Site-Specific Benzo[a]pyrene Diol Epoxide–DNA Adducts Inhibit Transcription Elongation by Bacteriophage T7 RNA Polymerase†

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ABSTRACT: Benzo[a]pyrene, an extremely potent procarcinogen and mutagen, is metabolized to a variety of products, including the ultimate carcinogen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. This product of biotransformation reacts with DNA, forming a series of adducts principally at the N^2 position of guanine that differ in their stereochemistry and exhibit unique biological properties. In order to gain a better understanding of the effects on RNA synthesis of these adducts, we used purified bacteriophage T7 RNA polymerase to transcribe a series of templates containing one of four stereoisomerically pure BPDE-guanine lesions—(+)-*trans*-, (−)-*trans*-, (+)-*cis*-, or (−)-*cis*-*anti*-N^2-BPDE-guanine—or no damaged bases. To construct suitable double-stranded oligodeoxynucleotides for these studies, we annealed an 11-mer containing a site-specific stereoisomerically pure N^2-BPDE-guanine adduct, a 37-mer, and a 10-mer to a complementary 58-base sequence of single-stranded DNA. The oligomers were ligated, purified, and reannealed. The resulting DNA template contained the promoter for T7 RNA polymerase and a BPDE adduct at position +16 following the transcription initiation site. The results of the transcription assays clearly demonstrate that each of the adducts inhibits elongation by T7 RNA polymerase, but they do so to significantly different extents, depending on the stereochemical characteristics of the BPDE-modified guanine. The order of inhibition is (+)-*trans* > (−)-*trans* > (+)-*cis* > (−)-*cis*, when the amount of full-length transcript for each is compared to that obtained for an unmodified template. Furthermore, premature termination of RNA synthesis occurs at or near the site of the BPDE lesion as evidenced by the formation of discrete, truncated transcripts. These results might be related to the fact that the pyrenyl moiety of the *trans*-BPDE adducts is situated in the minor groove of double-stranded DNA, but is quasi-intercalated into the double helix in the case of the *cis* stereoisomers. Our results are in agreement with previous data showing that DNA randomly damaged with BPDE is poorly transcribed; they also add a new level of complexity to understanding the influence of these adducts on DNA-dependent enzymatic RNA synthesis by showing a strong effect of lesion stereochemistry on the inhibition of elongation.

Benzo[a]pyrene is a ubiquitous byproduct of incomplete combustion and is one of the most potent carcinogens known (Albert & Burns, 1977; Selkirk et al., 1982; Conney, 1982). In mammals, it is metabolized to a variety of products, including the ultimate DNA-damaging agent BPDE[1] (Sims & Grover, 1974; Weinstein et al., 1976; Koreeda et al., 1978). This biotransformation results in a metabolite that exists in two diastereomeric forms, each having a pair of enantiomers: (+)-*anti*-BPDE, (−)-*anti*-BPDE, (+)-*syn*-BPDE, and (−)-*syn*-BPDE (Figure 1) (Yang & Gelboin, 1976; Yang et al., 1976; Singer & Grunberger, 1983). The biological effects of these different stereoisomers are strikingly different (Conney, 1982). For example, the (+)-*anti*-BPDE enantiomer is significantly more tumorigenic than the (−)-*anti*-BPDE isomer when applied to mouse skin (Slaga et al., 1979); likewise, it causes tumors in the lungs of newborn mice, but the (−)-*anti*-BPDE conformer does not (Buening et al., 1978). The stereochemistry of BPDE is also responsible for dramatic differences in mutagenicity in several bacterial and mammalian test systems: (+)-*anti*-BPDE is more mutagenic than (−)-*anti*-BPDE or either of the *syn*-BPDE stereoisomers in mammalian cells; however, (−)-*anti*-BPDE is several times more mutagenic in some of the *Salmonella typhimurium* strains than (+)-*anti*-BPDE (Wood et al., 1977; Burgess et al., 1985; Stevens et al., 1985).

The covalent binding of BPDE to cellular DNA can give rise to mutations and probably plays a critical role in the initiation stages of carcinogenesis (Singer & Grunberger, 1983). This binding occurs via either *cis* or *trans* opening of the epoxide ring, leading to the formation of mostly guanine and adenine adducts (Meehan & Straub, 1979; Jeffrey, 1985; Cheng et al., 1989). The *anti*-BPDE enantiomers form primarily (+)-*trans*-, (−)-*trans*-, (+)-*cis*-, and (−)-*cis*-*anti*-N^2-BPDE-guanine adducts, and these are shown in Figure 1 (Meehan & Straub, 1979; Cheng et al., 1989). Recently, oligodeoxynucleotides 9–18 bases in length containing stereochemically and positionally defined *anti*-N^2-BPDE-guanine lesions have been synthesized (Cosman et al., 1990; Geacintov et al., 1991; Hruszkewycz et al., 1992; Mao et al., 1992; Margulis et al., 1992; Shibutani et al., 1993). Structural data for several of these *anti*-N^2-BPDE-guanine oligonucleotide adducts have been obtained using high-resolution NMR

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[1] Abbreviations: BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (+)-*anti*-BPDE, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, which is the 7*R*,8*S*,9*S*,10*R* configuration; (−)-*anti*-BPDE, (−)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, which is the 7*S*,8*R*,9*R*,10*S* configuration; (+)-*syn*-BPDE, (+)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, which is the 7*S*,8*R*,9*S*,10*R* configuration; (−)-*syn*-BPDE, (−)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, which is the 7*R*,8*S*,9*R*,10*S* configuration; bp, base pairs; nt, nucleotides; rNTPs, ribonucleoside triphosphates.

