1398–1405 Nucleic Acids Research, 1995, Vol. 23, No. 8

In vitro replication by prokaryotic and eukaryotic polymerases on DNA templates containing site-specific and stereospecific benzo[*a*]pyrene-7, 8-dihydrodiol-9,10-epoxide adducts

Parvathi Chary and R. Stephen Lloyd*

Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, TX 77555, USA

Received December 1, 1994; Revised and Accepted March 9, 1995

ABSTRACT

DNA adducts of the environmental carcinogen benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) interact stereospecifically with prokaryotic and eukaryotic polymerases *in vitro*. Toward understanding the capacity to replicate past different diastereomers of BPDE at specific sites in DNA, six deoxyoligonucleotides, each 33 bases long, were constructed with stereochemically defined BPDE adducts on adenine N^6 at position two of the human N-*ras* codon 61. Four polymerases that were studied under single encounters with the template–primer complex terminated synthesis one base 3′ to the lesion with all the adducted templates. When multiple encounters between polymerase and substrate were permitted, each of the polymerases analyzed revealed a unique pattern for a given adducted template. The general replication pattern was encompassed under two categories, reflecting the significance of the R and S configurations of C_{10} of the pyrenyl ring attached to the single-stranded DNA template. Furthermore, within each of these categories, every polymerase demonstrated distinct quantitative differences in product accumulation at a given site, for the various adducted templates. Among the polymerases utilized in this study, exonuclease-deficient Klenow fragment of polymerase I (*exo*⁻ KF) exhibited the most efficient translesion synthesis resulting in ~16% full-length products with the modified templates bearing adducts with C_{10}-S configuration. In contrast, chain elongation with bacteriophage T4 DNA polymerase bearing an active 3′→5′ exonucleolytic activity was most strongly inhibited by all six BPDE-adducted templates. Misincorporation of A opposite the adduct occurred in all the templates when polymerized with Sequenase, whereas *exo*⁻ KF preferentially incorporated C opposite the C_{10}-R BPDE adducts and A opposite the C_{10}-S BPDE adducts.

INTRODUCTION

Benzo[*a*]pyrene, a polycyclic aromatic hydrocarbon (PAH), is metabolically activated to bay region 7,8-dihydrodiol-9,10-epoxides (BPDE) that initiate mutagenesis and carcinogenesis after covalently binding to DNA in both bacterial and mammalian cells (1–6). The mutagenic potential of these diol epoxides is dependent on a variety of interactions including ones between the stereospecific (and site-specific) carcinogen and the template, the nature of the polymerase involved in replication past the adduct, and the efficiency of DNA repair within the cell (2). DNA containing *ras* genes generates a transforming oncogene when incubated with BPDE and transfected into NIH 3T3 cells (7,8). *Ras* gene activation can be initiated by a single point mutation, usually resulting in the alteration of amino acid residue 12 or 61 of the protein encoded by these genes (7). This occurs mainly due to DNA lesions formed by *-cis-* and *-trans-* opening of the epoxide group of BPDE adducts at the exocyclic N^2 position of guanine, at N^6 position of adenine, or N-7 and O^6 positions of guanine or N^4 position of cytidine. These events thus lead to several possible products, many of which are potentially mutagenic (3,9).

Our earlier investigations on the *in vivo* consequences of stereospecifically and site-specifically placed BPDE lesions involved a prokaryotic model system (10). This required the construction of six stereochemically defined BPDE adducts on adenine N^6 at position two of N-*ras* codon 61 within an 11-base oligodeoxynucleotide that was inserted into a single-stranded vector and the mutation spectrum was then examined in a repair-deficient background. All the six BPDE adducts generated exclusively A→G mutations at frequencies ranging from 0.26 to 1.20% (10).

In vitro replication studies are also important, especially given the likelihood that mutagenicity caused by these stereospecific bulky adducts could be dependent upon the DNA polymerase that encounters the lesion (11,12). Several such studies with bulky lesions report blockage in the vicinity of the damaged site (13–16). However, the correlation between the orientation/tilt of the bulky adduct and the *in vitro* replication pattern with a given polymerase is not well established. Recent NMR studies and observations of stereoselective resistance to digestion of the BPDE modified templates by phosphodiesterases I and II determined the general

* To whom correspondence should be addressed

directionality of the individual stereoisomers at N^2 position of guanine (17–19). Solution conformation of BPDE-adducted double-stranded oligonucleotides at N^2 guanine revealed that the (+)-*anti-trans* isomer bearing the S configuration at C_{10} position is directed toward the 5′ end of the modified strand. In contrast, the (–)-*anti-trans* isomer (C_{10}-R configuration) is oriented toward the 3′ end of the adducted strand (17). The general orientation of these adducts is in agreement with those deduced from exonuclease digestion studies with dG-BPDE-adducted single-stranded DNA (18). However, conclusions on the orientation of dA-BPDE C_{10}-R and C_{10}-S adducts on single-stranded templates based on exonucleolytic assays were different from those obtained by NMR analysis of the duplex structure (19,20). Inferences made on the tilt of these adducts by primer extension studies using HIV-I RT were in agreement with those obtained from double-stranded structures, thus suggesting that hydrolytic enzymes recognize single-stranded oligonucleotides differently than polymerases at template–primer complexes (19,20). The present study was undertaken to further investigate if polymerases in general follow this pattern. There is also a recent report on the action of polymerases on BPDE-dA adducted templates that offers support to our finding (21).

