# Primer Extension by Various Polymerases Using Oligonucleotide Templates Containing Stereoisomeric Benzo[*a*]pyrene−Deoxyadenosine Adducts†

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ABSTRACT: Four isomeric benzo[*a*]pyrene−deoxyadenosine adducts, corresponding to the products of trans opening of the epoxide ring in the four configurationally isomeric benzo[*a*]pyrene dihydrodiol epoxides by the amino group of deoxyadenosine, were separately introduced into each of two 16-mer sequence contexts. The sequences were from the *supF* gene, and the site of the adducted adenine was known, for some hydrocarbon dihydrodiol epoxides, to be a hotspot for mutation in Context I and a coldspot for mutation in Context II. Using primers complementary to the 3′ ends of these oligonucleotides, the abilities of several polymerases to replicate these templates *in vitro* were investigated. Each adduct proved to be an effective block to primer extension such that only with high concentrations of exo⁻ Klenow fragment was any bypass of adducts seen. DNA polymerase α and HIV-1 reverse transcriptase were blocked 3′ to the adduct when the configuration at $C_{10}$ of the hydrocarbon was *S*, and some introduction of thymine opposite the adenine adduct was seen with the *R* configuration. Incorporation of a nucleotide opposite the adduct occurred more readily with Sequenase and the Klenow fragment, and the mutagenic introduction of adenine was apparent in most cases. This corresponded to the A → T transversions frequently seen in mutation studies with hydrocarbon dihydrodiol epoxides that react extensively with adenine in DNA. Overall, it was clear that sequence context, adduct stereochemistry, and the choice of polymerase all influenced the polymerization reaction. With these *in vitro* systems, no major differences correlating with the differing tumorigenicities of the isomeric dihydrodiol epoxides or with the hotspot or coldspot nature of the sequences were detected.

Polycyclic aromatic hydrocarbons are widespread environmental contaminants that exert their carcinogenic action through covalent interactions with cellular macromolecules (Brookes & Lawley, 1964). These interactions are mediated by reactive metabolites identified as bay region dihydrodiol epoxides (Sims et al., 1974; Jerina & Daly, 1976). Reaction with nucleic acids occurs primarily with the amino groups of the purine bases, following the chemical selectivity first described for the 7-(bromomethyl)benz[*a*]anthracenes (Dipple et al., 1971). Whereas the dihydrodiol epoxides formed from benzo[*a*]pyrene preferentially react with deoxyguanosine residues in nucleic acids (Koreeda et al., 1976, 1978; Jeffrey et al., 1976), those derived from the more potent carcinogen 7,12-dimethylbenz[*a*]anthracene (Dipple et al., 1983) and from benzo[*c*]phenanthrene (Agarwal et al., 1987; Dipple et al., 1987) react extensively with deoxyadenosine as well as deoxyguanosine, suggesting that deoxyadenosine adducts might be intrinsically more potent biologically (Bigger et al., 1983; Wei et al., 1991).

Some investigations of the mechanisms through which chemical damage in DNA might be translated into mutagenic events have involved studies of polymerase action *in vitro* on templates containing specific carcinogen adducts (Moore & Strauss, 1979; Moore et al., 1981). Recently, it was found that primer extension by Sequenase was blocked by the presence of deoxyguanosine adducts from benzo[*a*]pyrene 7(*R*),8(*S*)-dihydrodiol 9(*S*),10(*R*)-epoxide. Extension was inhibited at the nucleotide neighboring the adduct (*i.e.*, 3′ to the adduct), and inhibition was more effective with the adduct in which the epoxide ring was opened cis than with the trans adduct (Hruszkewycz et al., 1992). In studies with the exo⁻ Klenow fragment of DNA polymerase I and oligonucleotides containing the cis- or trans-opened deoxyguanosine adducts from either enantiomer of the benzo[*a*]pyrene dihydrodiol epoxide diastereomer in which the benzylic hydroxyl and the epoxide are trans (referred to here as DE-2[1] and also known as the *anti*-diol epoxide), some degree of lesion bypass was recorded and each stereoisomeric adduct elicited a somewhat different response (Shibutani et al., 1993).

To probe further the effects of adduct structure, sequence context, and polymerase on primer extension *in vitro* using templates containing benzo[*a*]pyrene adducts, we have incorporated the trans deoxyadenosine adduct derived from

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[1] Abbreviations: DE-1 and DE-2, vicinal dihydrodiol epoxides in which the benzylic hydroxyl and epoxide oxygen are cis and trans, respectively; RT, reverse transcriptase; CPG, controlled pore glass; A, T, G, and C, deoxyribonucleotides derived from adenine, thymine, guanine, and cytosine, respectively; CD, circular dichroism; DTT, dithiothreitol; dNTP, deoxyribonucleoside triphosphate.

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each of the four configurationally isomeric benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxides into two different sequence contexts. We have then examined the action of a series of polymerases on the eight oligonucleotide templates containing these adducts. *In vitro*, each adduct largely or completely blocked primer extension catalyzed by each polymerase. However, the principal site of polymerase arrest and the nucleotide preferentially incorporated opposite the adduct, if any, depended upon the nature of the polymerase, the sequence context, and the absolute configuration at the site of attachment of the adenine amino group to the benzo[*a*]-pyrene residue.

## EXPERIMENTAL PROCEDURES

*Materials.* [$\tau$-$^{32}$P]ATP was obtained from Amersham Corp. Sequenase (Version 2.0), the Klenow fragment of *Escherichia coli* DNA polymerase I, exonuclease-free Klenow (exo$^-$ Klenow), and T4 polynucleotide kinase were obtained from USB Corp. Human DNA polymerase $\alpha$ was obtained from Molecular Biology Resources, Inc. HIV-1 reverse transcriptase (HIV-1 RT), as described by Clark et al. (1990), was a gift from Dr. Stephen H. Hughes (ABL—Basic Research Program, NCI-FCRDC). Deoxyribonucleoside triphosphates were obtained from Promega. Oligonucleotides containing benzo[*a*]pyrene deoxyribonucleoside adducts were prepared and purified by HPLC as described below. Two 16-mer sequences, 5′-TTT(•A)GAGTCTGCTCCC-3′ (Context I) and 5′-CAG(•A)TTTAGAGTCTGC-3′ (Context II), which correspond to regions 137−122 and 141−126, respectively, in the *supF* gene of a vector used extensively for mutation studies, were used. The (•A) signifies the adducted adenine nucleotide. Three primers, an 8-mer, 11-mer, and 12-mer, complementary to the 3′ end of the templates were prepared for each sequence context and were labeled at the 5′ end with [$\tau$-$^{32}$P]ATP using T4 polynucleotide kinase.