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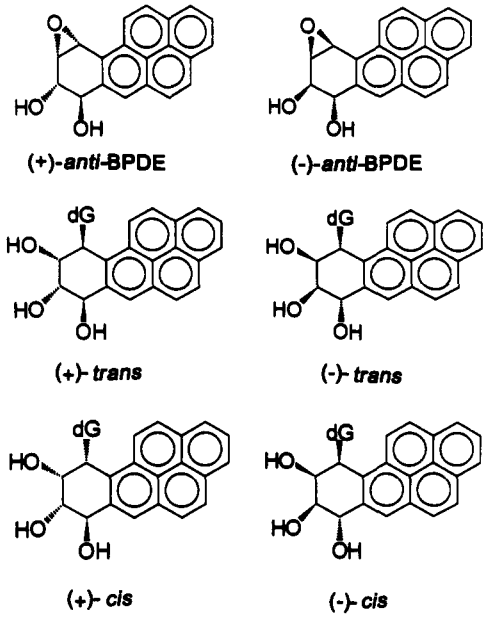

FIGURE 1: The enantiomers of *anti*-BPDE are shown. (+)-*anti*-BPDE gives rise to *anti*-N^2-BPDE-dG lesions having only (+) configurations as represented by (+)-*trans* and (+)-*cis*; (−)-*anti*-BPDE yields only the (−) configuration adducts as represented by (−)-*trans* and (−)-*cis*. *syn*-BPDE and the products that result from its reaction with DNA are not shown because it was not used in these investigations.

techniques, and their conformational properties have been characterized using NMR distance constraints and computational strategies with potential energy minimization (Cosman et al., 1992, 1993; de los Santos et al., 1992). In the 11-mer 5′-d(CCATCG^{BPDE}CTACC), the pyrenyl moiety of (+)-*trans*-*anti*-N^2-BPDE-guanine lies in the minor groove of a B-form DNA duplex and is situated over a deoxyribose in the complementary strand, with its long axis pointing toward the 5′ end of the modified strand (Cosman et al., 1992); interestingly, the (−)-*trans*-*anti*-N^2-BPDE-guanine adduct has a similar conformation, but the pyrenyl group is oriented toward the 3′ end of the altered strand (de los Santos et al., 1992). The pyrenyl moiety in (+)-*cis*-*anti*-BPDE adducts is inserted into the B-DNA double helix and stacks with neighboring bases on the same strand, with a displacement of the guanosyl moiety into the minor groove; this disrupts normal base-pairing at the site of the lesion (Cosman et al., 1993). This type of conformation is thus different from classical intercalation in which the normal Watson–Crick base pairs are not disturbed, and the helix is merely stretched by the modified base. The fine structure for the (−)-*cis*-*anti*-N^2-BPDE-guanine adduct has not been fully clarified, but spectroscopic studies suggest that its arrangement is similar in overall aspects to that of the (+)-*cis* adduct (Geacintov et al., 1991). The striking variations in the orientations of the (+)-*trans* and (−)-*trans* isomeric *anti*-N^2-BPDE-guanine lesions in the minor groove of B-form DNA was predicted for a different alternating GC sequence by energy minimization computer modeling techniques using the Program Duplex without any input of experimental parameters (Singh et al., 1991).

Various biological events such as replication and transcription are likely to be affected adversely by the presence of BPDE adducts in DNA. In addition, enzymatic processes associated with their clearance might be influenced by the stereochemical configuration of the BPDE-guanine lesion and a particular adduct's effect on DNA synthesis and gene expression. Indeed, this is supported by recent evidence demonstrating that transcription and DNA repair are intimately connected, with certain adducts being more efficiently cleared from the transcribed strands of active loci than elsewhere in the genome (Scicchitano & Hanawalt, 1992; Hanawalt & Mellon, 1993). It has been suggested by Hanawalt (1991) that those DNA lesions that pose blocks to message elongation by RNA polymerases are subject to preferential removal. The validity of this hypothesis can be verified in part by meticulously investigating the process of transcription with adduct-containing DNA templates.

Most investigations concerning the behavior of DNA polymerases, RNA polymerases, or DNA repair at BPDE damage have used randomly modified DNA with a multiplicity of lesions, rather than well-characterized site-specific modifications and stereoisomerically defined adducts. The utilization of DNA molecules containing stereoisomerically defined lesions at unique sites is a powerful tool for unraveling the relationships between specific structural features and the biological consequences of individual adducts (Basu & Essigmann, 1988; Singer & Essigmann, 1991). We have made use of oligodeoxynucleotides, specifically 5′-d(CCATCG^{BPDE}CTACC), containing stereoisomerically pure site-specific *anti*-N^2-BPDE-guanine lesions to synthesize double-stranded DNA suitable for transcription by T7 RNA polymerase. This is the same BPDE-modified oligonucleotide sequence that was used in duplex form in previous NMR structural studies (Cosman et al., 1992, 1993; de los Santos et al., 1992). Bacteriophage T7 RNA polymerase was chosen to initiate these investigations because it is well characterized, its promoter is clearly defined, and the purified enzyme is commercially available (Chamberlin & Ryan, 1982). By fixing the precise position and configuration of the modified base, we have been able to demonstrate that single *anti*-N^2-BPDE-guanine adducts present on the transcribed strand of the DNA template impede elongation catalyzed by T7 RNA polymerase. Furthermore, the inhibition of transcription, as assessed by the amount of full-length transcript obtained as a function of time, differs dramatically, depending on the stereoisomeric configuration of the BPDE lesions.

MATERIALS AND METHODS

Oligodeoxynucleotides, Enzymes, and Chemicals. Oligodeoxynucleotides containing BPDE adducts were synthesized, purified, and characterized as previously described (Cosman et al., 1990). Unmodified oligodeoxynucleotides were purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX). Radiolabeled compounds were procured from Du Pont NEN Research Products (Boston, MA). All enzymes and biochemical reagents were obtained from United States Biochemical Corp. (Cleveland, OH) or Promega (Madison, WI); all other chemicals, reagents, and supplies were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO).