MATERIALS AND METHODS

Enzymes, BPDE-adducted oligonucleotides and radiolabeled nucleotides

The T4 polynucleotide kinase and T4 DNA ligase were obtained from New England Biolabs Inc. (Beverly, MA). Klenow fragment (6 U/μl) was purchased from Life Technologies, Inc. (Bethesda, MD), human polymerase α (1.5 U/μl) from Molecular Biology Resources, Inc (Milwaukee, WI) and Sequenase 2.0 version (13 U/μl) from US Biochemical (Cleveland, OH). Klenow fragment *exo*$^-$ was a gift from Drs M. F. Goodman and L. Bloom, University of Southern Califomia, Los Angeles, CA, rat polymerase β was the gift of Dr S. H. Wilson, University of Texas Medical Branch, Galveston, TX and T4 polymerase holoenzyme (gene products 43, 44/62 and 45) was generously supplied by the laboratory of Dr B. Alberts, University of California, San Franscisco, CA. The six stereochemically defined BPDE adducts were constructed on adenine N^6 at position two of N-*ras* codon 61 within an 11-base oligodeoxynucleotide by the post-oligomerization strategy (22,23) and kindly provided by Drs C. M. and T. M. Harris, Vanderbilt University, Nashville, TN. (Fig. 1). Deoxynucleoside triphosphates (100 mM) were obtained from Pharmacia (Piscataway, NJ), and [γ-^{32}P]ATP (3000 Ci/mmol) utilized for 5′ end-labeling of oligodeoxynucleotides was purchased from Dupont NEN (Boston, MA).

Construction of BPDE-adducted 33mers for chain extension reaction

The six BPDE-adducted 11mers were analyzed for their purity on 15% polyacrylamide sequencing gels prior to ligation with a 22mer, using a 27mer as a scaffold (24). The 33mer sequence utilized was 5′-CGGACAAGAAGAATTC GT C GTGACTGG-GAAA AC-3′. The adducted base on the template is underlined. This oligonucleotide was used for *in vitro* primer extension assays with a [γ-^{32}P]ATP (3000 Ci/mmol) 5′ end-labeled 17mer primer (5′-GTTTTCC CAGTCACGAC-3′).

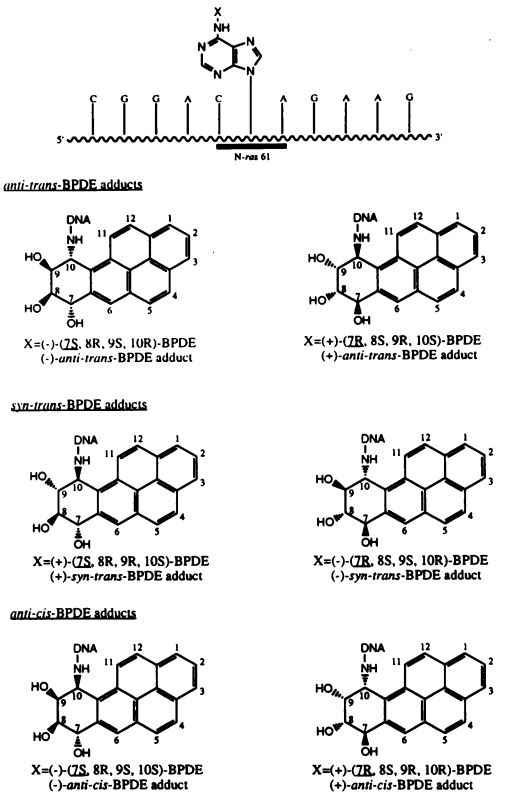


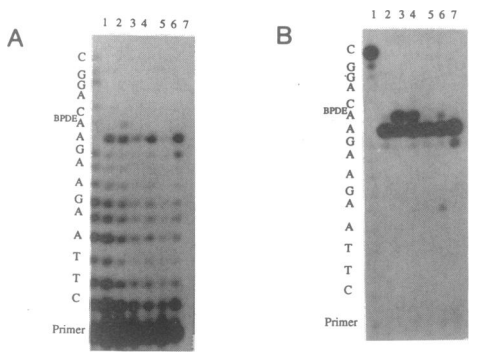
Figure 1. Structures of stereochemically specific adenine N^6-BPDE adducts attached to the second position of N-*ras* codon 61 within an 11mer oligodeoxynucleotide that is bridged to a 22mer to form a 33mer template. Note that the (–)-*anti-trans*-, (–)-*syn-trans*- and (+)-*anti-cis*–BPDE stereoisomers exhibit an R configuration at the C_{10} position, whereas the (+)-*anti-trans*, (+)-*syn-trans*, (–)-*anti-cis*-BPDE stereoisomers reveal an S configuration at C_{10}—the position of attachment to DNA.

Polymerase arrest assays

The template (BPDE-adducted or non-adducted 33mer) primer (T·P) ratio was 5:1 (250:50 fmol) in which >99% of the primer annealed to the template. This was analyzed by separation of the annealed mixture on a 10% native polyacrylamide gel followed by quantitation using the PhosphorImager (Molecular Dynamics, Sunnyville, CA). Chain elongation conditions have been previously described (24,25). Nucleotide incorporation opposite the adducted site was determined by utilizing a 27mer primer in the polymerase extension reaction and incubated with the individual nucleotide triphosphates (750 μM final concentration of each nucleotide) along with one polymerase per reaction at concentrations used for the multiple-hit conditions. Primer extended products were separated on 15% polyacrylamide sequencing gels and quantified by the PhosphorImager (Molecular Dynamics).

