Template Activity of Calf Thymus DNA Modified by a Dihydrodiol Epoxide Derivative of Benzo[*a*]pyrene†

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ABSTRACT: The purpose of the present study was to determine the effects of covalent binding to DNA of a reactive derivative of benzo[*a*]pyrene on template activity during in vitro transcription with RNA polymerase. Calf thymus deoxyribonucleic acid, modified by reaction with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, was transcribed with *Escherichia coli* DNA-dependent RNA polymerase. With increasing levels of modification, there was a progressive inhibition of transcription. The inhibition was much greater under conditions where continuous reinitiation of transcription occurred than under conditions where only one RNA chain was synthesized per initiation site. This suggested that the modified sites block the movement of polymerase along the template and prevent recycling of the enzyme. Consistent with this interpretation were analyses of RNA transcripts on sucrose density gradients which showed a progressive decrease in average RNA chain length as the extent of template modification increased. In contrast to the inhibitory effect on chain elongation, evidence was obtained that the modified DNA had an increase in the number of initiation sites for transcription. These results are consistent with separate physical studies indicating that modification of DNA by this benzo[*a*]pyrene derivative can induce small localized regions of denaturation.

In the accompanying paper (Pulkrabek et al., 1977), we have described certain physical properties of DNA modified by in vitro reaction with a dihydrodiol epoxide derivative of benzo[*a*]pyrene (BPDE).[1] It was of interest to also examine the effects of this modification on the template activity of DNA and to relate the findings of such studies to the structural changes previously elucidated. We were particularly interested in knowing whether modification of DNA by this derivative of benzo[*a*]pyrene (BP) affected its in vitro transcription by *E. coli* DNA-dependent RNA polymerase since this appears to be a very sensitive parameter that is altered when DNA is modified by AAF (Troll et al., 1968; Yamasaki et al., 1977; for a review of this subject, see Kaplan and Weinstein, 1976), by aflatoxin (Saunders et al., 1972), by alkylating agents (Hendler et al., 1970; Mamet-Bratley, 1971; Degre-Couve and Mamet-Bratley, 1973; Muramatsu et al., 1973), or by ultraviolet irradiation (Hagen et al., 1970; Sauerbier et al., 1970). For example, the covalent binding of AAF to DNA, resulting from in vitro incubation with *N*-acetoxy-AAF, causes a marked inhibition of RNA synthesis which is associated with premature chain termination and no inhibition or even a slight enhancement in initiation of transcription (Yamasaki et al., 1977). In the present paper we have, therefore, studied the transcription of BP-modified DNA templates, in two different systems. In one, RNA polymerase is allowed to recycle continuously on the DNA template, while in the other only a single round of initiation is permitted (Cedar and Felsenfeld, 1973). In this way, the initiation and elongation phases of transcription are distinguishable. The results we report here show that, as with AAF, BP modification of DNA results in premature chain termination and shorter RNA chains, as well as an increased number of polymerase initiation sites on the template.

Materials and Methods

Materials. The ± isomer I of BPDE was kindly supplied by Dr. Ronald Harvey. Nucleoside triphosphates were from Schwarz/Mann. Deoxyribonucleoside triphosphates were purchased from Calbiochem. [^{3}H]UTP (27 Ci/mmol) was from New England Nuclear. Calf thymus DNA was from Worthington; DNA-dependent RNA polymerase was prepared from *E. coli* B cells (Miles) according to the procedure of Burgess (1969). All other chemicals were reagent grade or better.

Preparation of BP-Modified Nucleic Acids. Native calf thymus DNA was reacted in vitro with BPDE, the noncovalently bound material extracted, and the extent of modification calculated from the ϵ_{350} of the modified material, as previously described (Pulkrabek et al., 1977). A control sample of native DNA was prepared by incubation under the identical conditions as those used for preparation of the modified sample, but the BPDE was omitted. All procedures were carried out under subdued light to prevent photodecomposition.

Transcription without Reinitiation. The reaction was carried out according to Cedar and Felsenfeld (1973) with some modifications. The initiation reaction mixtures contained in either 0.1 or 0.5 mL: 10 mM Tris-HCl, pH 7.9 at 25 °C; 1 mM $MnCl_2$; 0.08 mM each of ATP and GTP and 0.02 mM [^{3}H]-UTP (1500 cpm/pmol); 3 units of *E. coli* DNA dependent RNA polymerase; and 1 μg of DNA. To allow initiation, the latter mixture was incubated at 37 °C for 15 min and stopped by the addition of 0.032 mL of 1.6 M ammonium sulfate per 0.1 mL of reaction mixture. This raised the salt concentration to 0.4 M which prevented further chain initiation by polymerase (Hyman and Davidson, 1970). Propagation was then begun by adding 0.06 mM CTP and 5 mM $MgCl_2$. When a

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[1] Abbreviations used: BP, benzo[*a*]pyrene; BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BP-nucleic acid or BP-modified nucleic acid, nucleic acids containing covalently bound derivatives of BP following in vitro reaction with BPDE; EDTA, ethylenediaminetetraacetate; AAF, 2-acetylaminofluorene; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: In Vitro Template Activity of Calf Thymus DNA Modified with Benzo[*a*]pyrene. Transcription Done under Nonreinitiating Conditions.