*Synthetic Oligonucleotides.* The oligonucleotides containing benzo[*a*]pyrene−dAdo adducts were prepared by use of a postoligomerization modification procedure (Kim et al., 1992) from the 16-mers 5′-TTT(6-FP)GAGTCTGCTCCC-3′ (Context I) and 5′-CAG(6-FP)TTTAGAGTCTGC-3′ (Context II) bound to controlled pore glass (CPG), in which 6-FP represents a 6-fluoro-9-(2-deoxy-$\beta$-D-*erythro*-pentofuranosyl)purine residue. The activated intermediate, 6-fluoro-9-[[5-*O*-(4,4′-dimethoxytrityl)-3-*O*-(*N*,*N*-diisopropylamino)-($\beta$-cyanoethoxy)phosphinyl]-2-deoxy-$\beta$-D-*erythro*-pentofuranosyl]purine (6-FP phosphoramidite) was synthesized as reported for hydrocarbon-modified 6-FP phosphoramidite (Lakshman et al., 1991). Fluorinated oligonucleotides were typically prepared (Lakshman et al., 1992) by automated synthesis of the appropriate 12-mer on 155 mg (14.4 $\mu$mol) of CPG (170 Å, loaded with 93 $\mu$mol/g $N^4$-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-3′-succinic acid; Caruthers et al., 1987), followed by manual coupling of 40 mg (53 $\mu$mol, 3.7-fold excess) of 6-FP phosphoramidite for 16 h at room temperature in the presence of 250 $\mu$L of 0.5 M 1*H*-tetrazole in acetonitrile. Following manual coupling, end-capping with pyridine/4-(dimethylamino)pyridine/acetic anhydride in tetrahydrofuran was performed manually as described (Lakshman et al., 1992), and the final three residues were added by the automated procedure to yield CPG-bound fluorinated oligonucleotides with sequences corresponding to Contexts I and II (226 mg, 48.3 $\mu$mol/g, and 223 mg, 44.8 $\mu$mol/g, respectively). Postoligomerization modification was carried out by heating 1 $\mu$mol of each support-bound oligonucleotide with 5.1 mg of (±)-7$\beta$,8$\alpha$,9$\beta$-trihydroxy-10$\alpha$-amino-7,8,9,10-tetrahydrobenzo[*a*]pyrene (DE-1 aminotriol) or (±)-7$\beta$,8$\alpha$,9$\alpha$-trihydroxy-10$\beta$-amino-7,8,9,10-tetrahydrobenzo[*a*]pyrene (DE-2 aminotriol) and 4 $\mu$L of triethylamine in 100 $\mu$L of dimethyl sulfoxide at 55 °C for 4 (DE-1) or 6 (DE-2) days. The glass beads were collected by centrifugation, washed three times with methanol by centrifugation, and heated overnight at 55 °C in ca. 0.5 mL of concentrated ammonium hydroxide solution. The beads were removed by centrifugation and washed twice with water. The ammonia solution and aqueous washes were combined, filtered, and lyophilized. Preliminary purification of the resultant oligonucleotides containing a terminal dimethoxytrityl group was performed on a Hamilton PRP-1 column (7 × 305 mm) eluted at a flow rate of 2.5 mL/min with a gradient from 10% to 100% solvent B in solvent A over 40 min, where solvent A is 0.1 M ammonium carbonate buffer at pH 7.5 and solvent B is a 1:1 mixture of A with acetonitrile at pH 7.5. Under these conditions both the DE-1- and DE-2-modified oligonucleotides corresponding to Context I eluted at ca. 25 min, whereas those corresponding to Context II eluted at 22−23 min. Separation of the diastereomeric dimethoxytrityl oligonucleotides derived from the enantiomeric dihydrodiol epoxides was not attempted. After removal of the dimethyoxytrityl group (0.5 mL of 80% acetic acid in $H_2O$, 45 min, room temperature) and evaporation of acetic acid, the oligonucleotides were redissolved in water, extracted with ethyl acetate, and concentrated. The modified oligonucleotides were subjected to HPLC on the Hamilton PRP-1 column eluted at a flow rate of 2.5 mL/min with a gradient from 20% to 40% solvent B in solvent A, as above, to separate two diastereomeric oligonucleotides in each case.

Configurational assignments for each member of the pairs of diastereomeric oligonucleotides derived from the racemic dihydrodiol epoxides were made on the basis of their CD spectra as well as enzymatic degradation of selected oligonucleotides to give adducted nucleosides of known configuration. The *early*-eluting oligonucleotides for both Context I and II exhibited positive CD bands in the 330−350 nm region (pyrene chromophore) whereas the *late*-eluting oligomers exhibited negative bands in this region. Comparison with the CD spectra of the monomeric dAdo adducts (Jeffrey et al., 1979; Cheng et al., 1989; Sayer et al., 1991) suggested that the *early*-eluting oligonucleotides had the 10*R*-configuration and the *late*-eluting oligonucleotides had the 10*S*-configuration. However, the characteristic intense band at ca. 280 nm observed with the nucleoside adducts (positive for 10*S* and negative for 10*R* adducts) could not be used for assignment of configuration since it was obscured by an overlapping positive band corresponding to DNA base interactions in the oligomers. To confirm the assignments based on the longer wavelength bands of the adducted oligonucleotides, the *late*-eluting oligonucleotides of Context I derived from DE-1 and DE-2 aminotriols (1.5−2.0 $A_{260}$) were each subjected to hydrolysis (Sayer et al., 1991) with deoxyribonuclease I (0.3 mg), followed by snake venom phosphodiesterase (0.1−0.2 unit), for ca. 40 h, and then alkaline phosphatase (3 units). Each adducted nucleoside was isolated by chromatography on a Beckman Ultrasphere $C_{18}$ column (4.5 × 250 mm) eluted at 1.2 mL/min with 50%

methanol in water for 2 min, followed by a linear gradient that increased the methanol concentration to 100% in 28 min, with detection at 345 nm. Retention times (15.3 min for DE-1 adduct; 14.6 min for DE-2 adduct) and CD spectra in methanol were identical to those for authentic standards of the trans dAdo adducts with the 10*S*-configuration derived from (7*S*,8*R*,9*S*,10*R*)-dihydrodiol epoxide-1 and (7*R*,8*S*,9*S*,-10*R*)-dihydrodiol epoxide-2, respectively.

