Mechanism of Rat Liver DNA Methyltransferase Interaction with *anti*-Benzo[*a*]pyrenediol Epoxide Modified DNA Templates†

Mathuros Ruchirawat, Frederick F. Becker, and Jean-Numa Lapeyre*

ABSTRACT: We investigated the methylation reaction catalyzed by 1500-fold purified rat liver DNA methyltransferase (DMase) on native *Micrococcal luteus* DNA (ML-DNA) and poly(dC-dG) templates containing covalently bound (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene (*anti*-BPDE), the strongly carcinogenic, principal metabolite of benzo[*a*]pyrene. Since eukaryotic DNA methyltransferases recognize the dinucleotide 5′d[CG] in DNA as a substrate for methylation, the model polynucleotide poly(dC-dG) was used to study in more detail the mode of interaction and effect on incorporation. With either of these BPDE-modified templates, a progressive inhibition of methylation was correlated with increasing amount of BPDE substitution. The effect of BPDE–dG adducts did not alter the apparent k_m with respect to the concentration of d[CG] in either unmodified or BPDE-modified poly(dC-dG) (k_m = 10 μM) but lowered the relative apparent V_{max}. In assays in which perturbation by salt of preformed enzyme–DNA complex is measured, no change in the relative stability to either unsubstituted or the carcinogen-modified template was noted, thus, excluding any change in the ionic component of this interaction. However, in competition-type experiments, BPDE–DNA is an inhibitor of the methylation reaction on native DNA. When BPDE–DNA is allowed to interact with the enzyme before the addition of native competitor DNA, the methylation rate is not stimulated, suggesting very tight hydrophobic binding of the enzyme to BPDE–DNA and an inhibition in the dissociation of DMase from the template following a methylation event. The interaction of DMase with poly(dC-dG) was also investigated from the size and rate of accretion of *Hha*I-resistant methylated regions in the polymer. These experiments indicated that the enzyme did not methylate the polymer processively. Rather, after each methylation event dissociation was followed by random rebinding for the next methylation event. The circular dichroism spectra of the poly(dC-dG) modified with BPDE excluded the possibility of a conformational shift from B-DNA to Z-DNA as a cause of the inhibition of the methylation reaction. Furthermore, the CD spectra indicated that the pyrene ring position of the adduct is situated in the minor groove of B-DNA to potentially interact with the enzyme. These data suggested that an altered dG-base configuration and nearly irreversible enzyme binding to BPDE-modified DNA regions are responsible for the inhibition in methylation kinetics.

The enzyme DNA methyltransferase (DMase)[1] catalyzes the transfer of methyl groups from the donor substrate *S*-adenosylmethionine (SAM) to the 5-position on deoxycytidine on an acceptor DNA substrate [reviewed by Lapeyre et al. (1984)]. At the very least, the specificity residing in the template for this transfer appears to be dinucleotide 5′d[CG] in either single-stranded or double-stranded configuration.

Recent studies using *Hpa*II/*Msp*I to monitor the methylation in d[CCGG] sequences, which are a small proportion of total methylatable sites in eukaryotic genes, have been consistent with the prediction that DNA methylation plays a role in eukaryotic gene expression [review in Razin & Riggs (1980), Ehrlich & Wang (1981), and Drahovsky & Boehm (1980)]. Although there are notable exceptions to this pattern (McKeon et al., 1980), it appears that specific demethylation of upstream flanking sequences may be necessary for transcriptional activity (Simon et al., 1983; Busslinger et al., 1983). Thus, the enzyme DMase may play a key role in the proposed mechanism of gene regulation in eukaryotes in which specific patterns of cytosine methylation must be transmitted to daughter cells with a high degree of fidelity to ensure the stability of differentiated states.

Of the several models for carcinogenesis, the theory proposed by Holliday (1979) predicts that tissue-specific patterns of methylation become altered by carcinogenic and/or oncogenic agents. This alteration in methylation would be a precondition for destabilizing and then altering the normal cellular phenotype by scrambling gene expression. One mechansim by which this may be achieved is direct chemical carcinogen inactivation or alteration of DMase (Cox, 1980; Wilson & Jones, 1983; Chan et al., 1983) or, as considered in this study, an indirect result of the covalent binding and modification of DNA by a carcinogen that is an acceptor substrate for the enzyme. Numerous recent studies on alterations in genomic methylation during carcinogenesis involving either diverse chemical carcinogens (Lapeyre & Becker, 1979; Boehm & Drahovsky, 1981a,b; Lapeyre et al., 1981; Feinberg & Vogelstein, 1983a,b) or oncogenic viruses (Desrosiers et al., 1979; Cohen, 1980) have lent support to this hypotheses.

The ultimate carcinogenic metabolite of the B[*a*]P is believed to be (+)-*anti*-BPDE (Buening et al., 1978) that is generated stereospecifically by microsomal activation of the parent compound (Sims et al., 1974; Huberman et al., 1976). *anti*-BPDE reacts with both nucleic acids and proteins (Koreeda et al., 1978) and is highly mutagenic in both mammalian (Wood et al., 1974) and bacterial test systems (Brookes, 1979). The *anti*-BPDE metabolite of B[*a*]P has skin tumor initiating activity (Slaga et al., 1977) and, in the lung, serves as a complete carcinogen (Kapitulnik et al., 1978).

† From the Department of Pathology, Section of Experimental Pathology, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030. *Received January 11, 1984.* This investigation was supported by U.S. Public Health Service Grant CA31487, awarded by the National Cancer Institute, and by a Fellowship in Toxicology awarded to M.R. by Conoco, Inc.

