Targeted A → T and G → T Mutations Induced by Site-Specific Deoxyadenosine and Deoxyguanosine Adducts, Respectively, from the (+)-*anti*-Diol Epoxide of Dibenz[*a,j*]anthracene in M13mp7L2[†]

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ABSTRACT: The studies described in this report directly examined the mutagenicity in *Escherichia coli* of both a deoxyadenosine (dAdo) and a deoxyguanosine (dGuo) adduct derived from (+)-*anti*-dibenz[*a,j*]-anthracene-3,4-diol 1,2-epoxide [(+)*anti*-DB[*a,j*]A-DE] that were site-specifically placed in a single-stranded M13mp7L2 replication vector. An 11-base oligonucleotide (5′-CTC ACG CTT CT-3′) containing either a single (+)*anti*-DB[*a,j*]A-DE−*trans*-N^2-dGuo or (+)*anti*-DB[*a,j*]A-DE−*trans*-N^6-dAdo adduct was successfully incorporated into single-stranded M13mp7L2 plasmid via ligation. In vitro studies using *E. coli* DNA polymerase I (Klenow fragment) indicated that both adducts were effective blocks for polymerase action. *E. coli* strains JM103 and JM103 *uvrA*6 were subsequently transformed with control (unadducted) and adduct-containing M13mp7L2 constructs followed by analysis of progeny DNA. In both JM103 and JM103 *uvrA*6 cells, plaque yields were markedly reduced with adduct containing vectors compared to control vectors. Activation of the inducible bacterial DNA repair system (SOS) by UV light only slightly increased the number of plaques recovered from either bacterial strain transformed with adduct-containing vectors. Targeted mutations were obtained with both adduct-containing vectors in both bacterial strains, whereas no mutations were detected in plaques recovered from control M13mp7L2 vectors. In JM103 cells, (+)*anti*-DB[*a,j*]A-DE−N^6-dAdo induced exclusively A → T transversions and (+)*anti*-DB[*a,j*]A-DE−N^2-dGuo induced exclusively G → T transversions. In JM103 *uvrA*6 cells, similar targeted transversion mutations were also obtained except that a few C deletions (i.e., ∼10% of the mutations) were detected immediately 3′ to the dAdo adduct. While mutagenesis was SOS dependent in JM103 cells [<0.15% (-SOS) vs ∼1.3% (+SOS)], it appeared to be SOS independent in JM103 *uvrA*6 cells (∼1−2% in the presence or absence of SOS induction). It is argued that adduct-induced G → T mutations can be rationalized by either misinformational or noninformational mechanisms. In contrast, A → T mutations are unlikely to arise via a misinformational pathway, which provides the strongest support to date that bulky DNA adducts can induce mutations via a noninformational pathway.

Polycyclic aromatic hydrocarbons (PAHs)[1] are widespread environmental contaminants and have been implicated in human cancer for over 200 years (Cartwright, 1984). PAHs must be metabolically activated to reactive electrophilic intermediates to exert their biological effects (Dipple et al., 1984; Hall & Grover, 1990) and, in general, are metabolically activated by the mixed function oxidases in a two-step oxidation process, ultimately forming bay-region diol epoxides. The electrophilic diol epoxides bind covalently to nucleophilic macromolecules in the cell, especially DNA, leading to the induction of mutations and the initiation of cancer (Dipple et al., 1984; Hall & Grover, 1990; Pelkonen & Nebert, 1982).

Our laboratories have been studying the biological activity of the environmentally prevalent PAHs, benzo[*a*]pyrene (B[*a*]P) (Jelinsky et al., 1995; Mackay et al., 1992; Rodriguez & Loechler, 1993a,b) and dibenz[*a,j*]anthracene (DB[*a,j*]A) (DiGiovanni et al., 1983; Harvey et al., 1988; Sawyer et al., 1988). DB[*a,j*]A (Figure 1), a nonalternant PAH, is an effective tumor initiator in mouse skin although less potent than B[*a*]P (DiGiovanni et al., 1983; Sawyer et al., 1988). This compound, like other PAHs, appears to generate its carcinogenic action through metabolic conversion of the parent hydrocarbon into diol epoxide derivatives (Baer-Dubowska et al., 1995; Nair et al., 1991; Sawyer et al., 1988). In particular, the (+)-*anti*-diol epoxide (Figure 1) is a more potent tumor initiator than the parent compound (Harvey et al., 1988; Sawyer et al., 1988). The *anti*-diol epoxide of DB[*a,j*]A binds extensively to both dGuo and dAdo residues when reacted in vitro with calf thymus DNA (ratio of ∼3:1, respectively) (Chadha et al., 1989; Nair et al., 1989). The major reaction products obtained with either racemic or pure (+)*anti*-DB[*a,j*]A-diol epoxide [(+)*anti*-DB[*a,j*]A-DE] arise

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[1] Abbreviations: PAHs, polycyclic aromatic hydrocarbons; (+)*anti*-DB[*a,j*]A, (+)-*anti*-dibenz[*a,j*]anthracene; (+)*anti*-DB[*a,j*]A-DE, (+)-*anti*-dibenz[*a,j*]anthracene-3,4-diol 1,2-epoxide; dAdo, deoxyadenosine; dGuo, deoxyguanosine; DMBA, 7,12-dimethylbenz[*a*]anthracene; ss, single-stranded; ds, double-stranded; kb, kilobase; bp, base pair; TEAA, triethylammonium acetate; ACN, acetonitrile; PEG, polyethyene glycol; TE, 10 mM Tris-base, 1 mM ethylenediaminetetraacetic acid, pH 8.0; dNTPs, deoxynucleotides; SOC, 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, and 0.37% glycose; PAGE, polyacrylamide gel electrophoresis.

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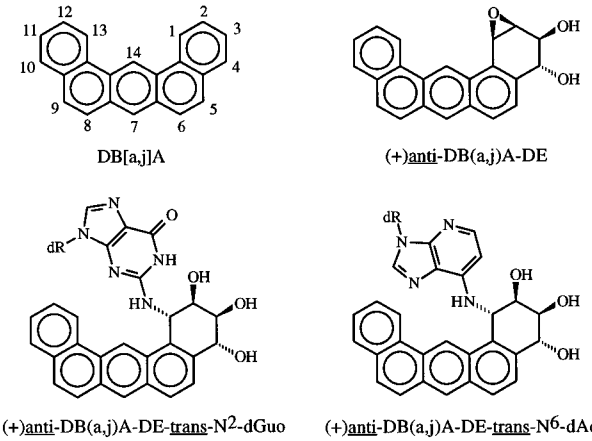

FIGURE 1: Chemical structures of DB[*a,j*]A, (+)*anti*-DB[*a,j*]A-DE, and the *trans*-N^2-dGuo and *trans*-N^6-dAdo adducts derived from (+)*anti*-DB[*a,j*]A-DE.

through *trans* addition of the exocyclic amino groups of dGuo and dAdo (Figure 1). Treatment of cultured mouse keratinocytes with DB[*a,j*]A produced adducts with both dGuo and dAdo residues covalently bound to the (+)-enantiomer of the *anti*-diol epoxide where the dAdo adducts were predominant (Nair et al., 1991). The (+)*anti*-DB[*a,j*]A-DE−*trans*-N^6-dAdo adduct was also found in mouse epidermal DNA after topical application of DB[*a,j*]A (Baer-Dubowska et al., 1995). The fact that c-Ha-ras mutations in skin tumors resulting from topical application of DB[*a,j*]A (Gill et al., 1992) revealed exclusively $A^{182} \rightarrow T$ transversions at codon 61 suggests an important role for dAdo adducts in tumorigenesis by this PAH. A useful approach to test further this hypothesis is through the use of a site-directed technique to determine directly whether specific DNA adduct species are capable of producing specific mutations in defined sequence contexts. This is particularly important for PAH dAdo adducts, for which little information currently exists (Chary et al., 1995).

