Bacteriophage T7 RNA Polymerase Transcription Elongation Is Inhibited by Site-Specific, Stereospecific Benzo[*c*]phenanthrene Diol Epoxide DNA Lesions†

Richard B. Roth,‡ Shantu Amin,§ Nicholas E. Geacintov,|| and David A. Scicchitano*,‡

Departments of Biology and Chemistry, New York University, New York, New York 10003, and American Health Foundation, Valhalla, New York 10595

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ABSTRACT: Benzo[*c*]phenanthrene diol epoxide (B[*c*]PhDE), the ultimate carcinogenic metabolite of the environmental pollutant benzo[*c*]phenanthrene, reacts with DNA primarily at the exocyclic amino groups of purines, forming B[*c*]PhDE−DNA adducts that differ in their stereochemical configurations and their effect on biological processes such as transcription. To determine the effect of these stereoisomers on RNA synthesis, *in vitro* T7 RNA polymerase transcription assays were performed using DNA templates modified on the transcribed strand by either a site-specific (+)-*trans*- or (−)-*trans*-*anti*-B[*c*]PhDE−N^6-dA lesion located within the sequence 5′-CTCTCACTTCC-3′. The results show that both (−)-*trans*-*anti*-B[*c*]PhDE−N^6-dA and (+)-*trans*-*anti*-B[*c*]PhDE−N^6-dA block RNA synthesis. Furthermore, both B[*c*]PhDE−dA stereoisomeric adducts lead to lower levels of initiation of transcription relative to that observed using an unmodified DNA template. In contrast to these results, placement of the adduct on the nontranscribed strand within the template does not impede transcription elongation. In addition to the assessment of the effect of the lesions on transcription elongation, the resulting transcripts were characterized in terms of their base composition. A high level of base misincorporation is detected at the 3′-ends of truncated transcripts, with guanosine being most frequently incorporated opposite the modified nucleotide rather than the expected uridine. This result supports the notion that translocation past a modified base in a DNA template relies in part on correct base incorporation, and suggests that stalling of RNA polymerases at damaged sites in DNA may well be dependent on both the presence of the lesion and the base which is incorporated opposite the modified nucleotide.

Benzo[*c*]phenanthrene is a member of the class of ubiquitous environmental pollutants called polycyclic aromatic hydrocarbons that are byproducts of incomplete combustion of organic materials. These compounds are converted by cellular enzymes to a variety of oxygenated derivatives, including highly reactive diol epoxides that react with sites in DNA and are sometimes mutagenic and carcinogenic (*1*, *2*). Important to the tumorigenicity of the diol epoxides is the fact that they exist as stereoisomers; hence, the resulting DNA adducts possess various three-dimensional orientations that influence to very different extents the structural features and biological properties of the regions of DNA in which they form (*3*).

In the case of benzo[*c*]phenanthrene, the ultimate carcinogenic metabolite is B[*c*]PhDE[1] that exists as *anti*-B[*c*]-PhDE and *syn*-B[*c*]PhDE diastereomers. The *anti*-B[*c*]PhDE diastereomer can be resolved into (+)-1*S*,2*R*,3*R*,4*S* and (−)-1*R*,2*S*,3*S*,4*R* enantiomers, and analogous stereoisomeric diol epoxides in the case of the (+)-*syn* diastereomers (*4*). The diol epoxides bind preferentially to the exocyclic amino groups of purines, with the predominant active site being the N^6 position of adenine (*5*−*9*). Upon binding to adenine, the epoxide ring opens either *trans* or *cis* to the epoxide group, forming stereoisomeric (+)-(1*R*)-*trans*-*anti*- and (−)-(1*S*)-*trans*-*anti*-B[*c*]PhDE−N^6-dA adducts (Figure 1) (*10*).

B[*c*]PHDE carcinogenicity studies demonstrate that its tumorigenic profile is quite complex, depending on the stereoisomeric configuration of the specific diol epoxide intermediate and the model system used in the studies (*4*, *11*). Likewise, the relative mutagenicity of each epoxide is also a function of the experimental system that is used (*11*−*13*), and recent mutagenesis studies further suggest that the configuration of the point of attachment of benzo[*c*]phenan-

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* To whom correspondence should be addressed: Department of Biology, New York University, 1009 Main Building, 100 Washington Square East, New York, NY 10003. Phone: (212) 998-8229. Fax: (212) 995-4015. E-mail: das2@nyu.edu.

‡ Department of Biology, New York University.

§ American Health Foundation.

|| Department of Chemistry, New York University.

[1] Abbreviations: B[*c*]PHDE, benzo[*c*]phenanthrene diol epoxide; (+)-*anti*-B[*c*]PHDE, (4*S*,3*R*)-dihydroxy-(2*R*,1*S*)-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene; (−)-*anti*-B[*c*]PHDE, (4*R*,3*S*)-dihydroxy-(2*S*,1*R*)-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene; (+)-*syn*-B[*c*]-PHDE, (4*S*,3*R*)-dihydroxy-(2*S*,1*R*)-epoxy-1,2,3,4-tetrahydrobenzo[*c*]-phenanthrene; (−)-*syn*-B[*c*]PHDE, (4*R*,3*S*)-dihydroxy-(2*R*,1*S*)-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene; TCR, transcription-coupled DNA repair; (+)-*anti*-B[*a*]PDE, (7*R*,8*S*)-dihydroxy-(9*S*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (−)-*anti*-B[*a*]PDE, (7*S*,8*R*)-dihydroxy-(9*R*,10*S*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; B[*a*]PDE, benzo[*a*]pyrene diol epoxide.