*Primer Extension Reactions.* The primers and templates, in a 1:1 molar ratio, were annealed by heating the samples to 85 °C for 10 min and then slowly cooling them to room temperature. Each polymerase reaction mixture had a final volume of 10 μL and contained either individual nucleoside triphosphates or all four nucleoside triphosphates.

The Sequenase reactions contained 1.3 pmol of primer/template complex in a solution of 8 mM $MgCl_2$, 32 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM dithiothreitol (DTT), and dNTPs (20, 200, or 670 μM). Reactions were initiated by addition of Sequenase (0.5−1.8 units). Reactions were held for 10 min or 1 h at room temperature or at 37 °C and were terminated by the addition of 6 μL of denaturing dye (95% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol FF).

Klenow fragment reactions contained 1.3 pmol of primer/template complex in a solution of 8 mM $MgCl_2$, 32 mM Tris-HCl (pH 7.5), 10 mM DTT, and dNTPs (20 or 670 μM). Reactions were initiated by the addition of Klenow fragment (0.3 unit) and were held for 5 min at room temperature.

HIV-1 RT reactions contained 0.3 pmol of primer/template complex in a solution of 8 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0), 75 mM KCl, 2 mM DTT, and dNTPs (100 μM). Reactions were initiated by the addition of HIV-1 RT (0.04 mg/mL) and were held for 2 h at 37 °C.

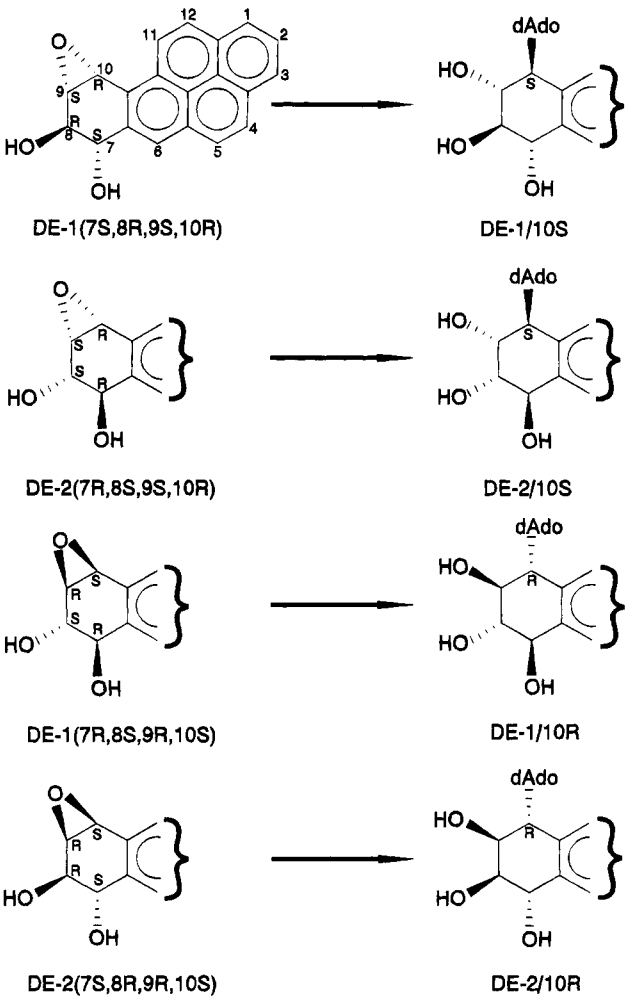
Human DNA polymerase α reactions contained 0.4 pmol of primer/template complex in a solution of 5 mM $MgCl_2$, 60 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.3 mg/mL bovine serum albumin, 0.1 mM spermine, and dNTPs (200 μM). Reactions were initiated by the addition of polymerase α (0.75 unit) and were held at room temperature for 1 h.

After incubation, samples were loaded onto a 20% denaturing polyacrylamide gel and were subject to electrophoresis at 2600 V for approximately 2 h until the bromophenol blue dye had migrated 20 cm. Gels were dried on a Bio-Rad slab dryer for 45 min at 80 °C, after which X-OMAT film was placed on the gel for 30 min and subsequently developed.

## RESULTS

The four deoxyribonucleoside adducts from trans opening of the epoxide ring of the four configurationally isomeric benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxides by the amino group of deoxyadenosine are illustrated in Scheme 1. In this scheme, they are each identified by labels indicating the diastereomeric dihydrodiol epoxide from which they were derived, *i.e.*, DE-1 or DE-2 [dihydrodiol epoxide diastereomer in which the benzylic hydroxyl group is cis (DE-1) or trans (DE-2) to the epoxide oxygen, also referred to as the *syn* and *anti* diastereomers, respectively], and by a designation of the absolute configuration at $C_{10}$ of the hydrocarbon residue, *i.e.*, 10*S* or 10*R*. Since all of the adducts studied here resulted from trans opening of the epoxide at $C_{10}$, absolute configuration at $C_{10}$ in the adducts is the reverse of that in the diol epoxide from which they are derived.

Scheme 1: Structures of the Four Configurationally Isomeric Benzo[a]pyrene−Deoxyadenosine Adducts




The two sequence contexts chosen for these studies were derived from the *supF* gene sequence that has been used extensively for mutation studies with hydrocarbon dihydrodiol epoxides (Bigger et al., 1992, 1991, 1990, 1989; Yang et al., 1987). In mutation studies with benzo[*c*]-phenanthrene dihydrodiol epoxides, which react extensively with deoxyadenosine residues (Agarwal et al., 1987; Dipple et al., 1987; Bigger et al., 1992), the adducted adenine in Context I was a hotspot for mutation whereas the adducted adenine in Context II was not mutated. HPLC retention times and configurational assignments for each of the eight oligonucleotides used in these studies are summarized in Table 1. The adducted oligonucleotide 16-mers or unadducted controls were hybridized with $^{32}P$-labeled complementary 11-mer or 12-mer primers and incubated with polymerase and deoxyribonucleoside triphosphates, and the radioactive products of primer extension were analyzed by gel electrophoresis. As indicated in the schematic diagram in Figure 1, this strategy allows examination of primer extension to the nucleotide 3′ to the adduct (using an 11-mer primer) and extension to the adduct itself (using a 12-mer primer).