Extensive studies of different carcinogenic compounds have revealed a strong correlation between mutagenicity and carcinogenicity (Andrews et al., 1978; Pelkonen & Nebert, 1982). Reactive metabolites of B[*a*]P are mutagenic (inducing base pair and insertion/deletion mutations) in both bacterial and mammalian systems (Bernelot-Moens et al., 1990; Brookes & Osborne, 1982; Carothers et al., 1990; Chen et al., 1990; Eisenstadt et al., 1982; Mackay et al., 1992; Malaveille et al., 1977; Mizusawa et al., 1981; Newbold & Brookes, 1976; Recio et al., 1987; Rodriguez & Loechler, 1993a,b; Roilides et al., 1988; Yang et al., 1987). The overall spectrum of mutations arising from the (+)-*anti*-diol epoxide of B[*a*]P [(+)*anti*-BPDE] is similar in bacteria and mammalian systems; the most frequent mutations are GC → TA transversions, although AT → TA and GC → CG mutations are also seen (Bernelot-Moens et al., 1990; Carothers & Grunberger, 1990; Chen et al., 1990; Eisenstadt et al., 1982; Yang et al., 1987). Mutagenesis of pure (+)-*anti*-BPDE was very recently examined in bacteria (Jelinsky et al., 1995; Mackay et al., 1992; Rodriguez & Loechler, 1993a,b). In particular, studies with site-directed vectors containing a single (+)*anti*-BPDE−*trans*-N^2-dGuo adduct yielded only G → T transversions in a 5′-TGC-3′ sequence context, but G → T, G → A, and G → C in a 5′-CGG-3′ sequence context (Jelinsky et al., 1995; Mackay et al., 1992). Recently, we reported the mutagenic spectrum of (+)*anti*-DB[*a,j*]A-DE (Figure 1) using a simple forward mutation system with the *supF* gene as the target (Gill et al., 1993c). Base substitution mutations predominated, particularly G → A transitions, but G → T and G → C transversions also were observed. Interestingly, the frequency of mutations at A·T base pairs in SOS-induced cells (~6% when normalized for target size) was lower than expected since the (+)*anti*-DB[*a,j*]A-DE−N^6-dAdo adduct (Figure 1) accounted for ~25% of all adducts in the pUB3 plasmid used in these studies.

To investigate mutagenesis by adducts derived from (+)-*anti*-DB[*a,j*]A-DE, we report on the construction of a single-stranded M13mp7L2 vector containing either a single dAdo or a dGuo adduct derived from (+)*anti*-DB[*a,j*]A-DE at a defined site. Replication of this vector in bacteria has allowed an initial evaluation of the mutagenic potency and specificity of these adducts. The data indicate that both adducts induced targeted transversion mutations (i.e., G → T and A → T transversion mutations for the dGuo and dAdo adducts, respectively) in the context of the specific sequence chosen for this study.

MATERIALS AND METHODS

Materials. (+)*anti*-DB[*a,j*]A-DE was synthesized as previously described (Harvey et al., 1988; Nair et al., 1989). This compound was stored at −20 °C in crystalline form. The 11-mer and 51-mer oligonucleotides were obtained commercially through National Biosciences, Inc. (Plymouth, MN). Snake venom phosphodiesterase (*Crotalus atrox*, type II) and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). *Eco*RI, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were obtained from New England Biolabs (Beverly, MA); T4 DNA ligase was purchased from Promega (Madison, WI). The *Escherchia coli* strains JM103 (F′ tranD36 $proAB^+$ $lacI^q$ lacZdeltaM15 delta (pro-lac) supE hsdR endA1 sbcB15 thi-1 strA lambda-] and JM103 *uvrA*6 (repair-deficient JM103, $uvrA^-$) were obtained from American Type Culture Collection (Rockville, MD). The 21-mer and 22-mer oligonucleotide probes used for screening plaques were obtained from Operon Technologies Inc. (Alameda, CA). Sequenase version 2.0 DNA-sequencing kit was purchased from Amersham Life Science Inc. (Arlington, IL). [γ-^{32}P]ATP and [α-^{35}S]-dATP were obtained from DuPont NEN (Boston, MA). Single-stranded M13mp7L2 was generously provided by Dr. C. W. Lawrence, Department of Biochemistry, University of Rochester School of Medicine and Dentistry. M13mp7L2 was amplified in *E. coli* JM101 K12 and purified by polyethylene glycol (PEG) precipitation of the supernatant. All other chemicals and reagents used were either molecular biology grade or high-performance liquid chromatography (HPLC) grade. All experimental procedures were carried out under yellow lights in order to minimize photodegradation of the chemical compounds and toxicity to UV-light sensitive cells.

Preparation of Oligonucleotides Containing either a Single (+)anti-DB[a,j]A-DE dAdo or dGuo Adduct. Prior to chemical modification with (+)*anti*-DB[*a,j*]A-DE, the 11-base oligonucleotide (5′-CTC ACG CTT CT-3′) was purified by reverse phase HPLC on an Altex ultrasphere octadecylsilane (ODS) column (10 × 25 cm) using a Shimadzu LC-6A high-performance liquid chromatography equipped with a SPP-6A UV detector (254 nm). The mobile phase was 0% acetonitrile (ACN) in 0.1 M triethylammonium acetate (TEAA) (pH 7.0) for 10 min and then 0−10% ACN

in 0.1 M TEAA over 10 min at a flow rate of 4.7 mL/min. The purified 11-mer was collected and dried in a Speed-Vac apparatus (Savant Instruments), redissolved in 50 mM potassium acetate (pH 5.2), and quantitated by determination of OD_{260}.

Approximately 1 mg of purified 11-mer was reacted with 300 μg of (+)*anti*-DB[*a*,*j*]A-DE [in 5 mL of 50 mM potassium acetate (pH 5.2)] at room temperature for 18 h with gentle mixing. The reaction was stopped by four consecutive extractions with *n*-butanol followed by two extractions with anhydrous ether to remove any unreacted diol epoxide or its hydrolysis products. Nitrogen gas was then used to evaporate residual anhydrous ether in the reaction solution.

Purification and Identification of Adducted Oligonucleotides. To separate the adducted oligonucleotides, aliquots of the reaction mixture were subjected to reverse phase HPLC on the same ODS column described above using both fluorescence detection (excitation, 293 nm; emission, 405 nm) and UV detection (254 nm). Separation was achieved using the following gradient system at a flow rate of 4.7 mL/min: step 1, 0−15% ACN in TEAA over 15 min; step 2, 15−20% ACN in TEAA over 45 min; and step 3, 20−30% ACN in TEAA over 40 min. Individual peaks obtained from HPLC runs were digested to mononucleosides sequentially using snake venom phosphodiesterase, followed by alkaline phosphatase digestion. The adducted mononucleosides were separated from unadducted mononucleosides using C_{18} Sep-Pak cartridges (DuPont NEN, Boston, MA). The samples containing the adducts were dried in a Speed-Vac apparatus and redissolved in 65% methanol in water. The DNA adducts were identified by cochromatography with authentic (+)*anti*-DB[*a*,*j*]A-DE DNA adduct markers as described previously (Nair et al., 1989).

Once identified, the adducted oligonucleotides of interest were subjected to electrophoresis to purify them further prior to vector construction. In this regard, the adducted and unadducted oligonucleotides were 5′-end-labeled using [γ-^{32}P]-ATP, followed by electrophoresis in a 20% native polyacrylamide gel. The gel was then exposed to X-ray film (Kodak X-OMAT-AR) for 20 min. A single band containing the corresponding adducted or unadducted oligonucleotide was excised, resuspended in oligonucleotide elution buffer [0.5 M ammonium acetate, 10 mM magnesium acetate (pH 6.7), and 0.1% sodium dodecyl sulfate (SDS)], and incubated overnight at 37 °C with shaking. The samples were then recovered according to published procedures (Sambrook et al., 1989). A small portion of each purified oligonucleotide was applied to a 20% polyacrylamide gel, followed by autoradiography to confirm its purity prior to use.

