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Effects of Bulky Polycyclic Aromatic Hydrocarbon Adducts on DNA Replication by Exonuclease-Deficient T7 and T4 DNA Polymerases

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ABSTRACT

***In vitro* DNA replication by exonuclease-deficient T7 DNA polymerase (Sequenase) and an exonuclease deficient T4 DNA polymerase was examined on a 244-nucleotide DNA template treated with three electrophilic polycyclic aromatic hydrocarbon (PAH) metabolites: racemic *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BaPDE), *trans*-2,3-dihydroxy-*anti*-1,10b-epoxy-10b,1,2,3-tetrahydrofluoranthene (FADE), or 3,4-epoxy-3,4-dihydrocyclopenta[*cd*]pyrene (CPPE). The DNA replication terminated opposite template guanines and, to a lesser extent, at template adenines, as expected, as purines were modified preferentially by the chemical treatments. Analysis of the products synthesized on the damaged templates indicated that bypass replication by Sequenase proceeded in three steps: (1) replication first terminated one base 3′ to each adduct; (2) a nucleotide was then incorporated opposite the PAH-modified base; and (3) replication continued at some sites to give full bypass of the lesions. The rate of lesion bypass was affected by the type of chemical adduct, the sequence context of the adduct, and the concentration of deoxynucleoside triphosphates. Short DNA repeats appeared to facilitate translesion replication.**

INTRODUCTION

ADDUCTS FORMED BY reactive intermediates derived from polycyclic aromatic hydrocarbons (PAHs), such as those formed by the metabolites of benzo[*a*]pyrene (B*a*P), fluoranthene (FA), and cyclopenta[*cd*]pyrene (CPP), have an important biological significance, as unrepaired or misrepaired adducts formed by these electrophiles may be misreplicated and give rise to genetic changes in cells. These genetic alterations are believed to be the first step in chemical carcinogenesis (Harris, 1991).

The most widely investigated chemical in the study of carcinogenesis is B*a*P, an abundant environmental pollutant that is highly mutagenic (Mazur and Glickman, 1988; Chen *et al.*, 1990; Keohavong and Thilly, 1992; DeMarini *et al.*, 1994). It is metabolized to a mixture of highly reactive forms, the most important of which are its (+)- and (−)-*anti*-diol epoxides (B*a*PDE). The B*a*PDE interacts covalently with DNA to form *trans* adducts with deoxyguanine at N-2 (Jeffrey *et al.*, 1977; Cheng *et al.*, 1989) and also, but to a lesser extent, at N-6 of deoxyadenine (Meehan *et al.*, 1977; Jeffrey *et al.*, 1979). In addition, B*a*PDE has been reported to form minor adducts by interaction of N-7 of deoxyguanine (Osborn *et al.*, 1981; Sage and Haseltine, 1984) and also with deoxycytidine (Meehan *et al.*, 1977; Straub *et al.*, 1977; Rill and Marsh, 1990). Reactive metabolites of other environmentally prevalent PAHs, such as the 3,4-epoxide of CPP (CPPE) and the *anti*-diol epoxide of FA (FADE), have been studied less than B*a*PDE (Fig. 1), although the mutagenic and carcinogenic potentials of both CPP (Wood *et al.*, 1980; Busby *et al.*, 1988; Nesnow *et al.*, 1994; Keohavong *et al.*, 1995) and FA (Van Duuren and Goldschmidt, 1976; Busby *et al.*, 1984; Babson *et al.*, 1986a; LaVoie *et al.*,

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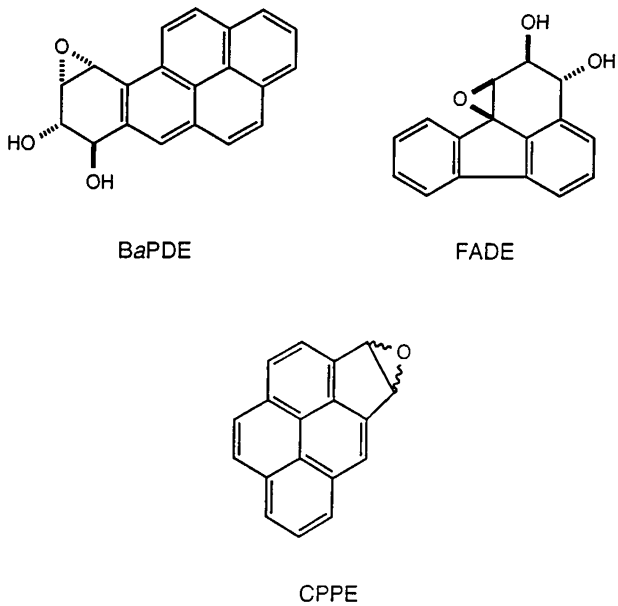

FIG. 1. Structural formulas of the major mammalian-formed mutagenic enantiomers of B*a*PDE and FADE. The major enantiomer of CPPE is unknown but is likely 3*S*,4*R* (Sahali *et al.*, 1992).

