Effects of Benzo[*a*]pyrene−Deoxyguanosine Lesions on DNA Methylation Catalyzed by EcoRII DNA Methyltransferase and on DNA Cleavage Effected by EcoRII Restriction Endonuclease†

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ABSTRACT: DNA methylation is an important cellular mechanism for controlling gene expression. Whereas the mutagenic properties of many DNA adducts, e.g., those arising from polycyclic aromatic hydrocarbons, have been widely studied, little is known about their influence on DNA methylation. We have constructed site-specifically modified 18-mer oligodeoxynucleotide duplexes containing a pair of stereoisomeric adducts derived from a benzo[*a*]pyrene-derived diol epoxide [(+)- and (−)-*r*7,*t*8-dihydroxy-*t*9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene, or B[*a*]PDE] bound to the exocyclic amino group of guanine. The adducts, either (+)- or (−)-*trans*-*anti*-B[*a*]P−N^2-dG (G*), positioned either at the 5′-side or the 3′-side deoxyguanosine residue in the recognition sequence of EcoRII restriction-modification enzymes (5′-...CCA/TGG...) were incorporated into 18-mer oligodeoxynucleotide duplexes. The effects of these lesions on complex formation and the catalytic activity of the EcoRII DNA methyltransferase (M.EcoRII) and EcoRII restriction endonuclease (R.EcoRII) were investigated. The M.EcoRII catalyzes the transfer of a methyl group to the C5 position of the 3′-side cytosine of each strand of the recognition sequence, whereas R.EcoRII catalyzes cleavage of both strands. The binding of R.EcoRII to the oligodeoxynucleotide duplexes and the catalytic cleavage were completely abolished when G* was positioned at the 3′-side dG position (5′-...CCTGG*...). When G* was at the 5′-side dG position, binding was moderately diminished, but cleavage was completely blocked. In the case of M.EcoRII, binding is diminished by factors of 5−30 but the catalytic activity was either abolished or reduced 4−80-fold when the adducts were located at either position. Somewhat smaller effects were observed with hemimethylated oligodeoxynucleotide duplexes. These findings suggest that epigenetic effects, in addition to genotoxic effects, need to be considered in chemical carcinogenesis initiated by B[*a*]PDE, since the inhibition of methylation may allow the expression of genes that promote tumor development.

The methylation of DNA is an epigenetic alteration of the genome that plays an important role in the regulation of gene expression in eukaryotes. Abnormalities in the levels of methylation and methylation patterns are one of the hallmarks of tumorigenesis (*1*, *2*). Although the mutational pathways associated with the reactions of chemical carcinogens with DNA leading to cancer have been widely studied (*3*, *4*), less attention has been paid to epigenetic mechanisms such as alterations in methylation patterns (*5*, *6*). Many polycyclic aromatic hydrocarbons, e.g., the widely studied benzo[*a*]-pyrene (*7*), have been classified as carcinogens (*8*) and are ubiquitous environmental pollutants. The polycyclic aromatic hydrocarbons are metabolized in vivo to highly genotoxic dihydrodiol epoxides that chemically bind to cellular DNA causing mutations, thus contributing to the initiation of cancer. Many of the polycyclic aromatic hydrocarbon diol epoxides, including the bay region diol epoxides of benzo-[*a*]pyrene, *anti*-B[*a*]PDE,[1] bind predominantly to the exocyclic amino group of guanine in DNA (*9*) to yield the stereoisomeric pair of adducts shown in Figure 1. The impact of such DNA adducts on site-directed mutagenesis (*10*−*12*) and on the interactions with a variety of enzymes and proteins has been assessed in vitro and includes DNA repair enzymes

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[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; B[*a*]PDE, *r*7,*t*8-dihydroxy-*t*9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene; DMTr, dimethoxytrityl; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FAM, 5(6)-carboxyfluorescein; G*, *trans*-*anti*-B[*a*]P-N^2-dG; M.EcoRII, EcoRII DNA methyltransferase; MTase, methyltransferase; PAGE, polyacrylamide gel electrophoresis; R.EcoRII, EcoRII restriction endonuclease; HPLC, high-performance liquid chromatography; T_m, melting temperature of oligodeoxynucleotide duplexes; K_d, dissociation constant.

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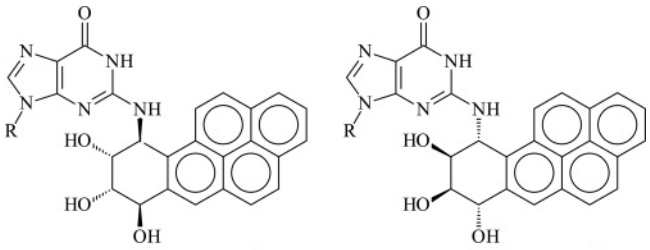

FIGURE 1: Chemical structures of the (+)- and (−)-*trans-anti*-B[*a*]P−N^2-dG adducts.

(*13*−*15*), T7 RNA polymerase (*16*), human DNA polymerases κ and μ (*17*, *18*), transcription factors (*19*−*21*), topoisomerases (*22*), and other enzymes. Furthermore, adducts derived from reactions of benzo[*a*]pyrene diol epoxides with DNA have been employed to probe their effects on enzyme−DNA interactions (*22*). As an example, steric hindrance effects exerted by the (+)- and (−)-*trans-anti*-B[*a*]P−N^2-dG lesions (Figure 1) permitted the detailed mapping of the minor groove interface between *vaccinia* topoisomerase and its DNA cleavage site (*22*). Although methylation appears to play an important role in carcinogenesis (*5*, *6*), the effects of carcinogen−DNA lesions on the binding of methyltransferases to their canonical recognition sequences and their catalytic activities have not yet been examined explicitly.

Here we examine the effect of (+)- and (−)-*trans-anti*-B[*a*]P−N^2-dG adducts positioned within the EcoRII recognition sequence (5′-...CCA/TGG...) on the functional behavior of EcoRII restriction-modification (R-M) enzymes. The monomeric EcoRII MTase (M.EcoRII) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the C5 position of the underlined cytosine of each strand of the 5′-...CCA/TGG... sequence. The structures of cocrystals of M.HhaI (*23*) and M.HaeIII (*24*) with their DNA substrates propose the two-domain organization for C5 MTases. The large domain encompasses the AdoMet binding site and the catalytic center, whereas the small domain contains amino acids that are involved in target recognition. The DNA substrates fit into the cleft between the two domains. All protein−DNA interactions that mediate sequence specificity are at the small domain of the enzyme−major groove interface (*23*, *24*). Recently, it was suggested that the discrimination between AT and GC base pairs is achieved by interactions between the large domain of M.EcoRII and the minor groove of DNA (*25*). The EcoRII endonuclease exists as a homodimer and belongs to the type IIE restriction endonucleases. This endonuclease simultaneously binds two copies of the recognition sequence (5′-...↓CCA/TGG...) and in the presence of a Mg^{2+} cofactor, cleaves (↓) two phosphodiester bonds in the two strands of only one of these two recognition sequences (*26*). R.EcoRII contains two domains as shown by biochemical and crystal structure analysis (*27*, *28*). These two domains are an endonuclease-like catalytic domain and a DNA binding effector domain. The effector domain initially binds one of the two recognition sequences, the effector DNA. This binding activates the endonuclease-like domain to bind and to cleave the second DNA recognition sequence, the substrate DNA. The impact of B[*a*]P−N^2-dG on the function of EcoRII restriction-modification enzymes is of intrinsic interest in understanding structure−function relationships in general. However, our main interest here was to evaluate how these two enzymes with the same recognition sequences but different catalytic activities and target sites within the (5′-...CCA/TGG...) recognition sequence would respond to the same pair of stereoisomeric adducts positioned at either the 5′- or 3′-side of the modified guanine (Table 1).

Table 1: Oligodeoxynucleotide Sequences Synthesized[a]

5′-GAGCCAACCTGGCTCTGA
5′-TCAGAGCCAGGTTGGCTC
5′-TCAGAGCMAGGTTGGCTC
5′-FAM-TCAGAGCCAGGTTGGCTC
5′-GAGCCAACCT[(−)G*]GCTCTGA
5′-GAGCCAACCT[(+)G*]GCTCTGA
5′-GAGCCAACCTG[(−)G*]CTCTGA
5′-GAGCCAACCTG[(+)G*]CTCTGA

[a] M = m^5dC; (−)G* is (−)-*trans-anti*-B[*a*]P−N^2-dG and (+)G* is (+)-*trans-anti*-B[*a*]P−N^2-dG.