[1] Abbreviations: AAF, 2-(acetylamino)fluorene; B[*a*]P, benzo[*a*]-pyrene; BPDE, benzo[*a*]pyrenediol epoxide; CD, circular dichroism; DMase, DNA methyltransferase; DTT, dithiothreitol; ML, *Micrococcus luteus*; SAM, *S*-adenosylmethionine; TCA, trichloroacetic acid; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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Although there is evidence for the formation of *anti*-BPDE adducts to deoxyguanosine and deoxyadenosine in DNA in vitro (Straub et al., 1977; Meehan et al., 1977) and a reversible, noncovalently bound complex with DNA (Meehan et al., 1982), the principal adduct which is formed both in vivo and in vitro is (7*R*)-*anti*-BPDE–*trans*-deoxyguanosine linked through the exocyclic N(2) position. In vivo studies on the carcinogenicity of B[*a*]P have demonstrated a good correlation on the formation of adducts to DNA but not to RNA or protein (Brookes & Lawley, 1964). In the study by Ashurst et al. (1983), the dose–response curve for the induction of papillomas by B[*a*]P was essentially parallel to the corresponding formation of BPDE–dG adducts, suggesting that this type of adduct may play a determinitive role in mouse skin carcinogenesis.

In this study, we investigated the mechanism of interaction of 1500-fold purified DNA methyltransferase from rat liver with DNA which has been reacted with *anti*-BPDE in vitro. This enzyme appears to be the only major DNA methyltransferase activity in rat liver and methylates preferentially double-stranded over single-stranded DNA. The purified activity has a molecular weight of 280 000 and an order of magnitude preference for hemimethylated sites in DNA, thus qualifying for the role of "maintenance methylase". Our results indicate that the mechanism for the inhibition of DNA methylation by covalent modification by BPDE is quite similar to that observed by Pfohl-Lezkowicz et al. (1981) for *N*-acetoxy-AAF, a reactive form of the carcinogenic arylamine 2-(acetylamino)fluorene (AAF), whose principal adducts are bound primarily (90%) to the C(8) and the remainder to exocyclic N(2) of deoxyguanosine. Our findings suggest that when engaged on DNA the enzyme is nearly irreversibly bound to exposed hydrophobic B[*a*]P adducts. With BPDE–DNA, the k_m for either modified or unmodified d[CG] dinucleotides does not seem altered, nor does the methylation complex exhibit any change in stability with respect to the perturbing effects of increasing salt in preincubation assays. However, when bound to B[*a*]P-modified regions in DNA, the enzyme is not able to freely dissociate and methylate competitor DNAs, and the movement of the methylase appears to be substantially blocked from further methylation along the DNA helix as determined from the reaction kinetics.

Materials and Methods

Poly(dC-dG) and poly(BrdC-dG) were obtained from P-L Biochemicals; DNase (DN-Cl) was from Sigma Chemical Co.; phosphocellulose (P-11) was from Whatman; hydroxylapatite (HPT) was from Bio-Rad; DEAE-Sephacel was from Pharmacia; AcA 34 was from LKB. *M. luteus* (67% G+C) DNA was purified by the procedure of Marmur (1961) from freeze-dried *Micrococcus luteus* (ML) cells from Cal-Biochem. [^{3}H]Methyl-*S*-adenosylmethionine (9 Ci/mmol) was from ICN. *anti*-(+)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-[7-^{14}C]BPDE) (55.6 mCi/mmol) was obtained through the NCI Chemical Repository, Midwest Research Institute. The reaction of *anti*-[^{14}C]BPDE with DNA was performed under N_2 at 37 °C in 0.01 M NaCl, 2 mM trisodium citrate, pH 7.5, and 5% Me_2SO for 1 h with carcinogen to DNA-P ratios ranging from 0.05 to 0.5. Nonreacted carcinogen was removed by one benzene–ethyl acetate extraction followed by two ethyl acetate, one phenol–$CHCl_3$, and two ether extractions and the polymer recovered by ethanol precipitation. The degree of bound BPDE was assessed by liquid scintillation counting of known quantities of reacted DNA and by spectrofluorometric determination on a Perkin-Elmer MFP44A with excitation set at 279 nm and emission at 380 nm for bound benzo[*a*]pyrene fluorophor (Hemminiki et al., 1980). Both methods gave comparable results for percent modification.

Preparation of Rat Liver Methyltransferase. The method for purification and characterization of the physical and catalytic properties of DMase is described elsewhere (M. Ruchirawat, F. F. Becker, and J.-N. Lapeyre, unpublished results). Briefly, with all steps at 0–4 °C, DMase was isolated from 0.63 M KCl extracts of liver nuclei as described by Simon et al. (1978). The crude methylase was subsequently purified by absorption to phosphocellulose, eluted at 0.6 M NaCl, and then absorbed onto a hydroxylapatite column that was developed with a linear gradient from 0.05 M potassium phosphate to 0.4 M potassium phosphate, pH 7.5. A single peak eluting at approximately 0.18 M potassium phosphate, pH 7.5, was dialyzed and chromatographed on DEAE-Sephacel equilibrated in 0.02 M Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol (buffer A) plus 0.025 M NaCl. A linear gradient to buffer A plus 0.3 M NaCl was developed, and a single peak eluting at approximately 0.08 M NaCl was concentrated onto a small hydroxylapatite column and recovered in a minimum volume of 0.25 M potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Fraction III enzyme was further purified by gel filtration on an AcA 34 column (1.5 × 90 cm) equilibrated in buffer A plus 0.1 M NaCl. Glycerol was added to 50% v/v and the solution stored in small aliquots at −20 or −70 °C for long-term storage. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin for standard. The DMase isolated by this procedure is about 1500-fold purified over the initial nuclear extract, has an apparent molecular weight of 280 000 by gel filtration, and preferentially methylates double- over single-standed DNA and hemimethylated over unmethylated double-stranded DNA.