1994) have been shown. Although relatively less reactive than B*a*PDE, FADE is structurally similar and also forms *trans* deoxyguanine-N-2-yl adducts (Babson *et al.*, 1986b). Similarly, CPPE forms adducts primarily at N-2 of deoxyguanosine, but these are of the *cis* configuration (P. L. Skipper and S. R. Tannenbaum, personal communication).

In vitro DNA replication assays with chemically treated DNA templates or templates synthesized with modified nucleotides have been used to study how PAH adducts are converted to mutations. In reactions with *Escherichia coli* DNA polymerase I (DNA pol I), replication is observed to terminate one base immediately 3′ to a B*a*PDE-modified guanine (Moore and Strauss, 1979) and to an aminofluorence (AF)-modified guanine (Belguise-Valladier *et al.*, 1994; Belguise-Valladier and Fuchs, 1995). With the exonuclease-deficient (exo$^-$) Klenow fragment (KF), replication also terminates one base 3′ to a B*a*PDE-modified adenine, but replication also terminates opposite the lesion and bypasses the lesion fully under conditions where the enzyme could have multiple encounters with the modified DNA template (Chary and Lloyd, 1995). In reactions with T7 (exo$^-$) DNA polymerase (Sequenase), replication of PAH-modified DNA templates was observed to terminate one base 3′ to PAH lesions and opposite the lesions (Chary and Lloyd, 1995; Hruszkewycz *et al.*, 1991; Hruszkewycz and Dipple, 1991; Hruszkewycz *et al.*, 1992; Thrall *et al.*, 1992; Shibutani *et al.*, 1993). The termination one base 3′ to the adduct and the complete bypass, or translesion synthesis, were also observed when exo$^-$ KF and Sequenase were used to replicate AF-modified guanines within an oligonucleotide containing the recognition sequence for the *Nar*I endonuclease (Belguise-Valladier *et al.*, 1994; Belguise-Valladier and Fuchs, 1995).

Because chemical modification of bases may destabilize the N-glycosidic bond and produce apurinic/apyrimidinic (AP) sites, replication of AP-containing DNA templates is also relevant to the study of PAH-damaged DNAs. Replication of DNA pol I, DNA polymerase α, and AMV reverse transcriptase stops either before or opposite the AP lesions (Sagher and Strauss, 1983; Goodman *et al.*, 1993). DNA polymerases tend to insert dAMP preferentially opposite AP sites (Sagher and Strauss, 1983; Strauss, 1991).

The blocked or slow replication of damaged DNA templates is predicted from our understanding of DNA polymerase function. Incorporation of a nucleotide opposite a damaged base or AP site will be a slow reaction because of the absence of strong template coding signals; hence, replication will be observed to pause *before* the modified template base. If a nucleotide is incorporated opposite a damaged base, further extension will also be slow because of difficulties in extending a distorted and frequently less stable primer terminus; thus, replication will be observed to pause opposite the damaged template base. Although the nucleotide incorporated opposite the lesion is likely incorrect and, thus, a potential mutation, further extension, i.e., full bypass replication, may be required in order to fix the mutation.

We report here that Sequenase, T7 (exo$^-$) DNA polymerase, has the ability to fully replicate some PAH adducts at certain DNA sites. A T4 (exo$^-$) DNA polymerase was also examined, but this enzyme could not fully replicate PAH-damaged DNA under our reaction conditions. Translesion replication by Sequenase appeared to be stimulated by local DNA environments that contained short repeated sequences.

MATERIALS AND METHODS

PAH epoxides and diol epoxides

Racemic *anti*-BaPDE was purchased from the NCI Chemical Carcinogen Repository maintained by Midwest Research Institute (Kansas City, MO). The FADE was synthesized from > 99.9% pure *trans*-2,3-dihydroxy-2,3-dihydrofluoranthene (Day *et al.*, 1992) by epoxidation with 3-chloroperbenzoic acid in $CHCl_3$ and purified by medium-pressure chromatography (dry N_2) over DEAE cellulose (Rastetter *et al.*, 1982; Day *et al.*, 1992) MS (*m/z*, % base): 253 (100, $[M+H]^+$). The CPPE was synthesized from 2 mg of CPP (NCI Chemical Carcinogen Repository) that was previously purified to >99% by several flash SiO_2 chromatographic runs (petroleum ether). All reactions and handling procedures were performed in subdued light. Epoxidation was achieved in 30 min at 0°C using a freshly prepared dry 0.034 M acetone/CH_2Cl_2 solution of dimethyldioxirane (Beach *et al.*, 1993). Solvent was removed in a Speed-vac to give CPPE as a pale residue: MS (*m/z*, % base): 243 (100, $[M+H]^+$), 214 (11, $[M-CO]^+$).

Mass spectrometry

Pneumatically assisted electrospray mass spectral analyses of synthetic CPPE and FADE were performed with a Perkin-Elmer/Sciex API *1* mass spectrometer equipped with an atmospheric pressure ionization source and an articulated Ion-Spray interface maintained at 5 kV. The orifice was operated at 60 V. High-purity N_2 flowing at 0.6L/min and heated to 55°C served as the curtain gas, and high-purity air maintained at 40

psi was used for nebulization. Samples were introduced with a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a Hewlett-Packard 1040A diode array detector, connected in tandem to the mass spectrometer with glass capillary tubing. Samples were dissolved in 9:1 THF-Et_3N, mixed with 10 parts of CH_3OH containing 0.01% CH_3CO_2H, then quickly injected into the LC system in a flowing (13 μL/min) stream of CH_3OH containing 0.01% CH_3CO_2H.

