efficiencies were estimated after subjecting the ligation products (∼100 ng) to agarose gel electrophoresis (*20*) and transferring the DNA to nitrocellulose filters by Southern blotting. A radioactive probe (5′-GGCGAAAGGGG-GATGTGC, 1 ng/mL hybridization solution) that was complementary to M13 sequence was used to detect closed circular and linear DNA, and a phosphorimager (Molecular Dynamics) was used to quantify these products.

Primer Extension Reactions. Scaffold in ligation mixtures was digested with exonuclease III (0.1 unit) diluted 1:500 in ligase dilution buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM DTT, 60 mM KCl, 50% glycerol) at 37 °C for 30 min followed by inactivation of the enzyme at 70 °C for 20 min. From these solutions, aliquots equivalent to 15 fmol of M13 DNA in 11 μL of ligation reaction buffer (66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 66 μM ATP) were annealed with primer, 5′-GGCGAAAGGGG-GATGTGC (0.1 pmol), for 10 min at 37 °C, transferred to room temperature for 2−3 min, and then kept on ice. Nucleoside triphosphates and Sequenase (4 units) to initiate reactions were added sequentially such that the final solution was 56 mM Tris-HCl, pH 7.6, 12 mM $MgCl_2$, 25 mM $NaCl_2$, 11.5 mM DTT, 0.8 μM each of dCTP, dGTP, and dTTP, and 0.2 μM [α-^{32}P]dATP (10 μCi), all in a total volume of 19.4 μL. Solutions were held at 37 °C for 30 min. Reactions were terminated by the addition of 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol Blue) and analyzed by electrophoresis on 6% denaturing polyacrylamide gels at 80 W for 1.7 h. Gels were dried and exposed to X-ray films. Aliquots (0.75 pmol of DNA in 15 μL of ligation buffer) of exonuclease III digested unadducted ligation mixtures were sequenced using the same primer (2 pmol), a Sequenase version 2.0 Sequencing Kit, and [α-^{32}P]dATP. These reactions were analyzed on the same gels as the primer extension reactions.

Transfection of E. coli with M13 DNA. E. coli SMH 77 cells were SOS-induced by irradiation with 40 J/m^2 UV at 1 J m^{-2} s^{-1} immediately before they were made competent by the $CaCl_2$ method (*37*). The cells were transfected with control- or adduct-containing vector (either 20 ng of DNA/50 μL or 20 ng of DNA/100 μL of competent cells per plate), mixed with top agarose, poured onto 2 × YT agar plates, and grown overnight at 37 °C.

Detection of Wild-Type or Mutant Progeny. M13 DNA from each plate was transferred to four nitrocellulose filters and immobilized by baking the filters 2 h at 80 °C in a vacuum oven. The filters were washed with 3 × SSC, 0.1% SDS for 1.5−2 h and prehybridized for ≥1.5 h at 37 °C. Hybridization with radiolabeled probes (1 ng/mL hybridization solution), complementary to the DNA surrounding the adduct site and containing A, C, T, or G opposite the adduct site [5′-TCTAA(**C**/**A**)**B**(**A**/**C**)TGCAC for the 5′-CA(**T**/**G**)-A(**G**/**T**)TTAGAGTCTGC sequences and 5′-CAGACT(**C**/**A**)-**B**(**A**/**C**)AACAC for 5′-TT(**T**/**G**)A(**G**/**T**)AGTCTGCTCCC where the base labeled **B** was either T, A, G, or C], was started at 37 °C followed by slow cooling to room temperature overnight with constant agitation. The filters were then washed with 6 × SSC (4 × 30 min) at room temperature followed by a 30 min wash at the stringent temperature [35.5 °C for the 5′-CA(**T**/**G**)A(**G**/**T**)TTAGAGTCTGC sequences and 38 °C for the 5′-TT(**T**/**G**)A(**G**/**T**)AGTCTGCTCCC sequences] and exposed to autoradiography films overnight with an intensifying screen at −70 °C. Occasionally, these probes did not give a clear result for some plaques, and in such cases, the assignment was made by sequencing.