Construction of Single-Stranded M13mp7L2 Vectors Containing Site-Specific DNA Adducts. ss M13mp7L2 vectors containing either (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo or (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^6-dAdo adducts were constructed following procedures similar to those previously reported (Banerjee et al., 1990), and construction is summarized in Figure 2. Single-stranded M13mp7L2 (100 μg) was treated with *Eco*RI (8 u/μg of DNA) at room temperature overnight and then separated on a 1% agarose gel. Linearized M13mp7L2 was excised and recovered by electroelution at 100 V for 55 min, and the samples were then extracted with phenol/chloroform. Following purification, ^{32}P-5′-end-labeled oligonucleotides were annealed with a 2-fold excess

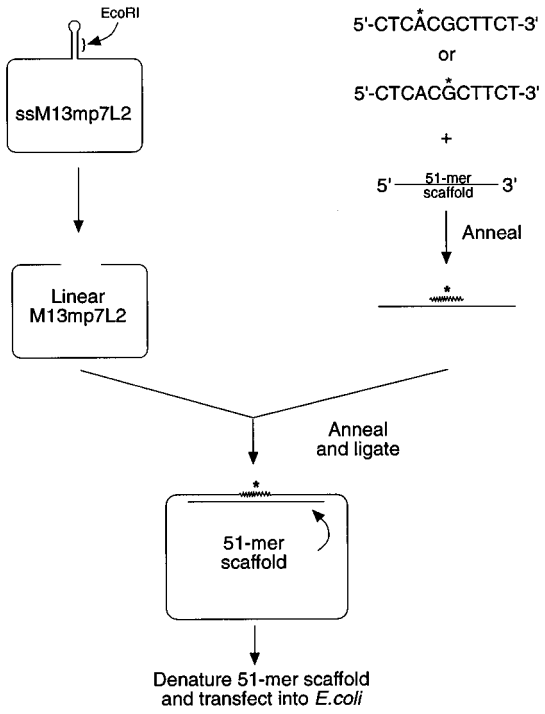

FIGURE 2: Strategy for construction of site-specific vectors using M13mp7L2. ss M13mp7L2 was linearized with *Eco*RI and purified by electroelution. Each purified adducted or unadducted 11-mer was annealed with a 51-mer scaffold at room temperature over 5 h before ligation into linear ss M13mp7L2. The ligation reaction was carried out at 16 °C overnight in the presence of 0.15 u/μL (final concentration) T4 DNA ligase. The ligation products were then purified in a Microconcentrator-30, followed by extraction with phenol/chloroform. The circular DNA constructs were heated at 80 °C for 3 min to denature the 51-mer scaffold prior to transformation of *E. coli.*

of a 51-mer scaffold whose internal sequence was complementary to the 11-mer. The annealing reaction was carried out in 25 mM NaCl at an initial temperature of 80 °C for 5 min followed by room temperature for at least 5 h. Approximately 4.8 μg of purified linear M13mp7L2 was denatured at 80 °C for 5 min and then chilled on ice for 10 min prior to addition of the annealed oligonucleotide mixture (100-fold molar excess). The ligation reaction was initiated by addition of 0.15 u/μL T4 polynucleotide ligase (final concentration), and the mixture was incubated overnight at 16 °C. The ligated products were purified using a Microconcentrator-30 (Amicon, Inc., Beverly, MA) to remove any unannealed 11-mer and 51-mer scaffold, followed by extraction with phenol/chloroform. Two sets of each of the purified ligation products were prepared for a 1% agarose gel to verify that the ligation was successful; one was heated at 80 °C for 3 min to denature the 51-mer scaffold from the ligated vectors prior to electrophoresis, and the other was not. Ligation efficiencies for each 11-mer were calculated as follows. The radioactivity of the ligation product was converted to picomoles of ligation product, and then picomoles of ligation product was divided by the initial picomoles of linear M13mp7L2 used in the ligation reaction.

DNA Polymerase Stop Assay. Approximately 0.5 μg of each ligated product was annealed to 0.5 pmol of a ^{32}P-5′-end-labeled universal primer (5′-GTTTTCCCAGTCACGAG-3′) that was complementary to M13mp7L2 at approximately 40 bp upstream of the 11-mer insertion position and then incubated with 10 u of DNA polymerase I (Klenow fragment) and dNTPs to a final concentration of 20 μM in

reaction buffer at 37 °C for 15 min. The reaction was stopped by the addition of the loading dye (95% formamide with xylene cyanol and bromophenol blue). The samples were heated to 95 °C for 3 min, placed on ice for 10 min, and then run on a 8% polyacrylamide/8 M urea sequencing gel for analysis.

SOS Induction and Transformation of E. coli JM103 and JM103 uvrA6 Cells. Fresh cultures of both JM103 and JM103 *uvrA*6 were made competent for transformation as previously described (Gill et al., 1992). Briefly, the cells were divided into two groups: SOS-induced and non-SOS-induced. A 254 nm germicidal lamp was used to induce a SOS response in both JM103 and JM103 *uvrA*6. The UV dose was predetermined in a series of Weigle reactivation experiments (data not shown). For JM103, the UV dose was 45 J/m^2; for JM103 *uvrA*6, the UV dose was 8 J/m^2. UV dosage to cells was monitored with a UVX radiometer (UVP, Inc., San Gabriel, CA). Approximately 2 ng of purified ss M13mp7L2 vector construct was heated at 80 °C for 3 min to remove the 51-mer scaffold from the ligated constructs and then immediately mixed with 100 μL of competent JM103 or JM103 *uvrA*6 cells. Transformation was accomplished by electroporation as described previously (Gill et al., 1992). Following electroporation, cells were allowed to recover for 1 h and 30 min at 37 °C.

Analysis of Progeny DNA Using Differential Hybridization Screening. Individual plaques were randomly selected and transferred to duplicate gridded plates seeded with a lawn of approximately 2 × 10^6 cells of either JM103 or JM103 *uvrA*6. After overnight incubation, one set of plates was blotted with 0.45 μm, 85 mm nylon membranes (MSI, Westborough, MA), followed by cross-linking with UV light. The membranes were soaked in 2 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate buffer) for 5 min with gentle shaking to break cells before prehybridization. Prehybridization was carried out in 6 × SSC, 5 × Denhardt's solution, and 5 μg/mL salmon sperm DNA at 37 °C for 2 h. A ^{32}P-5′-end-labeled probe (P_{21-3}, 5′-CCA GTG AAT TAG AAG CGT GAG-3′) that spanned the site of ligation was added to the solution and allowed to hybridize at 37 °C overnight. Under low-stringency conditions, membranes were washed in 6 × SSC twice at 37 °C for 20 min and exposed to X-ray film overnight. Following the first exposure, membranes were subjected to a high-stringency wash in 1 × SSC/0.1% SDS at 57 °C for 20 min, followed by a 2 min wash at 62 °C, and then exposed to X-ray film for 2 days. The plaques that gave an obvious signal on the first film and a very faint or, in most cases, no signal on the second film were selected and sequenced. A second probe (P_{22-2}, 5′-GCC AGT GAA TTC ACT GAA TCA T-3′) was used to detect self-ligated DNA that did not contain the 11-mer insertion. Progeny DNA of this type was excluded from the data. The hybridization and washing conditions were the same as those for the P_{21-3} probe described above.

Isolation and Sequencing of Differentially Hybridized Plaque DNA. Individual plaques that gave a differential hybridization signal were suspended in 2 mL of LB (Luria-Bertani) broth and allowed to stand at room temperature for 2 h to release the plasmid DNA from the top agarose. Overnight culture of either JM103 or JM103 *uvrA*6 cells (50 μL) was added to the cell suspension and incubated at 37 °C with vigorous shaking overnight. The plasmid DNAs from overnight cultures were then purified by polyethylene glycol (PEG) precipitation and phenol/chloroform/isoamyl alcohol extraction. The isolated DNA was sequenced using the dideoxy chain termination method (Sanger, 1977) and a Sequenase Version 2.0 Kit according to the protocol given by the manufacturer. The sequencing primer was complementary to a sequence 40 bp upstream from the 3′ ligation site of M13mp7L2.