Single and multiple encounters

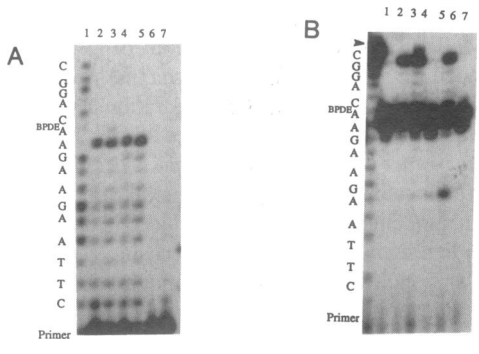
The random event of enzyme binding to the T·P complex was calculated for single and multiple encounters using the Poisson distribution. For single-hit events, sufficient enzyme was added for short time periods such that <35% of the T·P complex interacted with the polymerases. In contrast, multiple encounters were defined



C

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(−) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of fully extended primer	96.4	0.3	0.4	0.34	0.3	0.2	0.27
% of blockage opposite adduct	1.02	4.1	45.2	28.9	5.5	8.6	1.6
% of blockage 1 base 3' to adduct	2.6	95.6	54.6	70.7	94.2	91.2	98.2

Figure 2. Polymerase arrest assay of BPDE-adducted templates by the *exo*$^+$ Klenow fragment. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (−)-*anti-trans*-BPDE-adducted 33mer; lane 4, (−)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (−)-*anti-cis*-BPDE-adducted 33mer. (**A**) Reaction products under single-hit encounters ranging from 0.1 to 0.4 (1.25 fmol, 10 s). (**B**) Multiple-hit encounters ranging from 2.9 to 3.8 (25 fmol, 30 min). (**C**) The quantitative representation of the arrested products following multiple-hit conditions.



C

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(-) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of blunt end addition product	75.6	1.6	3.2	4.4	1.6	1.2	1.0
% of fully extended primer	15.2	3.7	18.8	16.6	3.7	13.8	1.1
% of blockage opposite the adduct	1.7	14.8	5.8	9.5	8.6	8	4.6
% of blockage 1 base 3' to adduct	5.5	74.2	69.5	65.6	76.9	70.4	77.1
% of blockage 2 bases 3' to adduct	1.4	5.5	2.5	3.5	8.3	3	15.5
% of blockage 6 bases 3' to adduct	0.6	0.2	0.2	0.4	0.9	3.6	0.7

Figure 3. Replication pattern of BPDE-adducted templates by *exo*- Klenow fragment. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (−)-*anti-trans*-BPDE-adducted 33mer; lane 4, (−)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (−)-*anti-cis*-BPDE-adducted 33mer. (**A**) Replication products under single-hit encounters ranging from 0.1 to 0.8 (8 fmol, 20 s). (**B**) With multiple-hit encounters ranging from 3.6 to 4.0 (4 pmol, 30 min) fully extended products were formed in lanes 3, 4 and 5 alone. (**C**) Quantitative representation of the results of (B) pointing to the clear demarcation in replication pattern between lanes 3, 4 and 6 versus lanes 2, 5 and 7.

as those events in which at least 95% of the primers were extended. These conditions would be predicted to reflect at least three polymerase hits per T·P complex. This was accomplished by greatly increasing the incubation period and enzyme concentration. The percentage of unextended primers (for both single- and multiple-hit conditions) and products resulting from differential blockage at varied positions 3′ and 5′ to the adducted site (under multiple hit conditions) were quantitated using the PhosphorImager.

RESULTS

Characterization of DNA synthesis by Klenow fragment

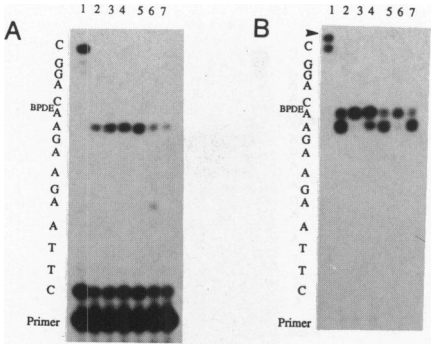
The Klenow fragment (KF), a moderately processive polymerase and 3′→5′ exonuclease was used on all six BPDE adducted templates under both single- and multiple-hit conditions (Fig. 2A and B) (26–28). Under single-hit conditions replication with all the adducted templates terminated one base 3′ to the site of the lesion. Whereas, under multiple-hit conditions, although none of the adducted templates permitted the synthesis of fully extended products, (−)-*anti-trans*-, (−)-*syn-trans*- and (+)-*anti-cis*-BPDE-adducted templates allowed the incorporation of nucleotides one base 3′ and opposite the site of the lesion (category 1). However, most of the extended primers which were complexed with the (+)-*anti-trans*-, (+)-*syn-trans*- and (−)-*anti-cis*-BPDE-adducted templates terminated one base prior to the damaged site (category 2) (Fig. 2B). The quantitation of the termination bands are presented in Figure 2C.