RESULTS

The strategy used to evaluate the influence of immediately adjacent nucleotides on the mutagenic properties of hydrocarbon−deoxyribonucleoside adducts was to determine mutagenicity for both cis B[*c*]PhDE-2/1*S* and cis B[*c*]PhDE-2/1*R* in each of the four oligonucleotide 16-mer sequences shown in Figure 1. Although trans-opened N^6-dAdo adducts and oligonucleotides of B[*c*]PhDE-2 have been described (*38*−*41*), the present report describes the first synthesis of oligonucleotides containing cis-opened N^6-dAdo adducts of this diol epoxide. A novel aminohydroxylation procedure (*27, 28*) for bond formation between N^6 of a protected dAdo derivative and (±)-*trans*-3,4-dihydroxy-3,4-dihydrobenzo-[*c*]phenanthrene (*29, 30*) provided an extremely facile and efficient method to accomplish the crucial step in constructing the appropriate adducted phosphoramidite for solid-phase synthesis of the desired oligonucleotide sequences. Since a racemic diol was used in this synthetic step, diastereomeric oligonucleotide pairs were produced, and these were separated by HPLC.

The configuration at the point of attachment of the hydrocarbon residue to dAdo in each oligonucleotide was established from the circular dichroism (CD) spectra of the adducted nucleosides obtained from enzymatic hydrolysis of the oligonucleotides to nucleosides. It was noted in the course of hydrolyses of Contexts I(A) and II(A) that, in contrast to the 1*S*-diastereomers, the 1*R*-diastereomers of cis-opened B[*c*]PhDE-2−dAdo adducts in these single-stranded oligonucleotides strongly resisted digestion with DNase I, snake venom phosphodiesterase, and alkaline phosphatase, but the products obtained after this incomplete hydrolysis were readily digested to the monomer level by spleen phosphodiesterase. In concert with this observation, resistance of dAdo adducts to enzymatic release from DNA by digestion

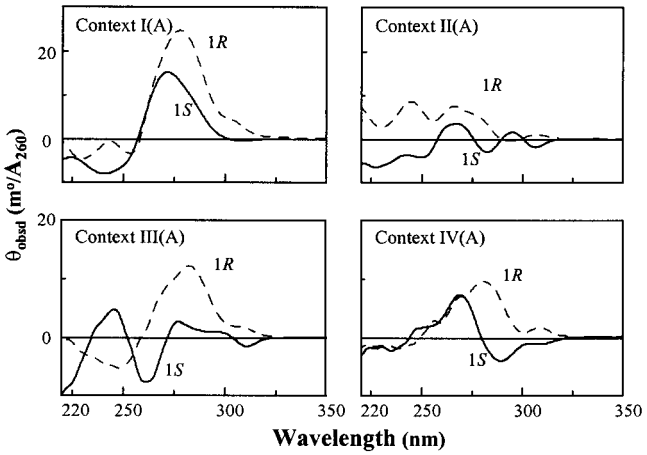

FIGURE 2: Circular dichroism spectra of the synthesized oligonucleotides containing cis-opened B[*c*]PhDE-2 adducts in 0.02 M phosphate buffer, pH 7, with the ionic strength adjusted to 0.1 M with NaCl. Spectra are normalized to 1.0 absorbance unit at 260 nm. Absolute configurations were assigned by enzymatic hydrolysis of both diastereomers of Contexts I(A) and II(A), and of the *early-eluting* diastereomers of III(A) and IV(A), to the known nucleoside adducts (see text).

with snake venom phosphodiesterase (*42*) has previously been found to be greater for DNA containing B[*c*]PhDE−dAdo adducts with 1*R* configuration at the site of adduct attachment than for those with 1*S* configuration (*35*).

