Cytosine Methylation in a CpG Sequence Leads to Enhanced Reactivity with Benzo[a]pyrene Diol Epoxide That Correlates with a Conformational Change*

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Daniel J. Weisenberger and Louis J. Romano‡
From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202

Benzo[a]pyrene (B[a]P) is a widespread environmental carcinogen that must be activated by cellular metabolism to a diol epoxide form (BPDE) before it reacts with DNA. It has recently been shown that BPDE preferentially modifies the guanine in methylated 5′-CpG-3′ sequences in the human p53 gene, providing one explanation for why these sites are mutational hot spots. Using purified duplex oligonucleotides containing identical methylated and unmethylated CpG sequences, we show here that BPDE preferentially modified the guanine in hemimethylated or fully methylated CpG sequences, producing between 3- and 8-fold more modification at this site. Analysis of this reaction using shorter duplex oligonucleotides indicated that it was the level of the (+)-trans isomer that was specifically increased. To determine if there were conformational differences between the methylated and unmethylated B[a]P-modified DNA sequences that may be responsible for this enhanced reactivity, a native polyacrylamide gel electrophoresis analysis was carried out using DNA containing isomerically pure B[a]P-DNA adducts. These experiments showed that each adduct resulted in an altered gel mobility in duplex DNA but that only the presence of a (+)-trans isomer and a methylated C 5′ to the adduct resulted in a significant gel mobility shift compared with the unmethylated case.

Benzo[a]pyrene (B[a]P) is a well studied polycyclic aromatic hydrocarbon (for reviews see Refs. 1 and 2) that is ultimately converted by the P450 mixed oxygenase system (1, 3) to one of four diastereomeric diol epoxides: (+)-anti-BPDE, (−)-anti-BPDE, (−)-syn-BPDE, and (−)-syn-BPDE (4, 5). The (±)-anti-BPDE forms are thought to be the most biologically relevant (6), and these enantiomers display very different mutagenicities depending on the host system: the (−)-anti form is more mutagenic in bacteria, (7) whereas (+)-anti-BPDE is more mutagenic in mammalian cells (7, 8) and is widely considered to be the ultimate carcinogenic form of BPDE (9).

Regardless of the stereochemistry of anti-BPDE, it is highly reactive, and the major adducts are formed by the cis or trans opening of the epoxide at the C-10 position by the exocyclic amine of guanine (10). The four major guanine adducts are shown in Fig. 1A (11). BPDE also reacts to a lesser extent with the N-6 position of adenine (11) residues to form similar enantiomeric mixtures.

The (+)-trans-anti-B[a]P-dGuo adduct is the major form produced following either in vivo or in vitro treatment, and NMR solution studies have shown that this adduct resides in the minor groove of DNA pointing toward the 5′-end of the adducted DNA strand (12). The (−)-trans adduct is also positioned in the minor groove but points in the 3′ direction (13). Phosphodiesterase digestion of single-stranded DNA oligomers containing the trans adducts have also shown these same adduct orientations (14). NMR studies indicate that both of the cis isomers are more intercalated into the helix, with the (+)-cis pointing toward the minor groove and the (−)-cis isomers pointing toward the major groove (15, 16).

Studies involving isomerically pure B[a]P-DNA adducts have shown that their presence in duplex or single-stranded DNA can result in DNA bending that is dependent on the stereochernistry of the adduct and on the sequence context (17–20). Polyacrylamide gel electrophoresis (PAGE) studies have shown that B[a]P-DNA adducts migrate with anomalously slow rates (21), with the (+)-trans adduct the most retarding, and have suggested that this adduct induces significant DNA bending (17–19). Other studies have concluded that the sequence context on the 5′- and 3′-side of the damaged guanine is also a factor in determining the extent of bending (18, 20).