DNA	Modified bases (%)	UTP incorp (pmol)[a]	Incorp relative to native DNA (%)
Native	0	298.8	100
Mock modified	0	276.3	92.5
BP-modified	0.2	223.5	74.8
BP-modified	0.5	166.8	55.8
BP-modified	1.5	135.4	45.3
BP-modified	2.2	77.8	26.0
BP-modified	4.5	32.8	11.0

[a] [^{3}H]UTP, specific activity 1500 cpm/pmol; each value is an average of three experiments. Blanks assayed in the absence of template averaged less than 2 pmol and have been subtracted. Additional details are given in Materials and Methods.

TABLE II: In Vitro Template Activity of Calf Thymus DNA Modified with Benzo[*a*]pyrene. Transcription Done under Reinitiating Conditions.

DNA	Modified bases (%)	UTP incorp (pmol)[a]	Incorp relative to native DNA (%)
Native	0	756.0	100
Mock modified	0	869.0	114.9
BP-modified	0.2	448.5	59.3
BP-modified	0.5	352.1	46.5
BP-modified	1.5	202.1	26.7
BP-modified	2.2	111.2	14.7
BP-modified	4.5	24.5	3.2

[a] [^{3}H]UTP, specific activity 8 cpm/pmol; each value is an average of three experiments. Blanks assayed in the absence of template averaged 5 pmol and have been subtracted. Additional details are given in Materials and Methods.

0.1-mL reaction mixture was used, incorporation was determined after 20 min at 37 °C by adding 2 mL of cold 10% Cl_3CCOOH containing 0.05 M sodium pyrophosphate and 0.2 mg of yeast tRNA as carrier. After 15 min on ice, the precipitates were collected on Millipore filters and counted. When 0.5-mL reaction mixtures were used, 0.05-mL aliquots were taken at various intervals and processed as described above.

Transcription with Reinitiation. Reaction mixtures contained in 0.1 mL: 10 mM Tris-HCl, pH 7.9 at 25 °C; 1 mM $MnCl_2$; 0.08 mM each of ATP, GTP, CTP, and [^{3}H]UTP (8 cpm/pmol); 3 units of *E. coli* DNA dependent RNA polymerase; and 1 μg of DNA. Incubation was at 37 °C for 20 min. Processing of samples was as described above.

Sucrose Density Gradient Analysis. The procedure was essentially as previously described (Cedar and Felsenfeld, 1973). Reaction mixtures of 0.5 mL were incubated for 20 min as described under "transcription without reinitiation". Samples of 0.2 mL each were made 7.5 mM in EDTA and 0.5% in sodium dodecyl sulfate before being layered on 5 mL of a 5–20% linear sucrose gradient in 10 mM Tris-HCl, pH 7.9 at 25 °C; 1 mM EDTA; and 0.1 M NaCl. Centrifugation was for 3 h at 50 000 rpm in a Spinco SW65 rotor at 20 °C. Eukaryotic ribosomal RNA was used as a sedimentation marker in a parallel gradient. Approximately 25 fractions were collected from each gradient and the acid-precipitable radioactivity was measured. A control sample incubated in the absence of template was also centrifuged and the acid-precipitable counts in each fraction subtracted from the corresponding fraction in each gradient to give the net cpm incorporated into RNA.

Results and Discussion

Transcription of BP-Modified DNA. Since DNAs modified by AAF in vitro (Troll et al., 1968; Yamasaki et al., 1977; Zieve, 1973), or in vivo (Grunberger et al., 1973; Yu and Grunberger, 1976), have altered template activities during transcription, we measured the effects of BP modification of DNA templates on the overall synthesis of RNA. This was studied under two different conditions, nonreinitiating and reinitiating. In the former, initiation was carried out in low salt and propagation in high salt (Cedar and Felsenfeld, 1973). High salt conditions prevent the RNA polymerase from reinitiating on the DNA template, thus assuring that only one RNA chain per initiation site will be synthesized. In the other "reinitiating" system, the enzyme can recycle and initiate several chains per site.