The CD spectra of the single-stranded oligonucleotides (two diastereomers of each sequence) are shown in Figure 2. Unlike oligonucleotides containing dAdo adducts of benzo[*a*]pyrene diol epoxides, whose CD spectra generally exhibit long-wavelength pyrene bands which are positive for 10*R* and negative for 10*S* absolute configuration at the point of attachment of the hydrocarbon to the base (*26*), the present B[*c*]PhDE-modified oligonucleotides exhibited no features that were a priori clearly diagnostic of the configuration of the adducts. However, after assignment of configuration by enzymatic hydrolysis, relationships between absolute configuration and the CD spectra of the oligonucleotides became apparent. Notably, the 1*R* oligonucleotide adducts consistently displayed a more intense, positive band at ∼280 nm, analogous to the positive band observed in this region for the monomeric nucleoside adducts (*34*), as well as a positive shoulder near 300 nm.

After purification by HPLC and gel electrophoresis, all adducted oligonucleotide 16-mers and the unsubstituted control sequences were essentially homogeneous, as demonstrated by the electrophoretic analysis of end-labeled aliquots (Figure 3). The presence of the cis B[*c*]PhDE-2−dAdo adducts reduced the electrophoretic mobility of the adducted oligonucleotides in relation to the unmodified control oligonucleotides, but all eight adducted oligonucleotides had similar mobilities. However, in Context I(A), the oligonucleotide with 1*S* configuration at the point of attachment of dAdo to the hydrocarbon moved a little slower than the corresponding oligonucleotide with 1*R* configuration, whereas in Context II(A), this situation was reversed. Relative retention times on reverse-phase HPLC for each pair of diastereomeric oligonucleotides were sequence-dependent (see Table 1). In most cases, the oligonucleotides containing a 1*R* adduct eluted ahead of those containing a 1*S* adduct, but the pair of adducts having the sequence of Context II(A) exhibited the reverse order of elution.

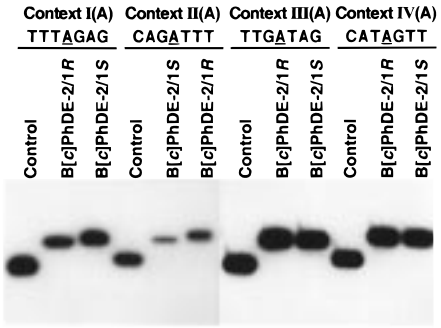

FIGURE 3: Polyacrylamide gel electrophoresis of end-labeled oligonucleotides after purification by HPLC and gel electrophoresis. See Figure 1 for complete 16-mer sequences and structures of cis B[*c*]PhDE-2/1*S* and cis B[*c*]PhDE-2/1*R*. Controls are the unmodified 16-mer sequences.

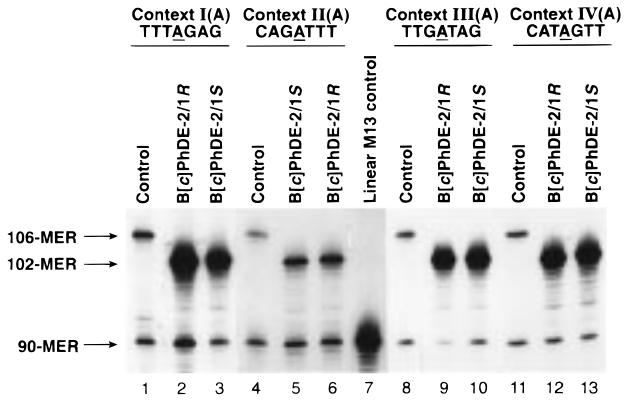

FIGURE 4: Polyacrylamide gel electrophoresis products from [α-^{32}P]-dATP-labeled primer, after primer extension reaction of M13 vectors containing Contexts I−IV(A) using Sequenase; unmodified, cis B[*c*]PhDE-2/1*R* adducted, and cis B[*c*]PhDE-2/1*S* adducted oligonucleotide, as shown.