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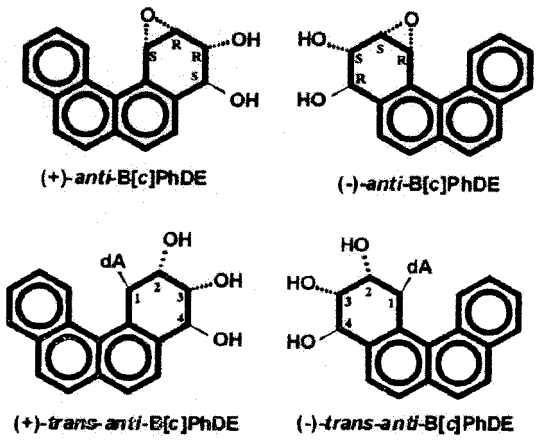

FIGURE 1: Enantiomeric forms of the *anti* diastereomer of B[*c*]-PhDE. The (+)- and (−)-*trans-anti* isomers of B[*c*]PhDE are shown. Opening of the epoxide ring and subsequent bonding to deoxyadenosine result in the formation of isomers differing only in their stereochemical orientations. The C2 hydroxyl group can assume the *trans* or *cis* conformation relative to the deoxyadenosine−B[*c*]PhDE bond, but only the *trans* forms studied here are shown.

threne to the base greatly influences its mutagenicity, with the *S* configuration being more mutagenic than the *R* configuration (*14*).

The reaction of an agent like B[*c*]PhDE with the DNA of a cell can adversely affect essential biological processes such as replication and transcription by causing base misincorporations that result in mutations in daughter DNA or the production of RNA molecules that function improperly. To ameliorate the potentially harmful consequences resulting from DNA damage, cells have evolved methods for removing lesions and abnormal base pairs from the genome (*2*). In certain instances, TCR is employed to remove the damage. TCR is characterized by the more rapid clearance of certain types of DNA lesions from expressed genes relative to quiescent domains in the genome, and it can be attributed directly to the preferential removal of damage from the transcribed strand over the nontranscribed strand in active genes (*15*−*20*). It has been proposed that only lesions that block transcription elongation are removed by TCR and that a stalled RNA polymerase provides the signal for clearance of damage from the transcribed strand of a gene (*21*).

Recent investigations concerning transcription elongation past DNA lesions have made use of site-specifically modified DNA to determine the effects of a diverse range of DNA-damaging agents on RNA synthesis. Among these studies are those utilizing a B[*a*]PDE lesion covalently attached to the N^2 position of a specific guanine residue in the DNA template in a stereospecific manner. These site-specific B[*a*]-PDE−N^2-dG adducts partially block transcription, resulting in a mixture of full-length and truncated transcripts (*22*). The full-length transcripts resulting from bypass do not contain misincorporated bases, and the truncated transcripts usually end with an incorrectly inserted purine. These results suggest that both the three-dimensional orientation of the lesion and the base incorporated during transcription affect the ability of RNA polymerase to bypass the adduct and may well influence TCR.

In the experiments described here, oligodeoxynucleotides modified by either a (+)-(1*R*)- or (−)-(1*S*)-*trans-anti*-B[*c*]-PhDE−DNA adduct located at the N^6 position of deoxyadenosine within the sequence 5′-CTCTCACTTCC-3′ were used to construct 58 bp DNA templates that are suitable for transcription by bacteriophage T7 RNA polymerase. Two sets of DNA templates were prepared. The first set contained the B[*c*]PhDE−dA adduct on the transcribed strand, and the second set contained the adduct positioned on the nontranscribed strand. To determine the influence each stereoisomer has on RNA synthesis, *in vitro* transcription assays were performed using T7 RNA polymerase. T7 RNA polymerase is an ideal enzyme for *in vitro* studies because it is thoroughly characterized (*23*−*25*). It is a single-subunit, DNA-directed RNA polymerase capable of transcription initiation, elongation, and termination without ancillary factors (*26*), and it requires a minimal promoter of 17 bp for reliable initiation (*27*). In addition, T7 RNA polymerase has a thumb−palm−fingers motif, which is commonly found in viral, bacterial, and eukaryotic nucleic acid polymerases (*28*). The results obtained in these studies show that B[*c*]PhDE−dA lesions pose blocks to transcription elongation, and they do so in a stereochemical-dependent fashion.

MATERIALS AND METHODS

Oligodeoxynucleotides. The 11-base oligodeoxynucleotides, including those modified by B[*c*]PhDE, were synthesized as described by Laryea et al. (*29*). All other oligomers were purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX).

Enzymes, Reagents, and Supplies. T7 RNA polymerase was purchased from GibcoBRL (Grand Island, NY); single-strand DNA binding (SSB) protein and T4 RNA ligase were obtained from Amersham Life Science (Cleveland, OH), and all other enzymes were acquired from GibcoBRL, Promega, Inc. (Madison, WI), or Sigma Chemical Co. (St. Louis, MO). Radioactive nucleotides were procured from DuPontNEN (Boston, MA). Ultrafiltration systems were obtained from Amicon, Inc. (Beverly, MA), and all other supplies were obtained from Fisher Scientific (Pittsburgh, PA), Sigma Chemical Co., or Promega, Inc.