Chemicals and enzymes

Modified T7 DNA polymerase (Sequenase version 2.0) and sequencing kits were purchased from United States Biochemical (Cleveland, OH). Exonuclease-deficient T4 DNA polymerase, the D112A + E114A-DNA polymerase, was prepared as described (Reha-Kranz and Nonay, 1993). The 2′-deoxynucleoside 5′-triphosphates (dATP, dCTP, dGTP, dTTP) were purchased from Pharmacia (Piscataway, NJ). Oligonucleotides (primer 1,5′-CATATATTAAATATACTCAC-3′, used for *in vitro* DNA replication assays, and primer 2,5′-TCCTGATTTTATTTCTGT-3′ and primer 13,5′-CTACTGTTGCCACTAAAAAG-3′, used for PCR amplification) were obtained from National BioScience (Plymouth, MN). Radioactive nucleotide [α-^{32}P]-dATP (3 kCi/mmol) was purchased from NEN Research Products (Boston, MA). The *Taq* DNA polymerase was obtained from Perkin-Elmer (Norwalk, CT).

Preparation of DNA template

Genomic DNA was isolated from the human male lymphoblastoid cell line TK6 (Skopek *et al.*, 1978) by phenol:chloroform extraction. The genomic DNA was stored at −75°C in 10 mM Tris HCl (pH 7.5) and 1 mM EDTA (TE buffer). To prepare the exon 3 fragment of the *HPRT* gene, 2 μg of genomic DNA was amplified for 26 cycles in a 100-μL reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM each of primers 2 and 13, and 2 U of *Taq* DNA polymerase. The resulting 244-bp PCR fragment contained exon 3 and flanking intron sequences at each end. The fragment was purified by gel electrophoresis and stored in TE buffer. The DNA concentration was estimated by ethidium bromide staining of electrophoresis bands and comparison with DNA bands of known concentration.

Treatment of template DNA with electrophilic PAH derivatives

The synthetic PAH metabolites were dissolved in dry 19:1 tetrahydrofuran:triethylamine and then mixed with the DNA template (5 ng/μL) in TE buffer. In order to determine the optimal conditions for treatment, the DNA was reacted with various concentrations of each chemical. Then B*a*PDE was added to give concentrations ranging from 0.05 to 10 μM; FADE and CPPE were added to give concentrations ranging from 0.5 to 500 μM. Adduction reactions were carried out at 37°C for 2 h. Unreacted and hydrolyzed PAH derivatives were removed by precipitating the DNA three times with ethanol. The treated DNA was then dissolved in TE buffer, aliquoted, and stored at −75°C until analyzed.

DNA replication/reactions

The first step of the DNA replication assay was to label the primer strand. Labeling was performed in the presence of a very low dNTP concentration to allow incorporation of only a few nucleotides, including the radiolabeled nucleotide. This reaction was performed with DNA (untreated or electrophile-treated) 5 ng/μL in 50 μL of reaction mixture containing 10 mM Tris HCl (pH 8.0), 10 mM dithiothreitol, 5 mM $MgCl_2$, 1 μM primer 1, 1 pM each dCTP, dGTP, and dTTP, and 0.2 μM [α-^{32}P]-dATP (3 kCi/mmol). The mixture was boiled for 2 min and cooled to 37°C to allow the primer to anneal. The DNA polymerase, either 2 U of Sequenase or 0.3 U of T4 (exo$^-$) DNA polymerase (equivalent protein concentrations), was then added, and the reactions were incubated at 37°C for 1 min. The labeled DNA was then used for further study as described in Results.

Reaction products were analyzed by PAGE. Each DNA was denatured at 90°C for 2 min, chilled quickly on ice, and separated on a 7% denaturing polyacrylamide gel for 3 and 7 h so that the short and long sequences synthesized could be separated with a sufficiently high resolution for subsequent densitometric quantitation. The gel was dried and subjected to autoradiography.

Quantitation of DNA replication block

The level of DNA replication block and bypass replication at each site of the template was estimated by using a semiquantitative approach. The intensities of the bands appearing in the autoradiograms were measured by scanning densitometry and analysis with the Collage Program (Fotodyne, Hartland, WI).