All C5 MTases that methylate the 5-position of cytosine in double-stranded DNA exhibit considerable amino acid sequence homologies (*30*). Because of this, the mechanism of methyl transfer is likely to be similar in bacterial and mammalian enzymes. We thus selected the prokaryotic M.EcoRII MTase as a model system to start investigating the relationship between biological DNA methylation and carcinogenic DNA modifications. Furthermore, the (5′-...CCA/TGG...) sequence, in addition to the CpG sequences, was found to be a target of eukaryotic MTases (*31*, *32*).

In this work, we report for the first time how methylation is impacted by a pair of stereoisomeric polycyclic aromatic hydrocarbon carcinogen-modified DNA adducts (Figure 1) positioned at either one or the other of the guanine sites in the EcoRII recognition sequence. We selected the bulky stereoisomeric (+)- and (−)-*trans-anti*-B[*a*]P−N^2-dG adducts for study because much is known about their conformational properties from NMR and other biophysical studies (*33*−*35*). The bulky pyrenyl residues are positioned in the minor groove and extend into sites opposite to the modified guanine residue within the oligodeoxynucleotide duplexes. Therefore, these lesions provide opportunities to probe interactions between the DNA minor groove and the M.EcoRII and R.EcoRII enzymes.

Depending on the position of the adducts in the recognition sequence, the binding of M.EcoRII to the adduct-modified recognition sequences is diminished by factors of 5−30, while the catalytic activity is reduced 4−80-fold, or is even blocked entirely. The B[*a*]P−N^2-dG lesions also diminish the binding of R.EcoRII to its DNA recognition sequences, again in a site-selective manner. However, cleavage of these lesions is completely blocked. Sequences with the lesions at the 5′-guanine are effectors of cleavage in contrast to those at the 3′-guanine.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. AdoMet and AdoHcy were purchased from Sigma (St. Louis, MO). [CH_3-^{3}H]AdoMet (77 Ci/mmol, 13 μM) was from Amersham Biosciences (Little Chalfont, U.K.). [γ-^{32}P]ATP (1000 Ci/mmol) was bought from Izotop (Obninsk, Russia). DNA methyltransferase EcoRII (52 μM) and the dimer of the restriction endo-

Table 2: Properties of the B[*a*]PDE-Modified Oligodeoxynucleotide Duplexes as Substrates of M.EcoRII

Designation	Oligodeoxynucleotide duplex	K_d, nM [a]		V_o, nM/min
		EMSA	Fluorescence polarization	
U	5'-GAGCCAA**CCTGG**CTCTGA 3'-CTCGGTT**GGACC**GAGACT	2.5± 0.5	1.9± 0.1 [b]	883±53
$(-)G_1^*$	5'-GAGCCAA**CCT[(-)G*]G**CTCTGA 3'-CTCGGTT**GGA**—**C**—**C**GAGACT	20± 4	21.8± 0.8	<1
$(+)G_1^*$	5'-GAGCCAA**CCT[(+)G*]G**CTCTGA 3'-CTCGGTT**GGA**-—**C**—**C**GAGACT	12± 2	15.2± 0.7	<1
$(-)G_2^*$	5'-GAGCCAA**CCTG[(-)G*]**CTCTGA 3'-CTCGGTT**GGAC**—**C**—GAGACT	63± 5	57.2± 2.2	10±3
$(+)G_2^*$	5'-GAGCCAA**CCTG[(+)G*]**CTCTGA 3'-CTCGGTT**GGAC**—**C**—GAGACT	10± 2	8.5± 1.2	19±1

[a] Determined for ternary complexes M.EcoRII/AdoHcy/DNA. [b] Corresponds to the duplex FAM-U.

nuclease EcoRII (2.4 μM) were overexpressed as N-terminally His_6-tagged protein and purified by chromatography on a nickel chelate column as described (*36*). T4 polynucleotide kinase was obtained from MBI Fermentas (Vilnius, Lithuania). Buffers A−G were prepared using Milli-Q water: buffer A, 10 mM Tris−HCl (pH 7.6) and 50 mM NaCl; buffer B, buffer A containing 5 mM $CaCl_2$; buffer C, buffer A containing 5 mM DTT and 1 mM EDTA; buffer D, buffer A containing 5 mM $MgCl_2$; buffer E, buffer A containing 5 mM $MgCl_2$ and 7 mM DTT; buffer F, 50 mM Tris−H_3BO_3, pH 8.3, and 2 mM EDTA; buffer G, buffer C containing 0.1 mg/mL acetylated bovine serum albumin.

Oligodeoxynucleotides. The oligodeoxynucleotide sequences employed are shown in Table 1. The unmodified oligodeoxynucleotides 5′-GAGCCAACCTGGCTCTGA, the complementary strand 5′-TCAGAGCCAGGTTGGCTC, and the fluorescein-labeled sequence 5′-FAM-TCAGAGCCAGGTTGGCTC-3′ were obtained from Syntol (Moscow, Russia). The fluorescein label was introduced at the 5′-end of the oligodeoxynucleotide by means of an aminoalkyl linker containing six methylene groups. The oligodeoxynucleotides were purified by electrophoresis on denaturing 20% polyacrylamide gel (PAGE) and desalted by passing the solutions through C18 Sep-Pak cartridges (Waters). The sequences were hot-labeled via the standard ^{32}P-5′-phosphorylation of oligodeoxynucleotides using T4 polynucleotide kinase and [γ-^{32}P]ATP.

B[a]PDE-Modified Oligodeoxynucleotides. The site-specifically modified oligodeoxynucleotides 5′-GAGCCAACCT[(−)G*]GCTCTGA, 5′-GAGCCAACCT[(+)G*]GCTCTGA, 5′-GAGCCAACCTG[(−)G*]CTCTGA, and 5′-GAGCCAACCTG[(+)G*]CTCTGA contained single (−)- and (+)-*trans*-*anti*-B[*a*]P−N^2-dG lesions (G*, Figure 1 and Table 1) at either the 5′-side or the 3′-side 2′-deoxyguanosine at the G_1G_2 dinucleotide step in the 5′-GAGCCAACCTG_1G_2-CTCTGA sequence. They were synthesized by automated DNA synthesis methods utilizing the appropriate 5′-*O*-DMTr-3′-*O*-phosphoramidites derived from racemic mixtures of the 7*R*,8*S*,9*S*,10*R* and 7*S*,8*R*,9*R*,10*S* enantiomers of B[*a*]PDE (*37*). Oligonucleotides 5′-GAGCCAACCT[(−)G*]GCTCTGA and 5′-GAGCCAACCT[(+)G*]GCTCTGA complexed with their complementary strands are denoted as the $(-)G_1^*$ and $(+)G_1^*$ duplexes in Table 2; the analogous duplexes derived from 5′-GAGCCAACCTG[(−)G*]CTCTGA and 5′-GAGCCAACCTG[(+)G*]CTCTGA are denoted as $(-)G_2^*$ and $(+)G_2^*$, respectively (Table 2).

The pairs of diastereomeric oligodeoxynucleotides 5′-GAGCCAACCT[(−)G*]GCTCTGA and 5′-GAGCCAACCT-[(+)G*]GCTCTGA were separated by reversed-phase HPLC at 60 °C utilizing an X Terra C18 column (Waters). The mixture of 5′-GAGCCAACCTG[(−)G*]CTCTGA and 5′-GAGCCAACCTG[(+)G*]CTCTGA oligodeoxynucleotides was poorly resolved (∼70% purity) after collecting the leading edge of the early-eluting peak and the trailing edge of the late-eluting peak. These samples (5.5 nmol) were annealed with their complementary strand 5′-TCAGAGCCAGGTTGGCTC (6 nmol) in 55 μL of buffer B by heating to 70 °C for about 5 min, followed by slow cooling to 5 °C to obtain the oligodeoxynucleotide duplexes $(-)G_2^*$ and $(+)G_2^*$, respectively. These double-stranded DNA samples were subjected to further purification using nondenaturing 8% PAGE. The oligodeoxynucleotide duplexes were prepared in buffer D, and the buffer used for the electrophoresis was 0.05 M Tris−H_3BO_3, pH 8.3, and 5 mM $MgCl_2$. After separation of the stereoisomeric B[*a*]PDE-modified oligodeoxynucleotides by HPLC in the case of the 5′-GAGCCAACCT[(−)G*]GCTCTGA and 5′-GAGCCAACCT[(+)G*]GCTCTGA sequences, or by nondenaturing 8% PAGE in the case of the $(-)G_2^*$ and $(+)G_2^*$ duplexes, all samples were subjected to further purification using denaturing 20% PAGE, followed by desalting on Sep-Pak C18 cartridges (Waters) to obtain individual stereoisomeric, modified oligodeoxynucleotides. The compositions of the modified sequences were verified by mass spectrometry using a Bruker Daltonics OmniFlex MALDI TOF MS instrument.