Methylation of DNA. The standard assay mixture in 200 μL consists of 3 μCi of [^{3}H]methyl-SAM (2 μM in dilute acetic acid immediately neutralized with NaH_2PO_4 before use), variable amounts of salt but generally 0.025 M NaCl where the enzyme has optimal activity, and enzyme protein in an assay buffer containing 20 mM Tris-HCl, pH 7.4, and 0.5 mM DTT. The incubation is carried out at 37 °C for 30 min to 2 h (the linear phase of the reaction of Figures 1–3). With this highly purified preparation of DMase, no contaminating RNA or protein (as self) methylation is detected, and the addition of EDTA to prevent nuclease degradation could be dispensed with. Nonetheless, the reactions are stopped by the addition of 12 μL of 5 N NaOH followed by 10 μL of 1 mg/mL sonicated calf thymus DNA and heating at 60 °C for 15 min, which removes any methylated RNA contaminants and destroys SAM. The reactions mixtures are directly precipitated with an equal volume of 20% trichloroacetic acid (TCA) and 40 mM sodium pyrophosphate. After 30 min on ice, the DNA is trapped by filtration on GF/C (Whatman) glass-filter disks which are washed 3 times with 5% TCA and twice with 95% ethanol and oven-dried at 60 °C before liquid scintillation counting by standard double-label techniques on ^{3}H and ^{14}C channels (when present). The specific activity of the rat liver DMase was determined by assaying known amounts of enzyme protein in 0.02 M Tris-HCl, pH 7.4, 1 mM DTT, and 1 mM EDTA under enzyme-limiting conditions on double-stranded poly(dC-dG). One unit of activity is defined as the incorporation of 1 pmol of [^{3}H]methyl group/h at 37 °C under standard assay conditions. Poly(dC-dG), a homogeneous polymer of d[CG] sites, has been found to be the methyl acceptor template of choice since base sequence effects

found in natural DNAs are negated. It also permits direct quantitation of the effects of template modification from the determination of kinetic parameters expressed as the concentration of d[CG] sites (or modified d[CG] dinucleotide sites). Other methyl acceptor templates may be utilized, but higher concentrations of these DNAs have to be used since the concentration of d[CG] dinucleotide sites is lower.

Spectroscopy. Circular dichroism spectra (CD) were recorded on a Jasco-Durrum SP-CD instrument calibrated to 0.3133° at 290 nm by using a 0.1% solution of *d*-10-camphorsulfonic acid (K & K Laboratories). The ellipticities were measured at 22 °C in quartz Supracil cuvettes with 1-cm optical path length. Subsequent measurements of UV absorption spectra were recorded in Cary 118 with the same cuvettes. Extinction coefficients used to calculate molar ellipticity of the various DNAs are 6800 cm^{-1} M^{-1} for ML-DNA, 7100 cm^{-1} M^{-1} for poly(dC-dG), and 6400 cm^{-1} M^{-1} for poly(BrdC-dG) (Pohl & Jovin, 1972). The DMase assay buffer used in CD studies consisted of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 0.5 mM DTT.

Poly(dC-dG) Labeling and Restriction Digest. Poly(dG-dG) (1 μg/5 μL) was heat denatured at 100 °C for 5 min and cooled on wet ice. Five microliters of [α-^{32}P]dGTP (300 Ci/mmol, 10 Ci/mL; ICN), was added to 45-μL reaction mixture containing the heat-denatured poly(dC-dG), 10 units of *Escherichia coli* DNA polymerase I (New England Biolabs), 100 μM dCTP, and 5 μM dGTP in 25 mM $MgCl_2$–0.5 mM DTT. The reaction mixture was incubated at 14 °C for 16 h and terminated by the addition of EDTA to 5 mM. Equal aliquots were methylated in vitro for various times and reactions stopped by one phenol–$CHCl_3$ extraction followed by an ether extraction. The product was recovered by ethanol precipitation and was digested to completion with 10-fold excess of *Hha*I for 1 h at 37 °C in 10 μL. *Hha*I from New England Biolabs was used according to supplier's instructions. The partial digest was composed of 0-, 1-, 5-, and 30-min aliquots of unmethylated *Hha*I-digested poly(dC-dG) by using 2-fold excess of enzyme. The oligodeoxyribonucleotide products of digestion were sized on a 30 × 20 × 0.1 cm, 12% polyacrylamide gel run in 50 mM Tris–borate, pH 8.3, and 1 mM EDTA and, after vacuum drying, autoradiographed on Kodak XRP-1 X-ray film at −70 °C.

Results

Methylation of Native DNA and Poly(dC-dG) Modified by BPDE. Double-stranded *M. luteus* DNA (67% G+C) and poly(dC-dG) were reacted in vitro with *anti*-[^{14}C]BPDE to yield templates with variable amounts of BPDE–dG-modified bases. These templates were purified of unreacted *anti*-BPDE and used in subsequent experiments to assess the effect of B[*a*]P adducts in an in vitro methylation system that utilizes 1500-fold purified rat liver DMase.

In Figure 1 we compare the activity of DMase on 1 μg of input double-stranded poly(dC-dG) with 1 μg of unmodified native ML-DNA and 2% and 4.1% BPDE-substituted ML-DNA. Increased amount of BPDE modification progressively reduces the rate of enzymatic methylation and the extent to which the template can accept methyl groups. This relationship also holds for the kinetics of methylation of BPDE-modified poly(dC-dG) (data not shown). The dose–response curve for the acceptor capacity of BPDE–poly(dC-dG) template as a function of BPDE modification is shown in Figure 2. The general shape of the inhibition of the methyl acceptor activity as a function of dG modification in Figure 2 is hyperbolic. When cast into linear form by a double-reciprocal plot of the data, the value for complete inhibition of

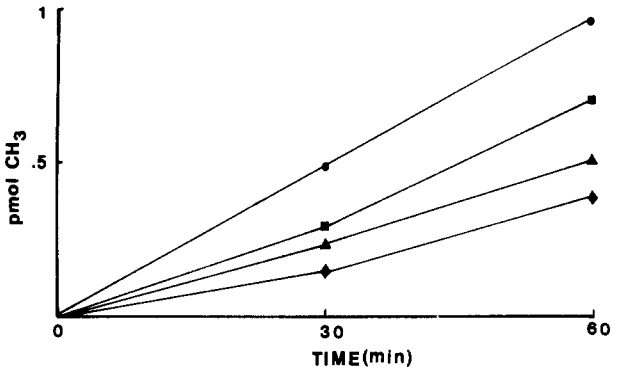

FIGURE 1: Time course of the enzymatic methylation of *anti*-BPDE-modified *M. luteus* DNA template. (●) Poly(dC-dG); (■) *M. luteus* DNA; (▲) BPDE 2% modified *M. luteus* DNA; (♦) BPDE 4.2% modified *M. luteus* DNA. The methylation reaction was performed under standard conditions as described under Materials and Methods.