Table I shows the results of total RNA synthesis under nonreinitiating conditions. Native and mock modified DNA gave virtually identical amounts of incorporation, while BP-modified DNA showed a progressive inhibition of incorporation with increasing extents of modification. A DNA sample with 1.5% modified bases showed approximately 55% inhibition of RNA synthesis when compared with native unmodified DNA. Table II indicates total RNA synthesis obtained under reinitiating conditions. Mock modified DNA showed stimulation of transcription (14%) when compared with a sample of native DNA. On the other hand, BP-modified samples were markedly inhibited in their template capacities. The sample in which there was only 0.2% modification showed about 40% inhibition. This value, as well as others in Table II, is significantly greater than the comparable values obtained under nonreinitiating conditions (Table I).

The finding that carcinogen modification of DNA caused a greater inhibition of transcription when assayed under conditions of reinitiation is in agreement with results obtained in separate studies with DNA modified with the aromatic amine carcinogen *N*-acetoxyacetylaminofluorene (Yamasaki et al., 1977) and also with results reported by others (Troll et al., 1968; Zieve, 1973; Millette and Fink, 1975). As expected, with native DNA total RNA synthesis was greater under reinitiating conditions since the enzyme is allowed to recycle. Thus, native DNA synthesized approximately 2.5 pmol of RNA under reinitiating conditions for every 1 pmol synthesized under conditions without reinitiation (Tables I and II). If movement of the polymerase along the DNA template is blocked when it encounters sites of BP modification, and the enzyme cannot recycle, then the percent inhibition would be greater under reinitiating conditions than without reinitiation. Our results suggest that the polymerase remains attached to the template after encountering a BP molecule since if it detached from the template it would presumably be available for reinitiation and the inhibition of RNA synthesis would be less marked than that observed. This explanation assumes that BP modification of DNA results in preferential inhibition of elongation with little or no inhibition of initiation (see below).

Saturation Kinetics of Modified DNA. In related studies we have shown that BP modification of DNA causes small localized regions of denaturation in native DNA (Pulkrabek

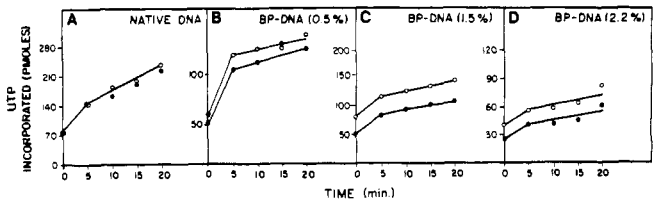

FIGURE 1: Saturation kinetics of BP-modified DNA. RNA synthesis was performed in 0.5 mL under nonreinitiating conditions as described in Materials and Methods. (●) Three units of RNA polymerase; (○) 6 units of RNA polymerase. (A) Native DNA; (B) 0.5% modified BP-DNA; (C) 1.5% modified BP-DNA; (D) 2.2% modified BP-DNA.

et al., 1977). Denaturation of DNA resulting from chemical modification by other carcinogens has also been described (Troll et al., 1969; Fuchs and Daune, 1972, 1973; Levine et al., 1974). Partial denaturation of DNA is known to increase the number of initiation sites for *E. coli* RNA polymerase (Maitra and Hurwitz, 1965; Tada and Tada, 1969), and thus we might expect to find an increase in the number of initiation sites in BP-DNA. We looked, therefore, at the kinetics of transcription under nonreinitiating conditions, in the presence of an amount of RNA polymerase which saturated all of the initiation sites on native DNA. With a fixed amount of native DNA, the concentration of enzyme used was saturating since doubling the enzyme concentration did not increase incorporation (Figure 1A). Identical concentrations of polymerase were used in transcription reactions with BP-DNAs containing either 0.5, 1.5, or 2.2% modification. Figure 1 indicates that an amount of polymerase which saturated all sites on the control native DNA was not saturating for any of the BP-modified templates. Although other explanations have not been excluded, this result, taken together with our other findings, suggests that BP modification does increase the number of initiation sites, presumably by producing localized regions of denaturation. When BP-DNA is used as a template, the increased number of initiation sites may be masked by the more marked inhibition of chain elongation (see below).