Synthesis of DNA Templates. The synthesis of DNA templates modified on the transcribed strand is depicted in Figure 2. The 5′-ends of the 37-mer and an unmodified or B[*c*]PhDE-modified 11-mer were phosphorylated using T4 polynucleotide kinase and 0.8 mM [γ-^{32}P]ATP. (To ensure an equal specific activity among the templates prepared for transcription, a single stock solution of [γ-^{32}P]ATP was prepared and used in all phosphorylation reactions.) Unincorporated ATP and residual salts were removed by filtering the samples through Sephadex G-25. A phosphorylated 37-mer, one phosphorylated 11-mer, a 10-mer, and a complementary 58-mer were mixed in 40 mM Tris-HCl (pH 7.6) containing 20 mM $MgCl_2$ and 50 mM NaCl; the three shorter oligomers were present at a 2-fold molar excess over the complementary 58-mer for the preparation of each template to ensure that each 58-mer had all three oligomers annealed to it. To anneal the oligomers, the samples were heated at 85 °C for 20 min and cooled slowly to 4 °C. The oligomers were ligated using T4 DNA ligase with 1 mM ATP and 5 mM DTT for 16 h at 15 °C. The ligated products were analyzed by 12% denaturing PAGE in the presence of 8 M urea, and the position of each full-length construct was

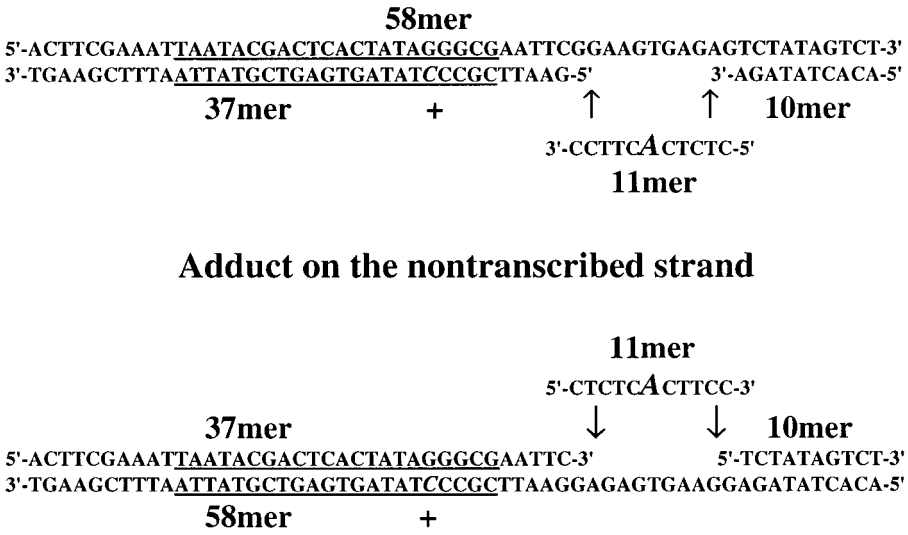

FIGURE 2: DNA template design. DNA templates modified on the transcribed strand or the nontranscribed by a B[*c*]PhDE−DNA adduct were constructed by annealing three oligomers (37-mer, 11-mer, and 10-mer) to a complementary 58-mer, and then ligating them. By using either the (+)-*trans*- or (−)-*trans-anti*-B[*c*]PhDE-modified 11-mer or unmodified 11-mer, three unique DNA templates were assembled. Each contains a 22 bp T7 RNAP promoter, which is underlined. Transcription initiation begins at the position 1 start site, which is denoted by the +, and the arrow indicates the direction of elongation. The modified adenine, which is denoted by the *A*, was placed at position 16.

determined by autoradiography. The resulting modified 58-mers were excised and eluted from the gel using 0.5 M ammonium acetate and 0.1 mM magnesium acetate (pH 7.0). The modified oligomers were purified using a Microcon-10 microconcentrator ultrafiltration system. Liquid scintillation counting in a Beckman (Fullerton, CA) LS-3800 liquid scintillation counter was used to determine the amount of DNA recovered from each sample.

The protocols used to synthesize DNA templates modified on the nontranscribed strand were identical to those discussed for the construction of DNA modified on the transcribed strand, except that the 10-mer was phosphorylated prior to ligation rather than the 37-mer (Figure 2).

Preparation of Double-Stranded DNA Templates. Each purified 58-mer prepared by ligation was annealed to its complement in 40 mM Tris-HCl (pH 7.6) with 20 mM $MgCl_2$ and 50 mM NaCl as described previously. A 20-fold molar excess of the complementary strand was used to ensure that all the B[*c*]PhDE-modified or control 58-mer was in the double-strand form. To inhibit RNA synthesis of single-strand DNA, the annealed template DNA was incubated with 80 pmol of SSB protein per picomole of template for 1 h at 37 °C in 40 mM Tris-HCl (pH 8.0) containing 8 mM $MgCl_2$, 25 mM NaCl, and 2 mM spermidine.

In Vitro T7 RNAP Transcription Assays. Transcription reactions were carried out at 37 °C in 100 μL volumes containing 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine, 5 mM DTT, 200 μM ATP, 200 μM CTP, 200 μM GTP, 4 μM [α-^{32}P]UTP (4 Ci/mmol), 20 units of RNasin, 1 pmol of DNA template, and 25 units (0.5 pmol) of T7 RNAP. To ensure an equal specific activity for [α-^{32}P]UTP among the transcription reactions, a stock solution containing each of the buffer components, except for DNA template and T7 RNAP, was prepared and divided equally among the reactions. Prior to the addition of T7 RNAP, an aliquot of the reaction mix was removed as a control for monitoring RNA synthesis; additional samples were withdrawn at specific times following addition of the polymerase and the reactions quenched with 2 μL of 0.5 M EDTA (pH 8.0). The resulting transcripts were analyzed by 15% denaturing PAGE in the presence of 8 M urea, and the bands were quantified with a Bio-Rad (Hercules, CA) GS-525 molecular imager.