Specificity of 3′→5′ exonuclease deficient KF

The mutant form of KF (D355A, E357A) which lacks 3′→5′ exonuclease activity generally allows for a better elongation of templates containing DNA adducts relative to its *exo*$^+$ counterpart (25,29,30). Like the 3′→5′*exo*$^+$ KF, under single-hit conditions all the adducted templates terminated one base prior to the site of the lesion (Fig. 3A). When replication was observed under multiple-hit conditions there was severe blockage with all the adducted templates not only one base 3′ to the lesion but also, to some extent, opposite the adduct. However in stark contrast to results seen for *exo*$^+$ KF, the (−)-*anti-trans*-, (−)-*syn-trans*- and (+)-*anti-cis*-BPDE-adducted samples promoted full-length products at levels similar to the non-adducted template. In addition, the unadducted strand and the (−)-*syn-trans*-BPDE-adducted template revealed a +1 blunt end addition product (Fig. 3B) (25). These data are quantitatively represented in Figure 3C.

Effect of BPDE-adducts on Sequenase progression

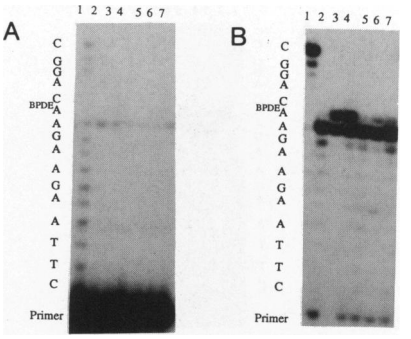
Sequenase version 2.0 (modified T7 DNA polymerase) was selected for its high degree of processivity and its lack of 3′→5′ exonucleolytic activity (31,32). Under single-hit conditions both the non-adducted and adducted templates exhibited strong pause sites 10 bases 3′ to the site of lesion, followed by very processive extension until one base 3′ to the lesion in all adducted templates. No translesion synthesis was observed (Fig. 4A). Under multiple-hit conditions all the primers on the adducted templates were extended up to the site of lesion. However the extent of termination one base prior to the damaged site was greater with the (+)-*anti-trans*-, (+)-*syn-trans*- and (−)-*anti-cis*-BPDE-adducted templates (Fig. 4B). Furthermore, with the non-adducted sample, a non-template directed +1 addition at the 3′ terminus



C

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(-) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of blunt end addition product	44.5	0.11	0.18	0.04	0.26	0.1	0.1
% of fully extended primer	44	0.13	0.2	0.1	0.09	0.2	0.18
% of blockage opposite adduct	4.5	41.5	95.8	73.1	34	82.8	29.9
% of blockage 1 base 3′ to adduct	6.9	58.2	3.8	26.8	65.6	16.9	69.7

Figure 4. Effect of BPDE adducts on replication by Sequenase. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (–)-*anti-trans*-BPDE-adducted 33mer; lane 4, (–)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (–)-*anti-cis*-BPDE-adducted 33mer. (**A**) Single-hit encounters ranging from 0.1 to 0.4 (25 fmol, 30 min). (**B**) Multiple-hit encounters ranged from 2.3 to 3.0 (1 pmol, 60 min). Quantitative analyses (**C**) revealed a pattern wherein lanes 3, 4 and 6 were similar, and 2, 5 and 7 were similar.



C

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(-) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of fully extended primer	72.3	2.7	0.9	0.4	0.3	1.0	2.0
% of blockage 4 bases 5′ to adduct	12.8	0.6	0.2	0.2	0.1	0.3	0.6
% of blockage opposite to adduct	1.8	3.9	34.8	22.4	5.2	14.3	4.4
% of blockage 1 base 3′ to adduct	9.9	78.9	57.3	70.3	84.8	77.2	82.5
% of blockage 2 bases 3′ to adduct	3.2	13.9	6.8	6.8	9.6	7.2	10.5

Figure 5. Primer extension of BPDE adducts by human polymerase α. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (–)-*anti-trans*-BPDE-adducted 33mer; lane 4, (–)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (–)-*anti-cis*-BPDE-adducted 33–mer. (**A**) Single-hit encounters ranging from <0.1 to 0.1 (7.5 fmol, 30 min). (**B**) Multiple-hit encounters range from 2.1 to 3.2 (75 fmol, 60 min). (**C**) A quantitative estimation of the extended products.

was observed as with *exo*⁻ KF. Quantitative estimation of these products are shown in Figure 4C.

Primer extension on BPDE-adducted templates by human polymerase α

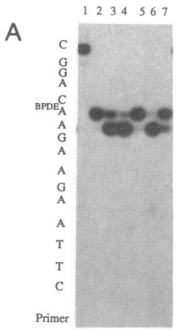
Human DNA polymerase α-primase is the only nuclear enzyme complex capable of binding single-stranded DNA and initiating the synthesis of a new strand of DNA *de novo* (33). It is essential for replication of both the leading and lagging strands in a eukaryotic cell, and exhibits low to moderate processivity. This enzyme was chosen for the present study to determine its ability to bypass stereospecifically oriented bulky adducts on a DNA template. Under single-hit conditions all the adducted templates forming truncated products one base 3′ to the site of lesion (Fig. 5A). Under multiple-hit conditions the predominant extension products still terminated one base 3′ to the adduct. However, in concert with the general pattern observed by other polymerases examined herein, the (–)-*anti-trans*-, (–)-*syn-trans*- and (+)-*anti-cis*-BPDE-adducted templates formed additional products that truncated opposite the site of the lesion (Fig. 5B). These data are quantitatively shown in Figure 5C.