*Sequenase.* In initial studies at room temperature using the 11-mer primer and 10 min incubations, Sequenase incorporated the correct complementary nucleotide 3′ to the

Table 1: Retention Times and Absolute Configurations of Modified Oligonucleotides[a]

| parent diol epoxide | retention time (min) | absolute configuration at $C_{10}$ |
|---|---|---|
| 5′-TTT(•A)GAGTCTGCTCCC-3′ (Context I)[b] | | |
| (7*R*,8*S*,9*R*,10*S*)-DE-1 | 9.6 | *R* |
| (7*S*,8*R*,9*S*,10*R*)-DE-1 | 11.5 | *S* |
| (7*S*,8*R*,9*R*,10*S*)-DE-2 | 10.1 | *R* |
| (7*R*,8*S*,9*S*,10*R*)-DE-2 | 11.9 | *S* |
| 5′-CAG(•A)TTTAGAGTCTGC-3′ (Context II)[c] | | |
| (7*R*,8*S*,9*R*,10*S*)-DE-1 | 6.9 | *R* |
| (7*S*,8*R*,9*S*,10*R*)-DE-1 | 8.8 | *S* |
| (7*S*,8*R*,9*R*,10*S*)-DE-2 | 8.3 | *R* |
| (7*R*,8*S*,9*S*,10*R*)-DE-2 | 9.9 | *S* |

[a] For chromatographic conditions, see text. [b] Assignments based on enzymatic hydrolysis of the *late*-eluting oligonucleotides from DE-1 and DE-2 to monomeric dA adducts of known absolute configuration. [c] Assignments based on comparison of CD spectra at 330−350 nm with those of Context I oligonucleotides.

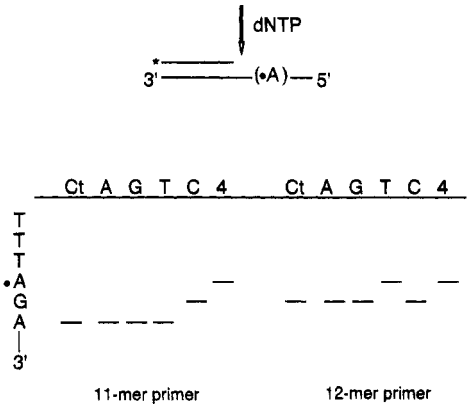



FIGURE 1: Schematic diagram illustrating the system used to study incorporation of nucleotides opposite the base 3′ to the adduct (11-mer primer) or opposite the adduct (12-mer primer). After polymerization, samples were analyzed by gel electrophoresis. The sequence of the template is shown at the left of the gel, with the adduct symbolized by the filled circle. Each oligonucleotide was incubated with the individual nucleotide triphosphates (A, G, T, C), or with all four nucleotide triphosphates (4). An unreacted control (Ct) is included to show the initial primer length. In this model, the 11-mer primer shows correct incorporation of dCTP opposite the template G, and extension opposite the adduct when all four nucleotides are supplied. The 12-mer primer shows correct incorporation of dTTP opposite the adducted adenine, but even when all four nucleotides were supplied, no further primer extension was observed.

adduct position, and further polymerization was then blocked by the adduct (Figure 2). In concert with this, no primer extension was detected when the 12-mer primer was used in such studies. With either primer, similar data were obtained using templates containing the four different adducts (*e.g.*, Figure 2) or templates derived from the two different sequence contexts (*i.e.*, Context I and Context II). Moreover, differences in nucleoside triphosphate concentration (20, 200, or 670 $\mu$M) had no significant effect on the reactions under these conditions.

In contrast, increasing the reaction time to 1 h did influence polymerization. Thus, with either Context I or Context II templates in the presence of all four nucleotide triphosphates, the 11-mer primer was extensively elongated to a 12-mer as seen with 10 min incubations, but with the 1 h incubation some extension to a 13-mer was also apparent. This extension was minimal at 20, but more pronounced at 200 and 670 $\mu$M nucleoside triphosphate though even at these higher nucleotide concentrations, only a small fraction of primer was elongated to a 13-mer.

To determine which nucleotide was incorporated opposite the adducts, experiments were undertaken using the 12-mer primer, individual nucleoside triphosphates at 670 $\mu$M, and the 1 h incubation period (Figure 3). The longer reaction time decreased fidelity in the reaction with the control oligonucleotide such that some misincorporation of A and C accompanied the incorporation of T opposite the unadducted adenine. In reactions with oligonucleotides containing adducts, the increased reaction time allowed the mutagenic incorporation of A opposite the adducted adenine, forming a 13-mer for each of the four adducts (Figure 3). No extension of the 13-mer was observed, and the 12-mer primer was only partially converted to 13-mer using any of the four adducted oligonucleotide templates.

*Klenow Fragment.* Since the Klenow fragment retains a 3′→5′ exonuclease activity, primer can be degraded if the correct deoxyribonucleotide is not supplied so that in incubations with single deoxyribonucleoside triphosphates, bands that are shorter than the starting primer were seen (Figure 4). Thus, using unadducted Context I as a template, significant degradation of the primer was observed when either dATP or dGTP was the sole nucleotide present. Such degradation was not found when dCTP was present because the primer ends in a C and this could be easily replaced if it was removed. The Klenow fragment can misincorporate a nucleotide, but will excise this before further elongation occurs using its proofreading mechanism (Eger et al., 1991). This ease of misincorporation in the presence of only one base is responsible for the primer extension bands that are visible in the control reactions containing the noncomplementary deoxyribonucleotides, but it was clear that the correct nucleotide (T) was incorporated most extensively in the control reactions (Figure 4). Using the Klenow fragment, the 12-mer primer, and 5 min incubations at room temperature, some differences in the findings obtained using the two different sequence contexts and using different adducted templates in the same sequence context were seen.