The density recorded for bands corresponding to the replication block before and opposite the adduct at each site during an incubation time extending from 10 sec to 2 h with the DNA polymerases was used to estimate the extension half-time for replication bypass. We observed that, during an incubation time of 10 sec with either Sequenase or T4 (exo$^-$), all primer-extended sequences were blocked at the base 3′ to the adduct at most sites of the template (see Fig. 2). The extension half-time for bypass replication was then estimated as the time required for the DNA polymerase to incorporate a nucleotide opposite the adduct in 50% of the products. This corresponded to a decrease of 50% of the sequences blocked at the base 3′ to each adduct during the 10-sec incubation time. At many sites of the template, this decrease was concomitant with a progressive increase of sequences extended with an additional nucleotide following an extended incubation time up to 1 or 2 h. Therefore, at the extension half-time for these sites, the total amount of sequences synthesized should be equally distributed among the products blocked at the base 3′ to the adduct and those containing an additional nucleotide. This assumed, however, that these latter sequences were not extended rapidly beyond the adduct. At some sites, However, the 1-nt addition reaction proceeded rapidly and had already occurred in a small fraction of the sequences synthesized during the 10-sec incubation time with Sequenase (Fig. 2), and extension beyond the adducts may have also occurred in a fraction of these sequences. Therefore, we approximated the extension half-time for these sites as the time needed to detect a decrease of 50% in the total intensity

recorded at the 10-sec incubation time for both the sequences blocked at the base 3′ to the adducts and those already extended with an additional nucleotide.

RESULTS

Sequenase-catalyzed DNA replication on a BaPDE-treated template

Figure 2A shows the pattern of sequences synthesized by Sequenase using untreated template (lane 5) and template treated with 0.2 μM (lane 1), 0.4 μM (lane 2), 0.8 μM (lane 3), or 1.5 μM (lane 4) B*a*PDE, all under our standard reaction conditions employing 200 μM of each dNTP and a 5-min incubation time to extend the labeled primer. The untreated template (lane 5) yielded the full-length product of 244 nt (the major band at the top of the gel) and a minor product (indicated by the diagonal line in lane 5), estimated to be 224 nt long on the basis of the dideoxy sequencing ladder (lanes A, C, G, T). Multiple shorter primer-extension products were observed in addition to the full-length product with the B*a*PDE-treated templates (lanes 1–4). These shorter products resulted from DNA replication termination when the DNA polymerase encountered B*a*PDE-modified bases on the template strand.

Comparison of the primer-extension products with the dideoxy sequencing ladder showed DNA replication termination opposite each guanine position of the template (dideoxy sequencing ladder "C" lane). Thus, the treated DNA contained subpopulations of modified DNAs representing each template guanine. Termination of replication opposite template guanines was expected, because the predominant interaction of B*a*PDE is with guanine (Jeffrey *et al.*, 1977). At some adducts in the template, however, such as those indicated by double diagonal lines in lane 2, primer extension was observed to terminate both before and opposite template guanines.

Effect of reaction time and dNTP concentration on the replication of BaPDE-damaged DNA

In order to learn more about the replication of BaPDE-damaged DNA, we performed a time course study from 10 sec to 30 min (Fig. 2B). In short reaction times, 10 sec to 1 min, DNA replication was observed to terminate predominantly one base 3′ to guanines and some adenines (positions 244, 277, and 283). With longer reaction times, 5 min to 30 min, DNAs terminating one position before damaged bases were extended by one nucleotide. For example, gel bands corresponding to primer extensions terminating one base 3′ to damaged guanines at positions 226, 250, and 289 disappeared with time, whereas DNAs one nucleotide longer appeared. These results suggest that replication of the BaPDE-modified DNA by Sequenase involved two steps: (1) rapid DNA replication from the primer to the base preceding each adduct; and (2) slow incorporation of a nucleotide opposite the adducted base.

The length of the incubation time needed for incorporation of a nucleotide opposite the lesion was not uniform. Extension half-times—the time required for Sequenase to incorporate a nucleotide opposite the modified base in 50% of the products—are listed in Table 1. More rapid nucleotide incorporation was observed opposite modified bases at positions 207, 234, 255, 260, 266, and 268. Slower rates were detected at positions 226, 250, and 289.