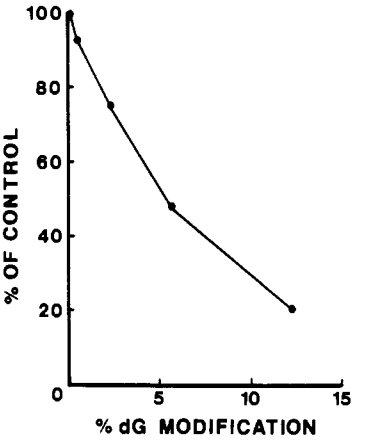

FIGURE 2: Dose–response curve for the inhibition of extent of enzymatic methylation as a function of BPDE modification of dG in poly(dC-dG) template.

enzymatic methylation extrapolates to approximately 20% dG substitution or one BPDE adduct per five base pairs.

Mechanism of DMase Interaction with BPDE-Modified Templates. The nature of the inhibition of DMase action on BPDE modified DNA was investigated by template competition experiments and by experiments on the effect of ionic strength on the stability of preformed DMase/DNA complexes. In Figure 3 a template competition experiment is shown for the methylation of control poly(dC-dG) where, after 30 min of methylation, an equal mass of 5.8% dG (left panel) or 9.5% dG (right panel) modified poly(dC-dG) is added to the methylation system. The addition of the carcinogen-modified template slows the rate of methylation to that which is intermediate between the rate for the control unmodified template and that of the carcinogen-modified templates, though the amount of template is doubled. This implies that the DMase once engaged in methylating normal poly(dC-dG) undergoes a dissociation from this template to commence methylation of the lower efficiency, carcinogen-modified template.

In Figure 4 the same experiment was performed in reverse order of addition of the reaction components in order to determine the relative rates of DMase dissociation from the normal and BPDE-modified templates. After 30 min of methylation of either BPDE–poly(dC-dG) 5.8% dG modification (panel A) or BPDE–poly(dC-dG) 12.1% dG modification (panel B), 0.5 μg of competitor poly(dC-dG) is added to each reaction, and the time course of methylation is determined over the next hour. Since the rates of methylation

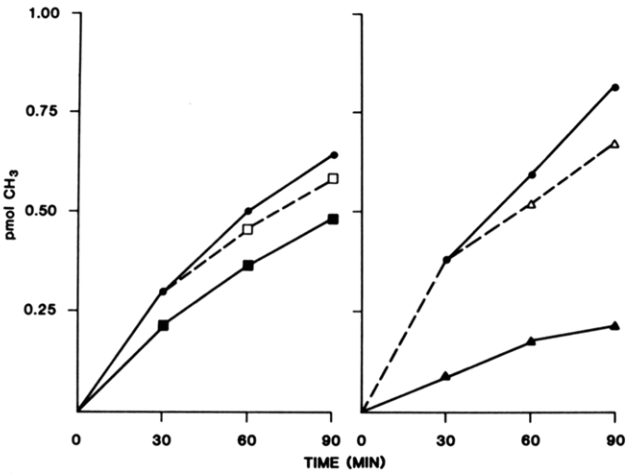

FIGURE 3: Effect of competitor *anti*-BPDE-modified poly(dC-dG) templates on enzymatic methylation of poly(dC-dG). Unmodified poly(dC-dG) was methylated at 37 °C in vitro under standard assay conditions. After a 30-min incubation, the reaction was split into two portions. An equivalent amount of BPDE–poly(dC-dG) was added to one portion while the other received an equal volume of buffer. The time course of the reaction was followed over the next 60 min. Left panel: (●) poly(dC-dG); (□) poly(dC-dG) + BPDE–poly(dC-dG), 5.8% dG modification; (■) BPDE–poly(dC-dG), 5.8; dG modification. Righ panel: (●) poly(dC-dG); (△) poly(dC-dG) + BPDE–poly(dC-dG), 9.5% dG modification; (▲) BPDE–poly(dC-dG), 9.5% dG modification.

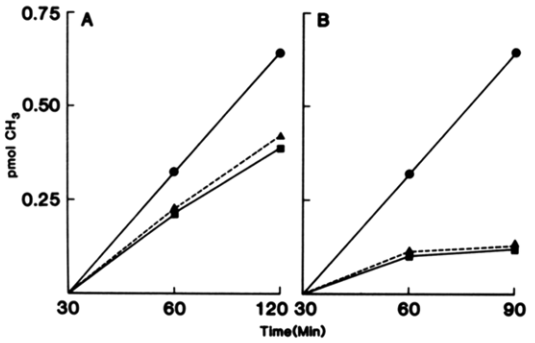

FIGURE 4: Time course of competitive DNA methylation of BPDE-modified poly(dC-dG) templates. Panel A: (●) unmodified poly-(dC-dG); (▲) BPDE–poly(dC-dG) 5.8% modified dG template allowed to react with DMase for 30 min prior to the addition of 0.5 μg of unmodified poly(dC-dG); (■) BPDE–poly(dC-dG) 5.8% modified dG template, no addition. Panel B: (●) unmodified poly(dC-dG); (▲) BPDE–poly(dC-dG) 12.1% modified dG template allowed to react with DMase for 30 min prior to the addition of 0.5 μg of unmoidified poly(dC-dG); (■) BPDE–poly(dC-dG) 12.1% modified dG template, no addition.

are approximately 50% and 20% of control poly(dC-dG) methylation (Figure 2), the rate is expected to increase proportionally to the degree that DMase is freed from the BPDE-modified template to methylate the active competitor template. Thus, if by 1 h after the addition of competitor half of the enzyme has dissociated from the BPDE template, the overall rate of methylation would be expected to increase somewhere to an intermediate value between rate of methylation of the control and that of BPDE-modified template. As seen in Figure 4, the rate after poly(dC-dG) addition is slightly increased for the 5.8% dG modified template and not changed for the template containing a higher degree of modification (12.1% dG modification). These results suggest that the effect of B[*a*]P adduct on dG in DNA is to impair the mechanism by which DMase dissociates to move along DNA helix.