For example, reactions for the DE-1/10*S* and DE-2/10*S* adducts in Context I showed primer degradation when dATP, dGTP, or dTTP was the sole nucleotide present whereas such degradation was not seen in the presence of dTTP when the template contained the DE-1/10*R* or DE-2/10*R* adducts in Context I. The reason for this difference is that DE-1/10*S* and DE-2/10*S* did not allow any substantial nucleotide incorporation opposite these adducts (even when incubation was extended to 120 min) while DE-1/10*R* and DE-2/10*R* allowed all the primer to be extended to a 13-mer by nonmutagenic incorporation of T opposite these adenine adducts. Additionally, for Context I, only in the case of the adducts with the *R*-absolute configuration at $C_{10}$ was there clear evidence of some mutagenic incorporation of A opposite the deoxyadenosine adducts (Figure 4A). This A incorporation was much less extensive than the T incorporation, so that in the presence of all four nucleoside triphosphates, T incorporation would presumably be the major, but not necessarily exclusive reaction. No product longer than a 13-mer was detected. With each of the four adducts in Context I, the original primer band was transformed into a double band in incubations with dGTP. It appeared that some exonucleolytic removal of the terminal C of the primer

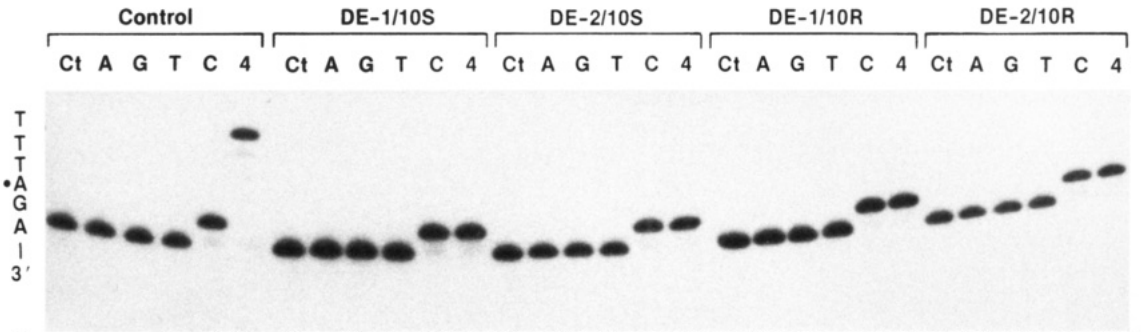



FIGURE 2: Sequenase reaction (0.5 unit) on Context I template with 11-mer primer. Samples were incubated with 20 $\mu$M nucleotide triphosphates for 10 min at room temperature.

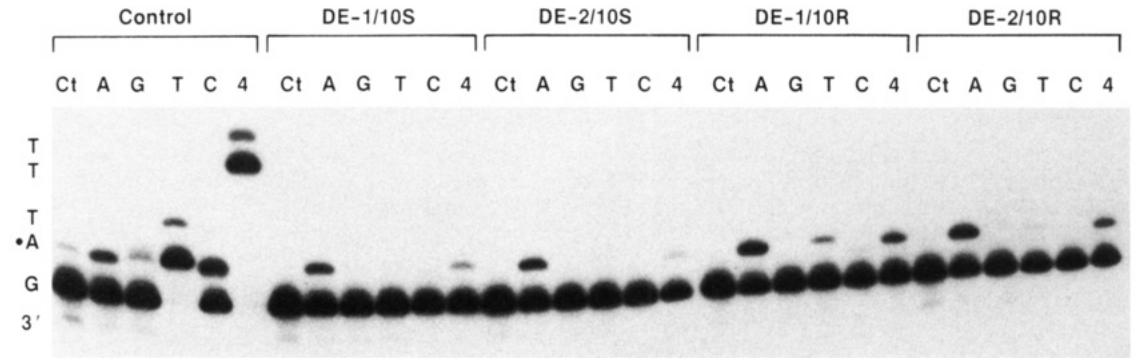



FIGURE 3: Sequenase reaction (0.5 unit) on Context I template with 12-mer primer. Samples were incubated with 670 $\mu$M nucleotide triphosphates for 1 h at room temperature.

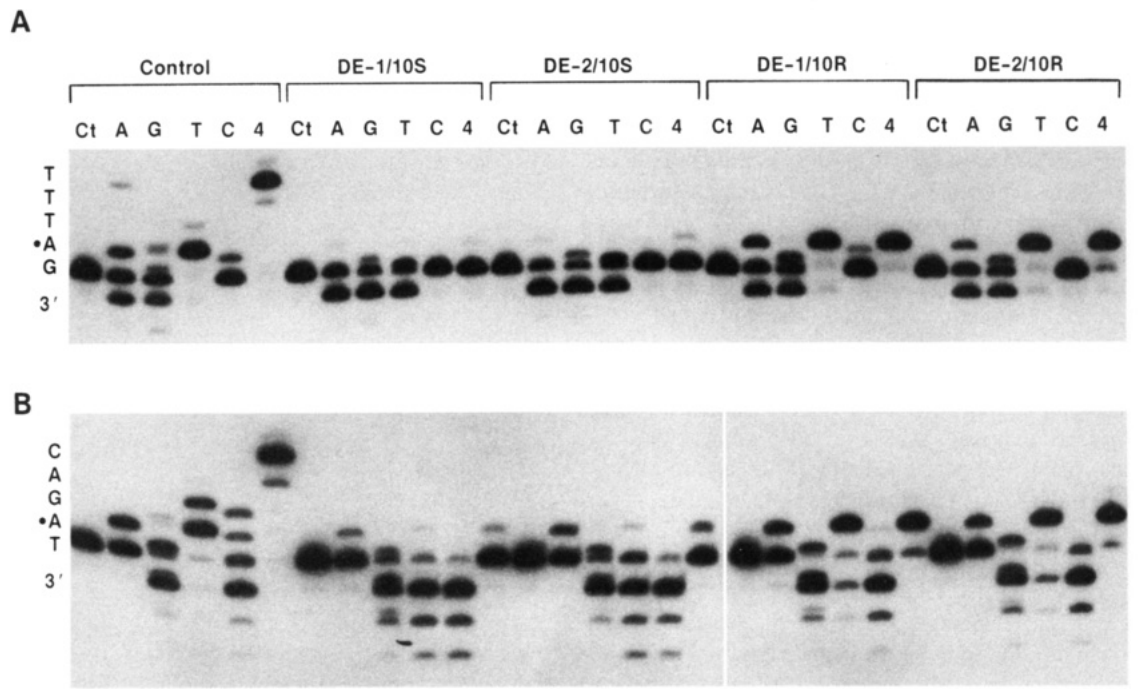



FIGURE 4: Reactions with the Klenow fragment (0.3 unit) on Context I template (panel A) or Context II template (panel B) with the 12-mer primer. Samples were incubated with 20 $\mu$M nucleotide triphosphates for 5 min at room temperature.

was followed by extension with G to form a second 12-mer that separated from the original 12-mer upon electrophoresis.