Recently, it has been shown that the reactivity of BPDE is influenced by the methylation status of CpG sequences in the p53 gene (22, 23). It is well established that this gene is mutated in nearly one-half of all cancers (24) and contains six codons that are major mutagenic hot spots. Five out of these six are in methylated CpG sequences (25), and when the DNA binding sites for BPDE were measured in the p53 sequence in human lung cells (26) or in plasmid DNA (22) it was found that binding was not only targeted to these same sequences but also that methylation of the 5′-C was required for the enhanced reactivity (22). In vitro studies have also shown that numerous carcinogens, including BPDE and N-acetoxy-N-acetyl-2-aminofluorene, show enhanced (2–5-fold) reactivity toward purified p53 DNA when the CpG sequences are methylated (23).

In the present study, we have used a purified system composed of BPDE and methylated and unmethylated duplex oligonucleotides to study the effect of methylation on the reactivity of BPDE at CpG sequences. In addition, the effect of methylation on B[a]P structure in DNA was also studied using a PAGE analysis. It was found that the specific presence of both a 5-mC and a (+)-trans-B[a]P-dGuo adduct (the major adduct formed in vivo) resulted in a substantial change in the DNA
structure and that the level of this adduct was specifically increased when the CpG was methylated.

**EXPERIMENTAL PROCEDURES**

**Materials**—All DNA oligomers were purchased from Midland Certified Reagent Co. (Midland, TX). Racemic (+)-anti BPDE was purchased from the National Cancer Institute Chemical Reference Standard Repository (Kansas City, MO). T4 polynucleotide kinase and [α-32P]dideoxy-ATP were purchased from Amersham Pharmacia Biotech, and terminal deoxynucleotidyl transferase and DraI enzymes were obtained from Promega. [γ-32P]ATP was purchased from ICN Radiochemicals. All other general reagents and chemicals were obtained from Fisher and VWR.

**BPDE Modification Reactions**—The BPDE modification reactions used to determine preferential binding involved the 51-mer oligomer shown in Fig. 1B (top strand). 100 pmol of the 51-mer (containing either a methylated or unmethylated CpG site) was 5’-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP (7,000 Ci/mmol). In a separate reaction, the oligonucleotide was 3’-end-labeled with terminal deoxynucleotidyl transferase and DraI enzymes were obtained from Promega. [γ-32P]ATP was purchased from ICN Radiochemicals. All other general reagents and chemicals were obtained from Fisher and VWR.

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**N-Acetoxy-N-acetyl-2-aminofluorene (N-AAAF) Modification Reactions**—The reaction of N-AAAF with the 51-mer shown in Fig. 1B was carried out as described for BPDE except as noted. The radiolabeled 51-mer was purified by denaturing polyacrylamide gel electrophoresis, and approximately 60 pmol were hybridized to a stoichiometric equivalent of the gel-purified complementary oligomer containing a methylated CpG (Fig. 1B, bottom strand). The annealing reactions (50 μl) contained 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol. The reaction mixtures were incubated at 80 °C for 5 min and then slow cooled to room temperature over 60 min. 3-pmol aliquots of each labeled duplex DNA were incubated with 0–1.2 mM N-AAAF in a 30-μl reaction volume containing final concentrations of 2 mM sodium ascorbate, pH 7.0, in a 60-μl total volume. The annealing mixtures were incubated at 80 °C for 5 min and then slow cooled to room temperature over 60 min. 3-pmol aliquots of each labeled duplex DNA were incubated with 0–1.2 mM N-AAAF in a 30-μl reaction volume containing final concentrations of 2 mM sodium ascorbate, pH 7.0, and 20% ethanol. The reaction was allowed to proceed for 16 h in the dark at room temperature, after which time a small aliquot was removed, the DNA was reacted with 5 units of DraI, and the PAGE analysis was carried out as described for the BPDE reaction. The AAF-modified species

FIG. 1. A, structures of the anti-B[a]P-guanine adducts. B, sequence of the DNA duplex used for determining the difference in reactivity of BPDE toward methylated and unmethylated 5’-CpG-3’ sequences (indicated by the arrows). Following cleavage with DraI, a methylated 28-mer and an unmethylated 24-mer are generated.
migrate slightly slower than the unmodified oligonucleotides and were identified by comparing the gel mobilities with that of AAF-modified and HPLC-purified standards. The relative percentages of AAF modification at each site were measured as a function of the concentration of N-AAAf in the reaction relative to the total number of guanines present.