In vitro replication events with rat polymerase β

DNA polymerase β (β-pol) is the simplest naturally occurring eukaryotic polymerase. It is a monomeric protein of 335 amino acids (39 kDa) lacking exonuclease activity. β-pol is one of five mammalian DNA polymerases and it is believed to function primarily in the repair of the damaged DNA (34,35). It works in a distributive manner normally, but is capable of gap-filling synthesis on single-stranded DNA templates in a processive fashion for up to 6 nucleotides, if there is a nearby 5′ phosphate end on the downstream primer (36). Owing to its putative role in repair, this enzyme was chosen to study its replicative capacity past bulky adducts. Single-hit conditions did not yield meaningful results due to the distributive nature of the enzyme. The polymerases under multiple-hit conditions terminated one base 3′ or opposite the site of lesion with all the adducted templates (Fig. 6A). The quantitation of the termination products is given in Figure 6B.

Role of T4 DNA polymerase holoenzyme

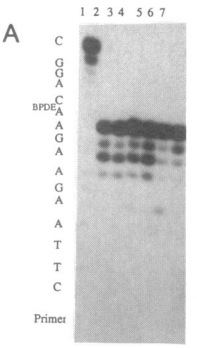
T4 DNA polymerase holoenzyme contains a polymerase activity (43 protein), a DNA-dependent ATPase (44/62 protein), a 'sliding clamp' capability (45 protein) and single-stranded DNA binding ability (32 protein) (37,38). It also possesses 3′→5′ exonucleolytic activity and has the distinction of being among the most accurate polymerases known (39). This enzyme, being analogous to the DNA polymerase holoenzyme complexes in both prokaryotic and eukaryotic replication systems, served as a good choice to study the *in vitro* replication pattern on site-specific and stereospecific BPDE-adducted templates. Multiple-hit conditions alone were studied due to the number of accessory proteins involved in assembling the holoenzyme. Unlike any of the other polymerases examined, there were significant pause sites 1–3 bases 3′ to the adduct (Fig. 7A). Although the general pattern qualitatively appears similar, quantitative estimation of all these truncated products revealed a slight increase in the replicated products two bases 3′ to the lesion in the (+)-*anti-trans*-, (+)-*syn-trans*- and (–)-*anti-cis*-BPDE-adducted templates (Fig. 7B). Increasing the incubation period to 90 min did not facilitate further elongation of primers complexed with the damaged templates. The non-adducted template however resulted in full-length products with as short an incubation period as 30 s.



B

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(-) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of fully extended primer	86.8	4.1	0.6	0.5	0.7	0.5	1.6
% of blockage opposite to adduct	5.2	**87.4**	24.5	13.9	**75.5**	19.8	**74.4**
% of blockage 1 base 3' to adduct	8.0	8.5	**74.9**	**85.5**	23.9	**79.7**	24.0

Figure 6. Interaction of rat polymerase β on BPDE-adducted templates. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (–)-*anti-trans*-BPDE-adducted 33mer; lane 4, (–)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (–)-*anti-cis*-BPDE-adducted 33mer. Multiple-hit encounters ranged from 3.0 to 3.7 (6 pmol, 30 min) (**A**). Values are quantitatively represented in (**B**).



B

Blockage Position	N-ras 61 non-adducted	(+) anti-trans BPDE adducted	(-) anti-trans BPDE adducted	(-) syn-trans BPDE adducted	(+) syn-trans BPDE adducted	(+) anti-cis BPDE adducted	(-) anti-cis BPDE adducted
% of fully extended primer	77.5	5.9	0.5	0.3	0.2	0.3	1.3
% of blockage 4 bases 5' to adduct	17.4	1.7	0.3	0.3	0.3	0.3	1.8
% of blockage 1 base 3' to adduct	1.1	**58.6**	**72.0**	**74.9**	**64.8**	**79.3**	**74.5**
% of blockage 2 bases 3' to adduct	0.6	**9.6**	7.2	8.0	**10.8**	8.0	**12.6**
% of blockage 3 bases 3' to adduct	1.0	**17.7**	**13.4**	**11.0**	**15.6**	**6.0**	**6.8**
% of blockage 4 bases 3' to adduct	1.4	6.0	5.9	4.5	7.0	2.4	2.1
% of blockage 6 bases 3' to adduct	1.0	1.5	0.7	1.0	1.3	3.7	0.9

Figure 7. Replication consequences of BPDE-adducted templates with T4 DNA polymerase. Lane 1, unmodified 33mer; lane 2, (+)-*anti-trans*-BPDE-adducted 33mer; lane 3, (–)-*anti-trans*-BPDE-adducted 33mer; lane 4, (–)-*syn-trans*-BPDE-adducted 33mer; lane 5, (+)-*syn-trans*-BPDE-adducted 33mer; lane 6, (+)-*anti-cis*-BPDE-adducted 33mer; lane 7, (–)-*anti-cis*-BPDE-adducted 33mer. (**A**) Multiple-hit encounters ranging from 4.3 to 5.3 (15 ng/μl 43p, 75 ng/μl 44/62p and 21 ng/μl 45p for 30 min). Quantitative representation of their values are tabulated in (**B**).

Nucleotide incorporation opposite the BPDE-adducted site

Primer extension studies on BPDE-adducted templates under single and multiple-hit conditions for each of the polymerases examined revealed either truncated-products that terminated in the vicinity of the damaged site or exhibited translesion synthesis leading to full-length products. Primers utilized in these analyses were 11 bases 3′ to the adducted site and used in a reaction mixture containing all four dNTPs. However, in order to precisely determine the nucleotide placed opposite each of the six BPDE-dA adducts with each of the polymerases under study, a 27mer primer was utilized that extended up to one base 3′ to the adduct and only a single dNTP per reaction mixture was used.