Oligodeoxynucleotide concentrations were estimated spectrophotometrically. The extinction coefficients ϵ_{260} of unmodified oligodeoxynucleotides were calculated as described by Cantor et al. (*38*). For modified oligodeoxynucleotides the ϵ_{260} values were calculated as the sum of ϵ_{260} of the unmodified strand and ϵ_{260} for the pyrenyl residue (14500 M^{-1} cm^{-1}) or FAM (21000 M^{-1} cm^{-1}).

Fluorescence Polarization Measurements. Fluorescence polarization measurements involving the labeled oligodeoxynucleotide duplex FAM-U (5′-FAM-TCAGAGCCAGGTTGGCTC/5′-GAGCCAACCTGGCTCTGA) were made

at 25 °C by means of a Beacon 2000 fluorescence polarization system (PanVera) with excitation at 488 nm and emission at 535 nm, using 10 × 75 mm borosilicate sample glass test tubes. The polarization (P) was defined in terms of the vertical (I_v) and horizontal (I_h) emission components and the expression:

$$P = (I_v - GI_h)/(I_v + GI_h)$$

where $G = I_v/I_h$ is an instrumental correction factor. The reaction mixtures were vortexed and allowed to equilibrate, and the polarization values were determined from at least five independent measurements.

Determination of the Amount of the Active Form of M.EcoRII by Binding-Site Titration. The FAM-U duplex (50 nM) and AdoHcy (1 mM) were preincubated in 0.5 mL of buffer G, and the fluorescence polarization value of the oligodeoxynucleotide duplex prior to the addition of M.EcoRII (P_0) was measured first. M.EcoRII was added in 100 nM aliquots to a final concentration of 900 nM, and the fluorescence polarization was then measured.

Determination of K_D by an EMSA. ^{32}P-Labeled oligodeoxynucleotide duplexes U (5′-GAGCCAACCTGGCTCTGA/5′-TCAGAGCCAGGTTGGCTC), (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* (10 nM) were incubated in the presence of 1 mM AdoHcy and varying M.EcoRII concentrations (5−100 nM) in 20 μL of buffer G containing 6% glycerol at room temperature for 5 min and at 0 °C for 5 min. The reaction mixtures were analyzed by nondenaturing 8% PAGE in 0.5× buffer F as described by Carey (*39*). Here, and in all other gel electrophoresis experiments, the gels were analyzed by autoradiography utilizing a Molecular Dynamics phosphorimager (Amersham Biosciences, Little Chalfont, U.K.) with ImageQuant 5.0 software. The radioactivity of free (c_{free}), total (c_{total}), and bound ($c_{bound} = c_{total} - c_{free}$) DNA was determined. The ratio (c_{bound})/(c_{total}) was calculated and plotted vs the protein concentration (E_0). The K_d values were calculated by fitting the data to the following equation, which is based on a standard bimolecular binding equilibrium (*40*):

$$\frac{c_{bound}}{c_{total}} = \frac{[ES]}{[S_0]} = \frac{1}{2[S_0]}([S_0] + [E_0] + K_d - \sqrt{([S_0] + [E_0] + K_d)^2 - 4[E_0][S_0]}) \quad (1)$$

where $[S_0]$ and $[E_0]$ are the DNA and M.EcoRII concentrations, respectively. The K_d values were generated from at least five independent experiments.

Determination of K_d by Fluorescence Polarization Measurements. The M.EcoRII/AdoHcy/FAM-U complex was preformed by incubating FAM-U (50 nM), M.EcoRII (30 nM), and AdoHcy (1 mM) in 0.5 mL of buffer G. The fluorescence polarization value was then measured. Aliquots (1−2 μL) of the competitor B[*a*]PDE-modified oligodeoxynucleotide duplexes (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* were added, and the fluorescence polarization was measured again. The concentration of B[*a*]PDE-modified oligodeoxynucleotide duplexes in competition experiments varied from 35 to 850 nM. The polarization values were determined at different competitor concentrations and fitted to the equation (*41*):

Table 3: Properties of the B[*a*]PDE-Modified Hemimethylated Oligodeoxynucleotide Duplexes as Substrates of M.EcoRII

Designation	Oligodeoxynucleotide duplex	V_o, nM/min
UM	5′-GAGCCAA**CCTGG**CTCTGA 3′-CTCGGTT**GGAMC**GAGACT	707±42
(−)G_1^*M	5′-GAGCCAA**CCT[(-)G*]G**CTCTGA 3′-CTCGGTT**GGA—M—C**GAGACT	2±1
(+)G_1^*M	5′-GAGCCAA**CCT[(+)G*]G**CTCTGA 3′-CTCGGTT**GGA—M——C**GAGACT	2±1
(−)G_2^*M	5′-GAGCCAA**CCTG[(-)G*]**CTCTGA 3′-CTCGGTT**GGAM—C——**GAGACT	30±4
(+)G_2^*M	5′-GAGCCAA**CCTG[(+)G*]**CTCTGA 3′-CTCGGTT**GGAM—C——**GAGACT	164±17

$$P = P_0 + \frac{P_{max} - P_0}{2[F]_t}\left\{K_1 + \frac{K_1}{K_2}[C]_t + [E]_t + [F]_t - \sqrt{\left(K_1 + \frac{K_1}{K_2}[C]_t + [E]_t + [F]_t\right)^2 - 4[F]_t[E]_t}\right\} \quad (2)$$

where $[E]_t$, $[F]_t$, and $[C]_t$ are the total concentrations of the enzyme, the fluorescence-emitting duplex FAM-U, and the nonfluorescent (in the ∼500 nm region) B[*a*]PDE-modified competitor oligodeoxynucleotide duplex, respectively, K_1 is the dissociation constant for the M.EcoRII/AdoHcy/FAM-U complex, K_2 is the dissociation constant of the complex M.EcoRII/AdoHcy/B[*a*]P−DNA, and P_0 and P_{max} are polarization values of the free and fully bound FAM-U oligodeoxynucleotide duplex.

The data points were fitted to binding isotherms using nonlinear regression by restricting parameters K_1 and K_2 within the intervals 1−7 and 1−100 nM, respectively. Both K_1 and K_2 were allowed to vary. The K_1 and K_2 values were generated from at least two independent experiments.

Methylation Assay. The efficiency of methylation was monitored by the radioactivity of tritium (CH_3-^{3}H) incorporated into the oligodeoxynucleotide duplexes (*42*). The reactions were carried out in 45 μL of buffer C, containing one of the oligodeoxynucleotide duplexes listed in Tables 2 and 3 (1 μM), M.EcoRII (30 nM), and [CH_3-^{3}H]AdoMet (1.3 μM). Reactions were started by adding the enzyme. After a 0.5, 1, 1.5, 2, 2.5, 3, 3.5, or 4 min incubation time at 37 °C, 5 μL of the reaction mixtures was pipetted onto DE81 (Whatman) paper disks and treated as described (*40*). The amounts of methylated DNA were computed as described (*42*). The relative methylation rates (V_0^{rel}) were calculated as the ratio of V_0 of the B[*a*]PDE-modified unmethylated (or hemimethylated) oligodeoxynucleotide duplexes to V_0 of the unmodified duplex U (or to V_0 of oligodeoxynucleotide duplex UM).

Binding of R.EcoRII to B[a]PDE-Modified Oligodeoxynucleotide Duplexes. P^{32}-Labeled oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* (0.1−1 μM) were incubated with endonuclease EcoRII (0.4−0.5 μM) in buffer B containing 7 mM DTT and 10% glycerol at 37 °C for 5 min and at 0 °C for 15 min. The reaction mixtures were then loaded on a nondenaturing 6% polyacrylamide gel in 0.5× buffer F.