Preparation of an Unmodified DNA Template Containing the Bacteriophage T7 RNA Polymerase Promoter. In order to optimize conditions for studying transcription past BPDE–DNA adducts, an unmodified template was prepared by annealing together an equimolar ratio of two complementary 58-mers. The sequence is identical to that shown in Figure 2, except that ligation was not necessary and no BPDE lesions were present. The annealing buffer consisted of 40 mM Tris·HCl, pH 7.6; 20 mM $MgCl_2$; and 50 mM NaCl; the mixture was heated to 80 °C and slowly cooled to 4 °C. This sequence was not radiolabeled.

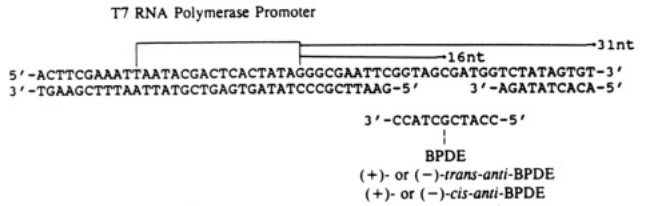

FIGURE 2: Site-specific BPDE-modified DNA templates containing a specific stereoisomer of BPDE on the transcribed strand have been designed as shown. The bacteriophage T7 RNA polymerase promoter has been incorporated into the sequence as well. The position of the specific stereoisomer of BPDE is indicated, and it is located at position +16 from the transcription initiation site. DNase footprinting data obtained by Basu and Maitra (1986) have shown that bacteriophage T7 RNA polymerase protects the promoter region over a 28 bp range that extends from −20 to +8. This suggests that the site of our adduct is sufficiently removed from the promoter region so as not to interefere with the actual process of initiation. The arrows indicate the direction of transcription; the full size of the transcript is 31 bases.

Preparation and Purification of Unmodified and BPDE-Modified DNA. The (−)-*cis-anti*-N^2-BPDE-guanine adducts tend to be labile in the presence of ultraviolet light; to prevent any deterioration of the adducts, we performed the synthesis reactions and transcription assays under yellow light. It is worth mentioning, however, that even when this precaution was not taken, our results were similar, suggesting that adduct decomposition is minimal during the relatively brief exposures to ambient, unfiltered laboratory lights.

An unmodified 37-base sequence and an unmodified or BPDE-modified 11-base oligodeoxynucleotide containing stereospecific BPDE-N^2-guanine lesions were phosphorylated at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP; the reaction was stopped by heating at 65 °C for 10 min, and unincorporated [γ-^{32}P]ATP was removed with a Sephadex G-25 spin column. The 5′ phosphorylated 37-mer, one of the 5′ phosphorylated unmodified or modified 11-mers, and a 10-mer were annealed to a complementary DNA sequence 58 bases in length at a ratio of 2:1 in 40 mM Tris·HCl, pH 7.6; 20 mM $MgCl_2$; and 50 mM NaCl (Figure 1). The annealing mixture was heated to 80 °C and slowly cooled to 4 °C; the three oligodeoxynucleotides were ligated with T4 DNA ligase and ATP at 15 °C for 16 h, and the reaction was stopped by the addition of EDTA. The resulting 58-base oligonucleotides containing a single BPDE adduct, or the unmodified 58-mer, were purified by denaturing 15% PAGE with 7 M urea. The gels were briefly exposed to X-ray film, and the bands corresponding to the 58-base oligodeoxynucleotides were excised and eluted from the polyacrylamide by soaking in 0.3 M sodium acetate, pH 7.5, overnight at 37 °C. The eluted BPDE oligonucleotides were precipitated with two volumes of ethanol and then dissolved in H_2O.

Preparation of BPDE–DNA Templates for T7 RNA Polymerase Assays. A purified 58-base oligodeoxynucleotide containing a specific stereoisomer of BPDE was annealed to a complementary 58-mer as described above. The annealed templates were treated with exonuclease I to remove single-stranded oligodeoxynucleotides, the solutions were then extracted with phenol, and the templates were precipitated in ethanol. The pellet was washed with 70% ethanol, and the double-stranded DNA templates were resuspended in H_2O.

T7 RNA Polymerase Transcription Assays. Unless stated otherwise, standard transcription assays were carried out in a 100-μL reaction mixture of 40 mM Tris·HCl, pH 8.0; 15 mM $MgCl_2$; 5 mM DTT; 0.5 mg/mL BSA; 200 μM each of ATP, GTP, and CTP; 4.8 μM [α-^{32}P]UTP (40 Ci/mmol); 2 pmol of template; 25 units of RNasin; and 20 units of T7 RNA polymerase. Transcripts were analyzed by 15% PAGE with 7 M urea and detected by autoradiography with X-ray film, or analyzed and quantified with a Model GS-250 Molecular Imager (Bio-Rad Laboratories; Hercules, CA).