Ligation of the unadducted 16-mers into the bacteriophage vector M13mp7L2 occurred with 20−40% efficiency (based on the amount of circular DNA as a fraction of the sum of linear and circular DNA). Ligation efficiencies for the B[*c*]-PhDE−dAdo adduct-containing constructs were somewhat lower (∼80−95% of those for the unadducted oligonucleotides). The presence of the oligonucleotide 16-mer in the M13 DNA constructs was verified by primer extension reactions. The primer used was complementary to bases 6365−6348 of the M13mp7L2 genome, and since the M13mp7L2 was cut with *Eco*RI at base 6275, extension of the primer to the *Eco*RI cut yielded a 90-mer product. Extension to the end of the 16-mer insert gave a 106-mer, and extension to the B[*c*]PhDE−dAdo adduct site gave a 102-mer product, as shown in Figure 4. Whereas primer extension, using constructs containing the unsubstituted oligonucleotides as templates, led primarily to the formation of both 90-mers and 106-mers, resulting from some failure of ligation at each end of the insert, all B[*c*]PhDE−dAdo adduct-containing constructs resulted in formation of only 90-mers and 102-mers. The absence of 106-mers indicated that all constructs contained the B[*c*]PhDE−dAdo adduct and that, in all cases, this caused complete failure of the polymerase to progress beyond the adduct. In fact, the polymerase largely failed to incorporate a nucleoside opposite the adduct, as has been reported for benzo[*a*]pyrene diol epoxide−dAdo adducts under similar conditions (*26*).

Table 2: Base Substitution Mutations for the cis B[*c*]PhDE-2/1*R* and cis B[*c*]PhDE-2/1*S* Adducts and Mutation Frequencies in Four Different Sequence Contexts[a]

	cis B[*c*]PhDE-2/1*R*				cis B[*c*]PhDE-2/1*S*			
sequence context	A→T	A→G	A→C	MF_{tot}[b]	A→T	A→G	A→C	MF_{tot}[b]
I(A) TT**T**A**G**AGTCTGCTCCC	38	15	4	0.62 ± 0.16	39	67	2	31.9 ± 5.1
II(A) CA**G**A**T**TTAGAGTCTGC[c]	39	41	2	5.6 ± 1.2	29	146	0	56.5 ± 5.6
III(A) TT**G**A**T**AGTCTGCTCCC	41	14	7	3.6 ±0.90	37	107	4	45.5 ± 5.5
IV(A) CA**T**A**G**TTAGAGTCTGC[d]	25	0	0	0.65 ± 0.25	40	2	5	11.9 ± 3.3

[a] No mutations were found in the TT**G**A**T**AGTCTGCTCCC or in the CA**T**A**G**TTAGAGTCTGC control sequences in the 2460 and 2525 plaques screened, respectively. For the TT**T**A**G**AGTCTGCTCCC control sequence, 1 A→T mutation was found in 10 741 screened plaques and 1 A→G mutation in 6458 screened CA**G**A**T**TTAGAGTCTGC controls. Thus, the background mutation frequency was less than 0.04% in all cases. Total number of plaques screened: TT**T**A**G**AGTCTGCTCCC sequence, 9140 for the 1*R* and 339 for the 1*S* adduct; CA**G**A**T**TTAGAGTCTGC, 1466 for the 1*R* and 310 for the 1*S*; TT**G**A**T**AGTCTGCTCCC, 1720 for the 1*R* and 325 for the 1*S*; and finally, for the CA**T**A**G**TTAGAGTCTGC sequence, 3845 for the 1*R* and 395 for the 1*S* adduct. [b] MF_{tot} is total mutation frequencies in percent ± (2 × the standard error) (for a 95% confidence interval) (*60*). [c] From the 1*S* adduct, one deletion of the adduct was found. [d] From the 1*R* adduct, one deletion of the adduct was found.