Evidence that enzyme translocation along a template involves a dissociation step is shown in Figure 5 where the

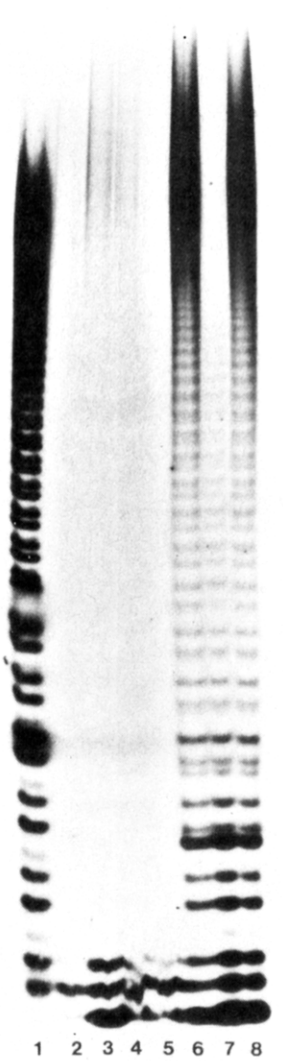

FIGURE 5: Size of *Hha*I-resistant methylated d[CG] sequences in poly(dC-dG) after enzymatic methylation. Equal aliquots of 0.15 μg of poly(dC-dG) replicated in the presence of [α-^{32}P]dGTP were methylated with 10 units of DMase for various times as indicated in each lane and subsequently digested with *Hha*I to completion. The size of *Hha*I-resistant oligonucleotides were sized on nondenaturing 12% polyacrylamide gel. Lane 1, partial *Hha*I digest control; lane 2, zero time unmethylated control; lanes 3–8, methylated for 5, 10, 15, 30, 45, and 60 min, respectively.

mechanism of the mode of interaction with the template was determined by measuring the size and rate of accretion of *Hha*I-digestible d[CG] dinucleotide units in poly(dC-dG) as a function of time. *Hha*I cleaves the sequence d[GCGC] and, as shown in lane 2 of Figure 5, poly(dG-dG) is mainly digested to tetranucleotide units. When the inner dC in the recognition sequence is methylated, *Hha*I cleavage is inhibited (McClelland, 1981). This inhibition is complete even when the recognition sequence is in a hemimethylated configuration (Gruenbaum et al., 1981). In Figure 5 the size of patches of *Hha*I resistant, hence methylated, dinucleotide units in poly-(dC-dG) was measured by *Hha*I digestion after various times of in vitro methylation with 10 units of purified DMase and sizing of the products on a 12% nondenaturing polyacrylamide gel. For the first 15 min of reaction, the size of dm^{5}C-resistant patches increases to, at most, hexanucleotide units. No appreciable protection of large oligonucleotide units occurs until 30 min of incubation although, as shown in Figure 1, the rate of methyl group transfer is linear with time over the first hour of incubation. These results indicate that the enzyme does not processively methylate sites in sequential order but either

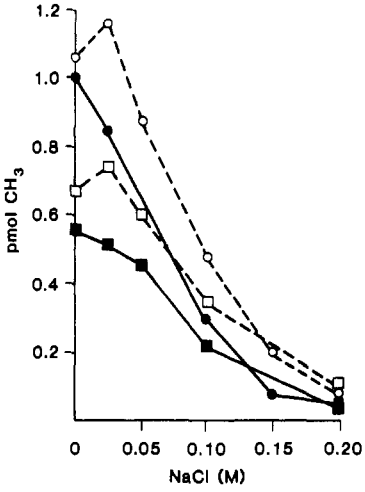

FIGURE 6: Effect of salt and preincubation on the activity of DMase on poly(dC-dG) and *anti*-BPDE modified template. Preincubation was carried out by the addition of template to DMase for 3 min at 37 °C prior to the addition of salt (open symbols). Closed symbols denote the absence of preincubation, e.g., salt added to the template before the enzyme at 37 °C. (●) Poly(dC-dG); (○) poly(dC-dG) preincubated; (■) BPDE–poly(dC-dG); (□) BPDE–poly(dC-dG) preincubated. The extent of modification was 5.8% of dG in BPDE–poly(dC-dG).

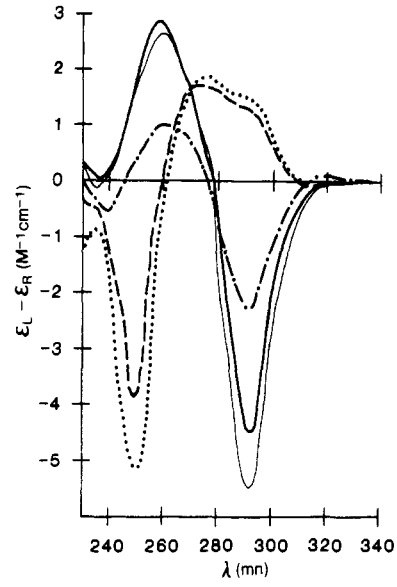

FIGURE 7: Circular dichroism spectra of BPDE-modified and unmodified poly(dC-dG) under various conditions. (---) Poly(dC-dG) in methylase assay buffer; (···) BPDE–poly(dC-dG), 9.5% dG modification in methylase assay buffer; (heavy line) poly(dC-dG) in 4 M NaCl; (-·-), BPDE–poly(dC-dG), 9.5% dG modification in 4 M NaCl; (—) poly(BrdC-dG) in methylase assay buffer.

obeys a random sequential mechanism in which it dissociates from the template, diffuses and rebinds or, alternatively, follows a facilitated one-dimensional diffusion (facilitated transfer) along the template before methylating another appropriate site (Berg et al., 1981). The degree to which adduction by BPDE inhibits this mechanism could not be measured by this method due to BPDE interference with *Hha*I cleavage of modified poly(dC-dG) templates (data not shown) (Boehm & Drahovsky, 1980).