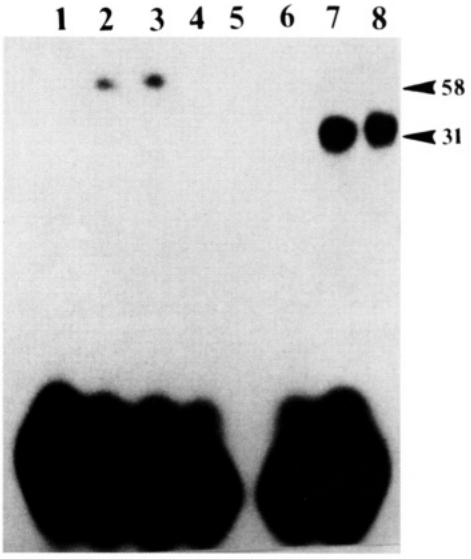

FIGURE 3: Transcription assay using denaturing PAGE and unmodified DNA as a template for T7 RNA polymerase. Characterization and optimization of the transcription assay were determined by using the unmodified, nonradioactive 58-mer as a template under a variety of conditions where certain components of the standard reaction mixture were omitted or changed. Unless indicated otherwise, the mixture analyzed in each lane of the gel shown contained 40 mM Tris·HCl, pH 8.0; 15 mM $MgCl_2$; 5 mM DTT; 0.5 mg/mL BSA; 200 μM each of ATP, GTP, and CTP; 4.8 μM [α-^{32}P]UTP (40 Ci/mmol); 2 pmol of unmodified 58-mer template; 25 units of RNasin; and 20 units of T7 RNA polymerase in a 50-μL volume that was incubated for 20 min at 37 °C. The arrows mark the positions of the expected 31-base transcript and the aberrant transcript located at 58 bases. (Lane 1) No DNA template, (lane 2) 58-base nontranscribed template strand, (lane 3) 58-base transcribed template strand, (lane 4) double-stranded template in the absence of RNA polymerase, (lane 5) double-stranded 58-mer template and no [α-^{32}P]UTP, (lane 6) double-stranded 58-mer template and 200 μM [α-^{32}P]UTP (5 Ci/mmol), (lane 7) double-stranded, unmodified 58-mer, (lane 8) transcription with double-stranded unmodified 58-mer, followed by chromatography with G-50 Sephadex to remove unincorporated [α-^{32}P]UTP. The template DNA was not radioactively labeled; therefore, it cannot be seen on the autoradiogram.

The 20 units of T7 RNA polymerase used in these experiments correspond to approximately 0.2–0.3 pmol of enzyme, assuming a specfic activity of 600 000 units/mg for purified polymerase and a molecular weight of 98 000 (Chamberlin & Ryan, 1982). This ensures that excess template is present in the reaction. The lower concentration of UTP reflected the need for high specific activity in order to obtain a signal. Assay conditions were adapted from Chen and Bogenhagen (1993) and Zhou and Deutsch (1993).

RESULTS

RNA Synthesis by Bacteriophage T7 RNA Polymerase Using a Short, Synthetic Double-Stranded DNA Template. An unmodified, nonradioactive template having the same sequence as that shown in Figure 2 was constructed by simply annealing two complementary 58-base oligodeoxynucleotides; it was then used to demonstrate that such a double-stranded segment of DNA could direct transcription by T7 RNA polymerase in the presence of rNTPs and proper buffer conditions. The results are presented in Figure 3. In the absence of template (lane 1), T7 RNA polymerase (lane 4), or labeled UTP (lane 5), synthesis of transcript was not

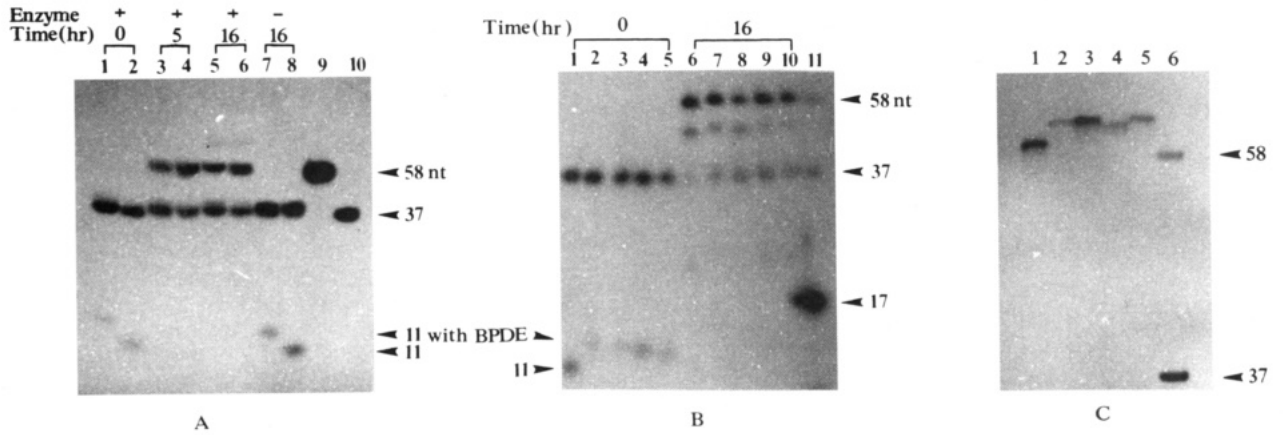

FIGURE 4: The synthesis of BPDE–DNA templates for T7 RNA transcription assays was carried out as described. The DNA template containing a different stereoisomer of BPDE at position 43 from the 3′ end was synthesized by annealing the 11-base sequence containing the BPDE-N^2-guanine adduct, a 37-mer, and 10-mer to a complementary 58-base oligonucleotide and ligating. Panel A: Time course for the synthesis of the unmodified and (+)-*trans* 11-mers analyzed by denaturing 15% PAGE. The ligation reaction was monitored by withdrawing a portion of the reaction mixture at various times as indicated above each lane; the presence or absence of T4 DNA ligase is also indicated. (Lanes 1, 3, 5, and 7) Reaction contains 11-base sequence with a (+)-*trans*-*anti*-N^2-BPDE-guanine adduct; (lanes 2, 4, 6, and 8) reaction contains unmodified 11 base sequence; (lanes 9 and 10) 58-base and 37-base single-stranded DNA markers. Panel B: Synthesis of 58-base oligonucleotides using an unmodified 11-mer or one of each of the 11-mers containing an *anti*-N^2-BPDE-guanine. The samples were analyzed by denaturing 15% PAGE. (Lanes 1 and 6) Unmodified; (lanes 2 and 7) (+)-*trans*; (lanes 3 and 8) (–)-*trans*; (lanes 4 and 9) (+)-*cis*; (lanes 5 and 10) (–)-*cis*; (lane 11) single-stranded DNA markers. Panel C: The 58-mers obtained in the gel shown in panel B were analyzed by denaturing PAGE (20%) at 2000 V. (Lane 1) Unmodified; (lane 2) (+)-*trans*; (lane 3) (+)-*cis*; (lane 4) (–)-*cis*; (lane 5) (–)-*trans*; (lane 6) single-stranded DNA markers. The 58-mer marker migrated slightly faster than the unmodified 58-mer in lane 1 because it was phosphorylated at the 5′ end; the label for the sample in lane 1 is at internal phosphates, and the 5′ end is not phosphorylated.

observed. Likewise, when the specific activity of the [α-^{32}P]-UTP was too low, no transcripts were seen (lane 6). Upon addition of all the essential components for transcription, RNA fragments of the expected size were synthesized, with the best results being obtained following removal of unincorporated [α-^{32}P]UTP with G-50 Sephadex as shown in lane 8. The newly appearing bands were degradable by RNase and were unaffected by DNase (results not shown). The optimal conditions for transcription assays for our experiments were found to be those that are described in Materials and Methods.