Density Gradient Analysis of Transcription Products. To directly monitor the effects of BP modification of DNA on chain elongation during transcription, we subjected the transcripts to sucrose density gradient centrifugation (Figure 2). Native DNA transcripts consisted of heterogeneous high-molecular-weight products ranging in size from about 30 to 7 S with a broad peak at about 20 S (Figure 2A). These values are similar to those obtained by others (Cedar and Felsenfeld, 1973; Cedar, 1975). Transcripts obtained from the mock modified DNA had a similar size distribution (data not shown here). On the other hand, transcripts obtained from BP-DNA containing a 0.5% modification showed a lower size distribution ranging from about 25 to 7 S (Figure 2B) with a peak at approximately 14 S, and those obtained from BP-DNA containing either 1.5 or 2.2% modification tended to be even smaller (Figures 2C and 2D). These results are consistent with the interpretation that BP modification tends to interrupt chain elongation during transcription. The results are qualitatively similar to those described with AAF modification of T7 DNA by Millette and Fink (1975). The actual size of the transcripts from the BP-DNA is greater, however, than one would expect if the BP derivatives were randomly distributed along the DNA template and if there was complete interference of chain elongation at every site of BP modification. Under the latter conditions, a 1% modification would yield transcripts that had a maximum chain length of less than 50–100 nucleotides, which would correspond to about 5 S. We observed that, when

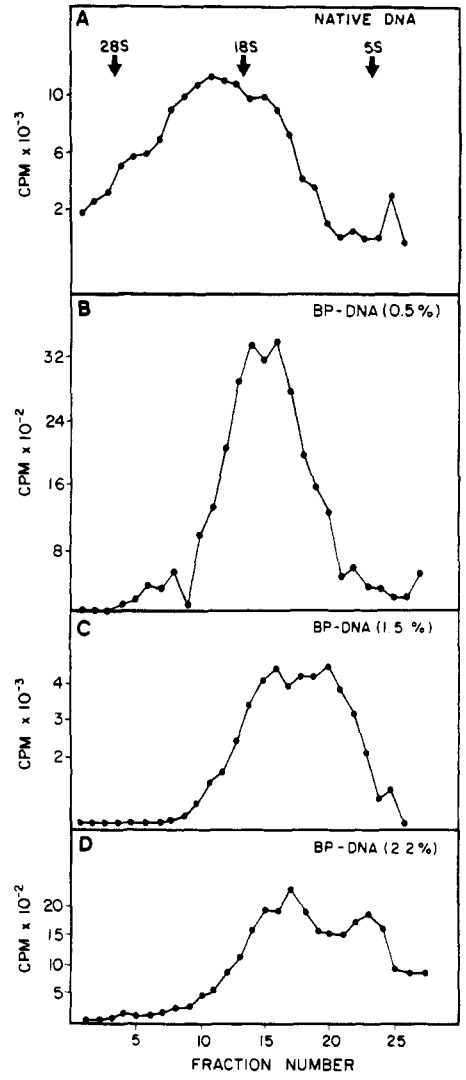

FIGURE 2: Sucrose density gradient centrifugation of RNA transcripts from BP-DNA templates. The experiments were performed as described in the text. (A) Native DNA; (B) 0.5% BP-DNA; (C) 1.5% BP-DNA; (D) 2.2% BP-DNA.

the data from Figure 2 were converted to chain length, as described by Cedar and Felsenfeld (1973), the mock-modified DNA sample and the native DNA sample yielded products with a number average nucleotide chain length of 1402–1450. The corresponding value for the 0.5% BP-DNA sample was 1259; for the 1.5% BP-DNA sample, 1016; and for the 2.2% sample, 750. Thus, although increasing modification of the template led to decreasing average chain length of the product, the average chain lengths were greater than predicted. A possible explanation is that the BP derivatives are clustered rather than scattered randomly on the DNA template, perhaps because the modification is "cooperative". Alternatively, the distribution may be random with the polymerase able to "bypass" some, but not all, of the sites of modification during the process of chain elongation. Further studies are required to clarify this aspect.

In vivo, the extent of covalent binding of the activated derivative of benzo[*a*]pyrene to cellular DNA is in the range of 1 BP residue per 10^4 to 10^5 nucleotides (Ivanovic et al., 1976). In the present paper, and in the related physical studies (Pulkrabek et al., 1977), the extent of modification was in the range of 1 carcinogen residue per 10^2 to 10^3 nucleotides. This high degree of substitution was required, particularly in some of the

physical studies, to observe an easily detectable change in the structure of the DNA, since with the methods employed we could only examine effects on the total DNA sample, rather than on the structure and function of single genes. It is difficult, therefore, to extrapolate from the present results to the in vivo situation. If, however, qualitatively similar, but quantitatively less extensive, changes occur in vivo when cells are exposed to BP, then the carcinogen could produce aberrations in the transcription of genetic information.

It is likely that the inhibitory effects of BP modification on DNA template function during transcription, demonstrated in the present study, also extend to DNA replication. This may explain the action of BP as a frame shift mutagen in *Salmonella typhimurium* (McCann et al., 1975). Further studies on the extent and fidelity of transcription and replication of BP-modified DNAs are currently in progress.

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