When the M13 constructs were transfected into SOS-induced *E. coli*, the adducted constructs consistently gave fewer plaques than the control constructs. In addition, the progeny yield was generally lower for constructs containing the cis B[*c*]PhDE-2/1*S* adducts (13−23% of unadducted control) than for constructs containing the cis B[*c*]PhDE-2/1*R* adducts (18−40% of unadducted control). The adducts that led to the lower progeny yields gave rise to the higher mutation frequencies, as shown in Table 2. The total base substitution mutation frequencies for constructs containing the cis B[*c*]PhDE-2/1*R* adduct were relatively low (0.62−5.6%) compared with those for constructs containing the cis B[*c*]PhDE-2/1*S* adduct (11.9−56.5%). The increased mutagenicity of the 1*S* adduct over the 1*R* adduct ranged from a high of ∼50-fold in Context I(A) to a low of ∼10-fold in Context II(A). However, for either adduct, mutation frequency was higher when the adduct was in a local **G**A**T** context [i.e., in Contexts II(A) or III(A)] than when it was in a **T**A**G** context [Contexts I(A) or IV(A)], suggesting that the immediately adjacent nucleotides were influential in determining mutation frequencies.

The frequency data for each adduct in the various sequence contexts present a less clear picture. For the cis B[*c*]PhDE-2/1*R* adduct, mutation frequencies in the two **T**A**G** sequence contexts were very similar (0.65 and 0.62%, respectively), and they were somewhat higher in the two **G**A**T** sequence contexts (3.6 and 5.6%, respectively). Thus, these data are consistent with a fairly prominent role for the immediately adjacent nucleotides in determining the overall mutation frequency for the cis B[*c*]PhDE-2/1*R* adduct.

For the cis B[*c*]PhDE-2/1*S* adduct, mutation frequencies were more disparate. In the two **G**A**T** contexts [II(A) and III(A)], they were 45.5 and 56.5%, and in the two **T**A**G** contexts [I(A) and IV(A)], they were 11.9 and 31.9%. However, comparison of mutation frequencies for either adduct in constructs with the same distal sequences [i.e., Contexts I(A) and III(A) and Contexts II(A) and IV(A)] suggests that in no case do the distal sequences play a dominant role in determining mutation frequencies. Overall, therefore, the implication of the frequency data is that the immediately adjacent nucleotides have a substantial effect on mutation frequency (frequencies for **G**A**T** were always higher than for **T**A**G** for a given adduct) but that the effect of these immediately adjacent nucleotides can be modulated by nucleotide sequences distal to the adduct. This modulating effect is greater perhaps for constructs containing the cis B[*c*]PhDE-2/1*S* adduct than for those containing the cis B[*c*]PhDE-2/1*R* adduct.