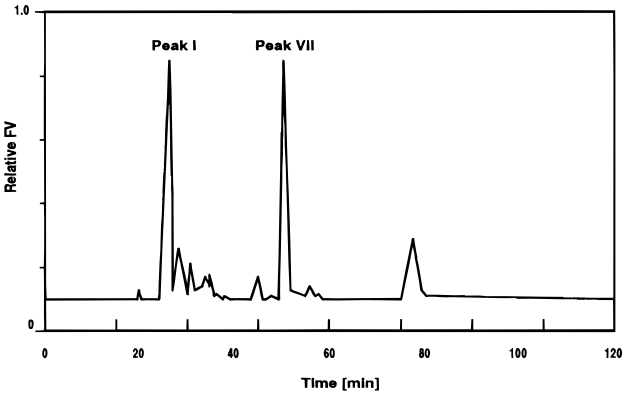

FIGURE 3: Separation of products obtained from reaction of (+)-*anti*-DB[*a*,*j*]A-DE with 5′-CTC ACG CTT CT-3′. The 11-mer (1.0 mg) was reacted with 300 μg of pure (+)*anti*-DB[*a*,*j*]A-DE for 18 h. The reaction was stopped by extraction with ethyl acetate followed by anhydrous ether to remove any unreacted diol epoxide or hydrolysis products. The adducted oligonucleotide products obtained were separated by reverse phase HPLC with a fluorescence detector (excitation, 293 nm; emission, 405 nm). The following gradient system was used to separate the adducted oligonucleotides: step 1, 10−15% ACN in TEAA over 15 min; step 2, 15−20% ACN in TEAA over 45 min; and step 3, 20−30% ACN in TEAA over 40 min. Peak I was identified as the 11-mer containing (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo; Peak VII was identified as the 11-mer containing (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^6-dAdo.

RESULTS

Initial Purification and Identification of Adducted Oligonucleotides. The 11-mer (5′-CTC ACG CTT CT-3′) was purified and reacted with (+)*anti*-DB[*a*,*j*]A-DE as described in Materials and Methods. The reaction mixtures were separated by reverse phase HPLC with fluorescence detection (excitation, 293 nm; emission, 405 nm) also as described in Materials and Methods. The adducted oligonucleotides were well-separated in this system as shown in Figure 3. Each individual peak was collected and dried, digested to mononucleosides using snake venom phosphodiesterase and alkaline phosphatase, and then passed through a C_{18} Sep-Pak cartridge. The DNA adducts (collected in the methanol phase after separation on the C_{18} Sep-Pak cartridge) were identified by cochromatography with authentic (±)*anti*-DB[*a*,*j*]A-DE DNA adduct markers (Figure 4A) using HPLC as previously described (Nair et al., 1989,1992). Peak I was identified as the 11-mer containing the (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo adduct (Figure 4B); peak VII was identified as the 11-mer containing the (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^6-dAdo adduct (Figure 4C).

Further Purification and Analysis of ^{32}P-End-Labeled Unadducted and Adducted Oligonucleotide. A 20% native PAGE was used to purify further the adducted oligonucleotides after the initial purification on HPLC. Following ^{32}P end labeling, the purity of oligonucleotides was then verified by a 20% PAGE followed by autoradiography. Only one band corresponding to the adducted oligonucleotide was detected on an overexposed film (Figure 5, >99.99% pure).

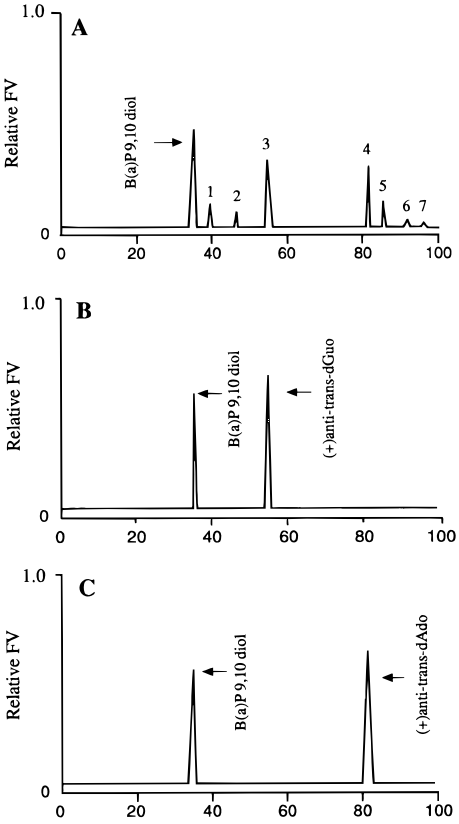

FIGURE 4: Identification of (+)- or (−)*anti*-DB[*a,j*]A-DE DNA adducts. (A) Marker DNA adducts prepared from calf thymus DNA reacted with (±)*anti*-DB[*a,j*]A-DE: peak 1, (−)*trans*-dGuo adduct; peak 2, (+)*cis*-dGuo adduct; peak 3, (+)*trans*-dGuo adduct; peak 4, *trans*-dAdo adduct; peak 5, (−)*trans*-dAdo adduct; peak 6, (−)-*cis*-dAdo adduct; and peak 7, (+)*cis*-dAdo adduct. (B) The adduct from peak I in Figure 3 following digestion with snake venom phosphodiesterase and alkaline phosphatase comigrates with the (+)*anti*-DB[*a,j*]A-DE−*trans*-N^2-dGuo adduct marker. (C) The adduct from peak VII in Figure 3 following digestion with snake venom phosphodiesterase and alkaline phosphatase comigrates with the (+)*anti*-DB[*a,j*]A-DE−*trans*-N^6-dAdo adduct marker. The B[*a*]P-9,10-diol was included as a marker in all three HPLC chromatograms shown. Adducted mononucleosides were separated from unmodified mononucleosides using C_{18} Sep-Paks. The gradient was 40% methanol in water for 50 min, 40−60% methanol in water for 50 min, and 60−100% methanol in water for 15 min, at a flow rate of 1 mL/min.

Note also in Figure 5 that the adducted oligonucleotides showed a decreased mobility in the gel. In addition, the dGuo adduct retarded migration to a greater extent than the dAdo adduct, which may be the result of conformational differences between the dAdo and dGuo adducts (Cosman et al., 1992, 1993a,b, 1995; de los Santos et al., 1993; Grunberger et al., 1979; Kakefuda et al., 1978; Schurter et al., 1995). No detectable instability of either adducted oligonucleotide was observed after storage for three months in the dark at −20 °C. The purified and quantitated oligonucleotides were then used to construct the ss M13mp7L2 vectors for mutagenesis studies.