**Synthesis and Purification of B[a]P-modified Oligonucleotides—16-mer sequences (Figs. 4–7) were purified by HPLC using a Varian 5000 HPLC with a Polychrom 9006 diode array detector on a Hypersil-ODS column (250 x 4.6 mm) in 20 mM NaPO₃, pH 7.0, using a gradient to 45% methanol at a flow rate of 1 ml/min. Each sample was then desalted using a Sep-Pak C-18 column. Approximately 40–50 OD units of purified DNA in 20 mM sodium phosphate, pH 7.0, in a 100–μl total volume were added to 100 μl of BPDE stock solution. The reactions were vortexed periodically over the 48–72-h incubation period at room temperature in the dark. The oligonucleotide products containing each of the four B[a]P adduct isomers were purified using the Hypersil-ODS column using the conditions previously described. Each oligonucleotide was then purified by denaturing 20% PAGE and then HPLC-purified as described above.

**PAGE Analysis of B[a]P-DNA Adducts—**The modified 16-mers (approximately 10 pmol) were 5′-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP. These single-stranded molecules were analyzed by 20% denaturing (containing 8 M urea) or nondenaturing gels. The 16-mer duplexes were formed by removing a small aliquot (1–2 pmol) from the heat-inactivated labeling reaction and incubating with a 5–10-fold excess of complement in annealing buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM MgCl₂, and 1 mM dithiothreitol) for 5 min at 80 °C. The samples were cooled to room temperature over a 60-min time period, and the duplex molecules were electrophoresed on 20% nondenaturing gels at 4 °C. Both native and denaturing gels were analyzed by Molecular Dynamics PhosphorImager.

**UV and CD Analysis of 16-Mer Oligomers—**UV and CD spectra were determined as described previously (27). DNA concentrations for both analyses were first determined by measuring the absorbance at 260-nm wavelength at 80 °C using an Aviv 14DS-UV spectrophotometer and integrating the known extinction coefficients for each DNA strand. Equal amounts of complementary DNA were mixed and annealed as previously described (5-μl total volume for UV, 1 μl for CD analysis). UV analysis of single-stranded DNA and DNA duplexes were obtained at room temperature on a Hewlett Packard 8452A diode array spectrophotometer. The CD spectra were generated at room temperature using a Jasco J-600 spectropolarimeter. The raw CD data were converted to Δε (m–1 cm–1) as described previously (27).

**Determination of B[a]P-DNA Adduct Distributions in Methylated and Unmethylated Duplex Oligonucleotides—**The methylated and unmethylated 16-mers shown in Fig. 6A were 5′-labeled with [γ-32P]ATP and T4 polynucleotide kinase. The labeled strand was annealed to its complement by incubating both strands for 5 min at 85 °C in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 50 mM NaCl, followed by cooling to room temperature. The labeled duplex DNA was purified on a 20% native polyacrylamide gel and desalted via Sep-Pak C-18 desalting columns (obtained from Waters, Inc., Milford, MA). Equal amounts of each duplex DNA (approximately 30 pmol) in a 50 mM NaPO₃, pH 7.0, buffer solution were incubated with 1 μl BPDE for 48 h in the dark at room temperature. To denature the 32P-labeled strand from the complement, a 25-fold excess of unlabeled 16-mer was added to each reaction mixture, heated at 90 °C for 4 min, and then cooled to room temperature. Small aliquots of the mixtures were then analyzed by 20% native PAGE. The identities of the bands in the gel were determined by comparing the mobilities of the bands in the reaction mixtures with those of 32P-labeled and isomerically pure B[a]P-DNA adducts of the same methylated and unmethylated 16-mer DNA molecules. The percentage of each isomer and the extent of BPDE reactivity in the two reaction mixtures were obtained by quantitation using a Molecular Dynamics PhosphorImager.