During the course of our studies using the 58 bp undamaged DNA template prepared by annealing two complementary oligodeoxynucleotides, we discovered that small amounts of RNA, larger in size than the anticipated transcript, were sometimes synthesized. The largest of the spurious transcripts was approximately equal to the total length of the template DNA used in these experiments, which led us to reason that excess unannealed DNA might be directing transcription even in the absence of a complete promoter. We tested this idea by using single-stranded 58-base DNA oligodeoxynucleotides as templates in the assay; these results are also presented in Figure 3. In the case where the single-stranded DNA corresponded to the nontranscribed strand of our double-stranded template, RNA was synthesized, and it was longer in length than the transcripts obtained from double-stranded templates (lane 2). When a single-stranded 58-mer corresponding to the transcribed strand of our template was used in the assay, another discrete band was obtained that was similar in size (lane 3). This observation is consistent with reports showing that T7 RNA polymerase can transcribe single-stranded DNA under suitable conditions (Chamberlin & Ryan, 1982). These results demonstrated that the presence of single-stranded DNA could potentially interfere with our assay by directing transcription by T7 RNA polymerase. It is for this reason that we employed exonuclease I, an enzyme that specifically digests single-stranded DNA, to remove residual single-stranded DNA from our templates that were prepared by ligation of several oligonucleotides as described in Materials and Methods.

Synthesis of BPDE–DNA Templates. In order to synthesize a template suitable for transcription by bacteriophage T7 RNA polymerase, each of the 11-mers—one unmodified and four modified with BPDE—were phosphorylated at the 5′ end with [^{32}P]phosphate and annealed to a complementary 58-mer along with a 5′ [^{32}P]phosphorylated 37-mer and a 10-mer (Figure 2). The oligomers were covalently joined with T4 DNA ligase. The progress of each ligation was monitored by withdrawing a portion of the reaction mixture at various times following the addition of the enzyme and analyzing each by PAGE; the results for the unmodified and (+)-*trans*-N^2-BPDE-guanine 11-mers are presented in Figure 4A. At 0 h or in the absence of ligase, none of the expected 58-base sequence was synthesized; at 5 and 16 h after addition of the ligase, product was formed. Figure 4B shows the reaction for all five 11-base sequences: At 16 h, the 58-base product was synthesized in all cases; an intermediate band 48 bases in length was also observed, most probably due to ligation of the 37- and 11-base sequences. The slower mobilities of the modified 11-mers with respect to the unmodified oligonucleotides have been previously observed and discussed (Mao et al., 1992). The retarding effect of the N^2-BPDE-guanine lesions on the migration rate of modified oligodeoxynucleotides is slightly moderated, but not entirely eliminated, as the DNA sequence becomes longer; therefore, we used this property of the modified oligonucleotides to demonstrate that the individual adducts were not lost during preparation of the 58-base sequences containing site-specific adducts (Figure 4C). The unmodified DNA has a faster mobility than the four oligomers containing BPDE adducts; furthermore, only a single band was observed in each lane, demonstrating that the products were indeed pure.

Transcription of Double-Stranded DNA Templates Containing anti-N^2-BPDE-Guanine Adducts. Following synthesis of the templates containing the bacteriophage T7 RNA polymerase promoter and a stereoisomerically pure BPDE adduct, transcription assays were performed; the results are shown in Figure 5. Transcripts of the expected length of 31 bases were found in all cases, regardless of whether the DNA