DNA Cleavage by R.EcoRII. Cleavage of the ^{32}P-labeled oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*,

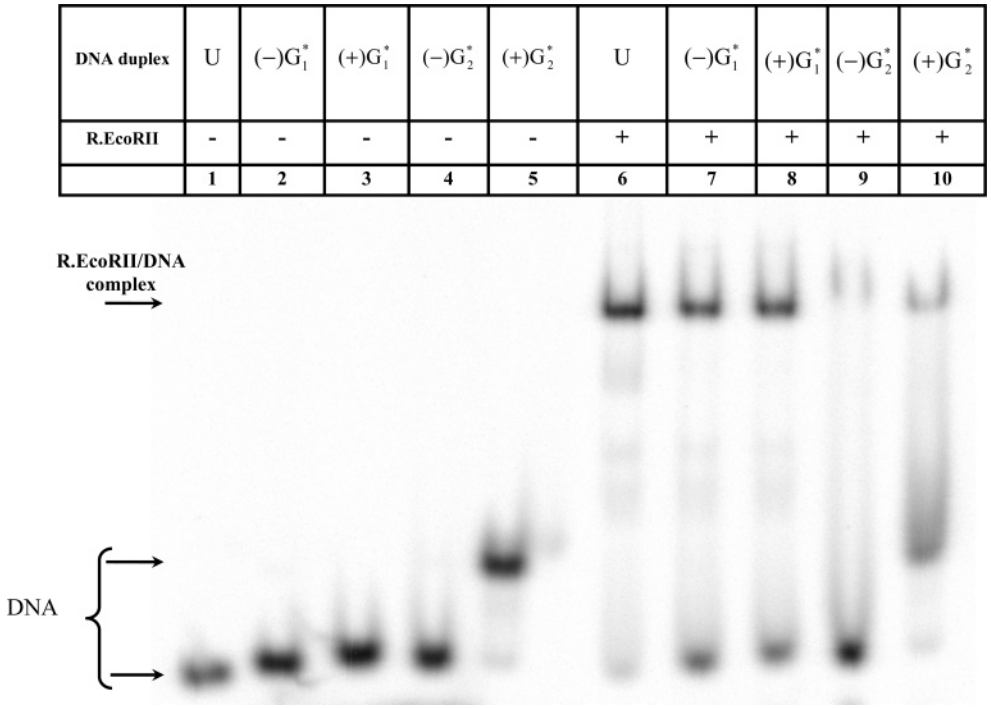

FIGURE 2: Binding of R.EcoRII to the B[*a*]PDE-modified oligodeoxynucleotide duplexes. The duplexes (0.25 μM) were incubated with R.EcoRII (0.2 μM) in buffer B and 7 mM DTT and loaded on a nondenaturing 6% polyacrylamide gel. The ^{32}P-label is in the upper strand of oligodeoxynucleotide duplexes (Table 2). Lanes: 1−5, oligodeoxynucleotide duplexes only; 6−10, duplexes with R.EcoRII enzyme.

and (−)G_2^* (0.35 μM) by R.EcoRII (4−1100 nM) was performed in buffer E at 37 °C for 30 min.

Cleavage of the ^{32}P-labeled oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* (0.35 μM) by R.EcoRII (2 nM) in the presence of the unmodified oligodeoxynucleotide duplex 5′-ACCTACCTGGTGGT/3′-TGGATGGACCACCA (3.5 μM) was accomplished in buffer E at 37 °C for 30 min.

Enzymatic reactions were stopped by addition of EDTA (10 mL), and the cleavage products were analyzed by denaturing 20% PAGE with 7 M urea.

Cleavage of phage T7 DNA (12 nM) by R.EcoRII (400 nM) in the presence of oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* (5 μM) was carried out in buffer E at 37 °C for 60 min. The reactions were stopped by heating at 65 °C for 10 min. The hydrolysis products were analyzed by 0.5% agarose gel stained with ethidium bromide.

RESULTS

We have concentrated on the bay region (+)- and (−)-*trans*-*anti*-B[*a*]P−N^2-dG adducts, introduced as single substituents into the EcoRII recognition sequence at 5′-side deoxyguanosine (G_1^* duplexes) or 3′-side deoxyguanosine (G_2^* duplexes) (Table 2). The length of the oligodeoxynucleotides was chosen as 18 base pairs, consistent with the possible DNA region which may be covered by C5-MTases (*43*, *44*) and R.EcoRII (*45*, *46*).

The upper sequences of the oligodeoxynucleotide duplexes (+)G_1^* and (−)G_1^* were easily separable by reversed-phase HPLC techniques. Peculiarities of purification of the B[*a*]PDE-modified oligodeoxynucleotides that are upper strands of duplexes (+)G_2^* and (−)G_2^* are discussed in the Supporting Information section. B[*a*]PDE-modified oligodeoxynucleotides were analyzed by denaturing 20% PAGE with 7 M urea (Supporting Information section, Figure 1S).

The melting curves for B[*a*]PDE-modified oligodeoxynucleotide duplexes (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* were cooperative in all cases with the T_m values ranging from 65 to 68 °C, being only 2−5 deg lower than that of the unmodified duplex U. Further details are provided in the Supporting Information section, Table 1S.

All B[*a*]PDE-modified oligodeoxynucleotide duplexes (Figure 2, lanes 2−5) migrate more slowly in nondenaturing 6% polyacrylamide gel than the unmodified oligodeoxynucleotide duplex U (lane 1), as noted previously (*47*). The mobilities of the oligodeoxynucleotide duplexes (−)G_1^* and (−)G_2^* are approximately equal, whereas the mobility of the (+)G_1^* duplex is somewhat lower. The mobility of the oligodeoxynucleotide duplex (+)G_2^* is strikingly lower than that of the (−)G_2^* and the related B[*a*]PDE-modified duplexes. This observation is in excellent agreement with previous reports showing that guanines flanking the (+)G* lesions on the 5′-side modified duplexes lead to anomalously slow electrophoretic mobilities as a consequence of DNA bending (*34*, *48*).

Determination of the Amount of the Active Form of M.EcoRII by Binding-Site Titration. We first determined how the presence of the bulky carcinogen substituents influences the site-specific recognition of the DNA by M.EcoRII and the methylation reaction catalyzed by this enzyme.

The concentration of the active form of MTases does not accurately correspond to the total protein concentration (*49*, *50*). The concentration of enzyme capable of binding the unmodified oligodeoxynucleotide duplex U in the presence of 1 mM AdoHcy was determined by two independent methods, EMSA and fluorescence polarization titration. In the first case, M.EcoRII was titrated with differing amounts

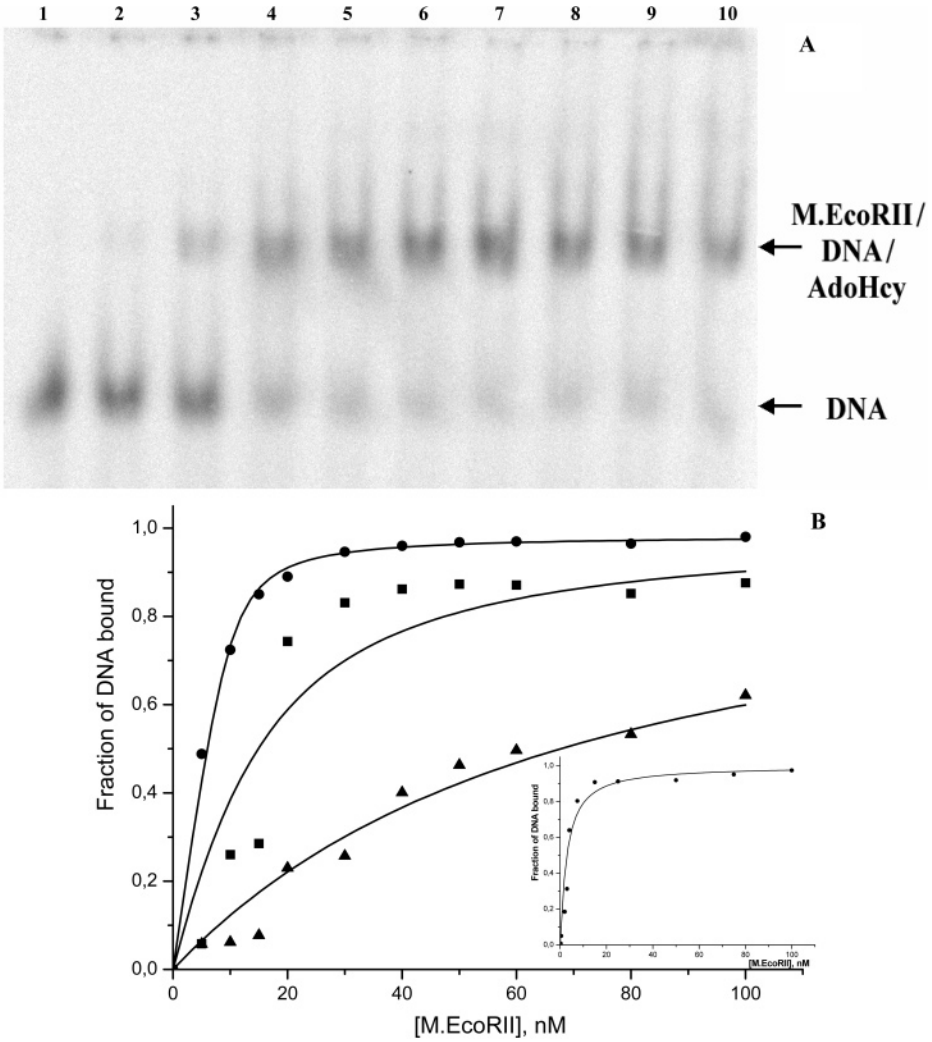

FIGURE 3: (A) Autoradiograph of a gel electrophoresis mobility shift assay of the complex formation of M.EcoRII with duplex (+)G_2^* and AdoHcy in buffer G containing 6% glycerol. Lanes: 1, duplex (+)G_2^* only; 2−10, duplex (+)G_2^* and increasing concentrations of M.EcoRII (10, 15, 20, 30, 40, 50, 60, 80, and 100 nM, from left to right). [AdoHcy] = 1 mM. (B) Binding isotherms for M.EcoRII complexes with AdoHcy and duplexes U, (−)G_2^*, and (+)G_2^* determined by EMSA. Oligodeoxynucleotide duplexes: unmodified U (●), (−)G_2^* (▲), and (+)G_2^* (■) (10 nM) were titrated with increasing amounts of M.EcoRII (5−100 nM range) in buffer G containing 6% glycerol. Solid lines were calculated according to eq 1, yielding the indicated values of K_d. Inset: oligodeoxynucleotide−duplex U (●) (1 nM) titrated with M.EcoRII (5−100 nM) in buffer G containing 6% glycerol.