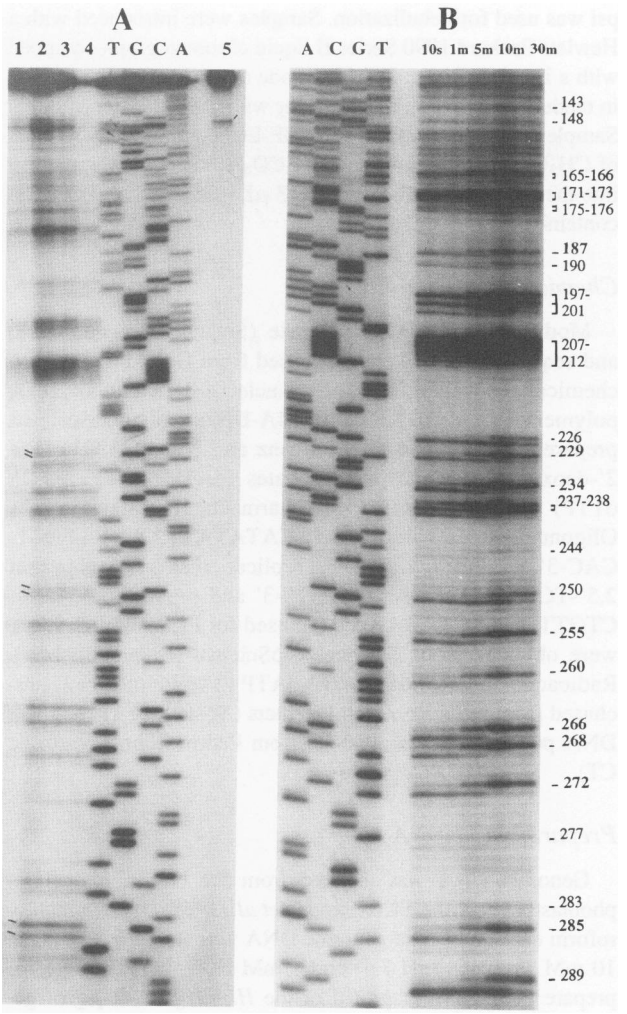

FIG. 2. Primer extension reactions by Sequenase on B*a*PDE-treated DNA template. The DNA template was a 244-nt fragment containing exon 3 of the human *HPRT* gene. A. The DNA was treated with B*a*PDE at 0.2 μM (lane 1), 0.4 μM (lane 2), 0.8 μM (lane 3), and 1.5 μM (lane 4). The treated DNAs and untreated DNA (lane 5) were used as templates in Sequenase-catalyzed DNA replication reactions. The reaction mixtures contained 0.2 mM dNTP and were incubated at 37°C for 5 min. The replication products were separated on a DNA sequencing gel. Lanes A, C, G, T are dideoxynucleotide sequencing products from the untreated template. The doublet lines in lane 2 indicate the positions at which DNA synthesis terminates before and opposite the adducts. The nucleotide sequence of the template corresponds to exon 3 (positions 135–318) of the human *HPRT* cDNA. B. A time course from 10 sec to 30 min. The DNA template was treated with 0.4 μM B*a*PDE.

The effect of dNTP concentrations was examined next. The extension half-times for reactions with 200 μM, 500 μM, 1 mM, 1.5 mM, and 2.5 mM of each dNTP were determined (Table 1). Increasing the dNTP concentrations in the reactions from 200 to 500 μM decreased the extension half-times by about 10-fold. Further increases in dNTP concentrations to 1

TABLE 1. EXTENSION HALF-TIMES (MIN) BY SEQUENASE FOR INCORPORATION OF NUCLEOTIDE OPPOSITE GUANINE- AND ADENINE-BaPDE ADDUCTS IN PORTION OF EXON 3 OF HUMAN HPRT GENE

Adduct Position	*Template Sequence*[b]	*dNTP Concentration*[a]			
		200 μM	*500 μM*	*1.0–1.5 mM*	*2.5 mM*
187	CATT G TAGC	5	0.2–1	0.2–1	1
190	TGTA G CCCT	5–10	0.2–1	0.2–1	1–5
197–201	CTCT GTGTG CT	5–10	0.2–1	0.2–1	1–5
207	TCAA G GGGG	1	0.2	0.2	1
212	GGGG G CTAT	5–10	0.2–1	0.2–1	1–5
226	CTTT G CTGA	30	5–10	1–5	5–10
229	TGCT G ACCT	5	0.2–1	0.2–1	1
234	TGCTGACCT G CTGG	1–5	0.2–1	0.2–1	1–5
238	GCTG G ATTA	5–10	0.2–1	0.2	1–5
244	TTAC A TCAA	5	0.2	0.2	1
250	CAAA G CACT	10–30	1–5	1	10
255	CACT G AATA	1–5	0.2–1	0.2–1	1
260	AATA G AAATA	1–5	0.2–1	0.2–1	1–5
266	AATA G TGAT	1–5	0.2	0.2	0.2–1
268	ATAGT G ATAG	1	0.2	0.2	0.2–1
272	GATA G ATCC	5	0.2	0.2	1
277	ATCC A TTCC	5	<0.2	<0.2	0.2
283	TCCT A TGAC	5	<0.2	<0.2	1–5
285	CTAT G ACTG	5	0.2–1	0.2–1	1–5
289	GACT G TAGA	10	1–5	1	1–5

[a]Approximate half-times for the incorporation of nucleotides opposite BaPDE-adducts, indicated by the underlined nucleotide in the second column, were estimated from time courses as shown in Figure 2B for reactions with 200 μM dNTP.

[b]The nucleotide sequences shown are for the template strand in the primer-extension reactions and are located in the nontranscribed strand exon 3 of the *HPRT* gene. Double underlining indicates short repeated DNA sequences.

and 1.5 mM had little effect on the extension half-times except for the positions that had the slowest extension times at lower dNTP concentrations: 226, 250, and 289. Higher dNTP concentrations, 2.5 mM, were inhibitory. As observed for the reactions with 200 μM dNTP, the extension half-times varied for the different adduct positions in the template (Table 1).