**RESULTS**

**BPDE Modification of Duplex DNA—**A 51-nucleotide-long duplex DNA oligonucleotide was prepared containing two CpG dinucleotides in identical sequences contexts, one containing a 5-mC and one unmethylated (Fig. 1B, top strand). This strand was labeled at either the 5′- or 3′-end with [32P] (see “Experimental Procedures”), equivalent amounts of each labeled duplex were mixed, and this mixture was then reacted with a molar excess of BPDE. The B[a]P-modified duplex was then cleaved with DraI to produce two labeled fragments, a 28-mer, which contained the methylated CpG, and a 24-mer, which was unmethylated. The modified products are the bands migrating slightly above the unmodified oligomers in the PAGE analysis shown in Fig. 2A. The extent of modification was determined by PhosphorImager analysis, and these levels are shown in Fig. 2B. From this analysis, it is clear that methylation of the CpG sequence leads to enhanced BPDE reactivity with the difference ranging from 3- to 4-fold over the range of BPDE concentration. A similar enhancement in BPDE reactivity was also seen if only the opposite strand contained a 5-mC.

Since the vast majority of cellular CpG sites would be methylated in both strands, an identical experiment was carried out where both strands were methylated in the CpG sequence located on the 3′-side of the 51-mer (Fig. 1B). As was found in the case of hemimethylation, there was a clear enhancement of reactivity for the methylated CpG sequence (Fig. 3A). Quantitation of the radioactivity present in the modified materials is shown in Fig. 3B, and the enhancement in this case ranged from 5- to 8-fold depending on the BPDE concentration. These enhanced levels of reactivity at methylated CpG sites are consistent with recently published data for in vitro reactivity of BPDE (22, 23). When the reaction was carried out comparing the effect of methylation in the complementary strand in which both sites in the modified strand were methylated, we found approximately a 2-fold enhancement (data not shown), consistent with the differences shown in Figs. 2 and 3.

**Structure Changes in Methylated CpG Sequences Modified by BPDE—**To understand the molecular differences that lead to the enhanced reactivity of methylated CpG sequences, we have attempted to measure the effects of CpG methylation...
on the structures of \( \text{BaP} \)-modified oligonucleotides. For these studies, we chose a 16-mer containing a single \( \text{CpG} \) site in a 5'-CGA-3' sequence context (Fig. 4A). Oligonucleotides of this length can be efficiently modified with \( \text{BPDE} \), and 16-mers containing each of the four \( \text{BaP} \)-dGuo isomers are easily separable by HPLC. Native (Fig. 4) or denaturing (not shown) PAGE analyses confirm that the isolated oligonucleotides are not cross-contaminated with the other isomers or unmodified materials and, as expected, run more slowly in the gel than the unmodified 16-mers (17, 18, 27). The mobility differences in methylated DNA as opposed to unmethylated DNA may be the result of a DNA structural distortion or a conformational change caused by the presence of both a 5-mC and a \( \text{BaP} \)-dGuo adduct isomer.

Each of these oligonucleotides was \( ^{32} \text{P} \)-labeled and hybridized to the complementary unmethylated 16-mer, and the duplexes were then analyzed by nondenaturing PAGE (Fig. 5). Under these gel conditions, there are no noticeable differences in the migration rates of unmethylated methylated versus the unmodified unmethylated duplex DNAs (Fig. 5, lanes c and d). This PAGE analysis also shows, as expected, that the modified oligomers exhibit a decrease in gel mobility compared with the unmodified DNA duplexes. The (+)-trans-\( \text{BaP} \)-dGuo modified duplex, since it is the most distorting \( \text{BaP} \)-dGuo adduct (12), gives rise to a significant decrease in PAGE mobility as compared with the less distorting minor adducts. Upon methylation, there is a significant difference in the migration rate of the (+)-trans-\( \text{BaP} \)-dGuo-modified duplex (compare lanes e and f), suggesting that there is a significant structural change in this particular duplex that results in a faster migration rate. Previous studies involving native PAGE analyses of unmodified DNA duplexes have shown similar magnitude mobility differences between duplex DNAs that are the result of DNA structural alterations, most notably those of DNA curvature and bending (28, 29). The methylation status of the remaining modified duplexes in Fig. 5 (lanes g–l) has little or no effect on the mobility of these samples. This suggests that although methylation causes either no change or a small structural change for the minor adduct isomers, a more significant structural change occurs in the (+)-trans-\( \text{BaP} \)-dGuo-modified duplex that results in the observed increased gel mobility. Interestingly, the (+)-trans-\( \text{BaP} \)-dGuo adduct alone is positioned on the outside of the helix pointing in the 5' direction, which is toward the 5-mC location (12).