As shown in Figure 8A the nucleotides incorporated opposite the site of lesion with a particular polymerase fell under two categories, based on the C_{10} configuration of BPDE relative to the template strand. In addition to this criterion, the presence or absence of exonucleolytic activity of the polymerases also classifies the specific nucleotide placement into two classes. Among the *exo*$^+$ enzymes, T4 DNA polymerases allowed no incorporation of nucleotides opposite the adducted site. However, with *exo*$^+$ KF, category 1 (C_{10}-R) exhibited an incorporation of T, whereas category 2 (C_{10}-S) showed no incorporation at all. In all cases, due to the inherent exonucleolytic activity, the length of the products formed with each of the nucleotides corresponded predominantly to the first 3′ position (relative to the adduct) of its respective complementary base on the template (Fig. 8B). Among the *exo*$^-$ enzymes, polymerases α and β showed no mismatch when a 28mer product was formed under multiple-hit conditions. In addition, polymerase β clearly showed that the hierarchy of the less preferred nucleotides fell under two categories that fit the classification based on the C_{10} configuration of the adduct (Fig. 8A). With *exo*$^-$KF, C was preferentially placed opposite the first category and A opposite the second category of BPDE-dA sites (Fig. 8A and C). With Sequenase, A was the predominant mismatch nucleotide opposite all the adducts. However, like pol β, a hierarchy of less preferred nucleotides followed a pattern that was unique to each of the two categories (Fig. 8A and D).

DISCUSSION

In order to understand how PAHs can give rise to mutations, a comparative *in vitro* investigation was undertaken to study the action of prokaryotic and eukaryotic DNA polymerases upon encountering bulky DNA adducts. These adducts not only have the potential to alter the base-pairing properties of the T·P complex, but also tend to impede DNA synthesis by the polymerase (40). Conformational differences caused by DNA polymerase–carcinogen adduct interaction therefore can have an impact in altering the site of termination of DNA synthesis, that could in turn be unique to individual polymerases (41). To characterize the effects of bulky adducts on DNA synthesis, several *in vitro* studies have been performed (13–15,21,42–44). With specific reference to BPDE-adducted templates, although investigations have mainly involved guanine residues, few studies included adenine adducts also, but both types of lesions blocked DNA replication with certain enzyme systems either at or one base prior to the site of the adducted template (15,16,21,45,46). The present study represents an extensive examination of the interaction of six polymerases with six stereochemically distinct BPDE adducts attached to the position N^6 of adenine at the second position of the N-*ras* 61 codon.

To determine the molecular details of DNA replication at the adducted site, *in vitro* reactions were conducted under both single

A

Polymerase Type	(+)*anti-trans* BPDEadducted template10S	(-)*anti-trans* BPDEadducted template10R	(-)*syn-trans* BPDEadducted template10R	(+)*syn-trans* BPDEadducted template10S	(+)*anti-cis* BPDEadducted template10R	(-)*anti-cis* BPDEadducted template10S
T4 DNA Polymerase	No incorpn.	No incorpn.	No incorpn.	No incorpn.	No incorpn.	No incorpn.
Exo+Klenow Fragment	No incorpn.	T	T	No incorpn.	T	No incorpn.
Exo-Klenow Fragment	A >>>T ≡ G ≡ C	C > A ≡ T > G	C > A ≡ T > G	A >>>T ≡ G ≡ C	C > A ≡ T > G	A >>>T ≡ G ≡ C
Sequenase	A >>> G ≡ T	A > G > T > C	A > G > T > C	A >>> G ≡ T	A > G > T > C	A >>>G ≡ T
Polymerase Alpha	No incorpn.	T >> A	T >> A	No incorpn.	T >> A	No incorpn.
Polymerase Beta	T >> A > C > G	T >> A > G	T >> A > G	T >> A > C > G	T >> A > G	T >> A > C > G