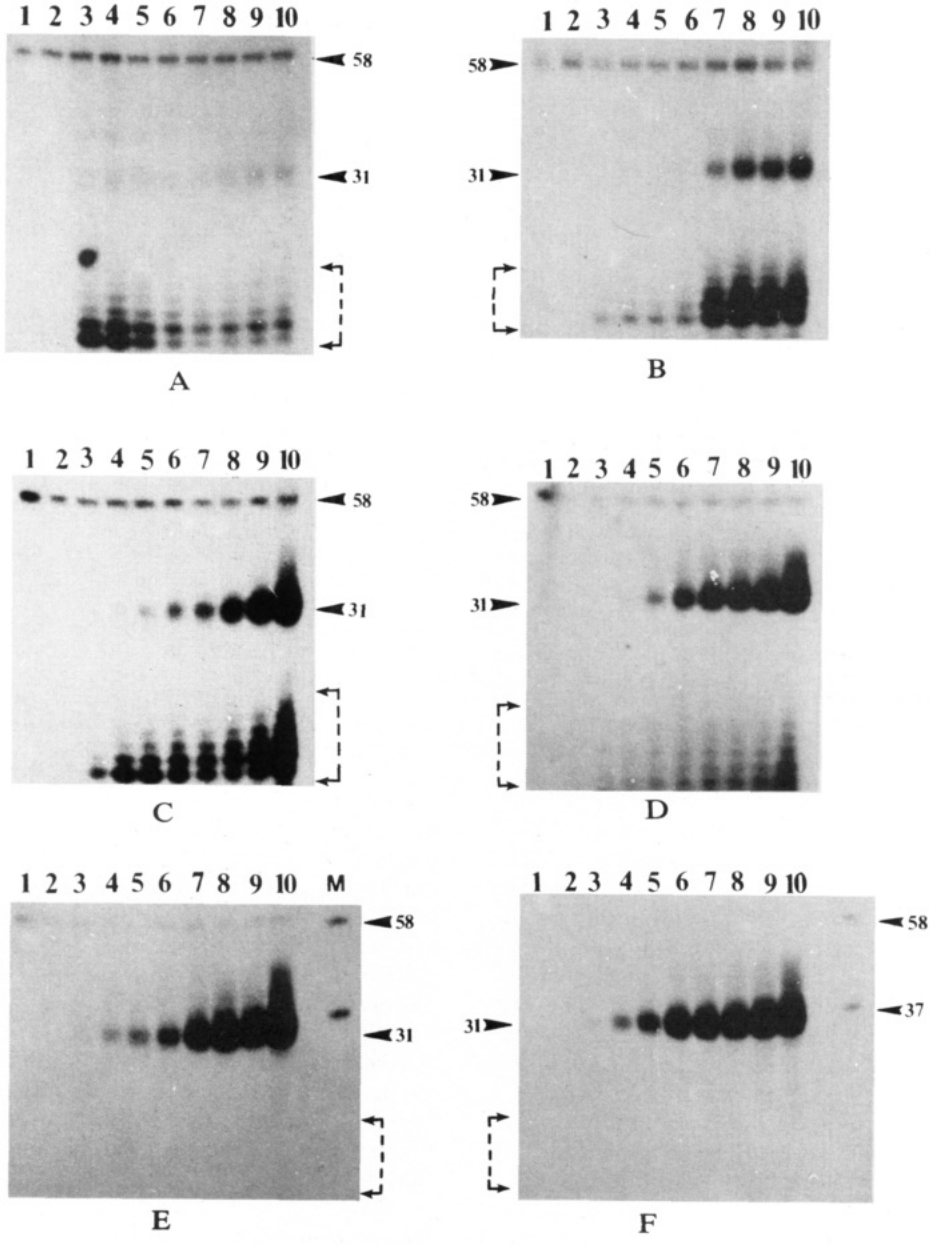

FIGURE 5: Transcription time courses for unmodified and modified DNA templates. These assays were performed as described in Materials and Methods. (Lane 1) DNA template alone; (lanes 2–10) DNA template transcribed for 0, 0.5, 1, 2, 4, 8, 15, 20, and 30 min, respectively; (lanes M) single-stranded DNA markers. The numbered arrows refer to the position of the template at 58 bases and the position of the transcript at 31 bases. The arrows connected by a hatched line show the region corresponding to the truncated transcripts. (A) (+)-*trans*, (B) (–)-*trans*, (C) (+)-*cis*, (D) (–)-*cis*, (E) template constructed with the unmodified 11-mer, (F) template prepared by annealing two nonradioactive complementary single-stranded 58-mers. The spurious band present in lane 3 of panel A is an artifact.

contained a BPDE lesion or not. In the templates having a BPDE adduct, however, several discrete, truncated transcripts were seen, suggesting that all four stereoisomers stall or block elongation by the T7 RNA polymerase. In the case of the (+)-*trans* adduct, the disappearance of the smallest discrete band formed might imply that the polymerase is actually stalled at the lesion but eventually bypasses it, perhaps with a low frequency, resulting in a complete RNA molecule. Note that in the case where the template was synthesized using an unmodified 11-mer as opposed to annealing two complementary 58-mers, the results are the same in that no truncated RNA is seen.

In an effort to compare the bypass rates among the BPDE-N^2-guanine–DNA adducts, we quantified the intensities of the bands corresponding to the full-length transcript and the template in each lane. By taking a ratio of these two values in conjunction with the specific activity and number of [^{32}P]-phosphates incorporated into each, we were able to estimate the relative amounts of full-length transcripts per DNA template at each time point. These data are presented in Figure 6; they demonstrate that the (+)-*trans*-BPDE adduct is significantly more inhibitory to the process of transcription than are the (–)-*trans*- and (+)-*cis*-BPDE adducts. The least inhibitory of all is the (–)-*cis*-N^2-BPDE-guanine. The values presented assume that the adenine residues near the site of damage at positions +13 and +19 after the transcription start site are correctly transcribed to uracil, resulting in the addition of two labeled phosphates. If misincorporation at these sites occurs, the intensity of the band corresponding to the full-length transcript would be lower. Even if this were the case, the variations in specific activity of the RNA would be modest and could not completely account for the observed differences. The results also suggest that multiple rounds of transcription are occurring in each case. If the RNA polymerase, which