An alternative to the transcription assay described herein was also developed and used when needed as described in the Results. In this case, the reactions were performed with 200 μM UTP in the absence of radioactive nucleotide. To radiolabel the RNA, resulting transcripts were phosphorylated at the 3′-end with [5′-^{32}P]pCp using T4 RNA ligase, and the products were resolved by 20% sequencing PAGE using 8 M urea as a denaturant.

Analysis of the 3′-Ends of Truncated Transcripts. RNA resulting from transcription assays performed without [α-^{32}P]-UTP, as described in the alternate protocol, was radiolabeled at the 3′-end with [5′-^{32}P]pCp using T4 RNA ligase (*30*). Following removal of the buffer components from transcription reaction mixtures by filtration through Sephadex G-25, [5′-^{32}P]pCp (0.66 μM) was ligated to the transcripts at 4 °C for 16 h in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 60 μg/mL BSA, 25 units of T4 RNA ligase, and RNA. The samples were resolved by 20% denaturing sequencing PAGE using 8 M urea, and the position of the truncated RNA was determined by autoradiography. Each truncated transcript was excised, eluted in 20 mM Tris-HCl (pH 7.6) and 0.1 mM EDTA, and purified using a Microcon-3 system. A Beckman LS-3800 liquid scintillation counter was used to quantify the RNA which was then digested to nucleoside 3′-monophosphates at 37 °C for 2 h in 50 mM sodium acetate (pH 4.5) containing 100 mM NaCl, 2 mM EDTA, 1000 dpm of

truncated transcript, 1 unit of ribonuclease T_1, 1 unit of ribonuclease T_2, and 50 μg/mL ribonuclease A. Each of the four unlabeled nucleoside 3′-monophosphates was added to the digested samples to a final concentration of 4 mM, and the nucleotides were resolved by two-dimensional thin-layer chromatography (TLC). Each sample was spotted onto the corner of a hydrated cellulose plate with a fluorescent indicator and allowed to dry for 5 min. The nucleotides were resolved in the first dimension for 2 h at ambient temperature using isobutyric acid, 0.5 M ammonium hydroxide, and 0.1 M EDTA in a 50:30:0.8 (v:v:v) ratio. The plates were dried overnight; the resolution of the nucleotides in the second dimension was performed at ambient temperature for 2 h, but the solvent that was used was 2-propanol, H_2O, concentrated HCl, and 0.1 M EDTA in a ratio of 70:15:15:1 (v:v:v:v) (*31*). The plates were then dried for several hours, and the positions of the nucleoside 3′-monophosphates were visualized under ultraviolet light. Molecular imaging was used to quantify the relative amounts of each of the four nucleotides.

RESULTS

Transcription of DNA Templates Modified on the Transcribed Strand. To study the effects of B[*c*]PhDE−DNA lesions on RNA synthesis, transcription reactions using bacteriophage T7 RNAP were performed in the presence of DNA templates modified by either a single, site-specific (+)-(1*R*)-*trans*- or (−)-(1*S*)-*trans*-*anti*-B[*c*]PhDE−N^6-dA adduct, or by using unmodified DNA. The results are shown in Figure 3. Full-length, 31-base transcripts were produced in all cases, but the presence of a B[*c*]PhDE−N^6-dA adduct significantly reduced the level of full-length RNA when compared to transcription of unmodified template. Furthermore, RNA synthesis of B[*c*]PhDE−dA-modified templates resulted in the production of truncated transcripts 15−18 bases in length, whereas no such transcripts were observed for the unmodified DNA. The presence of the truncated transcripts corresponds to the RNAP stalling at or near the site of the modified base that is located at position 16 on the transcribed strand.

To compare quantitatively the RNA produced from each DNA template, the amount of each transcript and template was estimated by molecular imaging. The specific activities of the DNA and RNA were used to calculate the relative amount of each transcript per unit of DNA template (Figure 3). Both enantiomers of the *anti*-B[*c*]PhDE−dA adduct substantially reduce the level of synthesis of the full-length transcript, but the degree of inhibition is somewhat stereospecific. The (−)-(1*S*)-*trans*-*anti*-B[*c*]PhDE−dA adduct impedes RNA synthesis to a slightly greater extent than the (+)-(1*R*)-*trans*-*anti*-B[*c*]PhDE−dA adduct.

The stereochemistry of the B[*c*]PhDE−dA adduct also affected the pattern of truncation (Figure 4). As determined by molecular imaging, RNA synthesis of DNA containing a (−)-1*S*-trans-*anti*-B[*c*]PhDE−dA adduct resulted in the formation of a predominantly 17-base transcript, representing 46% of the total truncated transcript (Figure 4, lane 10), but the presence of a (+)-(1*R*)-*trans*-*anti*-B[*c*]PhDE−dA adduct resulted in significant amounts of both 16- and 17-base RNA, 27 and 33% of the total truncated transcript, respectively (Figure 4, lane 5). In addition, the 15- and 16-base transcripts