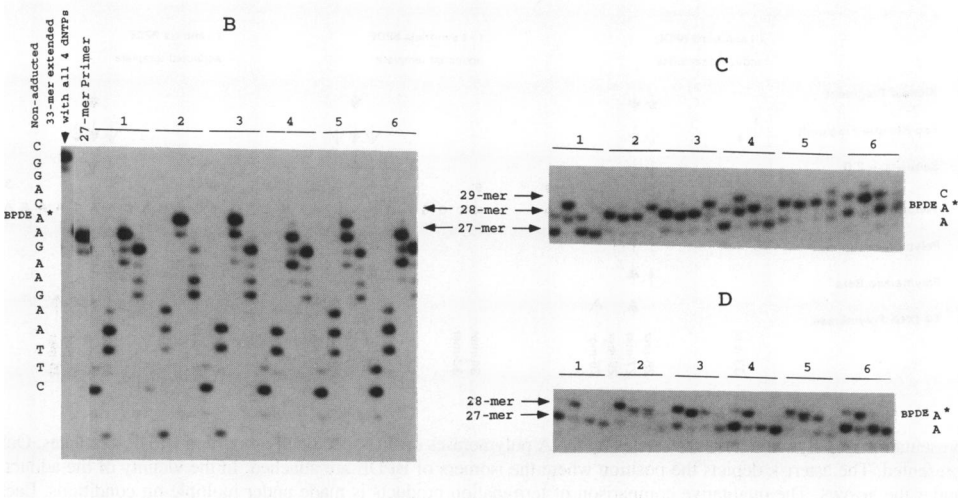


Figure 8. Nucleotide incorporation opposite BPDE-dA adducts by primer extension. (**A**) A tabular representation of the hierarchy of nucleotide preference opposite individual BPDE lesions with each of the six polymerases examined. The ratio of band intensity of the combined value of the 28 and 29mers:27mer primer was quantitated utilizing the PhosphorImager. The qualitative representation of the incorporated nucleotides is shown by the scale below. Scale: <1 is ≡, >1 is >, >2 is >>, >5 is >>>. (**B–D**) are autoradiographs of primer extension reactions of BPDE-adducted templates using *exo*+ and *exo*- KF and Sequenase, respectively. Sections 1–6 represent polymerase reactions with (+)-*anti-trans*, (–)-*anti-trans*, (–)-*syn-trans*, (+)-*syn-trans*, (+)-*anti-cis*, (–)-*anti-cis*-BPDE-adducted 33mer templates using a 27mer primer and 750 μM concentration of each of the dNTPs examined individually. Each set of four lanes represents G, A, T and C, respectively.

and multiple-hit conditions (47). As observed in Figures 2A–5A, single-hit events for each of the six stereochemically specific BPDE-adducted templates with each of the four polymerases examined resulted in primer extended products that terminated one base prior to the lesion. However, with five of the six polymerases tested under multiple-hit conditions, and with three of the six adducted templates examined (Figs 2B–5B, 6A and 7A), the polymerization products showed the incorporation of a nucleotide opposite the damaged site along with completely elongated products in a subpopulation (Fig. 3B). Each polymerase, tested on a given lesion, revealed a pattern that was distinct from the other enzymes examined. However, the general pattern of extension of the six adducted templates fell into two classes (Fig. 9). These results indicate that specific properties of a replicating polymerase are further influenced by the conformation of the damaged template. Similar observations have been made on interactions between DNA polymerases and adducts such as styrene oxide and aminofluorene (24,25,47,48).

There is clear evidence to show that PAHs with 10 S and 10 R configurations point in the opposite direction to each other and control enzymatic actions (17–19). Thus, these two categories of BPDE adducts demarcated on a structural basis not only regulate the resistance to exonucleolytic digestion by phosphodiesterase I and II, but also modulate polymerase function (18–21).

To further characterize the role played by the various polymerases, replication of adducted DNA could also be analyzed based on the presence or absence of 3′→5′ exonucleolytic, editing activity of the enzyme that controls the extent of misincorporation and elongation from the distorted template-primer structure (42,49). The proofreading 3′→5′ exonuclease activity competes with chain extension (42). On the contrary, low levels or lack of 3′→5′ exonuclease activity allows the priming of the nucleotide opposite the altered template site to remain in position such that elongation can occur past particular adducts, resulting in translesion synthesis (50). The polymerases used in this study include the KF and T4 DNA polymerase which exhibit a 3′→5′ exonucleolytic activity, whereas KF *(exo-)*, Sequenase, human polymerase α and rat polymerase β are devoid of this exonuclease function. As shown in Figures 2 and 7, the two *exo*+ polymerases do not extend beyond the site of the lesion. In addition, strong pause sites are seen 2–4 bases prior to the adducts even under multiple-hit conditions with the T4 DNA polymerase complex. In contrast, all the *exo*- polymerases examined extended the primers on three adducted templates up to the site of lesion (Figs 3–6) and,