of the ^{32}P-labeled duplex U followed by nondenaturing PAGE analysis. The data were analyzed according to the Scatchard equation as described in the literature (*40*, *49*). The amount of enzyme bound to DNA was determined to be only 11 ± 1% of the total enzyme molecules (data not shown). Alternatively, the fluorescein-labeled oligodeoxynucleotide duplex FAM-U was incubated with differing amounts of M.EcoRII, and the fluorescence polarization, *P*, was measured in each case. Then the value of *P* was plotted vs the total protein concentration (Supporting Information, Figure 2S). It was found that the enzyme has a binding efficiency of 11%, which is in excellent agreement with the estimates obtained from EMSA experiments. Thus, in all subsequent experiments, the concentration of active M.EcoRII was calculated as 11% of the total protein concentration.

Binding of M.EcoRII to B[a]PDE-Modified Oligodeoxynucleotide Duplexes. The determination of the dissociation constants (K_d) for the ternary complex M.EcoRII/AdoHcy/B[*a*]P−DNA was performed as outlined below.

The binding of ^{32}P-labeled B[*a*]P−DNA adducts (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* to M.EcoRII in ternary complexes with AdoHcy was analyzed by electrophoretic mobility shift assays. A representative autoradiograph of such EMSA experiments is depicted in Figure 3A for the (+)G_2^* duplex. In the case of this and all other duplexes studied by the same method, only one type of complex with M.EcoRII was evident, and this corresponds to one molecule of M.EcoRII subunit bound to one DNA molecule. Examples of some of the typical binding isotherms determined from EMSA data are shown in Figure 3B. A 10 nM concentration of oligodeoxynucleotide duplexes was employed in these experiments. The values of K_d estimated from the best fits of a simple 1:1 binding isotherm model (solid lines, Figure 3B) are summarized in Table 2. In the case of the unmodified duplex U (Table 2, inset in Figure 3B), the results are shown for a concentration of 1 nM DNA (this concentration was used to define more exactly the K_d value). It is evident that M.EcoRII forms tight complexes with the unmodified and

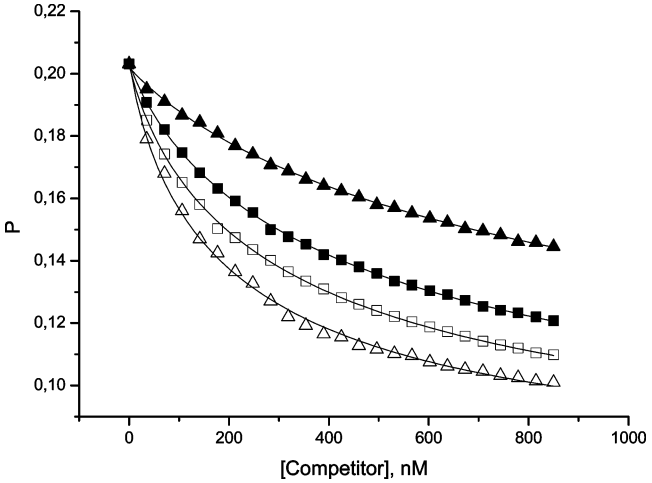

FIGURE 4: Competition titration of M.EcoRII/AdoHcy/FAM-U complexes by the modified duplexes (−)G_1^*, (+)G_1^*, (−)G_2^*, and (+)G_2^*. In each case, the fluorescein-labeled sequence is gradually displaced from the enzyme by the indicated carcinogen-modified duplexes that do not contain a fluorescein label, thus diminishing P. Concentrations: [FAM-U] = 50 nM; [M.EcoRII] = 30 nM; [AdoHcy] = 1 mM, in buffer G. Test competitors: (−)G_1^* (■), (+)G_1^* (□), (−)G_2^* (▲), and (+)G_2^* (△).

B[*a*]PDE-modified oligodeoxynucleotide duplexes (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^*. However, the bulky B[*a*]P residues clearly diminish the efficiencies of binding of M.EcoRII to the modified oligodeoxynucleotide duplexes. The K_d values for the ternary complexes M.EcoRII/AdoHcy/B[*a*]P−DNA are increased by factors of 4−25 in comparison with the unmodified duplex U. The largest K_d value and thus the weakest binding were observed in the case of the (−)G_2^* duplex (Table 2).

As shown in Figure 3B, the scatter in the data, particularly at the lower protein concentrations, can be significant. We therefore utilized a second, independent method for the determination of K_d values for the M.EcoRII/AdoHcy/DNA complexes using a competition titration−fluorescence polarization technique. This is a true equilibrium method since the experiments are carried out in aqueous solutions and are not influenced by potential gel cage effects, irreversible protein−DNA dissociation, or effects associated with varying enzyme concentrations within the gel (*41*). The fluorescence of the fluorescein-tagged duplex FAM-U was easily measurable, and the reproducibility was excellent (within a few percent). The experimental error for fluorescence polarization experiments is thus significantly lower than that in the EMSA experiments. At the excitation wavelength of 488 nm and emission measured at 535 nm, the weak emission of the covalently bound B[*a*]P residues was negligible (*51*). The 18-bp FAM-U duplex showed only a small decrease in the fluorescence yield upon binding to M.EcoRII. This observation suggests that the effect of the MTase on the fluorescence of the fluorescein fluorophore is small, and its presence at the 5′-terminus does not significantly interfere with the binding of the 18-mer substrates (*41*). This small change in the fluorescence yields was therefore neglected in the calculations of the K_1 and K_2 values. We observed a decrease of the degree of fluorescence polarization, *P*, as the unlabeled duplexes (+)G_1^*, (−)G_1^*, (+)G_2^* or (−)G_2^* were added to a preformed M.EcoRII/AdoHcy/FAM-U complex (Figure 4).

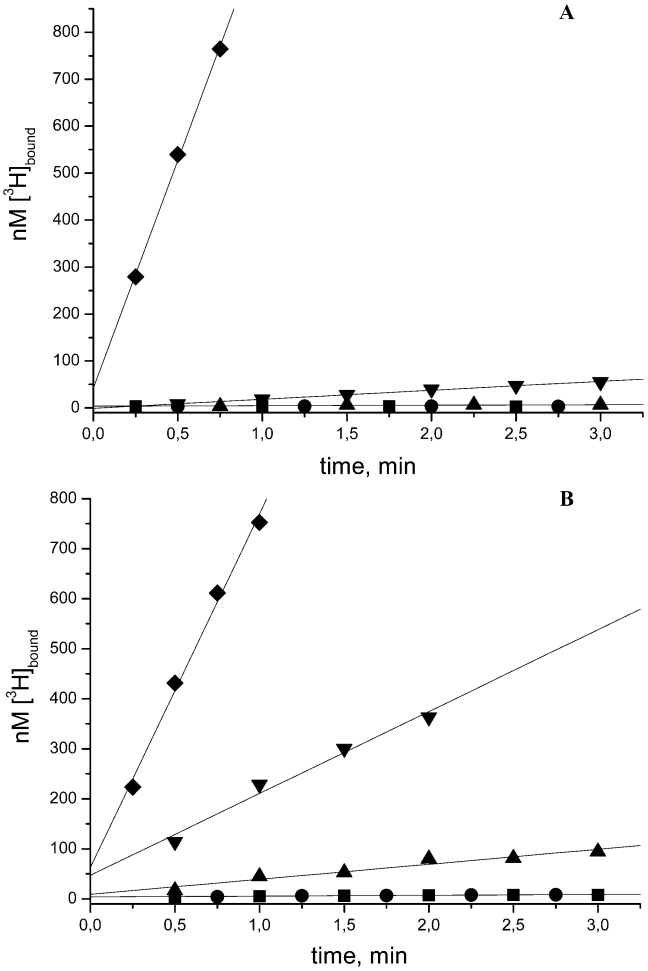

FIGURE 5: Steady-state kinetics of methylation of unmodified and B[*a*]PDE-modified oligodeoxynucleotide duplexes catalyzed by M.EcoRII. Panel A: U (◆), (−)G_1^* (■), (+)G_1^* (●), (−)G_2^* (▲), and (+)G_2^* (▼). Panel B: UM (◆), (−)G_1^*M (■), (+)G_1^*M (●), (−)G_2^*M (▲), and (+)G_2^*M (▼). The reaction mixtures contained DNA (1 μM), [CH_3-^{3}H]AdoMet (1.3 μM), and M.EcoRII (30 nM) in buffer C.