Ligation of the Unadducted and the Adducted Oligonucleotides into M13mp7L2. The oligonucleotides containing either (+)*anti*-DB[*a,j*]A-DE−*trans*-N^2-dGuo or (+)*anti*-DB[*a,j*]A-DE−*trans*-N^6-dAdo adducts and the unadducted 11-mer were annealed to a 51-mer scaffold and then ligated into purified linear ss M13mp7L2 (Figure 2). Ligation products were analyzed on a 1% agarose gel (Figure 6A, ethidium bromide-stained gel; Figure 6B, autoradiogram of

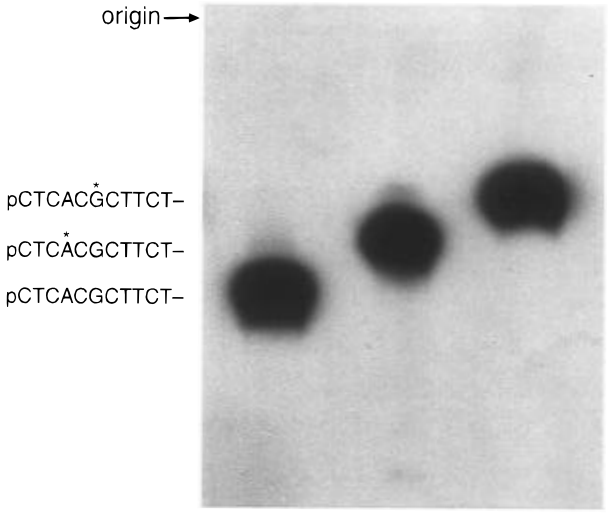

FIGURE 5: Characterization of ^{32}P-end-labeled adducted oligonucleotides by 20% PAGE after purification. The figure shows an overexposed autoradiogram of the gel used to confirm the purity of the oligonucleotides before ligation. * denotes the position of the adduct.

gel shown in panel A). The presence of circular ss DNA (top bands) indicated that the oligonucleotides were ligated into both the 5′ and 3′ ends of the linear ss M13mp7L2 vector. The lower bands are linear DNA containing the oligonucleotides ligated at only one end (either 5′ or 3′) of the linear M13mp7L2. The ligation efficiency for the adducted oligonucleotides was 4.0 and 3.1% for the dAdo- and dGuo-containing M13mp7L2 constructs, respectively, values ∼10-fold lower than that of the unadducted 11-mer (33.3%), as expected.

Characterization of M13mp7L2 Vectors Containing either the (+)anti-DB[a,j]A-DE−trans-N^2-dGuo (dGuo-M13mp7L2) or (+)anti-DB[a,j]A-DE−trans-N^6-dAdo (dAdo-M13mp7L2) Adducts. Previous preliminary experiments indicated that dGuo and dAdo adducts derived from (+)*anti*-DB[*a,j*]A-DE could effectively block DNA replication when inserted into M13mp19 vectors in a different sequence context (Gill et al., 1993a). In order to extend these earlier results with the present constructs and confirm that the dAdo or dGuo adducts of interest in our present study were located at the expected position after construction of the modified M13 genomes, polymerase stop assays were performed. As shown in Figure 7, DNA polymerase termination was observed primarily at the corresponding adduct site in both of the adduct-containing constructs. The exact sites of termination were determined by comparison with the DNA-sequencing ladder of the control M13mp7L2 construct in the adjacent lanes. Careful examination of Figure 7 also suggests that a small amount of translesional synthesis was observed with the dGuo adduct, although further work is required to confirm this observation.

Plaque Yields from Modified and Unmodified M13mp7L2 Constructs in SOS- and Non-SOS-induced cells. The plaque yields from M13mp7L2 constructs were quantified in SOS-induced and non-SOS-induced cells (both JM103 and JM103 *uvrA*6) after electroporation and are summarized in Table 1. Equal amounts of M13mp7L2 constructs, quantitated by agarose gels, and radioactivity incorporated into M13mp7L2 constructs were used for electroporation. Plaque yields from control M13mp7L2 constructs were considered as 100% in order to eliminate any inherent plaque-forming background due to the ligation event. Plaque yields from both of the

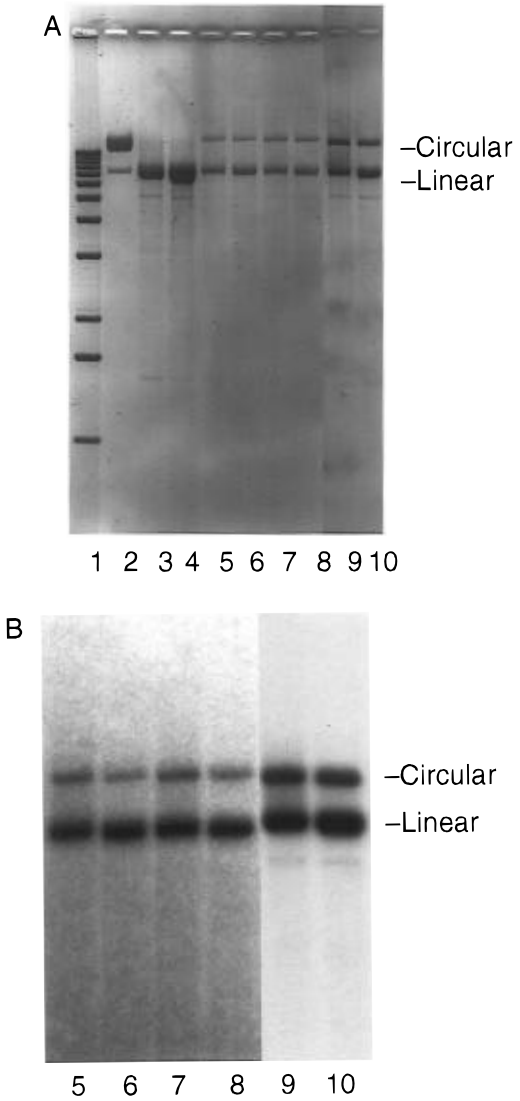

FIGURE 6: Analysis of M13mp7L2 ligation products by agarose gel electrophoresis and autoradiography. (A) Agarose gel (1%) of ligation products stained with ethidium bromide: lane 1, 1 kb DNA ladder (Gibco, BRL); lane 2, circular ss M13mp7L2 DNA marker; lanes 3 and 4, linear ss M13mp7L2 marker; lanes 5 and 6, ligation products containing the dAdo-adducted 11-mer; lanes 7 and 8, ligation products containing the dGuo-adducted 11-mer; lanes 9 and 10, ligation products containing the unmodified 11-mer; lanes 5, 7, and 9, no heat; and lanes 6, 8, and 10, DNA was heated to 80 °C for 3 min before the gel was loaded. (B) Autoradiogram of the gel shown in panel A. Lanes 5−10 in panel B correspond to lanes 5−10 in panel A.

adducted M13mp7L2 constructs were significantly lower than the plaque yield from the control M13mp7L2 construct. This was true for both bacterial strains and regardless of whether the SOS response had been induced. These results are consistent with the results from the DNA polymerase stop assays which indicated effective blockage of DNA replication by the adducted oligonucleotides. Relative plaque yields in JM103 were higher for both SOS- and non-SOS-induced groups compared with JM103 *uvrA*6 cells. In addition, plaque yields for both the dAdo-M13mp7L2 and dGuo-M13mp7L2 constructs were slightly higher in SOS-compared to non-SOS-induced cells using both JM103 and *uvrA*6 cells. Plaque yields for the dAdo-M13mp7L2 construct were also slightly lower than that of the dGuo-M13mp7L2 construct again regardless of SOS status in the bacteria.

Differential Hybridization Screening of Progeny DNA. An efficient differential hybridization method was developed to screen for mutations in progeny DNA. In this method, a ^{32}P-end-labeled 21-mer probe which included the sequence complementary to the 11-mer was used. Under low stringency, the probe hybridized to all of the progeny DNA containing the sequence corresponding to the originally inserted 11-mer. With these conditions, the probe hybridized to all progeny DNA containing either a perfect match or a single base substitution or deletion mutation within the 11-mer sequence of interest. After low-stringency hybridization, washing, and subsequent autoradiography, the membranes were then washed under high-stringency conditions. High-stringency conditions consisted of washing in 1 × SSC/0.1% SDS at 57 °C for 20 min and then at 62 °C for 2 min. Following high-stringency washing, membranes were subjected to a second round of autoradiography. After high-stringency washing, the progeny DNA that contained a single mutation or deletion within the 11-mer sequence no longer hybridized to the probe or exhibited only a very low signal as shown in Figure 8, panels B and D. Only the progeny DNA containing the normal sequence remained bound tightly to the probe and exhibited a strong signal on the second film. In separate experiments not involving the adducts, we constructed G → T, C, and A, as well as A → T, G, and C, mutants and showed that each could be detected by our mutant identification strategy. The G → T mutation in panel B of Figure 8 shows representative data.