The studies using Context II as a template followed the same general pattern as those with Context I, but some differences were apparent (Figure 4B). Thus, in Context II, the adducts with the *S*-absolute configuration at $C_{10}$ still did not incorporate substantial amounts of T opposite the adducts, but there were clear indications that some mutagenic incorporation of A occurred. In other respects, findings with Context II were similar to those with Context I although the exonuclease action on the primer differed because the terminal nucleotide in the primer was different. In summary, the adducts with *R*-absolute configuration at $C_{10}$ incorporated T > A opposite the adducts, whereas those with *S*-absolute configuration led to the incorporation of A opposite the adduct in Context II and did not allow any substantial incorporation when in Context I.

*Exonuclease-Free Klenow Fragment.* The results of experiments using exo$^-$ Klenow fragment were similar to, but not identical with, those using the original Klenow

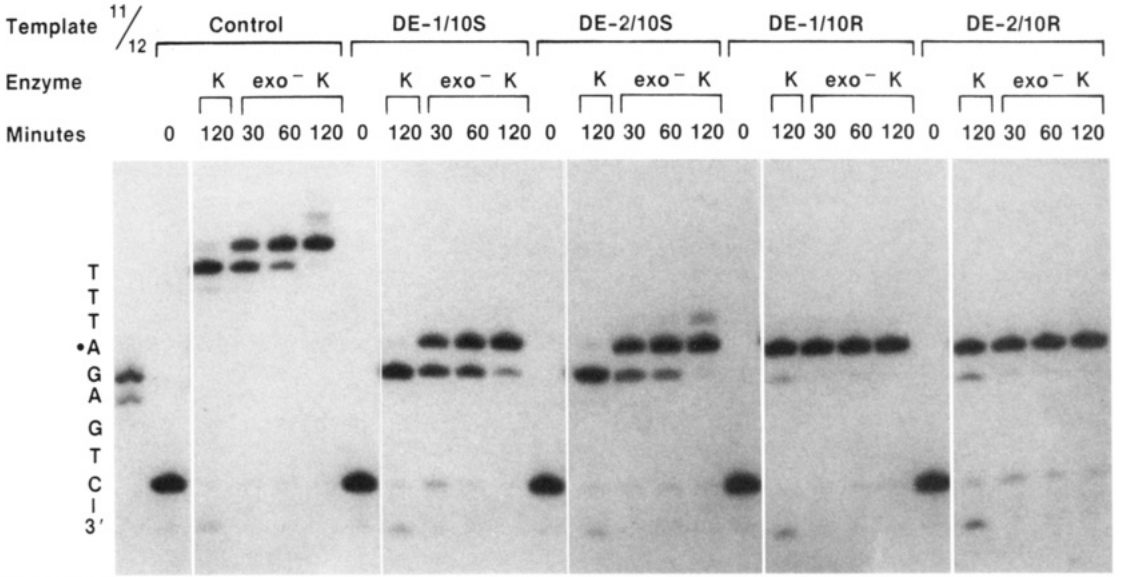



FIGURE 5: Comparison of reactions with the Klenow fragment (0.3 unit) and exo⁻ Klenow fragment (0.25 unit) on Context I template with the 8-mer primer. Samples were incubated with 20 $\mu$M of all four nucleotide triphosphates at room temperature for 30, 60, or 120 min. A mixture of the 11- and 12-mer primers were run as markers (11/12). For each oligonucleotide sample, only the 120 min Klenow reaction is shown.

fragment. The major similarity for these two enzymes was that incorporation of a nucleotide opposite adducts with the *R*-absolute configuration at $C_{10}$ (*i.e.,* DE-1/10*R* and DE-2/10*R*) was much more facile than incorporation opposite adducts with the *S*-absolute configuration. As illustrated in Figure 5, the Klenow fragment did not incorporate a nucleotide opposite DE-1/10*S* or DE-2/10*S* even after a 2 h incubation though, as shown earlier, incorporation opposite DE-1/10*R* and DE-2/10*R* was complete within 5 min (Figure 4). With the exo⁻ Klenow fragment, incorporation opposite the adducts with *R*-absolute configuration was again rapid (Figure 5). While incorporation opposite the adducts with *S*-absolute configuration was readily seen in the absence of exonuclease activity, it was clearly much slower, requiring 2 h to approach completion. It should be noted that with unadducted template exo⁻ Klenow fragment extends the primer one nucleotide beyond the length of the template whereas the Klenow fragment itself stops polymerization at the end of the template (Clark et al., 1987). In the experiments using adducts in Context I, incorporation opposite DE-1/10*S* appeared a little slower than that opposite DE-2/10*S* (Figure 5). In analogous experiments using Context II, this difference for DE-1/10*S* and DE-2/10*S* was a little more pronounced. The rate differences in incorporation opposite the different adducts were not due to differences in polymerase binding to the various primer/template complexes because the 8-mer primer was quickly elongated to a 12-mer in all cases (Figure 5). Apart from a faint band in the 2 h incubation with DE-2/10*S*, no substantial product longer than a 13-mer was seen.

An experiment using high levels of exo⁻ Klenow fragment and long incubation times (Shibutani et al., 1993) showed that some translesional synthesis (bypass) could occur (Figure 6). Bypass was most evident, however, in experiments using templates containing an adduct with the *S*-absolute configuration, *i.e.,* those that allowed the least ready extension of primer to a 13-mer. Based on the findings with the Klenow fragment, it seems likely that A was incorporated opposite the adducts with *S*-absolute configuration whereas the

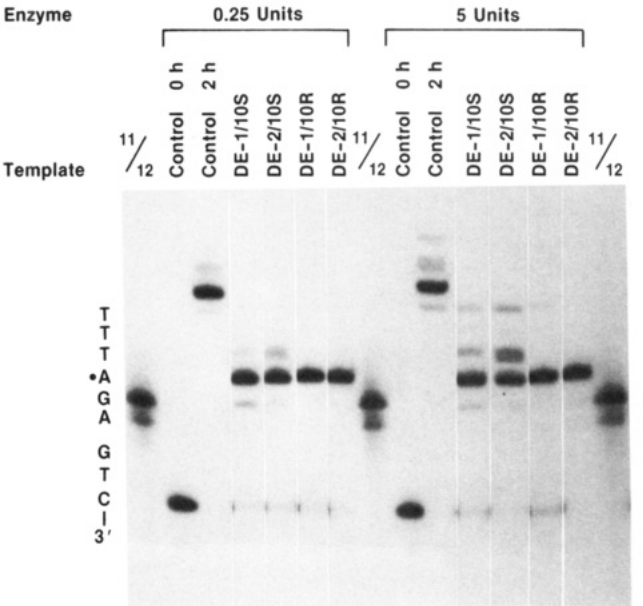



FIGURE 6: Comparison of reactions with exo⁻ Klenow on Context I template using 0.25 unit or 5 units of enzyme and the 8-mer primer. Samples were incubated at room temperature for 2 h with 200 $\mu$M nucleotide triphosphates. A mixture of the 11- and 12-mer primers were used as markers (11/12).

nucleotide incorporated most frequently opposite the adducts with *R*-absolute configuration would be T. Experiments with individual nucleoside triphosphates and the exo⁻ Klenow fragment did not allow the preferred nucleotides for incorporation opposite adducts to be determined, however, because of a lack of fidelity when only one nucleotide was supplied.