Since it might be expected that the interaction of DMase with a region containing BPDE adducts, which blocks the dissociation and translocation of the enzyme on the helix, might be detected as a strong binding complex, the following experiment was performed to examine the nature of this binding. The effect of salt on the stability of DMase/DNA complex formed at 37 °C in the absence of salt has been described by Drahovsky & Morris (1971) and interpreted on the basis that the enzyme "walks" on DNA. The DMase preparation used in the current experiments exhibits this classical response on single-stranded DNA and to a lesser degree on double-stranded *M. luteus* DNA and poly(dC-dG).

We examine the perturbing effects of salt on the stability of such a complex on control poly(dC-dG) compared with BPDE-poly(dC-dG) 5.8% dG modification. With preincubation of DMase with the template for 5 min at 37 °C before the addition of salt, a significant stabilization is observed when compared to the addition of salt to the template before incubation with DMase (Figure 6). This results in a curve shifted to the right showing an enhanced stability with a maximum activity at 25 mM NaCl for this preformed enzyme/DNA complex. When these curves are determined for BPDE-modified poly(dC-dG), the general shape and displacement of the preincubation curves are similar although the initial methylation acceptor levels are much lower for the carcinogen-altered template. This suggests that the component of ionic binding between DMase and the various sites on unmodified or carcinogen-modified template are not significantly different. Thus, the lower degree of dissociation on BPDE-modified templates is probably due to increased hydrophobic interactions in the binding of enzyme to polycyclic aromatic ring moieties in the DNA.

The kinetics of methylation of the control and 5.8% modified template was also determined with respect to the total concentration of d[CG] units in the polymers. Lineweaver–Burk plots of the data give single-value Michaelis–Menten parameters, with relative apparent V_{max} of reaction of 12.5 pmol/h for control poly(dC-dG) and 6.2 pmol/h for BPDE–poly(dC-dG) 5.8% dG modification. The apparent k_m for the reaction is unchanged: 10 μM for dinucleotide units in both the control and BPDE-modified polymer.

Circular Dichroism of BPDE-Modified Templates. On the basis of the finding that various carcinogens, notably AAF, may induce a conformational shift in d[CG] sites in DNA to a left-handed Z-DNA conformation (Sage & Leng, 1980; Santella et al., 1981), it remains a possibility that the effect of BPDE inhibition on enzymatic methylation could be attributed to such a conformational shift.

In Figure 7 the CD spectrum is shown for the BPDE–poly(dC-dG) 9.5% dG modification under various conditions. In the DMase assay buffer used in this study (20 mM Tris-HCl, pH 7.4, 0.5 mM DTT, and 50 mM NaCl), the CD spectrum of BPDE-modified poly(dC-dG) is conserved and typical of the B form of poly(dC-dG) with extremum at 276 nm, cross-over at 257–258 nm, and negative extremum at 240 nm. In 4 M NaCl, the BPDE-modified poly(dC-dG) spectrum adopts an inverted profile that does not, however, fully develop a negative extrema at 292 nm characteristic of poly(dC-dG) in Z form having $\Delta\epsilon$ of −4.4 M^{-1} cm^{-1}. For comparison purposes, the CD spectrum of poly(BrdC-dG) is shown in the methylase assay buffer. The conformation of poly(BrdC-dG) is totally in the Z conformation and independent of ionic strength. The BPDE-modified polymer does not display any evidence of being in Z conformation under conditions of the assay, but under conditions that favor Z conformation (4 M NaCl), the BPDE-modified poly(dC-dG) polymer appears to undergo a conformation change approaching that of a polymer in the Z form. As shown by Nordheim et al., the lag in developing the characteristic negative extremum at 292 nm is reminiscent of that for aflatoxin B_1, a carcinogen adduct that does not facilitate the B → Z transition. Since B → Z transition involves enthalpies of conversion from one confor-

mation to the other (van de Sande & Jovin, 1982; Roy & Miles, 1983), the conformation of BPDE-modified poly(dC-dG) observed in the methylase assay buffer could not be altered to Z or Z* by heating at 60 °C and cooling, indicating that the molecule was not in a metastable state but rather in a stable B conformation (data not shown). These CD data indicate that the BPDE modification does not destabilize or facilitate a transition for B → Z in regions of d[CG] units of DNA, and furthermore, a mechanism based on such a hypothetical shift in a conformation produced by B[*a*]P adducts inhibiting enzymatic methylation is excluded.

Discussion

In this study, we demonstrated that the normal postreplicational enzymatic methylation mechanism carried out in vitro with a highly purified preparation of rat liver DMase having the properties of a putative maintenance methylase is impaired by B[*a*]P adducts. The effect of BPDE-generated adducts on the efficiency of DNA templates to accept the transfer of methyl groups was reduced in a dose-dependent manner as a function of the degree of modification. Analysis of the kinetics of the methylation reaction indicated that BPDE modification did not affect the apparent k_m of the reaction but reduced the relative apparent V_{max}.