Mutation Frequency and Spectra. Using the method described in the preceding section, 3886 plaques from JM103 and 5698 plaques from JM103 *uvrA*6 cells transformed with either adducted or control M13mp7L2 constructs were screened. The DNA sequence of representative mutants is shown in Figure 9, and the results are summarized in Tables 2 and 3. No mutations were observed in control constructs with or without SOS induction in either bacterial strain ($<^1/_{530}$ and $<^1/_{568}$ in JM103 and $<^1/_{622}$ and $^1/_{480}$ in JM103 *uvrA*6, respectively). In contrast, targeted mutations were obtained with both adduct-containing constructs. Notably, targeted mutations in JM103 were highly dependent on SOS induction. In the absence of SOS induction, only one targeted mutation was obtained with the dGuo-M13mp7L2 construct (0.15%, $^1/_{638}$) and no mutations were found with the dAdo-M13mp7L2 construct ($<^1/_{568}$). In contrast, the mutation frequency was 1.2% ($^9/_{744}$) for the dAdo-M13mp7L2 construct and 1.3% ($^9/_{717}$) for the dGuo-M13mp7L2 construct in the presence of SOS induction. In JM103 *uvrA*6 cells, the mutation frequency was similar in the presence or absence of SOS induction: 1.3% ($^{17}/_{1294}$) for dAdo-M13mp7L2 and 1.8% ($^{25}/_{1382}$) for dGuo-M13mp7L2 with SOS induction and 1.0% ($^{10}/_{1030}$) for dAdo-M13mp7L2 and 1.3% ($^{12}/_{890}$) for dGuo-M13mp7L2 in the absence of SOS induction. The predominant mutation observed in both bacterial strains with dAdo-M13mp7L2 was an A → T transversion targeted to the adduct site, although a few C deletions ($^3/_{2224}$) immediately 3′ to the modified site were also observed in *uvrA*6 cells. With dGuo-M13mp7L2, only targeted G → T transversions were observed in both bacterial strains.

DISCUSSION

The present study has examined the mutagenicity of the (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo and the (+)*anti*-DB-[*a*,*j*]A-DE−*trans*-N^6-dAdo adducts site-specifically placed

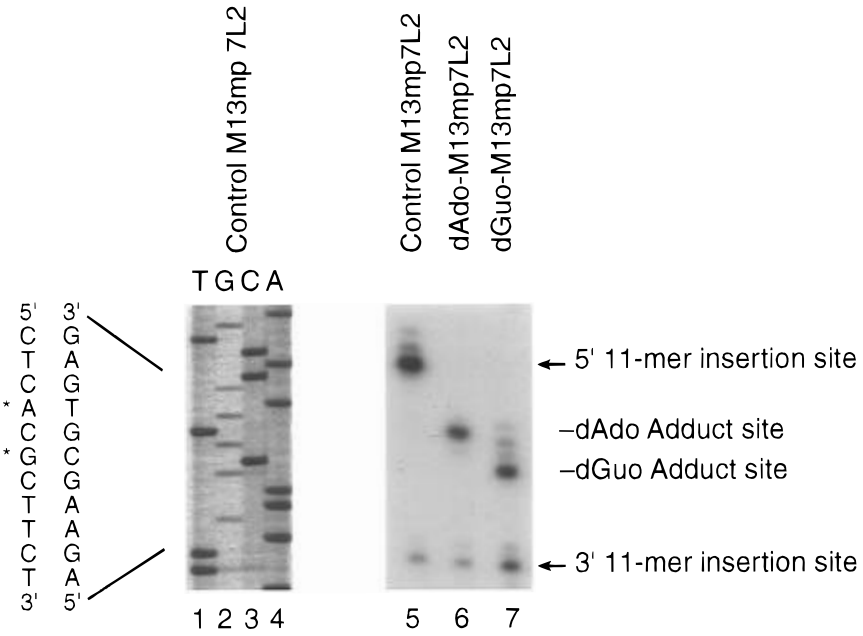

FIGURE 7: DNA polymerase stop assay with control M13mp7L2 (lane 5), dAdo-M13mp7L2 (lane 6), and dGuo-M13mp7L2 (lane 7). The first four lanes contain the sequencing ladder of control M13mp7L2. All vectors were annealed to the ^{32}P-end-labeled primer and incubated with *E. coli* DNA polymerase I (Klenow). The site of the dGuo or dAdo adduct is denoted on the right, and the sequence of the template strand is shown on the far left.

Table 1: Relative Plaque Yields for dAdo- and dGuo-M13mp7L2 Constructs

	survival, %[a] (JM103)		survival, %[a] (JM103 *uvr* A_6)	
constructs	−SOS	+SOS	−SOS	+SOS
control M13mp7L2[b]	100	100	100	100
dAdo-M13mp7L2[c]	6.0	7.8	0.8	1.0
dGuo-M13mp7L2[c]	7.9	11.8	3.9	4.8

[a] Values are based on an average of two separate experiments. Maximum variation was 18%. [b] Plaque yields from this group were considered as 100%. [c] % survival was calculated as follows. The plaque yields from the adduct-containing constructs were divided by the plaque yields from the control construct.

in a ss replication vector, M13mp7L2 (Banerjee et al., 1990). The long-term goals of these studies are to compare the mutagenicity of specific dAdo and dGuo adducts derived from (+)*anti*-DB[a,j]A-DE and to examine the effect of sequence context on mutagenesis by these adducts. The major findings of the current study are as follows:. (i) Site-specifically placed *trans*-N^2-dGuo and *trans*-N^6-dAdo adducts derived from (+)*anti*-DB[*a,j*]A-DE were effective blocks to DNA polymerase (Klenow fragment) action in vitro, blocking primarily at the adduct site under the experimental conditions used. (ii) Site-specifically placed *trans*-N^2-dGuo and *trans*-N^6-dAdo adducts derived from (+)-*anti*-DB[*a,j*]A-DE decreased plaque yield presumably by blocking DNA replication in vivo in both *E. coli* strains JM103 and JM103 *uvrA*6. (iii) Adduct-containing vectors induced targeted mutations with a low frequency, indicating that, for those vectors that were replicated, replication was correct most of the time. (iv) Targeted G → T and A → T mutations were exclusively obtained for both the dGuo and dAdo adduct. (v) Targeted transversion mutations were highly dependent on SOS functions in JM103 but not JM103 *uvrA*6 cells.

At present, relatively few studies examining the mutagenicity of specific PAH DNA adducts using site-specific approaches in vivo have been reported (Benasutti et al., 1988; Chary et al., 1995; Jelinsky et al., 1995; Kohotis et al., 1993; Mackay et al., 1992). The mutagenicity of the major (+)-*anti*-BPDE DNA adduct found in target tissues, (+)*anti*-BPDE−*trans*-N^2-dGuo, was studied in ds replication vectors. This adduct induced targeted G → T transversion mutations in the sequence 5′TGC-3′ (Mackey et al., 1992), but G → T, G → A, and G → C mutations in the sequence 5′-GGC-3′ (Jelinsky et al., 1995). The mutagenicity of all six possible stereoisomeric BPDE adducts formed with the N^6-position of dAdo was reported (Chary et al., 1995) using the same ss M13mp7L2 vector used in our present study. All six BPDE dAdo adducts induced exclusively A → G transition mutations when placed at the middle base in the sequence context of the N-ras 61st codon (5′-CAA). In our current study, mutations involve Ade insertion opposite both dGuo and dAdo adducts, giving exclusively transversion mutations (G → T and A → T, respectively) regardless of the excision repair capability of the bacterial strain. The sequence chosen for these initial studies was based, in part, on the ease of obtaining and separating the modified oligonucleotides but also with several sequence contexts in mind. In this regard, the dAdo adduct was placed in a sequence context where the 5′ base was the same as that found in the 61st codon of murine c-Ha-ras (Gill et al., 1992). In contrast, the dGuo adduct was placed in a sequence context where it was expected, on the basis of an earlier analysis of the mutational spectrum of (+)*anti*-DB[*a,j*]A-DE using the plasmid pUB3, to yield possibly more than one type of mutation (Gill et al., 1993c), including both G → T transversions and G → A transitions. The fact that only G → T and A → T transversion mutations were observed in the context of the 5′-CACG-3′ raises the interesting question of whether these DNA adducts are primarily noninformational lesions in this sequence context.