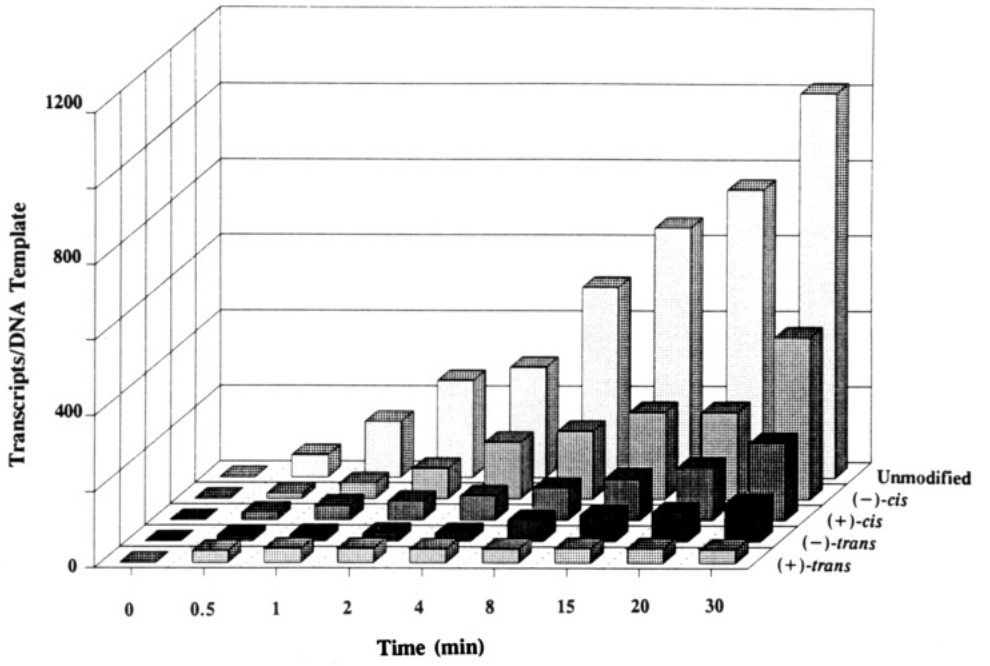

FIGURE 6: RNA synthesis by bacteriophage T7 RNA polymerase at BPDE-guanine adducts. The values for transcripts/DNA template were calculated by quantifying the bands for the full-length transcripts and the corresponding DNA template, and calculating the ratios for each. These results are the composite of two sets of experiments, with freshly prepared templates being employed each time.

is present in a molar amount approximately 10-fold less than the quantity of template, were only able to transcribe the template once, the lowest ratio of transcript to template would be 0.1; our values are higher than this in all cases.

DISCUSSION

Transcription at BPDE–DNA Adducts. We have investigated the behavior of bacteriophage T7 RNA polymerase at *anti*-N^2-BPDE-guanine adducts located at specific sites in a DNA template. The data presented here demonstrate that templates containing these lesions along with RNA polymerase promoters suitable for directing *in vitro* transcription can be constructed. When present on the transcribed strand of the DNA, the adducts impede elongation during RNA synthesis by bacteriophage T7 RNA polymerase. Of even greater interest are our results establishing that the stereoisomeric configuration of the BPDE adduct dramatically affects the degree to which transcription is impeded at or near the lesion, with the order of inhibition being (+)-*trans*- > (−)-*trans*- > (+)-*cis*- > (−)-*cis*-*anti*-N^2-BPDE-guanine. These findings add this group of adducts to the growing list of modified bases in DNA that have been shown to interfere with transcription by using site-specific analysis (Basu & Essigmann, 1988; Singer & Essigmann, 1991); among these are psoralen monoadducts (Shi et al., 1987), (acetylamino)fluorene (Chen, Y.-H., et al., 1991; Chen & Bogenhagen, 1993), and aminofluorene lesions (Chen, Y.-H., et al., 1991; Chen & Bogenhagen, 1993).

The synthesis of full-length RNA under our conditions could be attributable to a variety of causes. The most obvious explanation is that the RNA polymerase stalls at the site of damage, but that it eventually bypasses the adduct. This mechanism is supported by the ladder of transcripts that is obtained for each lesion. As noted earlier, the lowest band in the ladder for the (+)-*trans* moiety becomes less intense with time; this could happen if the polymerase actually continued to elongate an already existing piece of RNA. It is also possible that the full-length RNA molecules are generated by the polymerase transcribing small amounts of normal template contaminating the modified DNA, but this is unlikely for several reasons: The 58-mers that were synthesized were carefully purified by PAGE; no bands corresponding to unmodified template were detected in the preparations of the site-specifically damaged oligomers; and the adducts are stable, especially under the lighting conditions employed in this work.

When transcriptional bypass of a BPDE adduct occurs, there is the potential for miscoding at the site of damage. The best way to assess this is by sequencing the full-length RNA transcript using reverse transcriptase along with a suitable DNA primer and dideoxynucleoside triphosphates. Our full-length RNA is too short for such an analysis because the primer would extend very close to the potentially miscoded bases; hence, this issue will be addressed in future studies when a longer template will be prepared. Likewise, it is necessary to map the site(s) on the modified DNA where premature termination of elongation occurs relative to the position of the adduct. Our preliminary results suggest that the (+)-*trans*, (−)-*trans*, and (+)-*cis* lesions stall elongation beginning at the modified guanine; in contrast, the (−)-*cis* lesion inhibits transcription at the site three bases prior to the actual adduct.

As far as we could ascertain, reports of RNA synthesis directed by site-specific damaged templates with BPDE-guanine adducts have not yet been published; however, our observations concerning transcription stalling at site-specific BPDE adducts are consistent with previous reports describing reduced RNA synthesis due to the presence of random BPDE damage in DNA. Transcription of calf thymus DNA by *Escherichia coli* RNA polymerase was progressively inhibited with increasing levels of BPDE modification (Leffler et al., 1977). A similar dose-dependent inhibition was found for T7 RNA polymerase *in vitro* when M13 DNA exposed to BPDE was used as the template; furthermore, the damage reduced the synthesis of RNA to a much greater extent when it was present on the transcribed strand (Nath & Romano, 1991). Similar results showing diminished RNA synthesis were obtained when SP6 RNA polymerase was used to transcribe

a BPDE-damaged fragment of DNA containing a 5S rRNA gene (Thrall et al., 1992). Likewise, gene expression of a BPDE-modified plasmid reporter transfected into rat hepatocytes was inactivated by the presence of only one adduct (Koch et al., 1993). All these systems clearly show reduced transcription by several different RNA polymerases at random BPDE–DNA lesions. There is, however, an important caveat to bear in mind: Extrapolating results obtained for the behavior of one RNA polymerase at an adduct to other RNA polymerases is not necessarily valid. For example, eukaryotic RNA polymerase III and bacteriophage T7 RNA polymerase behave quite differently at site-specific 2-aminofluorene–guanine adducts and *N*-acetyl-2-aminofluorene–guanine adducts, and exhibit significant differences in rates of RNA synthesis. The former is primarily blocked at the modified sites, while the latter is able to bypass the adducts with significant efficiency (Chen, Y.-H., et al., 1991; Chen & Bogenhagen, 1993).