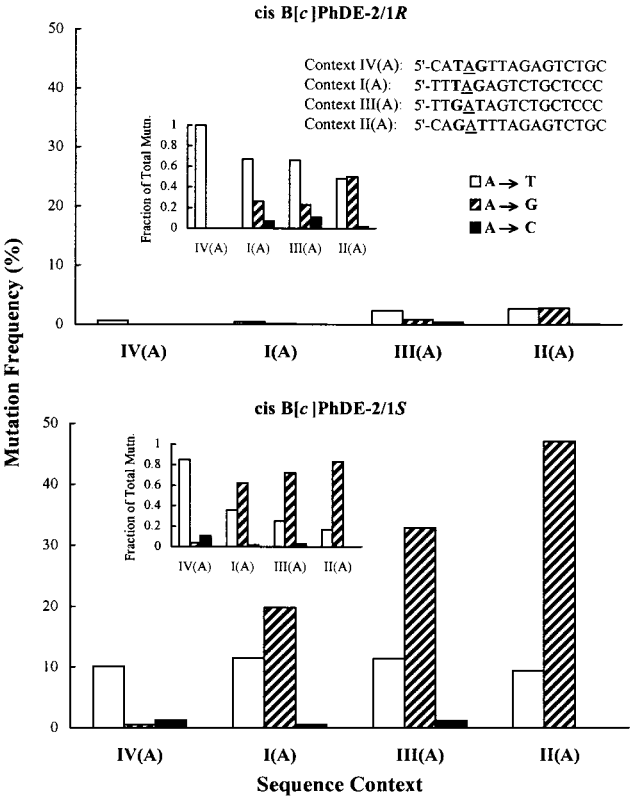

FIGURE 5: Mutation frequencies of base substitution mutations of the cis B[*c*]PhDE-2/1*R*− and cis B[*c*]PhDE-2/1*S*−dAdo adducts in Contexts I−IV(A). Inserted figures: distribution of base substitutions of the cis B[*c*]PhDE-2/1*R*− and cis B[*c*]PhDE-2/1*S*−dAdo adducts in Contexts I−IV(A).

Frequencies and distributions of individual base substitution mutations are summarized in Figure 5. Thus, for the cis B[*c*]PhDE-2/1*R* adduct, A→T mutation frequencies were similar in the two **T**A**G**-containing sequences. Though somewhat higher than the latter, A→T mutation frequencies were also similar in the two **G**A**T**-containing sequences, suggesting that the immediately adjacent nucleotides might exert a dominant effect. However, the frequencies for A→G transition mutations were different in each of the four contexts.

For the cis B[*c*]PhDE-2/1*S* adduct, A→T mutation frequencies were similar in all four sequence contexts, indicating that neither proximal nor distal sequence context exerted a

dominant effect. In contrast, the frequency of A→G mutations was different for each sequence context, but this again suggests that neither the proximal nor the distal context differences dominated the observed mutagenesis.

The distributions of base substitution mutations (insert in Figure 5) for the cis B[*c*]PhDE-2/1*R* adduct were similar in Context I(A) and Context III(A), i.e., two contexts with the same sequences distal to the adduct. Contexts I(A) and III(A) also resulted in similar distributions of mutations for the B[*c*]-PhDE-2/1*S* adduct. Thus, constructs containing the 5′-TT-(**T**/**G**)A(**G**/**T**)AGTCTGCTCCC sequence contexts gave similar distributions of base substitution mutations, indicating that the effect of the distal nucleotide sequence was greater than that of the nucleotides immediately adjacent to the adducts in this regard. A similarity in base change mutation distribution was not observed for the CA(**T**/**G**)A(**G**/**T**)-TTAGAGTCTGC sequences [Context II(A) and Context IV(A)], however. For example, for the cis, B[*c*]PhDE-2/1*R* adduct, only A→T changes were found in one case, and there was an equal distribution of A→T and A→G changes in the other. For the cis B[*c*]PhDE-2/1*S* adduct, the major base change was A→T in Context IV(A) and A→G in Context II(A). Thus, for the CA(**T**/**G**)A(**G**/**T**)TTAGAGTCTGC contexts, the changes in immediately adjacent nucleotides were associated with substantial changes in mutation distribution with **TAG** favoring A→T transversions and **GAT** favoring A→G transitions.