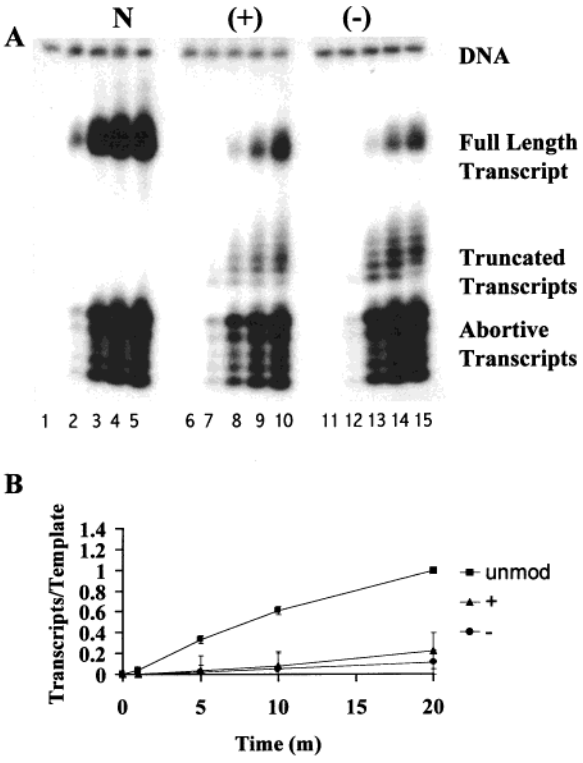

FIGURE 3: Transcription assay using B[*c*]PhDE−dA-modified DNA templates. (A) Autoradiogram resulting from transcription using DNA templates modified on the transcribed strand by a single, stereospecific B[*c*]PhDE−dA adduct to determine whether the stereochemistry of a lesion influences the level of inhibition. Transcription reactions were performed as described. Samples were removed from each reaction mixture at 0, 1, 5, 10, and 20 min and resolved by PAGE: lanes 1−5, transcription of unmodified DNA template N at 0, 1, 5, 10, and 20 min, respectively; lanes 6−10, transcription of (+)-*trans*-modified DNA template (+) at 0, 1, 5, 10, and 20 min, respectively; and lanes 11−15, transcription of (−)-*trans*-modified DNA template (−) at 0, 1, 5, 10, and 20 min, respectively. The presence of a B[*c*]PhDE−dA adduct inhibited the synthesis of full-length transcripts and also resulted in the production of truncated transcripts. (B) Full-length transcript per unit of DNA template. The presence of a B[*c*]PhDE−dA adduct significantly reduced the level of synthesis of the full-length transcript. The degree of inhibition was stereospecific, with the (−)-*trans*-*anti*-B[*c*]PhDE−dA adduct (−) inhibiting transcription to a greater extent than the (+)-*trans*-*anti*-B[*c*]PhDE−dA adduct (+).

synthesized from (−)-(1*S*)-*trans*-*anti*-B[*c*]PhDE−dA-modified DNA were often extended into longer transcripts as evidenced by their disappearance with time, but these same length transcripts generated from the (+)-*trans*-*anti*-B[*c*]-PhDE−dA-modified template were not extended.

To verify that the inhibition of transcription caused by the presence of a B[*c*]PhDE−dA adduct was not actually the result of using 4 μM UTP, which is far lower than the K_M of UTP for T7 RNAP (60 μM), the concentration of this nucleotide was increased to 200 μM and [α-^{32}P]UTP was replaced with [α-^{32}P]CTP. This precaution was taken because the modified adenine in the DNA template should direct incorporation of a uridine into a transcript when proper Watson−Crick base pairing occurs, a process that could be influenced by a nucleotide concentration that is too low. The DNA templates do not encode cytidine near the site of the DNA adduct; therefore, it was chosen to replace UTP. Under these conditions, the results of transcription using [α-^{32}P]-CTP were the same as those found with [α-^{32}P]UTP, ruling

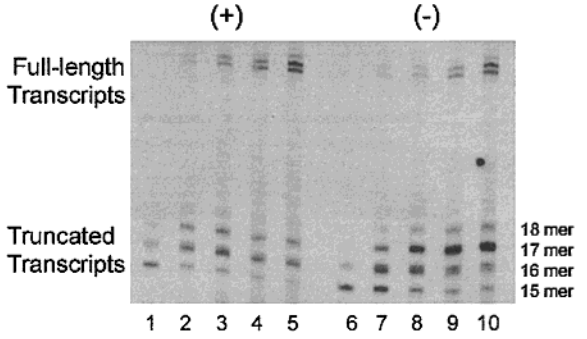

FIGURE 4: Transcription assay for resolving truncated transcripts. To resolve the truncated transcripts resulting from RNA synthesis of modified templates, a transcription assay was performed using the alternate procedure described in Materials and Methods. Samples were removed from each reaction mixture at various time points and resolved by 20% sequencing PAGE using 8 M urea as a denaturant: lanes 1−5, transcription of (+)-*trans-anti*-B[*c*]PhDE−dA-modified template (+) at 1, 5, 10, 20, and 60 min, respectively; and lanes 6−10, RNA synthesis off the (−)-*trans-anti*-B[*c*]PhDE−dA-modified template (−) at 1, 5, 10, 20, and 60 min, respectively. The pattern of truncated transcripts clearly differs between the modified templates. Two major truncated transcripts are produced from RNA synthesis off the (+)-*trans*-modified template, 16-mer and 17-mer; only one major transcript is produced from synthesis off the (−)-*trans*-modified template, 17-mer.

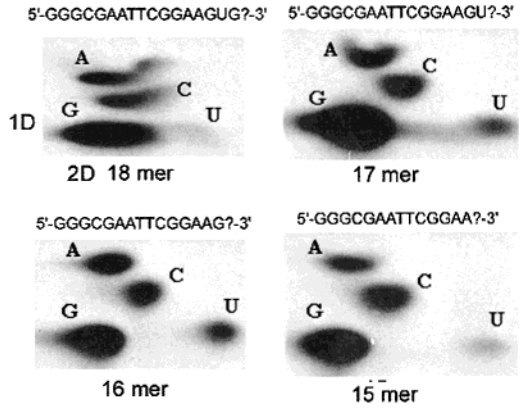

FIGURE 5: 3′-End base compositions of the 15−18-base transcripts at 5 min. Two-dimensional TLC was used to resolve and identify the 3′-ends of truncated transcripts resulting from transcription of the (+)-*trans-anti*-B[*c*]PhDE−dA-modified template as described in Materials and Methods. Depicted in this autoradiogram are the 3′-end base compositions of the 15, 16, 17 and 18 base transcripts at 5 min; the results for truncated transcripts from the (−)-*trans-anti*-B[*c*]PhDE−dA-modified template are not shown. Likewise, the resulting autoradiograms for 1, 10, 20, and 60 min are not presented for either B[*c*]PhDE stereoisomer. A, C, G, and U represent the adenosine, cytidine, guanosine, and uridine 3′-monophosphates, respectively.

out the possibility that the low UTP concentration was responsible for the reduction in the level of RNA synthesis (data not shown).