Our results were in agreement with studies by others that Sequenase can incorporate a nucleotide opposite PAH-damaged bases in a variety of DNA sequence contexts. Mutagenesis, however, likely requires full replication bypass of the lesion. Sequenase was tested for this activity. From previous experiments (Table 1), 1 mM dNTP appeared to be optimal for incorporation of a nucleotide opposite B*a*PDE-modified bases. Replication bypass activity was tested by extending the reaction times to 2 h (Fig. 3). At position 272, for example, primer-extension products terminating one base before and opposite the lesion were observed at 10 sec (lane a). By 1 min (lane b), most products terminated opposite the lesion. By 30 min (lane c), further extension was detected by the disappearance of the product terminated opposite the lesion. A similar pattern of an initial pause in replication before the modified base, another pause after nucleotide incorporation opposite the modified base, and then further extension was observed at the following positions: 187, 201, 226, 234, 237, 255, 260, 268, 272, 277, and 285. Poor or undetectable bypass extension was observed at positions 197, 229, 238, 266, and 289.

Effect of DNA sequence on translesion replication of BaPDE-damaged DNA

Relatively fast incorporation of a nucleotide across from a B*a*PDE-modified guanine and further extension were observed at four positions in the DNA template: guanines 234, 255, 260, and 268 (Table 1; Fig. 3). Guanine 260 is the only PAH-modified base in the DNA template that is flanked by four consecutive AT base pairs on both the 3′ and 5′ sides. The sequence–AATA is also repeated on both sides of the damaged base. A+T-Rich sequences also flank position 268, and the sequence–ATAG is repeated on both sides of the damaged base. However, A+T-richness does not always correlate with good bypass replication. For example, relatively fast nucleotide incorporation was observed opposite modified guanine 266, which is flanked by A+T-rich sequences (Table 1), but bypass extension was not detected (Fig. 3). Furthermore, relatively good translesion replication was observed at guanine 234, which is flanked by G+C-rich sequences on both sides; however, guanine 234 resides within a 5-nt repeat (Table 1). Relatively efficient translesion replication was also observed at guanine 255, but this residue is not flanked by direct repeats. Guanine 255 is flanked on the 3′ side by one of the repeats–AATA that flanks guanine 260. Thus, from the small sample of B*a*PDE-modified bases in our DNA template, the most efficient full bypass replication was correlated with

a local DNA environment containing repeats 4 or 5 nt in length.

Much slower replication of PAH-damaged bases was observed for guanines 226, 250, and 289 (Table 1; Fig. 3). At all three sites, slow incorporation of a nucleotide opposite the damaged base and slow continued extension were observed. The only distinguishing feature of the local sequence contexts was that at positions 226 and 250, the damaged base was followed by three consecutive thymidines at position 226 and by three consecutive adenines at position 250. Five guanines followed the damaged guanine at position 212, but bypass replication could not be analyzed in this region because modification of nearby guanines in other DNA templates masked the extension reaction opposite guanine 212.

Bacteriophage T4 DNA polymerase and T4 (exo$^-$) DNA polymerase-catalyzed DNA replication on the BaPDE-treated template

The experiments done with Sequenase were repeated with wild-type bacteriophage T4 DNA polymerase and with an exo$^-$ mutant, the D112A+E114A-T4 DNA polymerase (Reha-Kranz and Nonay, 1993). The same preparation of 0.4 μM B*a*PDE-treated template used for the Sequenase experiments was also used for the T4 DNA polymerase reactions. Wild-type T4 DNA polymerase produced only products that terminated one base preceding each PAH-modified guanine. There was no detectable incorporation of a nucleotide opposite the modified base, even at 750 μM dNTPs, a nucleotide concentration that produced optimal extension rates (data not shown). This result is in agreement with the data reported by Chary and Lloyd (1995) for the T4 DNA polymerase holoenzyme. They did not detect any nucleotide incorporation opposite PAH-modified adenines, presumably because any nucleotide incorporation was removed by the potent associated 3′ → 5′ exonuclease activity of the T4 DNA polymerase.

The T4 (exo$^-$) DNA polymerase yielded products terminating before and opposite modified template guanines, but the rates of nucleotide incorporation opposite the modified bases were slower than observed for Sequenase (Table 2). The DNA contexts that promoted faster nucleotide incorporation by Sequenase opposite modified bases did not necessarily stimulate nucleotide incorporation by the T4 DNA polymerase. For example, nucleotide incorporation opposite modified guanines 234 and 260 was relatively fast by Sequenase, but slow for the T4 (exo$^-$) DNA polymerase. The T4 (exo$^-$) DNA polymerase also could not extend primers that terminated opposite any of the modified bases; hence, full lesion bypass was not observed. This result is interesting because bypass of AP sites has been reported for another T4 (exo$^-$) DNA polymerase (Goodman *et al.*, 1993). Thus, the absence of full bypass replication of the PAH-treated DNA template by the T4 (exo$^-$) DNA polymerase indicates both that the DNA template contains predominately PAH-modified bases rather than AP sites and that the mechanism of bypass replication of PAH modified DNA differs from the replication of DNA with AP sites.

Effects of other electrophilic PAH derivatives on DNA replication

In order to test whether the results described above were specific to B*a*PDE adducts, we repeated the adduction and time-course DNA replication assays using CPPE- and FADE-treated DNAs. With the FADE-treated template, the pattern of products synthesized was very similar to the pattern observed with the B*a*PDE-modified template of Sequenase and for the T4 (exo$^-$) DNA polymerase (data not shown). With the CPPE-modified template, replication was blocked at some adenines and cytosines in addition to guanines (data not shown). Nucleotide incorporation opposite CPPE adducts proceeded more slowly than that opposite FADE and B*a*PDE adducts for Sequenase, and no translesion replication was observed. For the T4 (exo$^-$) DNA polymerase, no nucleotide incorporation opposite CPPE adducts was detected.