A "noninformational mechanism" of mutagenesis implies that a mutagenic lesion is uninterpretable and that rules other than simple base-pairing schemes govern base selection by

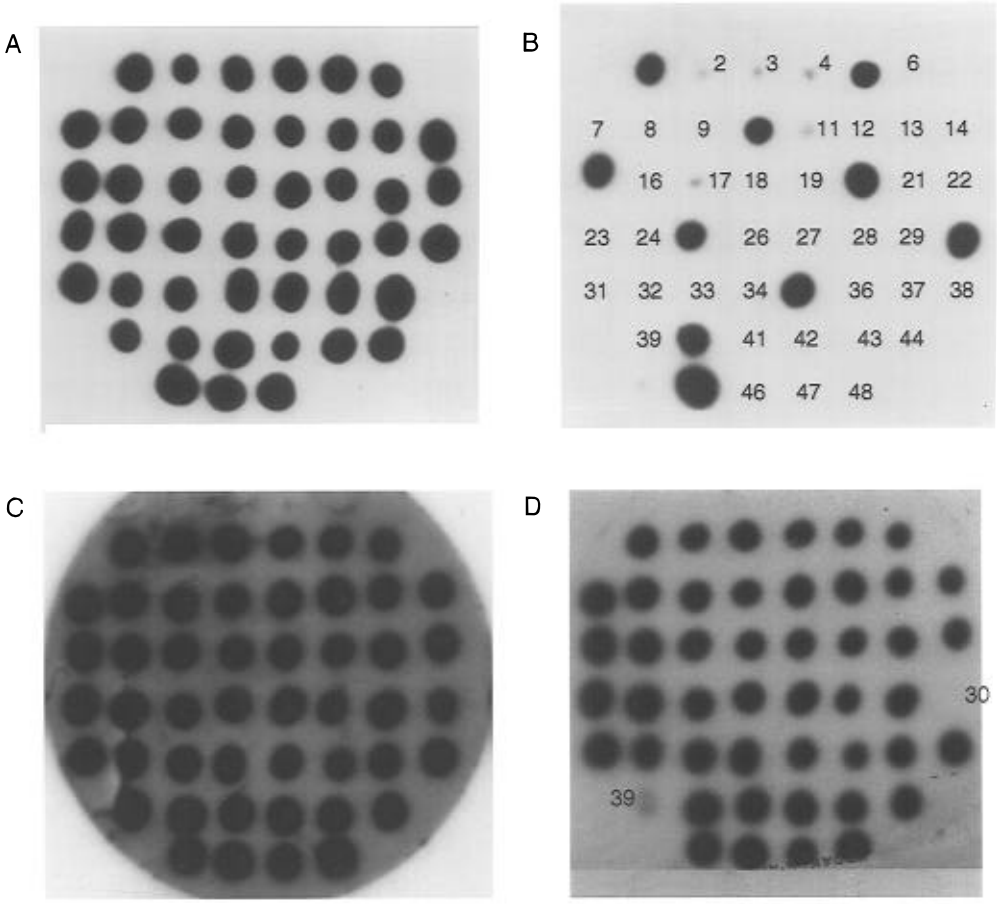

FIGURE 8: Differential hybridization for detection of mutations in the 11-mer sequence of progeny DNA. (A and B) Membranes containing progeny DNA from M13mp7L2 with a T substituted for the G in the unmodified 11-mer as a control to test this method. (C and D) Membranes containing progeny DNA from the dGuo adduct containing M13mp7L2 construct. After hybridization overnight with a 5′ ^{32}P-end-labeled probe (5′-CCA GTG AAT TAG AAG CGT GAG-3′), the membranes were subjected to low-stringency washing (6 × SSC, 37 °C for 20 min twice), shown in panels A and C. The same membranes were then subjected to high-stringency washing (1 × SSC/0.1% SDS, 57 °C for 20 min, followed by 62 °C for 2 min) to detect mutants (panels B and D). The progeny DNA that contained a base substitution mutation (G → T) no longer hybridized to the probe or, in some cases, exhibited a very low signal as depicted in panels B and D (2−4, 6−9, 11−14, 16− 19, 21−24, 26−29, 31−34, 36−39, 41−44, and 46−48 in panel B; 30 and 39 in panel D).

DNA polymerase. In *E. coli*, dAdo insertion opposite a lesion is frequently thought to be associated with noninformational pathways. Thus, adduct-induced G → T and A → T mutations may arise via noninformational mechanisms. Three different structures for G·A mispairs, which all appear reasonable and have two hydrogen bonds each, are known (Brown et al., 1989; Hunter et al., 1986; Kan et al., 1983; Kennard, 1985; Patel et al., 1984; Prive et al., 1987). This makes it easy to imagine that the carcinogen moiety of a dGuo adduct might facilitate pairing with dAdo to give a G → T mutation, which is a misinformational mechanism. Adduct-induced G → T mutations could also be due to a noninformational mechanism. In contrast, there are no reported structures for A:A mispairs, which are known to dramatically destablize the DNA helix and are not likely to be hydrogen-bonded (Ikuta et al., 1987). This is sensible given that there are no reasonable A·A mispairing structures possible [for a discussion, see Loechler (1994)]. Because of this, it is hard to envision how a bulky dAdo adduct would induce an A → T mutation by a simple A·A mispairing pathway; i.e., it seems unlikely that a DNA polymerase is misinterpreting a dAdo adduct as Thy during replication. Thus, the A → T mutations observed in this study provide some of the best data to support the existence of an adduct-induced noninformational mutational mechanism. The fact that the mutation frequency is low for both the dGuo and dAdo adducts (Tables 2 and 3) implies that the primary consequence of replication is no mutation, which is true of bulky adducts in general. This raises the question of how a bulky DNA adduct can be a noninformational lesion when it is correctly informational >90% of the time. Although the data in this paper do not provide insight into how this might be true, other data suggest that adducts can adopt multiple conformations in DNA, where each might be responsible for a different biological end point [Jelinsky et al., 1995; Rodriguez & Loechler, 1993b; reviewed in Loechler (1995)]. Vis-á-vis the work described herein, we envision one conformation being essentially informational and nonmutagenic and another(s) being noninformational and mutagenic. Although other possibilities have not been completely excluded, this is the model that is most consistent with all of our data and the one we currently favor.