Effect of Methylation on the 3'-Side of the BPDE Adduct or in the Complementary Strand—To determine if the specific se-
Effect of 5-mC in DNA on BPDE Reactivity and Structure

The presence of 5-mC in DNA was found to affect the reactivity of DNA with BPDE. To investigate this, methylated and unmethylated DNA were modified with BPDE, and the products containing each isomer were purified by HPLC. Each was then 32P-labeled and hybridized with equal amounts of complementary 16-mer, and analyzed by native PAGE as described under “Experimental Procedures.”

The PAGE analysis showed the effect of 5-mC on BPDE reactivity and structure. A, the sequences of the 5’- or 3’-methylated duplex 16-mers. B, the unmethylated 16-mer and two methylated 16-mers were modified with BPDE, and the products containing each isomer were purified by HPLC. Each was then 32P-labeled, hybridized with equal amounts of complementary 16-mer, and analyzed by native PAGE as described under “Experimental Procedures.”

To determine the effect of a 5-mC in the complementary strand, duplexes were prepared that were methylated opposite the B[a]P-dGuo adduct. Fig. 6A shows the PAGE analysis where the modified strand contained a 5-mC positioned 5’ to the B[a]P-dGuo adduct. For each of the possible combinations, a large mobility shift was observed only when the duplex contained a 5-mC in the (+)-trans-B[a]P-dGuo-modified strand (Fig. 6A, lanes g and i). None of the other isomers nor the presence of only a 5-mC in the complementary strand resulted in a significant mobility shift that could be observed in the native PAGE analysis. Similarly, if the 5-mC was positioned 3’ to the B[a]P-dGuo adducts and hybridized to a complement containing a 5-mC opposite the adduct, very little if any shift in mobility could be observed for any isomer (Fig. 6B).

Recently, the combination of a (+)-trans-B[a]P-dGuo adduct and a 5-mC on the 5’-side of the modified guanine is necessary to cause a significant methylation-induced mobility shift.

CD and UV Spectra of (+)-trans-B[a]P-dGuo DNA Duplexes—The minor groove positioning properties of the trans adducts and the intercalation properties of the cis adducts have been determined by NMR (12, 13, 15, 16) and were reported earlier by Geacintov and co-workers (30) using absorbance, fluorescence, and linear and circular dichroism measurements. In these experiments, an approximately 10-nm red-shifted absorbance for the B[a]P absorption maxima for duplexes containing the cis adducts was observed when compared with their single-stranded counterparts, indicating that the pyrenyl ring was intercalated into the duplex. In contrast, an approximately 10-nm blue shift was observed for the two duplexes containing the trans adducts, indicating that the adducts are outside the helix and exposed to solvent. To attempt to characterize the apparent structural changes that are present in the duplexes containing a 5-mC and a (+)-trans-B[a]P-dGuo adduct, UV and CD spectra were obtained for (+)-trans duplexes either methylated or unmethylated at the 5’-C in the 5’-CGC-3’ sequence context (Fig. 8). Neither the UV or CD spectra show any significant differences that can be attributed to the presence of the 5-mC, suggesting that methylation is not causing the intercalation of the (+)-trans adduct into the helix.