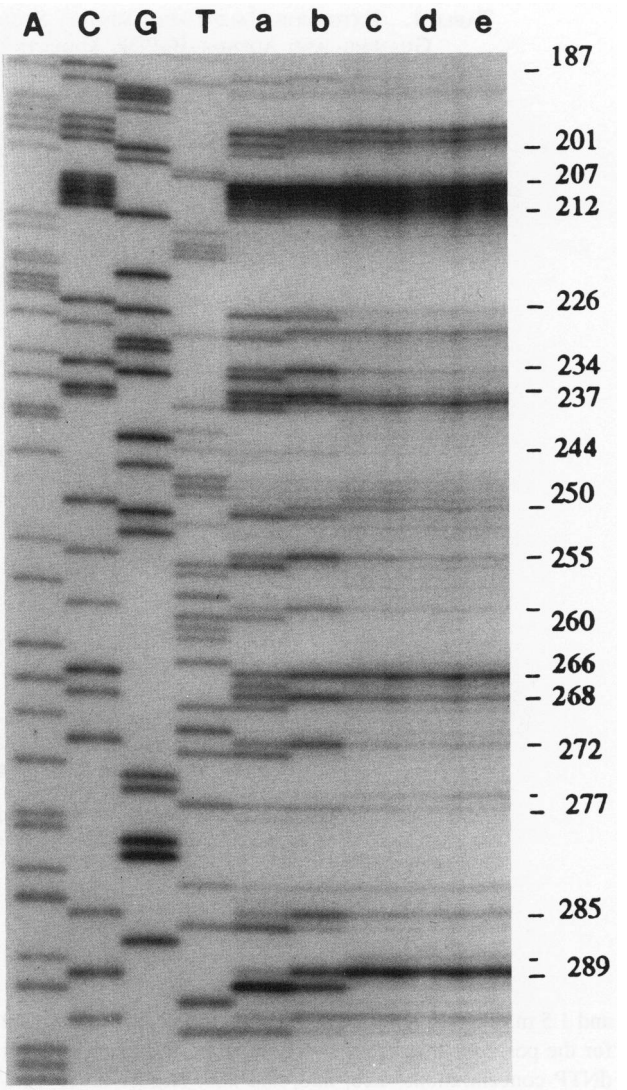

FIG. 3. Translesion replication by Sequenase. The DNA replication reactions were done at 37°C in the presence of 1.0 mM dNTP on B*a*PDE-treated DNA. The reactions were incubated 10 sec, 1 min, 30 min, 1 h, and 2 h (lanes a, b, c, d, and e, respectively).

DISCUSSION

An *in vitro* DNA replication assay was used to examine DNA polymerase function on templates damaged by bulky PAH

epoxide and diol epoxide adducts. The DNA template, a 244-base fragment containing exon 3 of the human *HPRT* gene, was treated with three chemicals: B*a*PDE, FADE, or CPPE. Three DNA polymerases were studied: T7 (exo$^-$) DNA polymerase (Sequenase), wild-type T4 polymerase (data not shown), and a T4 (exo$^-$) DNA polymerase. Analysis of the patterns of the replication products produced on the chemically treated templates demonstrated that replication by all three DNA polymerases was blocked initially opposite one base 3′ to each modified base, primarily guanines and, to a lesser extent, adenines (see Figs. 2 and 3). Treatment with CPPE produced more modifications of adenines and some cytosines, in agreement with earlier studies showing that CPPE induces mutations predominantly at GC basepairs, but also at AT basepairs at a frequency higher than observed with B*a*PDE (Eisenstadt *et al.*, 1982; Keohavong *et al.*, 1995).

We have extended previous results (Chary and Lloyd, 1995; Hruszkewycz *et al.*, 1991; Hruszkewycz and Dipple, 1991; Hruszkewycz *et al.*, 1992; Thrall *et al.*, 1992; Belguise-Valladier *et al.*, 1994) by studying bypass replication over time. Full replication of the damaged DNA template requires three steps. The first step is rapid extension from the primer to the nucleotide preceding the damaged base. The second step is incorporation of a nucleotide opposite the damaged base. The third step, detected only at certain sites, is further extension of the primer beyond the damaged base. Sequenase and the T4 (exo$^-$) DNA polymerase were both able to incorporate a nucleotide opposite PAH-modified bases, but only Sequenase was able to catalyze extension past some template lesions formed by B*a*PDE and FADE. Thus, B*a*PDE- and FADE-modified bases, in certain local DNA environments, were not absolute barriers to replication by Sequenase. It is interesting that the highly processive Sequenase, but not the T4 (exo$^-$) DNA polymerase, was able to catalyze full bypass replication. However, high processivity is not an absolute requirement for translesion replication, as a KF (exo$^-$) has also been reported to fully replicate some PAH-adenine lesions (Chary and Lloyd, 1995) and AF-modified guanines (Belguise-Valladier and Fuchs, 1995), and this DNA polymerase is not processive. Thus, bypass replication may be a characteristic of the type of DNA polymerase that encounters the lesion, and the mechanism of bypass replication may be different for various DNA polymerases. However, the two DNA polymerases with reported translesion activity—exonuclease-deficient KF and T7—are members of the same family of protein sequence-related DNA polymerases (Braithwaite and Ito 1993) and thus may share functional characteristics.