The decrease in *P* occurs because the rotational correlation time of the fluorophore is significantly smaller in the aqueous solution than in the environment of the enzyme complex, and thus *P* decreases as the FAM-U duplexes are displaced by the B[*a*]PDE-modified duplexes. The data in Figure 4 were each fitted to eq 2 using values of K_d that provided the best fits by nonlinear regression analysis. The K_d values thus obtained for the M.EcoRII/AdoHcy/FAM-U and the M.EcoRII/AdoHcy/B[*a*]P−DNA complexes are summarized in Table 2. The unmodified duplex FAM-U is characterized by the lowest K_d value and the strongest binding. The *trans-anti*-B[*a*]P−N^2-dG lesions clearly reduce the binding of M.EcoRII to the B[*a*]P−oligodeoxynucleotide duplexes by a factor of 5−30 in comparison with the unmodified duplex FAM-U (Table 2). These results are in good agreement with those obtained by the EMSA experiments.

Determination of the Susceptibilities to Methylation of Stereoisomeric B[a]PDE-Modified Oligodeoxynucleotide Duplexes. The initial reaction rates were determined for the duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* under steady-state conditions (Figure 5, Table 2). The amount of product formed depends linearly on time. The lesions cause either a significant decrease in the rates of reaction [duplexes

(+)G_2^* and (−)G_2^*] or block methylation altogether [duplexes (+)G_1^* and (−)G_1^*].

The extent of the decrease in the DNA methylation rates caused by *trans-anti*-B[*a*]P−N^2-dG lesions is much higher than the effect of the B[*a*]P residues on the binding affinities (Table 2). For example, for duplexes (+)G_1^* and (−)G_1^*, the binding affinities decreased by about a factor of 10, but no detectable methylation was observed. On the other hand, in the case of the (+)G_2^* and (−)G_2^* duplexes, analogous decreases in binding affinities were observed, but measurable rates of methylation also were observed, although smaller by factors of between 40 and 80, than in the case of the unmodified duplex U (Table 2). It is thus evident that rates of methylation are affected to a greater extent than the binding affinities.

The initial rates of methylation for hemimethylated oligodeoxynucleotide duplexes UM, (+)G_1^*M, (−)G_1^*M, (+)G_2^*M, and (−)G_2^*M (Table 3) were also determined. In these duplexes, the lower unmodified strand complementary to the modified strand has an m^5dC residue ("M" in Table 3) instead of the target dC. Hemimethylated DNA is a naturally occurring substrate for MTases. The lesions in the (+)G_1^*M, (−)G_1^*M, and (−)G_2^*M cause significant decreases in the rates of methylation in comparison to the unmodified UM duplex. It is noteworthy that, in contrast to the unmethylated oligodeoxynucleotide duplexes (+)G_1^* and (−)G_1^*, finite rates of methylation are observed in the oligodeoxynucleotide duplexes (+)G_1^*M and(−)G_1^*M (Tables 2 and 3). A greater rate of methylation was also observed for the hemimethylated substrates (+)G_2^*M and (−)G_2^*M in comparison to the unmethylated duplexes (+)G_2^* and (−)G_2^*.

Interaction of B[a]PDE-Modified Oligodeoxynucleotide Duplexes with EcoRII Restriction Endonuclease. The impact on the interactions of R.EcoRII with the 18-mer oligodeoxynucleotide duplexes G_1^* and G_2^* was examined. The binding of the oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)G_2^* to the enzyme was studied by EMSA in the presence of Ca^{2+}. Under these conditions, and at a DNA:R.EcoRII$_{monomer}$ molar ratio ~1:1, R.EcoRII forms a single prereactive complex with unmodified DNA substrates (*52*) as shown in Figure 2, lane 6. Both the (−)-*trans*- and (+)-*trans* adducts at the 5′-guanine in the GG dinucleotide in the recognition sequence [(−)G_1^* and (+)G_1^*] diminish but do not abolish the binding of R.EcoRII (lanes 7 and 8, Figure 2). However, the binding of this enzyme to the duplexes (−)G_2^* and (+)G_2^* with the lesion at the 3′-side guanine is almost completely abolished (lanes 9 and 10, Figure 2).

To cleave the DNA, R.EcoRII requires two copies of recognition sequences (*26*, *29*). To recall, one of the two recognition sequences (allosteric effector) binds in the effector domain of R.EcoRII. This promotes the binding of a second copy of the recognition sequence (substrate) within the catalytic R.EcoRII domain where cleavage occurs. In this connection, it is important to characterize the contribution of each of the two R.EcoRII domains to the interactions with the substrate/effector minor groove. The unmodified oligodeoxynucleotide duplex U is efficiently cleaved by R.EcoRII either in the lower strand (Figure 6A, lane 2) or in the upper strand (Figure 6B, lane 2). The short oligodeoxynucleotide duplexes containing the EcoRII recognition sequence are cleaved by the R.EcoRII because they are both allosteric effectors and substrates. Cleavage of all B[*a*]P-modified DNA duplexes was completely blocked for both unmodified (Figure 6A, lanes 5, 8, 11, and 14) and modified (Figure 6B, lanes 5, 8, 11, and 14) DNA strands. We wondered if cleavage of B[*a*]PDE-modified DNA would occur if we add the unlabeled short unmodified substrate (5′-ACCTACCTGGTGGT/3′-TGGATGGACCACCA) which one may consider an effector. However, in the presence of this duplex no detectable cleavage was observed in the case of any of the four B[*a*]P−N^2-dG adduct-containing duplexes G_1^* and G_2^* in the unmodified lower strands 5′-TCAGAGCCAGGTTGGCTC (lanes 6, 9, 12 and 15, Figure 6A). The cleavage in the modified upper strand 5′-GAGCCAACCTGGCTCTGA was completely blocked in both of the stereoisomeric G_2^* duplexes (lanes 12 and 15, Figure 6B), although a little cleavage in the modified strand was observed in the case of both stereoisomeric G_1^* duplexes in the presence of effector molecules (lanes 6 and 9, Figure 6B). In a control experiment in the presence of the same quantity of the effector, unmodified U was efficiently cleaved by R.EcoRII (Figure 6, lane 3).

We also investigated the cleavage of phage T7 DNA by R.EcoRII in the presence of the B[*a*]P−N^2-dG-containing oligodeoxynucleotide duplexes as effectors. Phage T7 DNA is resistant to R.EcoRII cleavage since its recognition sequences are separated by >1000 base pairs (*26*). However, cleavage of such resistant DNA is observed in the presence of our short unmodified 18-mer oligodeoxynucleotide substrate as cleavage effector as shown by agarose gel electrophoresis (Figure 7, lane 2) and (*53*). Only the oligodeoxynucleotide duplexes (+)G_1^* and (−)G_1^* preserve the ability to activate cleavage of phage T7 DNA. In summary, the B[*a*]PDE-modified oligodeoxynucleotide duplexes (+)G_1^* and (−)G_1^*, in contrast to (+)G_2^* and (−)G_2^*, bind to R.EcoRII and can activate the cleavage of EcoRII-resistant high molecular weight DNA. However, not all B[*a*]PDE−DNA adducts can be hydrolyzed by R.EcoRII either in the absence or in the presence of effector DNA.

DISCUSSION

Effects of B[a]P−N^2-dG Adducts on R.EcoRII Activity. At the present time, there is no information on the interaction of the EcoRII endonuclease with the DNA minor groove. In the absence of protein, both *trans-anti*-B[*a*]P−N^2-dG adducts are positioned in the DNA minor groove (*33*, *54*). Thus, the dramatic reductions in complex formation in the case of the two G_2^* duplexes (Figure 2, lanes 7−10) suggest that there are important contacts between R.EcoRII and the DNA minor groove. The catalytic activity of R.EcoRII was completely blocked regardless of the location of the bulky B[*a*]P lesions in the recognition sequence (in this case, B[*a*]P-modified DNA duplexes are both effectors of the cleavage and substrates). The behavior of this enzyme with respect to the bulky B[*a*]P lesions parallels that of other DNA hydrolyzing enzymes, e.g., the inhibition of the catalytic activities of viral HIV-1 integrase (*55*), topoisomerase type IB (*22*), and exonuclease III (*22*), providing that the B[*a*]P residue is positioned close to the site of cleavage.