In summary, these findings indicate that for the 1*R* adduct, the nucleotides immediately adjacent to the adduct have profound effects on the overall mutation frequency, whereas neither these nucleotides nor the sequence distal to the adduct independently has an overwhelming effect in the case of the 1*S* adduct. With regard to the frequencies of individual base change mutations, a substantial effect of the nucleotides immediately adjacent to the adduct was noted for A→T changes arising from the 1*R* adduct, but the frequencies of A→G mutations, the only other substantial frequency encountered, were not substantially dominated by either the proximal nucleotides or the distal nucleotides and they, therefore, depended on the combined effects of proximal and distal sequences. For both 1*R* and 1*S* adducts, the distribution of base substitution mutations was similar when the distal nucleotide sequences were the same in Contexts I(A) and III(A) despite differences in the nucleotides immediately neighboring the adducts. However, for Contexts II(A) and IV(A), despite having the same distal nucleotide sequences, one orientation of the immediately neighboring nucleotides skewed the distribution of mutations in favor of A→T transitions whereas the reverse orientation altered the distribution to emphasize A→G transversions (Figure 5). Thus, analysis of the present data indicates that sequence context effects on mutation are attributable to the combined effects of proximal and distal sequences, but that, in some circumstances, either the proximal or the distal sequences can have an apparently dominant effect.

DISCUSSION

Only a few site-specific mutation studies using PAH−dAdo adducts have been reported previously (*13*, *20*−*22*, *43*). Most of these used some version of the M13mp7L2 vector system (*44*), but different bacterial strains have been used for vector replication, or in one case, double-stranded M13 was replicated in a human cell extract (*43*). Lloyd and colleagues have used a repair-deficient *E. coli* strain and have studied trans-opened benzo[*a*]pyrene diol epoxide-1− or -2−dAdo adducts and cis-opened benzo[*a*]pyrene diol epoxide-2−dAdo adducts in a 5′-CGGA**C**A**A**GAAG sequence and found only A→G mutations (*13*). The mutation frequencies were relatively low but were highest for the cis adducts (up to 1.2%). They also reported different mutagenic responses in repair-deficient and repair-proficient *E. coli* strains for benz[*a*]anthracene diol epoxide adducts in the same sequence (*21*). Again, A→G mutations predominated. Min et al. (*22*) used different repair-deficient and repair-proficient strains of *E. coli* and reported a similar response in both strains for trans-opened dibenz[*a*,*j*]anthracene diol epoxide-2−dAdo adducts in a 5′-CT**C**A**C**GCTTCT sequence, i.e., exclusively A→T base substitution mutations (though a few deletions of the C 3′ to the dAdo adduct were also found in the repair-deficient strain; mutation frequencies were about 1.2% in SOS-induced cells). In Page et al. (*20*), trans-opened benzo-[*a*]pyrene diol epoxide−dAdo adducts (DE-1/10*S*, DE-1/10*R*, DE-2/10*S*, and DE-2/10*R*) in a 5′-TT**T**A**G**AGTCTGCTCCC sequence [Context I(A)] gave mostly A→T base substitutions. In a 5′-CA**G**A**T**TTAGAGTCTGC sequence [Context II(A)], these four adducts still gave mainly A→T mutations, but in this sequence, they also resulted in substantially more A→G mutations.

In the present study, the cis B[*c*]PhDE-2/1*R*−dAdo adduct gave either predominantly A→T transversion mutations or a more equal distribution of A→T and A→G base substitutions, depending on the sequence context used. The cis B[*c*]-PhDE-2/1*S*−dAdo adduct was more mutagenic and gave predominantly A→T mutations in one sequence context but predominantly A→G base substitutions in the other three. As mentioned in the introduction, a similar diversity in response has been reported for benzo[*a*]pyrene diol epoxide−dGuo adducts in different sequence contexts (*12*, *14*, *18*). Several reports suggest that the conformation of oligonucleotides containing benzo[*a*]pyrene diol epoxide−deoxyguanosine adducts may vary with sequence context (*45*−*51*), and presumably similar conformational dependence on sequence context might be exhibited by oligonucleotides containing B[*c*]PhDE−dAdo adducts. Loechler has argued that different adduct conformations might be responsible for different mutations arising in different sequence contexts (*52*). If this latter concept is correct, sequence-dependent changes in conformation could account for some of the findings reported herein.