The mechanism for this reduction in the steady-state rate of methylation was investigated by competition experiments and by measuring the size and rate of accretion of methylated sites on poly(dC-dG). In competition experiments, this translocation involves the dissociation and rebinding to another template with a characteristic template response to methylation. Experiments run with normal unmodified competitor template showed a strong inhibition in the dissociation reaction from templates containing low levels of BPDE modification which at higher levels of modification were completely blocked. Further evidence that movement along the helix involved a dissociation step between methylation events was provided by analyzing the size and accretion rate of dm^5C in poly(dC-dG) detected as *Hha*I-resistant stretches of $d[CG]_n$ oligodeoxyribonucleotides as a function of methylation. Under conditions of the assay, the rate of methyl transfer is linear over the first hours, yet a lag in the formation of adjacent (e.g., along one strand or staggered on both strands) methylated residues was detected, ruling out a processive mode for the methylation of double-stranded templates. This method could not distinguish between a dissociation following a methylation event that involves facilitated one-dimensional diffusion along the helix [facilitated transfer (Berg et al., 1981)] or a complete desorption, followed by a three-dimensional diffusion phase and then rebinding for the next transfer. It may be the case for natural DNAs, and in particular, for hemimethylated, de novo replicated DNA, that the enzyme functions in a processive manner. In competition experiments, it is clear that three-dimensional diffusion must take place. However, our preparation of DMase, which has a p*I* of 7.4–7.6 (M. Ruchirawat et al., unpublished results), demonstrates a "walking" response that is detected as a DMase/DNA complex with characteristic resistance to increased ionic strength on single-stranded DNA but a very weak response on duplex DNA (Drahovsky & Morris, 1971). From the enzyme's p*I*, one might not expect any strong electrostatic attraction to the helix. In the present case, an examination of the stability of preincubated DMase/DNA complex as to the perturbing effects of increasing ionic strength showed no change in the characteristic curves generated, whether the template was substituted with BPDE or not. This suggests that the impaired translocation along the helix or between helices, which involves a dissociation phase after a methylation event, is not a manifestation of increased ionic attractive forces but rather a strong hydrophobic interaction mediated by the aromatic pyrene ring structure of B[*a*]P with the enzyme.

In DNA, deoxyguanosine residues are the most highly susceptible to covalent modification by carcinogenic alkylating agents, which form principally N(7), O(6), and N(3) alkyl adducts (Singer, 1976). They are also subject to a variety of modifications by other carcinogen classes: the polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene metabolites, which form exocyclic N(2) adducts, and arylamine and azobenzene dye compounds, which principally form C(8), but also exocyclic N(2), adducts (Miller, 1978). In mammalian genomes the major modification of dC occurs in the dinucleotide 5′d[CG] and, to a lesser extent, in the outer C of d[CCGG] (Van der Ploeg & Flavell, 1980) and d[CC] (Browne & Burdon, 1977). Thus, although this dinucleotide is underrepresented in mammalian genomes (Bird, 1980), the formation of an adduct on dG has a high probability of altering a potential methylation site in several possible ways. One possibility is that the methylation site is no longer recognized as a "proper" site for methylation because of a change in configuration of the dG base. Conversely, the base configuration may not play an important role in enzyme recognition of the site, but specificity may depend upon the local template conformation. Methylation of dC or adjacent carcinogenic modification of dG may be sufficient to induce a partial or complete conformational change that the enzyme recognizes. A model for this is provided by the B → Z transition in poly(dm^5C-dG) that can be stabilized in Z form under physiological conditions (Behe & Felsenfeld, 1980; Klysik et al., 1981). Thus, agents the modify dG in unmethylated d[CG] sites may either directly prevent methylation (Z-DNA is not a substrate) or signal the enzyme that the site is already methylated because of its shift in conformation.

In this study we were also inerested in testing these possibilities using the model template poly(dC-dG) which can undergo B → Z conformational shifts. Under ionic conditions shown to stabilize poly(dm^5C-dG) but not poly(dC-dG) in Z form, we investigated the conformation poly(dC-dG) that had been modified with *anti*-BPDE and after methylation. Our results indicated that the normal B-DNA conformation was conserved. BPDE adduction in the range 2–12.1% dG modification did not induce or facilitate a conformational shift to the Z (Wang et al., 1979) or Z* (van de Sande & Jovin, 1982) family of left-handed DNA helices. Furthermore, after modification the B conformation was not in a metastable state since it could not be changed by a brief heating step at 60 °C (van de Sande & Jovin, 1982). From the CD results, the location of the pyrene ring would be in the minor groove of B-DNA for the BPDE adduct reacting via a trans addition to the exocyclic N(2). The evidence for tight hydrophobic binding by DMase to *N*-acetoxy-AAF-generated adducts which bind mainly via the C(8) but to a minor extent with the N(2) (Pfohl-Leszkowicz et al., 1981) and our findings that the 2-(hydroxyamino)fluorene generated adducts which bind solely via C(8) (Ruchirawat et al., 1984) implicate DMase interaction with functional determinants in the major groove. The present results with BPDE modification implicate interaction with determinants also in the minor groove of B-DNA, that can lead to impaired kinetics of enzymatic methylation, and tight hydrophobic binding of the enzyme affecting the release or dissociation phase between methylation events.

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Registry No. DMase, 9037-42-7; *anti*-BPDE, 63323-31-9; poly(dC-dG), 62081-33-8.

References

Ashurst, S. W., Cohen, G. M., Nesnow, S., Digiovanni, J., & Slaga, J. (1983) *Cancer Res. 43*, 1024–1029.

Behe, M., & Felsenfeld, F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619–1623.

Berg, O., Winter, R., & von Hippel, P. (1981) *Biochemistry 20*, 6929–6948.

Bird, A. P. (1980) *Nucleic Acids Res. 8*, 1499–1504.

Boehm, T. L. J., & Drahovsky, D. (1980) *Carcinogenesis (N.Y.) 1*, 729–731.

Boehm, T. L. J., & Drahovsky, D. (1981a) *Carcinogenesis (N.Y.) 2*, 39–42.

Boehm, T. L. J., & Drahovsky, D. (1981b) *Int. J. Biochem. 13*, 1225–1232.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brookes, P. (1979) *Mutat. Res. 39*, 257–284.

Brookes, P., & Lawley, P. D. (1964) *Nature (London) 202*, 781–784.

Browne, M. J., & Burdon, R. H. (1977) *Nucleic Acids Res. 4*, 1025–1037.