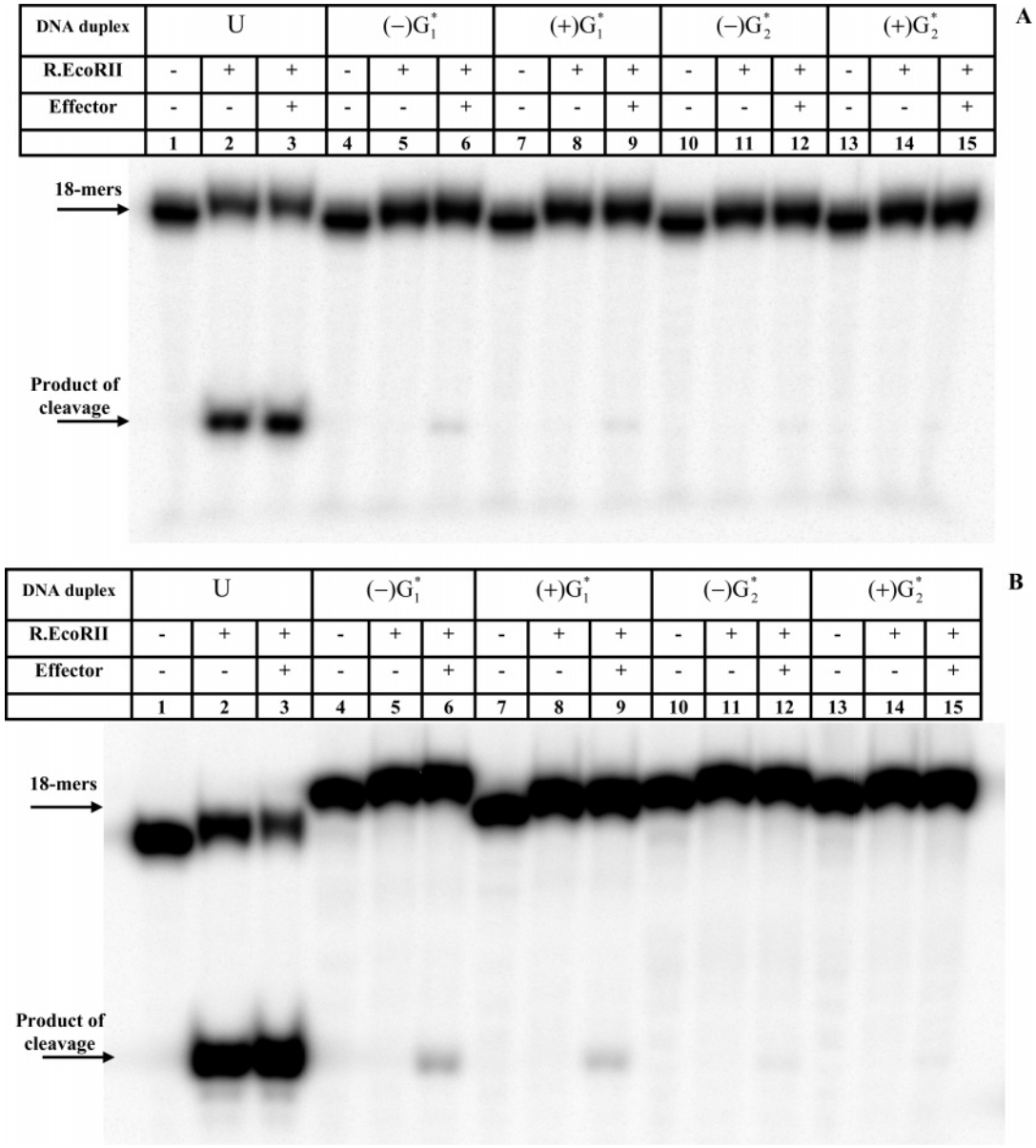

FIGURE 6: Cleavage of the (A) ^{32}P-labeled lower unmodified strand 5′-TCAGAGCCAGGTTGGCTC and (B) ^{32}P-labeled B[*a*]P-modified oligodeoxynucleotide strands in duplexes (−)G_1^*, (+)G_1^*, (−)G_2^*, and (+)G_2^* (0.35 μM) by R.EcoRII (2 nM) in the absence and in the presence of the unmodified oligodeoxynucleotide duplex 5′-ACCTA**CCTGG**TGGT/3′-TGGAT**GGACC**ACCA, used as the effector of cleavage (3.5 μM) at 37 °C, 30 min reaction time in buffer E. Conditions: denaturing 20% PAGE with 7 M urea in buffer F.

The B[*a*]P-modified adducts are not cleaved when a 14-mer unmodified substrate is taken as the effector (Figure 6, lanes 6, 9, 12, and 15). In this case the specific contacts with the effector domain of R.EcoRII are normal and only contacts with the catalytic domain of R.EcoRII are disturbed. Hence, contacts between the catalytic domain of R.EcoRII and the DNA minor groove appear to be important to the catalytic cleavage activity of R.EcoRII.

R.EcoRII forms complexes with (+)G_1^* and (−)G_1^* duplexes but not with the (+)G_2^* or (−)G_2^* duplexes. The observation that the G_1^* 18-mer duplexes promote the cleavage of phage T7 DNA unlike the G_2^* duplexes (Figure 7) is consistent with the observed differences in the binding of R.EcoRII to these two sequence isomers (Figure 2). The fact that no binding of the G_2^* duplexes with R.EcoRII and no cleavage of phage T7 DNA were observed with any of the G_2^* duplexes utilized as effectors (Figure 7) suggests that the contacts with the effector domain of R.EcoRII are disturbed. These observations support the proposal that there are interactions between the effector domain of the R.EcoRII and the DNA minor groove.

Effects of B[a]P−N^2-dG Lesions on the DNA Binding and the Activity of M.EcoRII. The presence of *trans-anti*-B[*a*]P−N^2-dG adducts positioned at either deoxyguanosine within the canonical recognition sequence of the M.EcoRII has a profound impact on the rates of methyl transfer to the cytosine residues within the same sequence. The binding affinities of the enzyme are diminished by factors of 5−30 (relative to the unmodified oligodeoxynucleotide duplex), depending on the adduct stereochemistry and position. However, the methylation is practically abolished with either the (+)-*trans* or (−)-*trans* adducts being positioned at the 5′-side deoxyguanosine in the G_1^* duplexes or diminished by factors of between 50 and 100 in the case of G_2^* du-

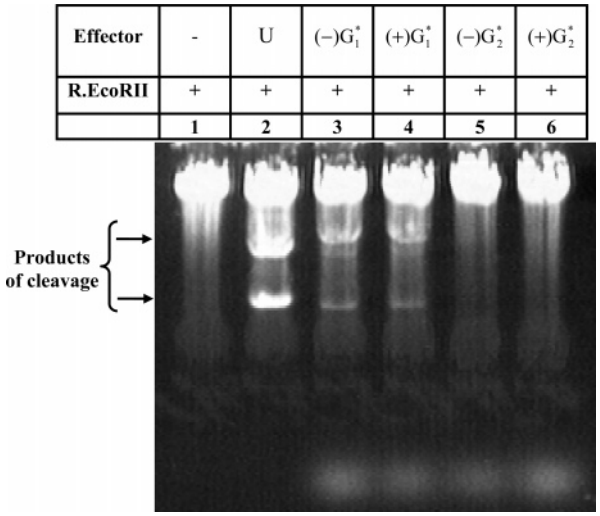

FIGURE 7: Cleavage of phage T7 DNA by EcoRII endonuclease in the presence of B[*a*]P−DNA adducts. The phage T7 DNA (12 nM) was incubated with R.EcoRII (400 nM) in the absence (line 1) or in the presence (lines 2−6) of indicated duplexes (5 μM) in buffer E at 37 °C for 60 min. Products of cleavage were monitored by 0.5% agarose gel stained with ethidium bromide.

plexes with the adduct at the 3′-side deoxyguanosine position in the 5′-...CCA/TGG... recognition sequence. Interestingly, the B[*a*]P lesions have a much greater impact on the catalytic activity of M.EcoRII than on its formation of a complex with the DNA substrates.