*HIV-1 Reverse Transcriptase.* With this enzyme it was found that a decrease in template level to 25% of that used in the Sequenase and Klenow experiments was necessary to obtain good primer extension using unadducted template. Under these conditions with Context I templates, differences were seen in primer elongation depending on the configuration of the adduct at $C_{10}$. Adducts DE-1/10*S* and DE-2/10*S* allowed complete extension of the 11-mer primer to a 12-

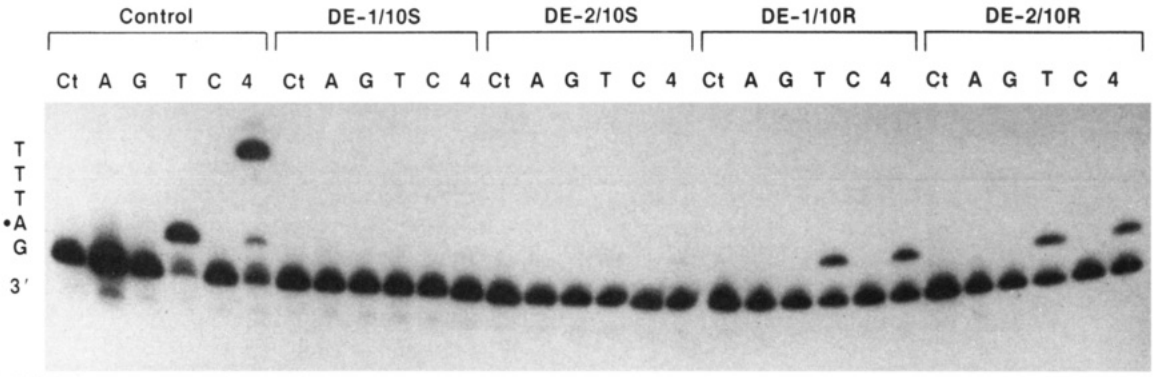



FIGURE 7: HIV-1 reverse transcriptase (0.04 mg/mL) reaction on Context I template with the 12-mer primer. Samples were incubated with 100 $\mu$M nucleotide triphosphates for 2 h at 37 °C.

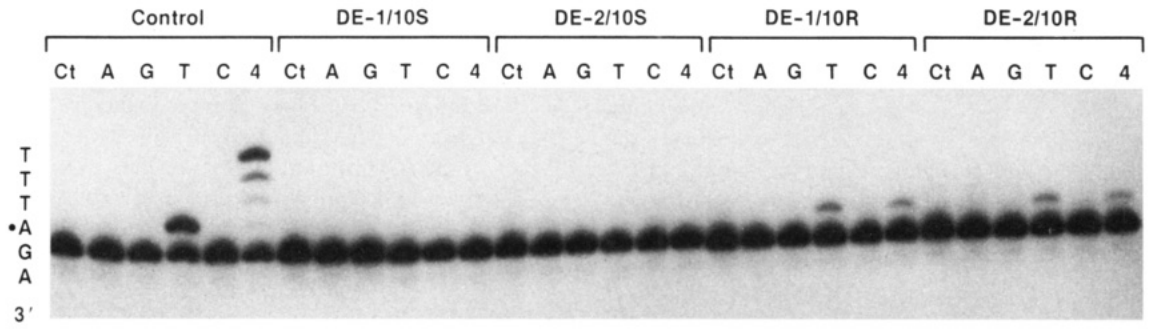



FIGURE 8: Human DNA polymerase α (0.75 unit) reaction on Context I template with the 12-mer primer. Samples were incubated with 200 $\mu$M nucleotide triphosphates for 1 h at room temperature.

Table 2: Summary of Nucleotides Incorporated by Primer Extension opposite Benzo[*a*]pyrene−Deoxyadenosine Adducts

| | templates | | |
|---|---|---|---|
| enzyme | *S* | *R* | sequence |
| Sequenase | A | A | |
| Klenow | no incorpn | T > A | |
| HIV-1 RT | no incorpn | T | Context I |
| Pol α | no incorpn | T | |
| Sequenase | A | A | |
| Klenow | A | T > A | |
| HIV-1 RT | no incorpn | no incorpn | Context II |
| Pol α | no incorpn | T | |

mer, but no incorporation opposite these adducts was detectable. The DE-1/10*R* and DE-2/10*R* adducted templates also allowed quick extension of the 11-mer primer to a 12-mer, but these adducts also allowed some incorporation of a nucleotide opposite the adduct. When the 12-mer primer was used in incubations with individual nucleotide triphosphates, little polymerization occurred with the adducts with *S*-absolute configuration, and it was clear that T was incorporated opposite the adducts with *R*-absolute configuration (Figure 7). The incorporation at position 13 was limited, and even with all four dNTPs present, further polymerization beyond position 13 was not observed. Sequence context influenced polymerization with HIV-1 reverse transcriptase. In similar experiments to those above using templates derived from Context II, all four of the adducted oligomers allowed complete extension of an 11-mer primer to a 12-mer, but there was no further extension of the primer to position 13 for any of these adducts.

*Human DNA Polymerase* α. Under the conditions used for HIV-1 RT approximately 50% full elongation of primer was obtained using an unadducted template and all four dNTPs. The eight adducted templates gave results that were largely similar to those obtained with HIV-1 reverse transcriptase, *i.e.*, the adducts with *S*-absolute configuration allowed primer extension only to a 12-mer (even after a 2 h incubation at 37 °C) whereas those with the *R*-absolute configuration allowed some additional extension to a 13-mer (Figure 8). However, the findings for both sequence contexts were essentially the same for polymerase α, whereas with reverse transcriptase no 13-mer was seen using the Context II templates.