Buening, M. K., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., & Conney, A. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5358–5361.

Busslinger, M., Hurst, J., & Flavell, R. A. (1983) *Cell (Cambridge, Mass.) 34*, 197–206.

Chan, J. Y. H., Ruchirawat, M., Lapeyre, J.-N., & Becker, F. F. (1983) *Carcinogenesis (N.Y.) 4*, 1097–1100.

Cohen, J. C. (1980) *Cell (Cambridge, Mass.) 19*, 653–662.

Cox, R. (1980) *Cancer Res. 40*, 61–63.

Desrosiers, R. C., Malder, C., & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3839–3843.

Drahovsky, D., & Morris, N. (1971) *J. Mol. Biol. 61*, 343–356.

Drahvosky, D., & Boehm, T. L. J. (1980) *Int. J. Biochem. 12*, 523–528.

Ehrlich, M., & Wang, R. Y. H. (1980) *Science (Washington, D.C.) 212*, 1350–1357.

Feinberg, A. P., & Vogelstein, B. (1983a) *Biochem. Biophys. Res. Commun. 111*, 47–54.

Feinberg, A. P., & Vogelstein, B. (1983b) *Nature (London) 301*, 89–92.

Gruenbaum, Y., Cedar, H., & Razin, A. (1981) *Nucleic Acids Res. 9*, 2509–2515.

Hemminki, K., Cooper, C. S., Ribeiro, O., Crover, P. L., & Sims, P. (1980) *Carcinogenesis (N.Y.) 1*, 277–286.

Holliday, R. (1979) *Br. J. Cancer 40*, 513–521.

Huberman, E., Sachs, L., Yang, S. K., & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 601–611.

Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M., & Conney, A. H. (1978) *Cancer Res. 38*, 354–358.

Klysik, J., Stridivant, S. M., Singleton, C. K., Zacharias, W., & Wells, R. D. (1983) *J. Mol. Biol. 168*, 51–71.

Koreeda, M., Moore, P. D., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., & Jerina, D. M. (1978) *Science (Washington, D.C.) 199*, 778–780.

Lapeyre, J.-N., & Becker, F. F. (1979) *Biochem. Biophys. Res. Commun. 87*, 698–705.

Lapeyre, J.-N., Walker, M. D., & Becker, F. F. (1981) *Carcinogenesis 2*, 873–878.

Lapeyre, J.-N., Ruchirawat, M., & Becker, F. F. (1984) in *Progress in Non-Histone Protein Research* (Liew, C. C., & Bekhor, I., Eds.) CRC Press, West Palm Beach, FL.

Marmur, J. (1961) *J. Mol. Biol. 3*, 208–218.

McClelland, M. (1981) *Nucleic Acids Res. 9*, 5859–5866.

McKeon, C., Ohkubo, H., Pastan, I., & de Crombrugghe, B. (1982) *Cell (Cambridge, Mass.) 29*, 203–210.

Meehan, T., Straub, K., & Calvin, M. (1977) *Nature (London) 269*, 725–727.

Miller, E. C. (1978) *Cancer Res. 38*, 1479–1496.

Nordheim, A., Hao, W. M., Wogan, G. N., & Rich, A. (1983) *Science (Washington, D.C.) 219*, 1434–1436.

Pfohl-Leszkowicz, A., Salas, C., Fuchs, R. P. P., & Dirheimer, G. (1981) *Biochemistry 20*, 3020–3024.

Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.

Razin, A., & Riggs, A. D. (1980) *Science (Washington, D.C.) 210*, 604–610.

Razin, A., & Friedman, J. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 25*, 33–52.

Roy, K. B., & Miles, H. T. (1983) *Biochem. Biophys. Res. Commun. 115*, 100–105.

Ruchirawat, M., Becker, F. F., & Lapeyre, J.-N. (1984) *Nucleic Acids Res. 12*, 3357–3372.

Sage, E., & Leng, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4597–4601.

Santella, R. M., Grunberger, D., Weinstein, I. B., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1451–1455.

Simon, D., Grunert, F., Acken, U. V., Doring, H. P., & Kroger, H. (1978) *Nucleic Acids Res. 6*, 2153–2167.

Simon, D., Stuhlmann, H., Jahner, D., Wagner, H., Werner, E., & Jaenisch, R. (1983) *Nature (London) 304*, 275–277.

Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252*, 326–328.

Singer, B. (1976) *Nature (London) 264*, 333–339.

Slaga, T. J., Viaje, A., Brachen, W. M., Berry, D. L., Fischer, S. M., Miller, D. R., & LeClerc, S. M. (1977) *Cancer Lett. 3*, 23–30.

Straub, K. M., Meehan, T., Burlingame, A. L., & Calvin, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5285–8289.

Strauss, B., Tatsumi, K., Kanan, P., Higgins, N. P., Ben-Asher, E., Altamirano-Dimas, M., Rosenblatt, L., & Bose, K. (1978) in *Polycylic Hydrocarbons and Cancer* (eds. Gelboin, H. V., & T'so, P. O. P., Eds.) Vol. 2, pp 177–201, Academic Press, New York.

Van der Ploeg, L. H. T., & Flavell, R. A. (1980) *Cell (Cambridge, Mass.) 19*, 947–957.

van de Sande, J. H., & Jovin, T. M. (1982) *EMBO J. 1*, 115–120.

Wang, A. H., Quigley, G. J., Kolpak, F. J., Cranford, J. L., Van Boom, S. A., van der Macel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.

Weinstein, I. B., Jeffrey, A. M., Leffler, S., Pulkrabek, P., Yanasaki, H., & Grunberger, V. (1978) in *Polycyclic Hydrocarbons and Cancer* Vol. 2 (Gelboin, H. V., & T'so, P. O. P., Eds.) Vol. 2, pp 3–36, Academic Press, New York.

Wilson, V. L., & Jones, P. A. (1983) *Cell (Cambridge, Mass.) 32*, 239–246.