Comparison of BPDE Reaction Products on Methylated and Unmethylated DNA—A prediction of the enhanced reactivity of the methylated sites shown in Figs. 2 and 3 and the structural analysis shown in Figs. 5–7 is that there is a specific increase...
in the formation of the (+)-trans-B[a]P isomer, since this isomer contained within a methylated CpG sequence specifically shows a conformational change relative to the identical unmethylated sequence. In order to test this hypothesis, two duplex oligonucleotides containing either a methylated or unmethylated CpG sequence were reacted with BPDE, the strands were denatured, and the products were analyzed by native PAGE (Fig. 9). This gel was able to separate each of the four possible products from each reaction as determined by comparison with authentic standards that were separated and analyzed by HPLC. These data were analyzed in two ways (Table I). First, the percentage of modification of each isomer relative to the unreacted material was determined, and it is evident from these data that not only does methylation cause an enhanced reactivity with BPDE as was also shown in Fig. 2 but also that this increase is specific for the formation of the (+)-trans isomer. The values shown in parentheses correspond to the relative amount of each isomer formed.

Modification of Duplex DNA with N-AAAFA Recent studies have indicated that other carcinogens, including N-AAAFA, show enhanced reactivity with methylated CpG sites in plasmid DNA (22, 23). Because of the specificity that is observed in the present study for the (+)-trans-B[a]P adducts, it seemed surprising that other unrelated carcinogens would display similar reactivities. To test this, the fully methylated duplex oligonucleotide shown in Fig. 1B was reacted with N-AAAFA and cleaved with DraI, and the products were analyzed by PAGE (Fig. 10). Unlike that which was observed with BPDE modification, no enhanced reactivity was observed in this case (Fig. 10B), suggesting that the results obtained in the prior studies that showed enhanced reactivity for N-AAAFA may have resulted from differences in the reactivity of the UvrABC endonuclease that was used to measure the adduct formation.

**Discussion**

Cytosine methylation is known to play an important role in numerous cellular processes, including cellular development, cell differentiation, genetic imprinting, X-chromosome inactivation, and tumorigenesis (31–35). In addition, DNA methylation also appears to be involved in mutagenesis, since transition mutation rates of cytosines at CpG sites are 10–40 times higher compared with unmethylated sites (36, 37). Because the majority of the mutations observed in human cancers at these sites are C:G $\rightarrow$ T:A transitions (24, 38) and 5-mC is known to deaminate 2–3-fold faster than unmethylated cytosine (39, 40), it has been suggested that most of these mutations arise by endogenous cytosine deamination at methylated CpG sites. However, in breast, liver, and lung tumors the percentage of C:G $\rightarrow$ A:T transversions is often found to be equal to or higher than these transition mutations (41). For example, in lung...
The CpG dinucleotide displays the strongest base-stacking interactions and the greatest degree of minor groove opening compared with each of the other DNA dinucleotides (49, 50). Most relevant to the present study, it has been shown through computational analyses (44) that cytosine methylation affords an increased helical stability resulting in increased hydrophobicity and molecular polarizability (51), and it may be these characteristics that lead to the increased reactivity of BPDE at these sites. In agreement with this prediction, the presence of multiple 5-methylcytosines in duplex DNA were shown to result in greater levels of noncovalent intercalation (52). Since BPDE is believed to first intercalate with DNA prior to forming a covalent adduct, an increase in intercalation or an altered structure may lead to the preferential modification seen in this study. However, it should be noted that the effect observed for DNA containing multiply methylated sites may not be a good predictor for the effect of methylation at a single CpG site.

To determine if methylation affected the structure of BPDE-modified DNA, a native gel analysis was used to measure conformational changes in the duplex oligonucleotides. It has been shown in numerous studies on DNA bending that differences in native gel mobility correlate with structural changes in DNA (28, 29). We find here that the presence of a 5-mC on the 5′-side of a (+)-trans-B[α]P-dGuo adduct specifically induced a mobility shift that was not observed for any of the other adducts, although the minor adduct did show a very slight change in mobility. Methylation was found to increase the gel mobility, suggesting that a more compact structure had formed, possibly caused by an increase in intercalation of the adduct into the DNA helix. However, the lack of a red shift in UV and CD analyses of DNA duplexes containing a (+)-trans adduct, which would be indicative of this enhanced intercalation, suggests that the explanation may not be as simple as this.