It is plausible that the limited amount of full-length RNA synthesized from B[*c*]PhDE−dA-modified DNA is the result of transcription of the residual unmodified template that is present. Although this possibility exists, it is very unlikely because the synthesis of the modified 11-mers and the purification of the DNA templates were carefully performed, and it is known that the B[*c*]PhDE−dA adducts are stable (A. Laryea, personal communication). In addition, the finding that the levels of 15- and 16-base truncated transcripts resulting from transcription of (−)-(1*S*)-*trans-anti*-B[*c*]-PhDE−dA-modified DNA diminish with time suggests that the RNAP stalls with a certain frequency at or near the site of the lesion but is then able to bypass it and continue elongation.

Transcription elongation is not inhibited by the presence of a B[*c*]PhDE−dA adduct on the nontranscribed strand of a DNA template as evidenced by the lack of truncated transcripts. However, the presence of the (+)-(1*R*)-*trans-anti*-B[*c*]PhDE−dA adduct on the nontranscribed strand led to an increase in the amount of full-length transcript that was approximately 3-fold greater than that of both the unmodified and (−)-(1*S*)-*trans-anti*-B[*c*]PhDE−dA-modified template (data not shown).

Analysis of RNA Synthesized from B[c]PhDE−dA-Modified DNA Templates. To determine whether the full-length and truncated transcripts differ in any way other than in length, an attempt was made to sequence the full-length RNA species. Although several methods were employed to determine the base sequences, including protocols involving nuclease digestion and reverse transcriptase, none proved to be successful. The limited amount of RNA obtained from transcription of DNA containing a B[*c*]PhDE−dA adduct precluded the inferring of an accurate nucleotide sequence. Also, the experimental system used in these studies produced a truncated transcript 15−18 bases in length, sizes that are too short to be sequenced by any of the methods described above.

The base composition of the truncated transcripts' 3′-ends was determined as described. The resulting RNA was digested to nucleoside 3′-monophosphates that were resolved by two-dimensional TLC to characterize the composition of the 3′-end nucleotide (Figure 5). The relative percentages of incorporation of each of the four bases could be determined using molecular imaging (Tables 1 and 2). Transcription of B[*c*]PhDE−dA-modified DNA templates produces truncated transcripts that usually end with a purine, predominantly a guanine, and the extent of guanine incorporation is dependent on the stereospecificity of the B[*c*]-PhDE−dA adduct. Truncated transcripts ending one base prior to the site of the lesion, at position 15, possess the proper 3′-end base guanine approximately 70% of the time. At position 16, a uracil would be incorporated opposite the site of the modified adenine if traditional Watson−Crick base pairing were obeyed. In fact, only 55% of the 16-base transcripts synthesized off (+)-(1*R*)-*trans-anti*-B[*c*]PhDE−dA-modified DNA ended with this base. Even more striking was the finding that only 11% of the 16-base transcripts produced from the (−)-(1*S*)-*trans-anti*-B[*c*]PhDE−dA-modified template ended with a uracil. A guanine should be incorporated opposite the cytosine at position 17; transcripts 17 bases in length synthesized off the (+)-(1*R*)-*trans-anti*-B[*c*]PhDE−dA-modified template contained a guanine at the 3′-end more than 90% of the time, while only 60% of the same length transcripts produced from the (−)-(1*S*)-*trans-anti*-B[*c*]PhDE−dA-modified DNA ended with this base. A similar disparity in the extent of guanine incorporation was seen with transcripts 18 bases in length. Although a thymine is located at position 18, transcription of (+)-(1*R*)-*trans-anti*-B[*c*]PhDE−dA-modified DNA yielded transcripts ending with a guanine 49% of the time, and more than 80% of the

Table 1: 3′-End Base Composition Analysis of Truncated Transcripts Synthesized from the (+)-*trans*-*anti*-B[*c*]PhDE−dA-Modified DNA Template[a]

time (min)	base	15-base transcript	16-base transcript	17-base transcript	18-base transcript
1	A	2	13	9	*22*
	C	1	0	5	12
	G	*97*	85	*82*	57
	U	0	*2*	4	9
5	A	9	12	10	*20*
	C	7	4	6	10
	G	*68*	34	*60*	54
	U	16	*50*	24	16
10	A	8	6	6	*23*
	C	11	3	3	3
	G	*63*	38	*66*	73
	U	18	*53*	25	0
20	A	5	3	2	*30*
	C	1	1	4	7
	G	*86*	66	*67*	60
	U	8	*30*	27	3
60	A	8	6	5	*16*
	C	12	3	5	15
	G	*69*	36	*60*	49
	U	11	*55*	31	20

[a] The 3′-end base compositions of the truncated transcripts from the 0, 1, 5, 10, and 60 min time points were quantified by molecular imaging, and the relative percentages of incorporation of each of the four bases were calculated. The italicized values represent the value for the bases which were expected to be incorporated at each position based on the sequence of the DNA template.