The variations in the methyltransferase activity are a function of adduct position, stereochemistry, and conformation. The spectroscopic characteristics (*54*) of the (+)-*trans*- and (−)-*trans*-*anti*-B[*a*]P−N^2-dG adducts in GG sequence contexts are of the minor groove type (F. Rodriguez, Y. Tang, and N. E. Geacintov, unpublished results), as they are in other sequence contexts (*33*, *54*). In these minor groove adduct conformations, the B[*a*]P residues point into either the 5′- [(+)-*trans* adduct stereochemistry] or the 3′-direction [(−)-*trans* adduct] of the modified strands. In the case of the minor groove *trans*-*anti*-B[*a*]P−N^2-dG adducts in the CG*C sequence context, the Watson−Crick base pairing is intact even at the B[*a*]PDE-modified G*•C base pairs (*54*). However, in recent studies with the same (+)-*trans* adducts positioned at either guanine in a ...GG... sequence context, the quality of the hydrogen bond at the lesion site was shown to be diminished, as evidenced by the abnormally low NMR connectivities of the imino proton to neighboring base pair protons (F. Rodriguez and N. E. Geacintov, unpublished results). Such a decrease in the quality of hydrogen bonding might enhance the flipping of the cytosine residue that is opposite the lesion, G*, out of the double helix to allow for its methylation (*30*). This mechanism has been confirmed by X-ray diffraction studies of M.HhaI (*23*), which demonstrated that, in the course of the methylation reaction, the target cytosine residue flips out of the DNA double helix and inserts itself into the active site pocket of the MTase. This model appears to be applicable to all cytosine-C5 methylating enzymes (*30*). It was shown for mismatched substrates that the affinity of DNA-M.HhaI binding correlates inversely with the stability of the target base pair (*56*). One therefore might expect that binding of DNA to M.EcoRII should increase when G* is positioned opposite the target cytosine in the M.EcoRII recognition sequence as a consequence of local destabilization of the cytosine−B[*a*]PDE-modified guanine base pair. However, our results are not in accord with such predictions since K_d values are increased in the presence of the minor groove B[*a*]P residues (Table 2). Instead, the major effect of carcinogen substitution seems to be associated with steric hindrance effects that diminish contacts that are vital to the MTase-catalyzed methylation mechanism.

The adverse effect of the minor groove B[*a*]P residues on the binding affinity and the associated K_d values in the modified G_1^* and G_2^* duplexes (Table 2) suggests that the formation of some important contacts between M.EcoRII and the duplexes are impeded by the B[*a*]P residue that is positioned in the minor groove. To achieve sequence specificity, the C5 MTases contact the DNA from the major groove side with residues located in the small domain of the MTase (*30*). This conclusion is based on the cocrystal structures of C5 MTases, HhaI and HaeIII, with DNA (*23*, *24*). Recently, it was suggested that M.EcoRII discriminates between its canonical recognition sequence (5′-...CCA/TGG...) and the site containing a GC or CG base pair in the center of the recognition sequence (CCG/CGG) by interactions in the minor groove (*25*). Our results agree with the possibility that M.EcoRII has some site-specific contacts within the DNA minor groove.

The binding affinities of other proteins to B[*a*]PDE-modified DNA sequences have been studied by other workers as well. For example, the transcription factor Sp binds more strongly to the B[*a*]PDE-modified unspecified DNA sequence (*20*), although it binds less effectively to its GC-box sequence when a (+)-*trans*-B[*a*]P−N^2-dG adduct is present (*19*). The binding of Fos-Jun proteins to BPDE-modified binding sites of the transcription factor AP-1 are diminished by ∼50% as compared to the unmodified sequence (*21*). However, the binding of the TATA box binding protein (TBP, a critical component of the transcription-initiating complex) to its recognition sequence can be enhanced dramatically in a stereochemical and site-selective manner, when different diastereomeric B[*a*]P−N^6-dA adducts are inserted into the TATA box element (*57*). Thus, although we have observed a decreased affinity of M.EcoRII in its binding to the recognition sequences containing B[*a*]P−N^2-dG adducts, the presence of such bulky B[*a*]P-derived DNA adducts does not always hinder the binding of a protein to its canonical recognition sequence.

The strong inhibition of methylation of the $(+)G_1^*$, $(-)G_1^*$, $(+)G_2^*$, and $(-)G_2^*$ duplexes by M.EcoRII suggests that the interaction of substrate with the catalytic center of M.EcoRII is strongly perturbed by the minor groove B[*a*]P residues. This conclusion is in accord with the proposed catalytic mechanism of C5 methylation that involves the interaction of the large domain of the enzyme with the minor groove of the DNA (*30*).

The methylation rates are generally greater when the 3′-side deoxyguanosine rather than the 5′-side deoxyguanosine in the recognition sequence (5′-...CCA/TGG...) is carcinogen-modified. However, the methylation efficiency seems to be relatively independent of the orientations of the bulky

residues toward the 5′- [(+)-*trans* adducts (*54*)] or the 3′-side [(−)-*trans* adduct (*58*)] of the modified guanines in the G_1^* and G_2^* duplexes (Table 2). It is noteworthy to mention that according to NMR studies cited the bulky pyrenyl residues in the (−)G_2^* duplex are positioned in the minor groove of B-form DNA and point toward its 3′-side and, thus, out of EcoRII recognition sequence. However, we also observed strong inhibition of methylation for this duplex. Taken together, this suggests that, for effective catalytic activity, M.EcoRII requires a rather extended region of the DNA within the minor groove spanning the recognition sequence.

To answer the question as to whether B[*a*]PDE-modified strands can be methylated, we investigated methylation of hemimethylated B[*a*]PDE-modified DNA duplexes (Table 3). In these oligodeoxynucleotide duplexes, the complementary strand 5′-TCAGAGCMAGGTTGGCTC contained an m^5dC (M) nucleotide instead of the target dC residue in the recognition sequence. Thus, the hemimethylated UM, (+)G_1^*M, (−)G_1^*M, (+)G_2^*M, and (−)G_2^*M duplexes (Table 3) contained only one target dC residue. We observed methylation of DNA duplexes (+)G_1^*M, (−)G_1^*M, (+)G_2^*M, and (−)G_2^*M although to a much lesser extent than in the case of the UM duplex. It is evident that a methyl group can be transferred to the DNA strand carrying a bulky B[*a*]PDE residue. The methylation is almost abolished in both the (+)- and (−)G_1^* duplexes and is reduced by factors of 50−100 in the cases of the (+)- and (−)G_2^* duplexes (Table 2). However, when hemimethylated, in the case of the G_1^*M duplexes, the observed catalytic rates are low but detectable. The rates are enhanced by factors of between 3 and 8 in the case of the (+)- and (−)G_2^*M duplexes relative to the unmethylated substrates (G_2^*) (Tables 2 and 3). These enhancements are observed even though the number of cytosine base substrates for methylation is reduced from two in the unmethylated duplexes to one in the hemimethylated oligodeoxynucleotide duplexes. The substitution of m^5dC for dC opposite the *trans-anti*-dG adduct in the oligodeoxynucleotide duplexes does not result in any detectable conformational changes in the adducts (*59*). Thus, it is unlikely that the methylation in the modified (+)- and (−)G_1^*M or (+)- and (−)G_2^*M duplexes is due to significant changes in the conformations of the lesions.

CONCLUSIONS

In this study, the impact of carcinogen-modified DNA on DNA methylation has been evaluated for the first time. It is shown that the introduction of *trans-anti*-B[*a*]P−N^2-dG lesions into the EcoRII recognition sequence moderately diminishes the binding of the prokaryotic enzyme, C5 MTase EcoRII. The methylation rates are also diminished and in some cases entirely abolished, depending on the position of the lesion within the recognition sequence (5′-...CCA/TGG...). In mammalian cells, methylation is an important epigenetic effect that contributes to the control of gene expression. When cytosine is methylated, the resulting 5-methylcytosines are believed to inhibit the binding of transcription factors to promoters, thus interfering with, or entirely silencing, the expression of the affected genes (*2*). Our results indicate that the formation of B[*a*]P−N^2-dG adducts in cellular DNA may critically affect the cell methylation status (both de novo and maintenance) and may dramatically affect methylation at critical promoter sites. Thus, the inhibition of methylation by the bulky B[*a*]P−N^2-dG and similar bulky adducts may contribute to the epigenetic promotion of tumor development, or at least lead to deleterious structural changes in chromosomes (*1*), by the activation of genes that are normally inhibited by methylation. To evaluate further the potential significance of such epigenetic effects, we consider it important to investigate the effects of B[*a*]P−N^2-dG adducts on methylation in CpG islands by eukaryotic MTases. Such experiments are presently in progress.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Purification procedures of the BPDE-modified oligodeoxynucleotides 5′-GAGCCAACCTG[(−)G*]CTCTGA and 5′-GAGCCAACCTG[(+)G*]CTCTGA, T_m values and hyperchromicities for oligodeoxynucleotide duplexes U, (+)G_1^*, (−)G_1^*, (+)G_2^*, and (−)7G_2^* (Table S1, Figure 1S), and determination of the amount of the active form of M.EcoRII by binding-site titration (Figure 2S). This material is available free of charge via the Internet at http://pubs.acs.org.

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