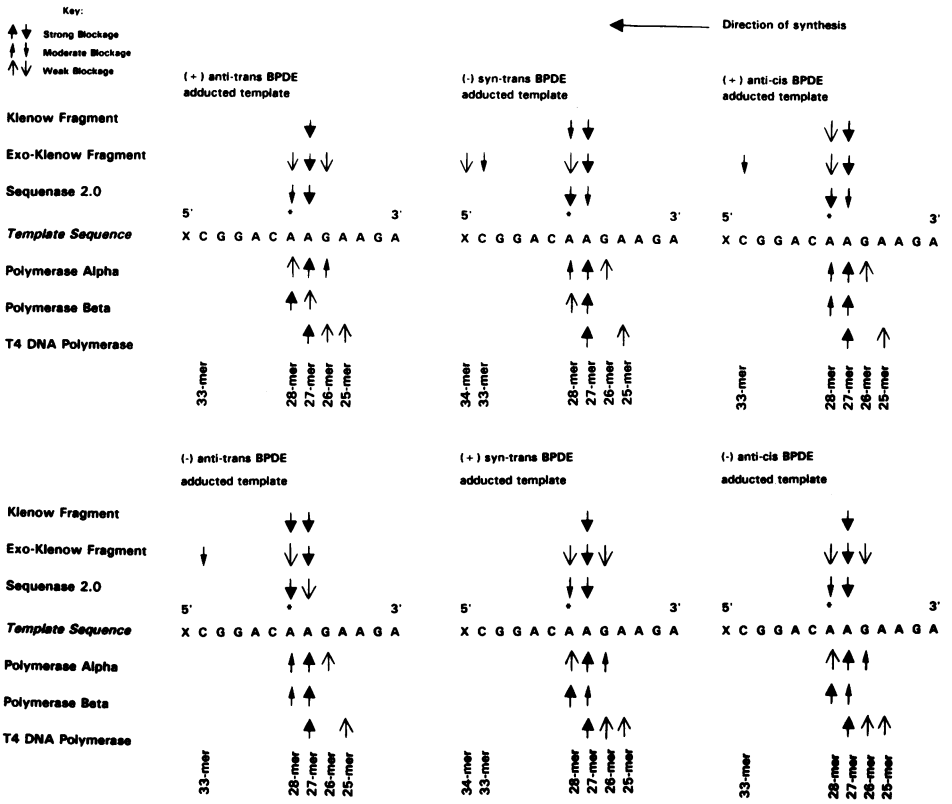


Figure 9. Schematic representation of polymerization arrest sites by DNA polymerases on site-specifically modified BPDE templates. Only a portion of the 33mer template sequence is represented. The asterisk depicts the position where the isomers of BPDE are attached. In the vicinity of the adduct, the sizes of termination products are indicated under the arrows. The qualitative comparison of termination products is made under multiple-hit conditions. Each of the six polymerases represents a unique termination pattern with individual BPDE-adducted templates. A common pattern is observed within (+)-*anti-trans*-, (+)-*syn-trans*- and (–)-*anti-cis*-BPDE-adducted templates, thus encompassing one group, whereas (–)-*anti-trans*-, (–)-*syn-trans*- and (+)-*anti-cis*-BPDE-adducted templates represent a common pattern which forms a second group, distinct from the first.

in some cases, even resulted in full-length products (Fig. 3). Thus, a spectrum of replication capability was observed even within the category that lacked 3′→5′ exonucleolytic activity.

The hierarchy for the *exo*⁻ polymerases was determined based on the criteria for their ability to extend the T·P complex. The polymerases ranked as follows in their degree of extension of the primer: *exo*⁻ KF > Sequenase > β pol > polymerase α. The *exo*⁻ KF was capable of forming fully extended products on (–)-*anti-trans*-, (–)-*syn-trans*- and (+)-*anti-cis*-BPDE-adducted templates and exhibited the best translesion synthesis (Fig. 3). These T·P complexes were the same as those that gave longer extension fragments with *exo*⁺ KF (Fig. 2). With Sequenase, all the T·P complexes revealed products that terminated opposite the adduct (Fig. 4). Primers extended by either polymerase α or β exhibited opposite *in vitro* replication patterns for the isomers within an enantiomeric pair. For example, with polymerase α the primers extended up to the site of lesion more predominantly with the (–)-*anti-trans*-, (–)–*syn-trans*- and (+)-*anti-cis*-BPDE-adducted templates (Fig. 5). In contrast, with polymerase β most of the extended primers with the (+)-*anti-trans*-, (+)-*syn-trans*- and (–)-*anti-cis*-BPDE-adducted templates terminated opposite the damaged site (Fig. 6). These observations suggest that exonuclease activity cannot be the only factor involved in elongation (50). Thus, the inequality in lesion bypass may be attributed to other parameters such as mechanistic differences in processivity of the enzyme and rate of polymerization. Furthermore, given that the two mammalian polymerases exhibit different replication patterns within a specific T·P complex, it is possible that these enzymes within a system could function synergistically, resulting in bypass of the damaged templates.

The dichotomy between the *exo*⁺ *and exo*⁻ polymerase function on adducted templates is further exemplified by nucleotide incorporation studies. The *exo*⁺ enzymes either exhibit no incorporation of a nucleotide opposite the lesion or exclusively insert a T (Fig. 8A). On the other hand, analyses of the products obtained with those *exo*⁻ enzymes that revealed a T predominantly opposite the lesion showed that other nucleotides can also be incorporated to a lesser extent. Furthermore, Sequenase exhibited misincorporation of A opposite all the dA-BPDE adducts examined, whereas *exo*⁻ KF placed C preferentially opposite the C_{10}-R adducts and A opposite the C_{10}-S adducts. It is possible that the adducts are directly in contact with these polymerases, leading to stabilization of a mispaired configuration in spite of improper hydrogen bonding (51,52).

In a recent study, the mutagenic introduction of adenine on BPDE-dA templates was also observed on different sequence contexts (21). However, distinct differences were observed between this study and our present report pertaining to nucleotide incorporation assays utilizing *exo*⁻ KF. Our results showed that full-length species were formed with the C_{10}-R adducted

templates with preferential insertion of C, whereas A was preferentially incorporated opposite the lesion in the truncated products of C_{10}-S BPDE templates. Christner *et al.* (21), on the other hand, could not firmly determine which nucleotide was incorporated opposite the adducts nor could substantial amounts of full-length products be obtained. This discrepancy could possibly be attributed to the utilization of a much longer template in our study (33mer:16mer).

In conclusion it appears that the interaction of bulky adducts with polymerases is not particularly sensitive to stereochemistry *(anti-trans, anti-cis* and *syn-trans)* except at the point of attachment to the nucleoside (C_{10}-R versus C_{10}-S). Furthermore, each polymerase appears to be unique in its interaction with the individual adducted templates. However, to evaluate the mutagenic potential of a given adduct within a cell, it will be necessary to gain a better understanding of *in vivo* replication and repair processes.

ACKNOWLEDGMENTS

We are grateful to Drs T. M. Harris and C. M. Harris for synthesis of the BPDE-adducted oligodeoxynucleotides, to Dr S. H. Wilson for his generous gift of rat polymerase β, to Drs B. M. Alberts and R. D. Schrock for providing T4 DNA polymerase holoenzyme, and Drs M. F. Goodman and L. Bloom for their gift of *exo*$^-$ KF. We are especially thankful to G. J. Latham and A. G. McNees for helpful comments during the course of this study. We are thankful to the personnel in the Recombinant DNA Laboratory, University of Texas Medical Branch, for providing the non-adducted templates, and to J. A. Daniels for the typing of this manuscript. This work was supported by United States Public Health Service grants ES05355 and ACS FRA 381.

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