Table 2: 3′-End Base Composition Analysis of Truncated Transcripts Synthesized from the (−)-*trans*-*anti*-B[*c*]PhDE−dA-Modified DNA Template[a]

time (min)	base	15-base transcript	16-base transcript	17-base transcript	18-base transcript
1	A	1	12	34	*22*
	C	1	0	0	2
	G	*98*	88	*63*	73
	U	0	*0*	3	2
5	A	2	46	23	*29*
	C	1	5	3	9
	G	*97*	45	*71*	61
	U	0	*4*	2	1
10	A	5	30	9	*13*
	C	3	7	0	4
	G	*92*	62	*91*	81
	U	0	*1*	0	3
20	A	6	17	3	*13*
	C	13	10	4	17
	G	*79*	67	*93*	66
	U	2	*6*	1	3
60	A	12	20	3	*11*
	C	20	19	3	7
	G	*66*	50	*92*	81
	U	2	*11*	2	1

[a] The 3′-end base compositions of the truncated transcripts from the 0, 1, 5, 10, and 60 min time points were quantified by molecular imaging, and the relative percentages of incorporation of each of the four bases were calculated. The italicized values represent the value for the bases which were expected to be incorporated at each position based on the sequence of the DNA template.

18-mers synthesized off the (−)-(1*S*)-*trans*-*anti*-B[*c*]PhDE−dA-modified template contained a 3′-end guanine.

DISCUSSION

Transcription of DNA Templates Modified by B[c]PhDE−dA Adducts. The presence of a B[*c*]PhDE−N^6-dA adduct on the transcribed strand of a DNA template significantly inhibited transcription elongation when compared to RNA synthesis off unmodified DNA. These are the first investigations concerning transcription elongation past site-specific B[*c*]PhDE−DNA adducts, and as such, they represent a novel set of experiments. This study adds another DNA adduct that has been shown through site-specific analysis to inhibit RNA synthesis (*22*, *32*−*43*). Furthermore, if the preferential removal of an adduct from the transcribed strand of an active gene is indeed dependent on the adduct's ability to block RNA synthesis, the results reported here indicate that B[*c*]PhDE−dA adducts should be subject to TCR. In fact, B[*c*]PhDE−DNA lesions have been shown to be repaired preferentially in the transcribed strand of the *dhfr* gene of CHO cells (*44*).

Both enantiomers of the *anti*-B[*c*]PhDE−dA adduct substantially affect transcription. Each reduces the synthesis of the full-length transcript. It is conceivable that the presence of a B[*c*]PhDE−dA adduct could stall or slow the polymerase at the site of the lesion or even affect promoter recognition and binding. Each of these possibilities would reduce the level of enzyme turnover and decrease the number of initiation events. In addition to a reduction in the level of synthesis of full-length RNA, transcription of templates modified by a B[*c*]PhDE−dA adduct also generated truncated transcripts 15−18 bases in length, which corresponds to RNAP blockage at or near the site of the modified base located at position 16. Both the degree and pattern of truncation were dependent on the stereochemistry of the adduct. The stereochemically defined differences in transcription inhibition and truncated transcript formation by B[*c*]-PhDE−dA adducts are analogous to those observed for B[*a*]PDE−N^2-dG lesions (*32*).

Characterization of the Transcripts Synthesized from B[c]-PhDE−dA-Modified DNA. Do the full-length and truncated transcripts resulting from transcription of B[*c*]PhDE−dA-modified DNA templates differ from one another in any way besides length? To determine this, attempts were made to sequence the full-length RNA. Previously, Chen and Bogenhagen sequenced the full-length transcripts resulting from transcription of DNA templates modified on the transcribed strand by AAF and AF adducts and found no base misincorporation (*36*). These results are in agreement with those of Choi et al. (*22*), who observed no misincorporated bases in the sequences of full-length RNA produced from (−)-(10*R*)-*trans*-, (+)-(10*R*)-*cis*-, or (−)-(10*S*)-*cis*-*anti*-B[*a*]PDE−dG DNA templates. However, due to the limited amount of full-length RNA synthesized off the (+)-(10*S*)-*trans*-*anti*-B[*a*]PDE-modified DNA, they were unable to determine the base sequence of this transcript. In studies reported here, several methods were employed to determine the base sequence of full-length RNA, but none proved to be successful because of the limited amount of RNA obtained from transcription of the B[*c*]PhDE−dA templates.

Although the full-length transcripts could not be sequenced, the compositions of the 3′-ends of truncated transcripts were determined. The truncated transcripts most frequently end with a misincorporated base, predominantly a guanine. These results are in agreement with those observed for truncated transcripts caused by the presence of a B[*a*]-PDE−dG lesion, although in their case the base most frequently incorporated was adenine (*22*).