DNA Replication at Site-Specific BPDE–DNA Adducts. Our data revealing that the (+)-*trans-anti*-N^2-BPDE guanine lesions impede transcription to a much higher extent than the other three adducts are in contrast to the results obtained for DNA synthesis at analogous N^2-BPDE-guanine lesions using DNA polymerase primer extension assays. Incorporation of deoxycytidilic acid opposite a BPDE adduct by Sequenase Version 2.0—which is genetically modified T7 DNA polymerase with no detectable exonuclease activity—is severely inhibited by both (+)-*cis*- and (+)-*trans*-N^2-BPDE guanine adducts; however, the (+)-*cis* conformer is a more effective block to primer extension (Hruszkewycz et al., 1992). Similar results were obtained for human DNA polymerase α (Hruszkewycz et al., 1992). The (−)-*cis*- and (−)-*trans*-N^2-BPDE-guanine lesions cause a greater amount of miscoding than do the isomers formed from (+)-*anti*-BPDE as evaluated by primer extension catalyzed by the Klenow fragment of *E. coli* DNA polymerase I; in all four stereoisomerically different adducts, deoxyadenylic acid was preferentially inserted opposite the lesion, and one- or two-base deletions were observed (Shibutani et al., 1993). It is important to realize that the assay systems for primer extension by DNA polymerases and the methods employed to study transcription by RNA polymerases are distinctly different: The former makes use of site-specific damage in single-stranded DNA with a primer suitable for elongation annealed to the 3′ end, and the latter employs double-stranded DNA with a promoter appropriate for the RNA polymerase being studied. The actual orientation of the individual adducts in the templates used in these two situations might be significantly different; however, recent experiments suggest that the orientation of the pyrenyl residues in single-stranded oligomers containing either (+)-*trans*- or (−)-*trans*-N^2-BPDE-guanine lesions relative to the 5′ → 3′ polarity (Mao et al., 1993) are similar to those found in duplexes (de los Santos et al., 1992).

Structural Relationships between BPDE-Guanine Lesions and Enzymatic Nucleic Acid Synthesis. It is tempting to speculate on the relationship between the conformation of an N^2-BPDE-modified guanine and its ability to modulate nucleic acid synthesis. The fundamental differences in orientation between the *cis* and *trans* adducts might explain their effects on transcription. The *trans* adducts could potentially impede elongation to a higher extent because the pyrenyl group lies in the minor groove, whereas the quasi-intercalated (+)- and (−)-*cis* adducts might have less effect on transcription (Cosman et al., 1992, 1993; de los Santos et al., 1992). Unfortunately, structural data based on high-resolution NMR analysis of the (−)-*cis* adduct are not yet available; in light of the particularly aberrant behavior of this lesion relative to the others, it will be of great interest to examine its structure–function relationship when the structure is finally determined. However, it is important to keep in mind that the conformational characteristics thus obtained are related to the presence of the modified bases in duplex DNA, not in transcription bubbles or at replication forks where the configurations could conceivably be quite different.

Transcription Elongation versus Termination: Implications for anti-N^2-BPDE-Guanine Adducts in DNA. The finding that various stereoisomers of N^2-BPDE-guanine in DNA incite T7 RNA polymerase to stall to differing degrees presents an interesting question: When is a stalled transcription complex perceived by the cell to be an actual block to elongation? In other words, when will the RNA polymerase actually proceed past the damaged site, as opposed to dissociating from the transcription complex, yielding truncated RNA? This is a perplexing question that is not easily addressed, but the consequences of a polymerase proceeding with RNA synthesis as opposed to aborting elongation have profound implications for cellular processes. Our data demonstrate that the T7 RNA polymerase transcription complex manages to bypass N^2-*anti*-BPDE-guanine adducts with varying degrees of efficiency, with truncated oligoribonucleotides being formed in each case. If the notion that preferential removal of an adduct from the transcribed strand of an active gene is dependent on its ability to block transcription is correct, a lengthy stall might be perceived by the cell as a signal to clear the adduct; hence, in this case, *trans* adducts would be repaired in active regions better than the *cis* lesions. This could be particularly relevant to the (−)-*cis* modification, which blocks elongation less efficiently than the other lesions: As a poor terminator of transcription, it would be less prone to preferential repair. The consequences of this slower removal from transcribed domains coupled to the higher miscoding potential when it is read by DNA polymerase (Shibutani et al., 1993) would make (−)-*cis-anti*-N^2-BPDE-guanine a particularly deleterious adduct. It is important to realize that our data do not demonstrate whether the truncated transcripts are readily released from the stalled transcription complex. DNase footprinting and filter binding assays should assist us in differentiating between these two possibilities.

It is worth noting that the reports concerning biased removal of BPDE adducts from discrete genetic loci are conflicting. Following exposure of diploid human fibroblasts to racemic BPDE, Chen et al. (1992) reported preferential removal of the resulting adducts from the transcribed strand of the *hprt* gene; these results are consistent with biases in mutagenesis arising from the nontranscribed strand in this locus (Chen, R.-H., et al., 1990, 1991). Tang and Zhang (1991), however, found no preferential removal of BPDE adducts from the *dhfr* locus of Chinese hamster ovary cells. Numerous scenarios could be developed to explain these findings, one of which is differential behavior of the RNA polymerases in these two cell lines when they encounter BPDE–DNA adducts, and the ultimate consequences of stalled transcription complexes on DNA repair. Of great interest and importance will be elucidation of the behavior of a variety of eukaryotic RNA polymerases at these lesions, with emphasis placed on the effect of transcription factors on elongation past BPDE–DNA adducts.

The repercussions of heterogeneous bypass of *anti*-N^2-BPDE-guanine DNA lesions by RNA polymerases based solely on their stereoisomeric configuration add a new dimension to

the impact of these moieties on biological processes, possibly including transcription-coupled DNA repair. It is not sufficient to investigate RNA synthesis at these or any adducts without first considering the stereoisomers involved. Furthermore, careful analysis of site-specific BPDE-damaged DNA will enhance and clarify the already existing data obtained using randomly damaged BPDE–DNA templates and will augment our understanding of the role of benzo[*a*]pyrene in the carcinogenic process.

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