## DISCUSSION

In these investigations, the action of a variety of polymerases on oligonucleotides that contain one of the four trans-opened deoxyadenosine adducts derived from the four configurational isomers of benzo[*a*]pyrene diol epoxide in two sequence contexts was examined. Of particular interest was an evaluation of the nucleotide inserted opposite these adducts by the polymerases since this could represent a mutagenic event. Because of the lack of fidelity in reactions with unadducted oligonucleotide controls, we were not able to identify the nucleotide inserted opposite the adducts for the exonuclease free Klenow fragment, but the findings for the other polymerases are summarized in Table 2. In general, the adducts derived from DE-1 or DE-2 elicited similar responses provided the absolute configuration of the adduct at $C_{10}$ was the same. The oligonucleotide templates in Table 2 are identified only, therefore, by their absolute configuration at $C_{10}$, *i.e.*, *R* or *S*.

Four different results were observed (Table 2). In several cases, the adduct appeared to block primer extension opposite itself. Alternatively, dTTP was incorporated (a nonmutagenic event), dATP was incorporated (a mutagenic event leading to an A → T transversion), or both of these nucleotides were incorporated to some extent. These dif-

ferent findings were dependent on the polymerase used, on the absolute configuration at $C_{10}$, and on sequence context. HIV-1 reverse transcriptase and human polymerase α were similar to one another in that only dTTP was incorporated opposite an adduct and this occurred detectably only with adducts with the *R*-absolute configuration at $C_{10}$.

Both Sequenase and the Klenow fragment allowed incorporation of dATP opposite the adducts. Such incorporation would be consistent with the observation that AT → TA transversions are the most prominent mutations at AT base pairs found for hydrocarbon dihydrodiol epoxides in general (Bigger et al., 1992, 1989; Carothers et al., 1990). Recently, it has been found that the *anti*-dihydrodiol epoxide of 5,6-dimethylchrysene induces extensive AT → GC transitions (Page et al., submitted). Very few mutations at AT base pairs have been found for benzo[*a*]pyrene dihydrodiol epoxide so far, presumably because of its tremendous selectivity for reaction with deoxyguanosine residues in DNA (Wei et al., 1993; Yang et al., 1987; Rodriguez & Loechler, 1993). In these *in vitro* studies, substantial extension of the primer beyond the adduct was not observed for any of the polymerases in Table 2. The differences seen for the different sequence contexts were less dramatic than the differences attributable to stereochemical differences and polymerase differences, but they do indicate that adduct sequence context can influence polymerization.

Several recent papers have established preferred conformations for benzo[*a*]pyrene−deoxyguanosine adducts in oligomers (Cosman et al., 1993a, 1992; Mao et al., 1993; De los Santos et al., 1992), but less information is available for deoxyadenosine adducts. A benzo[*c*]phenanthrene adduct, corresponding to the DE-2/10*R* structure here, has been found to intercalate its hydrocarbon moiety into DNA on the 5′ side of the adducted base (Cosman et al., 1993b). Based on exonucleolytic digestion studies, Cheh et al. (1990) have suggested that adducts with the *R*- or *S*-configuration at the site of attachment of hydrocarbon to nucleoside are oriented in opposite directions in DNA. The present experimental findings for the polymerases are that the *S*-absolute configuration blocks primer extension more effectively than the *R*-absolute configuration. Although polymerases copy templates from their 3′ end toward their 5′ end, it is not yet clear how the directionality of adducts affects the reaction. Polymerase arrest in response to bulky adenine adducts has been noted before for benzo[*c*]phenanthrene dihydrodiol epoxide- or 5-(hydroxymethyl)chrysene sulfate-adducts randomly introduced into DNA (Ross et al., 1993; Suzuki et al., 1991) or for 1-methylpyrene (Kokontis et al., 1993), styrene oxide (Latham et al., 1993), or 7-methylbenz[*a*]anthracene (Reardon et al., 1990) site-specifically placed in nucleotide sequences. In these present studies, primer extension opposite the nucleotide 3′ to the adduct was examined. For all adducts and both sequence contexts, the appropriate nucleotide (*i.e.*, C opposite G in Context I and A opposite T in Context II) was incorporated with each polymerase. In the specific case where a T is 3′ to a deoxyadenosine adducts as in Context II, we have previously observed misincorporation of G opposite T in addition to incorporation of A (Hruszkewycz et al., 1991; Hruszkewycz & Dipple, 1991). However, in attempts to extend these earlier studies, we have been unable to reproduce this misincorporation of G with freshly prepared oligonucleotide (identical to the original preparation) though the misincorporation can be reproduced with the original oligonucleotide. The basis for these differences is currently being investigated.

Previous studies of polymerase action on specific benzo[*a*]pyrene−deoxyribonucleoside adducts in oligonucleotides has focused on benzo[*a*]pyrene−deoxyguanosine adducts (Hruszkewycz et al., 1992; Shibutani et al., 1993). In general, these findings were similar to the present observations in that these bulky adducts primarily blocked primer extension, but some translesional synthesis was found in long incubations with large amounts of the exonuclease free Klenow fragment (Shibutani et al., 1993). A very recent report indicates that each of four benzo[*a*]pyrene−deoxyguanosine adducts, derived from the DE-2 isomer by cis or trans opening of the epoxide ring, inhibits elongation of transcripts by T7 RNA polymerase (Choi et al., 1994) and that the cis adducts are bypassed more readily than the trans adducts.

The present studies have compared the properties of four deoxyadenosine adducts derived from the trans opening of each of the four configurational isomers of benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide. The stereochemistry at $C_{10}$ is more important in determining polymerase response than the spatial orientation of the hydroxyl groups in the saturated 7,8,9,10-ring of the benzo[*a*]pyrene moiety since adducts with the *R*-absolute configuration at $C_{10}$ behaved similarly even though they were derived from different diastereomeric dihydrodiol epoxides. It is also clear that choice of polymerase and sequence context can also influence the outcome of these studies.

The benzo[*a*]pyrene dihydrodiol epoxide configurational isomer from which the DE-2/10*S* deoxyadenosine adduct was derived is by far the most tumorigenic isomer (Buening et al., 1978). However, with respect to the action of polymerases *in vitro* on these adducts in oligonucleotides, there was no major difference between the DE-2/10*S* adduct and the DE-1/10*S* adduct that is derived from a much weaker tumorigen. Thus, the differing tumorigenic potencies of the isomeric dihydrodiol epoxides may depend upon the yields of adducts formed or upon the properties of the more extensively formed deoxyguanosine adducts. In cellular systems, the polymerase action on these adducts is more complex than in the *in vitro* system used here. We are currently trying to introduce these oligonucleotides into a vector so that replication on these templates in cellular systems can be compared with these *in vitro* data.

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