Guanines were the primary target of the chemical treatments in our studies, but it appears that translesion replication was detected at adenines 244 and 277 (see Fig. 3). The full bypass replication detected in our studies with Sequenase and the absence of translesion replication detected in assays performed by Chary and Lloyd (1995) may indicate that PAH-modified guanines are easier to replicate than modified adenines or that some other aspect of the reaction conditions differed. The DNA template examined in this report was much longer, 244 nt, than the 33-nt template used by Chary and Lloyd and other investigators (Hruszkewycz *et al.*, 1991; Hruszkewycz and Dipple, 1991; Hruszkewycz *et al.*, 1992). In addition, our templates contained PAH-modified bases in a variety of DNA sequence contexts, some of which appeared to stimulate translesion replication.

Nucleotide concentration and the DNA sequences flanking both sides of the damaged base affected the rates of translesion replication. Very high dNTP concentrations, >750 μM, were required for efficient incorporation of a nucleotide opposite the damaged base by Sequenase and the T4 (exo$^-$) DNA polymerase (see Tables 1 and 2). Efficient translesion replication that was observed only for Sequenase (Fig. 3) was correlated with a local DNA environment that contained short, 4 to 5 nt, repeats. Also, A+T-richness, in addition to DNA repeats, may stimulate translesion replication at two modified guanines, numbers 260 and 268. The association of direct sequence repeats with translesion replication suggests that the mechanism of bypass replication involves DNA slippage. Yet monotonous runs of three Ts or As 5′ to the modified base, as at guanines 226 and 250, decreased the ability of Sequenase to incorporate a nucleotide opposite the damaged base (see Table 1). This mechanism of bypass replication involving slippage is not clearly understood. For lesion-induced slippage to occur, however, the nucleotide incorporated across from the lesion must, via slippage, form a correct basepair with the next template nucleotide, thus allowing the extension reaction to proceed efficiently.

In summary, our study using a 244-nt DNA template treated with B*a*PDE, CPPE, and FADE showed that DNA replication first terminated one base 3′ to each adduct followed by incorporation of a nucleotide opposite the modified base, consistent with previous reports using short oligonucleotide templates (Chary and Lloyd, 1995; Hruszkewycz *et al.*, 1991; Hruszkewycz and Dipple, 1991; Hruszkewycz *et al.*, 1992; Thrall *et al.*, 1992; Shibutani *et al.*, 1993; Belguise-Valladier

TABLE 2. EXTENSION HALF-TIMES (MIN) BY T4 (EXO$^-$)DNA POLYMERASE FOR INCORPORATION OF A NUCLEOTIDE OPPOSITE GUANINE- AND ADENINE-B*a*PDE ADDUCTS[a]

Adduct Position	*Template Sequence*[b]	*Time at dNTP 750 μM*
187	CATT G TAGC	>30
190	TGTA G CCCT	10–30
197–201	CTCT GTGTG CT	5–10
207	TCAA G GGGG	5
212	GGGG G CTAT	5–10
226	CTTT G CTGA	10–30
229	TGCT G ACCT	>30
234	TGCTGACCT G CTGG	30
238	GCTG G ATTA	10
244	TTAC A TCAA	30
250	CAAA G CACT	5–10
255	CACT G AATA	5–10
260	AATA G AAATA	30
266	AATA G TGAT	10–30
268	ATAGT G ATAG	5–10
272	GATA G ATCC	5–10
277	ATCC A TTCC	5
283	TCCT A TGAC	5
285	CTAT G ACTG	30
289	GACT G TAGA	>30

[a]Approximate half-times for the incorporation of nucleotides opposite BaPDE-adducts, indicated by the underlined nucleotide in the second column, were estimated from time courses.

[b]The nucleotide sequences shown are for the template strand in the primer-extension reactions and are located in the nontranscribed strand exon 3 of the *HPRT* gene. Double underlining indicates sequence repeats.

et al., 1994; Belguise-Valladier and Fuchs, 1995). Moreover, our results showed that replication continued at some sites, to give full bypass of the lesions, and that the rate of lesion bypass was affected by the type of PAH adduct, the sequence context of the adduct. These findings provide the means to design *in vitro* DNA replication assays to study the mechanism of bypass replication. Defined DNA templates can be constructed, and the *in vitro* products can be sequenced to determined which nucleotide is incorporated opposite the lesion. Although the T4 (exo$^-$) DNA polymerase could not fully replicate the PAH-modified templates, T4 DNA polymerase accessory proteins can be added in order to determine if a highly processive T4 (exo$^-$) DNA polymerase has the ability to catalyze translesion replication.

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