No studies on the conformation of cis-opened B[*c*]PhDE-2−dAdo adducts in DNA have been reported, though some NMR data are available for the corresponding trans-opened B[*c*]PhDE−dAdo adducts in double-stranded oligonucleotides [reviewed in (*53*, *54*)]. However, all dAdo adducts with bulky PAHs studied so far, including a cis adduct from benzo[*a*]pyrene diol epoxide (*54*), have shown modified classical intercalation in a **CAC** sequence. The PAH−dAdo adducts with *R* configuration at the site of attachment to the adenine component were inserted at the 5′-side of the modified adenine, and the ones with *S*-configuration were inserted at the 3′-side. If the cis adducts from B[*c*]PhDE-2 follow this pattern, the cis B[*c*]PhDE-2/1*R* adduct would be intercalated on the 5′-side of the adenine, and the cis B[*c*]-

PhDE-2/1*S* would be intercalated on the 3′-side of the adenine. If these conformations persist in the replication complex, encounters with polymerase during replication would be different for each adduct isomer, and perhaps it is reasonable that orientation of the hydrocarbon residue to the 3′-end of the template (the end from which the polymerase would approach) would be more toxic and mutagenic, as found in this study.

However, it is difficult to extrapolate from spectroscopic studies of adducts in double-stranded DNA to the mutation data because mutations are presumably being generated during replication when the DNA is no longer double-stranded. The existence of structure in *single-stranded* oligonucleotides is suggested by the different sensitivities of the oligonucleotides containing cis B[*c*]PhDE-2−dAdo adducts with either 1*S* or 1*R* configuration to enzymatic hydrolysis, as reported herein. Sensitivity of the 1*S* and resistance of the 1*R* oligonucleotide diastereomers to digestion by DNase I and snake venom phosphodiesterase were consistent with previous observations made on calf thymus DNA modified by reaction with several diol epoxides (*35*). Others have also reported sensitivities of *R* and *S* diastereomeric adducted oligonucleotides to enzymatic hydrolysis that are the same as those reported here (*55*, *56*). An attractive model (*35*) is that *R* dAdo adducts are oriented toward the 3′-end of the single-stranded oligonucleotide being cleaved, and thus block the action of snake venom phosphodiesterase, which proceeds from the 3′- to the 5′-end of the chain, whereas *S* dAdo adducts lie toward the 5′-end, and block the action of spleen phosphodiesterase, which proceeds along the chain in the opposite direction from the 5′- to the 3′-end. These suggested orientations for *R* and *S* dAdo adducts in single-stranded DNA are *opposite* to those generally observed in the NMR studies of double-stranded DNA. This interpretation suggests that the corresponding diastereomers of dGuo and dAdo adducts appear to be oriented in the *same* direction in single-stranded DNA, whereas NMR evidence shows that they are oriented in *opposite* directions (*53*, *54*, *57*, *58*) in double-stranded DNA.

CONCLUSION

The present study represents a first attempt to examine the internucleotide distances over which sequence context effects on base substitution mutations can operate. The data generated from these experiments indicate that there are situations in which the nucleotides immediately adjacent to the adduct have a dominant effect on the mutational response. However, there are related situations in which the surrounding sequences seem to play a more dominant role than the nucleotides immediately adjacent to the adduct. Generally, it seems likely that the combined effects of both distal and proximal nucleotides influence the mutational outcome of the experiment but that in certain circumstances the effects of one or the other seem dominant. Thus, in concert with earlier studies on the frequencies of deletion mutation (*24*, *59*), it is clear that the nucleotides immediately adjacent to an adduct are not the only important contributors to mutational effects. In the experiments reported here, nucleotide sequences were identical beyond 3 nucleotides on the 5′-side of the adduct and beyond 12 nucleotides on the 3′-side of the adduct. Thus, the dramatic sequence context effects reported here must be determined by nucleotides within the 16-nucleotide sequence that was varied. It will be important to determine if sequence changes throughout this window will be able to influence mutation.

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