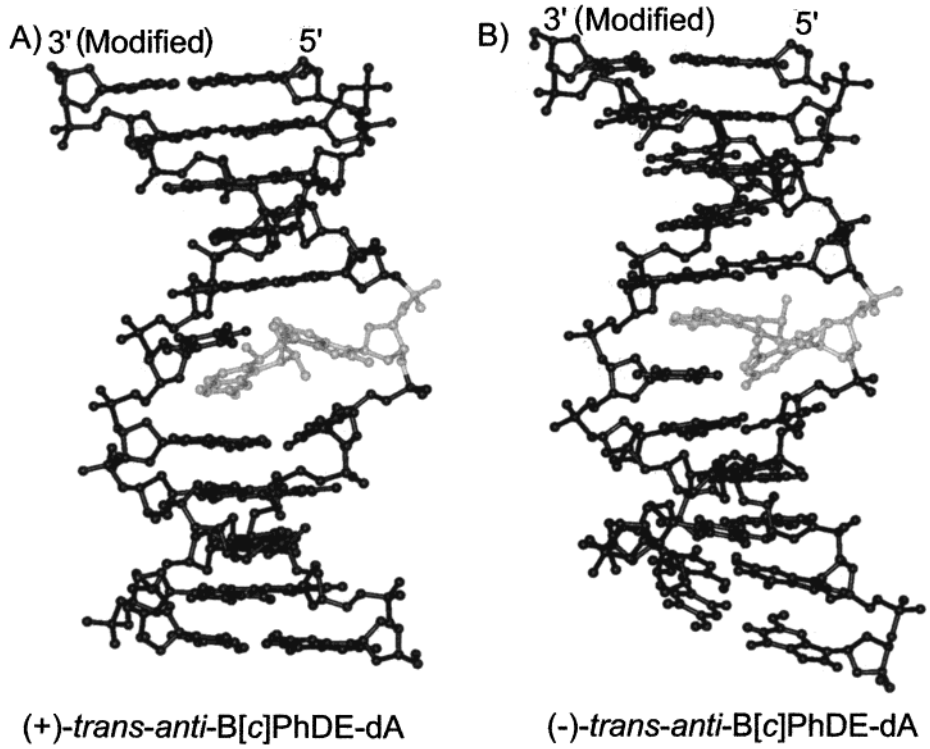

FIGURE 6: (A) Double-stranded DNA containing the (+)-*trans-anti*-B[*c*]PhDE−dA adduct, with the aromatic moiety intercalated on the 5′-side of the modified adenine. (B) Double-stranded DNA containing the (−)-*trans-anti*-B[*c*]PhDE−dA adduct, with the aromatic moiety intercalated on the 3′-side of the modified adenine. The base sequence that is shown corresponds to the modified 11-mer used in these studies. The 3′-end of the modified strand is indicated in each case (*3*, *48*, *49*).

RNAP Translocation at a Modified Base. The results presented in this study have been used to develop a model of RNAP translocation at a modified base. When RNAP encounters a DNA adduct, two possible events inhibit translocation of the polymerase: (i) the presence of the DNA adduct physically impedes the progression of the polymerase and (ii) the incorporation of an incorrect nucleotide into a growing transcript prevents RNAP translocation, thus deterring elongation. There are published data that support this idea. The ability of an RNA polymerase to detect a misincorporated base has been demonstrated for unmodified DNA templates, leading to the proposal that translocation of the polymerase from one site on the template to the next is an important discriminatory step for checking the fidelity of transcription (*45*). Other studies have also indicated that weakening of the DNA−RNA hybrid causes *Escherichia coli* RNA polymerase to arrest (*46*, *47*). A general mechanism based on our data and on these reports suggests that RNA polymerase can bypass a lesion in a DNA template provided that a residue is incorporated opposite the modified base that can permit translocation to occur. If, however, an incompatible base is placed opposite the modified site, the RNA polymerase stalls due to a weakened DNA−RNA hybrid and possibly dissociates, resulting in a truncated transcript.

Effect of Adduct Stereochemistry on Biological Activity. The three-dimensional orientations of the (+)-(1*R*)-*trans*- and (−)-(1*S*)-*trans-anti*-B[*c*]PhDE−DNA adducts in duplex DNA have been elucidated (Figure 6). For the sequence 5′-CTCTCA$^{B[c]PhDE}$CTTCC-3′ in double-strand DNA, both B[*c*]PhDE−dA enantiomers intercalate, but each does so in a different way. The phenanthrenyl group of the (+)-1*R*-*trans* adduct lies between the deoxyadenosine to which it is bound and the adjacent 5′-deoxycytosine (*48*), while the (−)-1*S*-*trans* phenanthrenyl adduct is positioned toward the 3′-side of deoxyadenosine next to the adjacent deoxycytosine (*49*). Neither adduct substantially disrupts base pairing between the modified deoxyadenosine and partner deoxythymidine, and base pairing of adjacent sites is not affected at all. During transcription, the RNAP translocates in a 3′ to 5′ direction, and as a result, it may encounter the (−)-1*S*-*trans* adduct just prior to reaching the adenine residue because the adduct is situated to the 3′-side of the modified base. In contrast, because the (+)-1*R*-*trans* adduct is positioned to the 5′-side of the modified nucleotide, the RNAP would encounter adenine prior to reaching the (+)-1*R*-*trans* adduct. Although this is speculative and the orientation of B[*c*]PhDE adducts in single-strand transcription bubbles is not known, it is conceivable that a difference in adduct orientation would result in a greater chance of the RNAP recognizing the modified adenine residue when the (+)-1*R*-*trans* adduct is present than when the (−)-1*S*-*trans* adduct is present. As a result, the polymerase would be able to incorporate a correct base opposite the (+)-1*R*-*trans*-modified adenine more often than opposite the (−)-1*S*-*trans*-modified adenine. On the basis of the model of RNAP translocation, this would suggest that the polymerase stalls less often at the (+)-1*R*-*trans* adduct than at the (−)-1*S*-*trans* adduct, resulting in more full-length transcript and less truncated transcript for the former, and this is indeed what the data indicate. Note that these results are consistent with replication studies showing that the (−)-1*S*-*trans* adduct reduces the overall efficiency of replication substantially more than the (+)-1*R*-*trans* adduct (*3*). These effects point to the greater biological impact of the (−)-(1*R*,2*S*,3*S*,4*R*)-*anti*-B[*c*]PhDE, which is by and large more tumorigenic than the (+)-*anti* enantiomer.

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