These altered structures that were observed were not specific to a single sequence, suggesting that the structural change was the result of the presence of both a 5-mC and a (±)-anti-B[α]P-dGuo adduct and not caused by a sequence-mediated unique structure. Moreover, a 5-mC in the complementary strand or 3′ to the adduct position had little or no effect on the gel mobility for any of the adduct stereoisomers. Taken together, these data indicate that the structural change that is observed is not simply the result of a simultaneous structural alteration caused by the presence of a 5-mC and a B[α]P adduct but instead indicate that there may be a unique conformation that is induced by the presence of a 5-mC positioned 5′ to a (+)-trans-B[α]P-dGuo adduct.

The fact that the trans isomer specifically induces the largest structural change in the methylated CpG sequence suggested that it might be this adduct whose formation is increasing. This hypothesis was tested by reacting two short duplex oligonucleotides that contained either a methylated or unmethylated CpG sequence. The relative amounts of each isomer were determined by native PAGE analysis and indicated that this prediction was valid, since there was a specific increase in only the (+)-trans material (Table I). Taken together with the structural analysis, these data may provide an explanation for the enhanced reactivity, possibly through a specific interaction between the incoming BPDE and the 5-mC positioned 5′ to the reacting guanine. The likelihood that an altered structure exists for the methylated (+)-trans isomer is supported by a recent study (53) that used molecular modeling to show that methylation of CpG sequence specifically increased the preference of only the (+)-trans isomer to convert from an anti to syn conformation.
Methylation of CpG sequences has also been suggested to effect the covalent binding of several other carcinogens that target guanine in duplex DNA. Mitomycin C was the first of these to be shown to target methylated CpG sequences, providing enhanced reactivity for both alkylation and cross-linking (54). More recently, two related cytotoxins, eseraminic A1 and C, were found to target CpG sequences in highly methylated locations (55). Methylation of CpG sequences was shown to enhance the levels of sunlight-induced cyclobutane pyrimidine dimer formation (56) but inhibit the formation of 6–4 photoproducts (57). Methylation has also resulted in decreased reactivity of both N-methyl and ethyl N-nitrosourea (58). From these results, it is not clear if there are specific interactions, electronic effects, or structures in the DNA that are responsible for these varied reactivities.

A recent study has suggested that benzog[chryosene diole epoxide, aflatoxin B1, and N-AAAF all have enhanced reactivity at methylated CpG sequences in vitro (23). However, these results could also be explained by a differing reactivity of the UvrABC endonuclease that is used in this analysis, since the reactivity of this enzyme is known to be influenced by the structure of the damaged site (59). The results presented here show that N-AAAF does not have enhanced reactivity at a fully methylated CpG sequence (Fig. 10). Moreover, the fact that it is the (+)-trans-8β-[p-dGuo isomer that is formed almost exclusively at methylated CpG sites (Fig. 9 and Table I) and that only this isomer shows a significant structural alteration in a methylated sequence (Figs. 5–7) suggests that methylation is inducing a structural change that participates in this reaction in a very specific manner rather than through a global change or electronic effect that targets many reactive species to these sites.

In conclusion, although most previous discussions of the role of 5-mC in tumorigenesis have suggested increased demethylation rate in DNA, this study has used a purified system to confirm other in vivo and in vitro analyses that have shown that methylated CpG sites react more readily with carcinogens. In addition, data has been presented that provides support for the hypothesis that the mechanism that causes this phenomenon is a structural change afforded by the presence of the methylated cytosine 5' to the modified guanine. Future studies will focus more closely on the nature of this structural change and the effect of these different structures on DNA repair and replication fidelity.

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