An interesting observation in the current study was the finding that both the dGuo and dAdo adducts derived from (+)*anti*-DB[*a*,*j*]A-DE induced similar mutations and mutation frequencies in the repair-deficient *E. coli* strain JM103 *uvrA*6 regardless of its SOS status. An important reason for choosing the ss M13mp7L2 vector for the current studies was that ss DNA is generally regarded as a poor substrate for DNA repair processes. However, we did not expect to see a similar mutation frequency in SOS-induced and non-SOS-induced JM103 *uvrA*6 cells. Possible explanations for

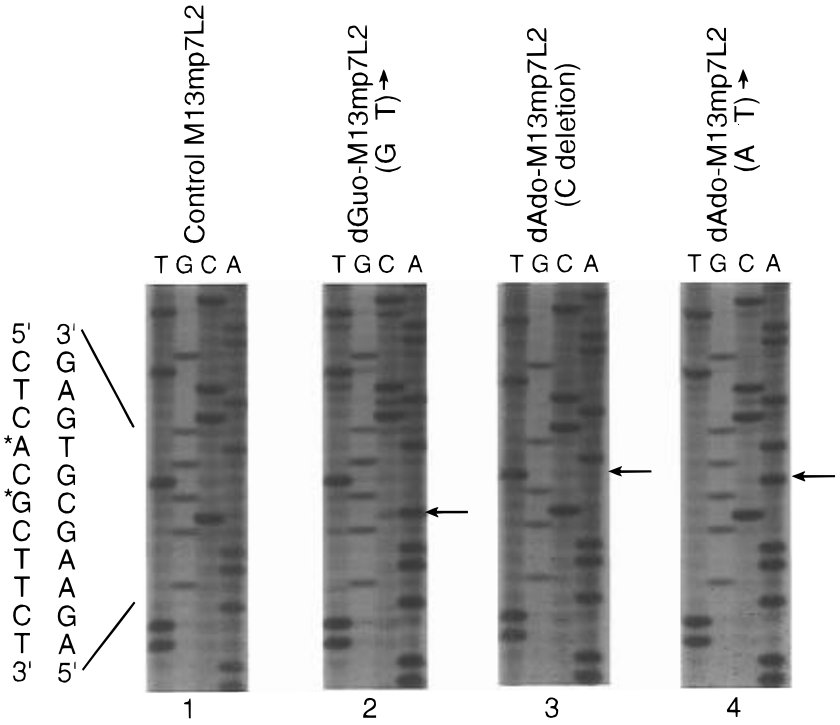

FIGURE 9: Representative dideoxy-sequencing autoradiograms showing sequences adjacent to and including the 11-mer of M13mp7L2 constructs: lane 1, control M13mp7L2; lane 2, dGuo-M13mp7L2 containing a G → T transversion targeted to the adduct site; and lanes 3 and 4, dAdo-M13mp7L2 containing one C deletion immediately 3′ to the modified site (lane 3) and an A → T transversion targeted to the adduct site (lane 4). The site of the dGuo or dAdo adduct is denoted on the right, and the sequence of the 11-mer template strand is shown on the far left.

Table 2: Frequency and Spectrum of Targeted Mutations from M13mp7L2 Constructs in *E. coli* Strain JM103

SOS induction	constructs	targeted mutation(s)	other mutations	number of transformants analyzed	number of mutants	mutation frequency, %
−SOS	control M13mp7L2	none	none	530	0[a]	<0.19
	dAdo-M13mp7L2	none	none	644	0[a]	<0.15
	dGuo-M13mp7L2	G → T	none	683	1	0.15
+SOS	control M13mp7L2	None	none	568	0[a]	<0.17
	dAdo-M13mp7L2	A → T	none	744	9	1.2
	dGuo-M13mp7L2	G → T	none	717	9	1.3

[a] No mutants were detected, and therefore, mutation frequency is $<1/530$, $<1/644$, and $<1/568$ for the control and dAdo-M13mp7L2 in the absence of SOS and the control M13mp7L2 in the presence of SOS induction, respectively.

Table 3: Frequency and Spectrum of Targeted Mutations from M13mp7L2 Constructs in *E. coli* Strain JM103 *uvrA*6

SOS induction	constructs	targeted mutation(s)	other mutations[a]	number of transformants analyzed	number of mutants	mutation frequency, %
-SOS	control M13mp7L2	none	none	622	0[b]	<0.16
	dAdo-M13mp7L2	A → T	deletion	1030	10[c]	1.0
	dGuo-M13mp7L2	G → T	none	890	12	1.3
+SOS	control M13mp7L2	none	none	480	0[b]	<0.20
	dAdo-M13mp7L2	A → T	deletions	1294	17[d]	1.3
	dGuo-M13mp7L2	G → T	none	1382	25	1.8

[a] All deletions are C deletions immediately 3′ to the dAdo adduct site. [b] No mutants were detected, and therefore, mutation frequency is $<1/622$ and $1/480$ for the control M13mp7L2 in the absence of SOS and the presence of SOS induction, respectively. [c] Mutations include nine A → T transversions and one C deletion. [d] Mutations include fifteen A → T transversions and two C deletions.

these results include the following. (i) The DNA repair defect in JM103 *uvrA*6 cells influenced the mutagenicity of these DNA adducts in non-SOS-induced cells. (ii) The experimental manipulations used in the current study (e.g., electroporation) led to induction of the SOS response in the non-UV-treated JM103 *uvrA*6 cells. (iii) The SOS response was not induced adequately in UV-exposed JM103 *uvrA*6 cells. The latter possibility does not seem likely at present since we performed Weigle reactivation (Weigle, 1953) experiments over a broad range of UV doses and found a significant induction of the SOS response at 8 J/m^2 as measured by this assay.

In addition to the in vivo mutagenesis experiments, we also examined the effect of both the (+)*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo and −*trans*-N^6-dAdo adducts on DNA polymerase action in vitro. For these experiments, we used *E. coli* DNA polymerase I (Klenow fragment) and the circular ss M13mp7L2 construct and a primer that hybridized 40 bases upstream of the 3′ 11-mer ligation site. These experiments demonstrated that both of the (+)*anti*-DB[*a*,*j*]A-

DE−*trans* adducts were effective blocks to the action of the polymerase in vitro and were primarily stopped at the site of the adduct in both cases. These data are similar to other studies indicating that bulky DNA adducts are good blocks to polymerase action in vitro (Basu et al., 1993; Belguise-Valladier et al., 1994; Chary et al., 1995; Chary & Lloyd, 1995; Christner et al., 1994; Comess et al., 1992; Hruszkewycz et al., 1991; Latham et al., 1993; Moore et al., 1980; Michaels et al., 1987; Reardon et al., 1990; Shibutani et al., 1993; Sun & Hurley, 1992; Thrall et al., 1992). In most studies, bulky adducts are stopped either one base before or at the adduct site. In previous studies using the same (+)-*anti*-DB[*a*,*j*]A-DE−*trans*-N^2-dGuo and −*trans*-N^6-dAdo adducts site-specifically placed in ds M13mp19 constructs (Gill et al., 1993b), we also observed effective blockage of *E. coli* DNA polymerase I (Klenow fragment) as well as with T4 DNA polymerase and Sequenase version 2.0 (Gill et al., 1993a); blockage occurred primarily at the adduct site, although significant blockage one base prior to the adduct site was observed with Sequenase version 2.0. As can be seen in Figure 7, little or no polymerase read-through could be detected with the dAdo adduct while some read-through was evident with the dGuo adduct. That these adducts are also effective blocks to DNA polymerase action in vivo is shown by the relative lethality of adduct-containing vectors in both JM103 and JM103 *uvrA*6 (Table 1). Interestingly, the data in Table 1 show that, in both bacterial strains, but especially the JM103 *uvrA*6 strains, the dAdo is more lethal than the dGuo adduct. These data might be consistent with the data in Figure 7 showing some read-though of the dGuo adduct compared to the dAdo adduct, although confirmation of this will require further experimentation.

In conclusion, we have shown that a specific dAdo adduct derived from the (+)*anti*-DB[*a*,*j*]A-DE is capable of producing A → T transversion mutations when specifically placed in a bacterial replication vector. Comparison with the corresponding dGuo adduct revealed that both adducts are mutagenic, although the dAdo adduct appeared to be more lethal in vivo. Evidence from experiments in the mouse skin tumorigenesis system indicates that the *anti*-diol epoxide of DB[*a*,*j*]A is an ultimate carcinogenic form of this carcinogen and that tumors arising from this compound or the parent PAH contain A^{182} → T transversion mutations in codon 61 of the c-Ha-ras gene (Gill et al., 1992). The current studies provide an important link with these mouse skin studies and show conclusively that a specific dAdo adduct found in vivo is capable of inducing an A → T transversion mutation in a model mutagenesis system. Future studies will now examine the effects of changing sequence context on mutagenesis by these adducts in an effort to further understand potential differences between dGuo and dAdo adducts as well as mechanism(s) for the specificity of mutations observed in target genes in vivo such as c-Ha-ras.

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