C5 Cytosine Methylation at CpG Sites Enhances Sequence Selectivity of Mitomycin C−DNA Bonding

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ABSTRACT: We have established that UvrABC nuclease is equally efficient in cutting mitomycin C (MC)−DNA monoadducts formed at different sequences and that the degree of UvrABC cutting represents the extent of drug−DNA bonding. Using this method we determined the effect of C5 cytosine methylation on the DNA monoalkylation by MC and the related analogues *N*-methyl-7-methoxyaziridinomitosene (MS-NMA) and 10-decarbamoylmitomycin C (DC-MC). We have found that C5 cytosine methylation at CpG sites greatly enhances MC and MS-NMA DNA adduct formation at those sites while reducing adduct formation at non-CpG sequences. In contrast, although DC-MC DNA bonding at CpG sites is greatly enhanced by CpG methylation, its bonding at non-CpG sequences is not appreciably affected. These cumulative results suggest that C5 cytosine methylation at CpG sites enhances sequence selectivity of drug−DNA bonding. We propose that the methylation pattern and status (hypo- or hypermethylation) of genomic DNA may determine the cells' susceptibility to MC and its analogues, and these effects may, in turn, play a crucial role in the antitumor activities of the drugs.

Mitomycin C (MC)[1] (Figure 1) is a potent antitumor drug; its antitumor activity has been attributed to its covalent bonding with DNA (*1*, *2*). During the past two decades, much work has been done on the chemistry of how MC alkylates guanine and the preferred sequence for DNA alkylation. One important finding is that the CpG sites are the preferred sites for MC bonding. This information is derived from studies using plasmid DNA isolated from *Escherichia coli* and chemically synthesized oligonucleotides (*3*−*9*).

Unlike *E. coli* DNA, in which the adenine residue at the -GATC- site and the internal cytosine at CC(A/T)GG are methylated (*10*, *11*), mammalian genomic DNA methylation occurs mainly at the C5 position of cytosine residues; 3−5% of cytosine residues are methylated, and 95% of this methylation occurs at CpG sites (*12*−*14*). Promoter regions of genes, particularly housekeeping genes and imprinted genes, are rich in CpG sites; ample evidence has demonstrated that C5 cytosine methylation can suppress the expression of these genes (*15*−*17*). Although the precise mechanisms of how methylation affects gene expression remain to be understood, it is known that methylated CpG sites affect nucleosome structure and histone interaction with deacetylase, and these interactions consequently elicit suppression of gene expression (*16*, *18*−*25*).

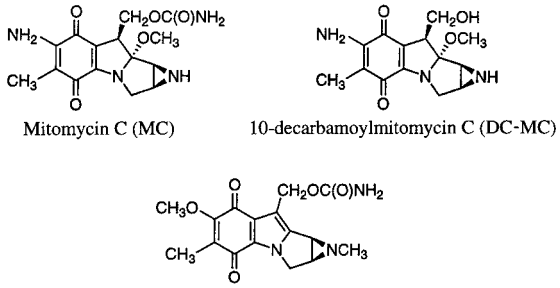

FIGURE 1: Chemical structures of MC, MS-NMA, and DC-MC.

Because MC preferentially bonds CpG sites and it appears that C5 methylation of CpG can dramatically affect gene expression, it is conceivable that most of the biological effects of MC could result from its interaction at CpG sequences. If this is the case, then, to understand the antitumor activity of this drug it is important to determine the effects of methylation at CpG sites on MC−DNA bonding. We have previously developed an approach using the UvrABC nuclease incision method to determine and map MC−DNA bonding at nucleotide resolution (*4*, *26*). In this report we have demonstrated that UvrABC nuclease incises MC−, MS-NMA−, and DC-MC−guanine monoadducts formed at methylated CpG and non-CpG sites with the same kinetics as drug−DNA adducts formed at other sequences; these results enable us to use this enzyme system to determine the effect of methylation on MC−DNA bonding at CpG sites as well as at other sequences. We have found

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[1] Abbreviations: MC, mitomycin C.; MS-NMA, *N*-methyl-7-methoxyaziridinomitosene; DC-MC, 10-decarbamoylmitomycin C; dTTP, thymidine triphosphate; APRT, adenine phosphoribosyltransferase; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid.

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that treatment of methylated DNA with MC, MS-NMA, and DC-MC (Figure 1) results in drug modification proceeding mainly at methylated CpG sites. The significance of this preferential drug−DNA bonding for MC antitumor activity is discussed.

MATERIAL AND METHODS

Materials. Mitomycin C (pure) was supplied by Bristol-Myers Squibb Co. (Wallingford, CT). Mitomycin A was kindly provided by Dr. M. Kasai (Kyowa Hakko Kogyo Co. Ltd.). 10-Decarbamoylmitomycin C was prepared by a procedure of Kinoshita et al. (*27*). *N*-Methyl-7-methoxyaziridinomitosene was prepared according to the method reported by Damishefsky and Egbertson (*28*). $Na_2S_2O_4$ was purchased from Fisher Scientific Co. Restriction enzymes, DNA polymerase I (Klenow fragment), and *Sss*I methylase were obtained from New England Biolabs. NACS Prepac cartridges (syringe format) were purchased from Bethesda Research Laboratories. All other chemicals and electrophoretic materials were obtained from either Sigma Chemical Co. or Bio-Rad Laboratories. The [α-^{32}P]dTTP (sp act. 3000 Ci/mmol) was purchased from DuPont−New England Nuclear.

DNA Fragment Isolation: ^{32}P End Labeling and Methylation. Plasmid pGem inserted with the adenine phosphoribosyltransferase (APRT) gene from Chinese hamster ovary (CHO) cells was purified via cesium chloride density centrifugation and dialyzed extensively against TE buffer [10 mM Tris·HCl (pH 8.0) and 1 mM EDTA] (*29*). The 284-bp fragment of exon III of the APRT gene was obtained by digesting plasmid DNA with restriction enzymes *Pst*I and *Stu*I. The band corresponding to the 284-bp fragment was isolated from a 1.4% agarose gel and cleaned by passing through a NACS Prepac column followed by ethanol precipitation. The 284-bp fragment was further cut with the restriction enzyme *Mse*I to generate a 188-bp fragment with a unique site for a single 3′-end-labeling by [α-^{32}P]dTTP as previously described (*30*). DNA methylation was carried out with bacterial *Sss*I methylase in the presence of *S*-adenosylmethionine (SAM) and NEB buffer 2, as recommended by the manufacturer. Only cytosines flanked by a 3′ G are methylated in this reaction (*31*).

Mitomycin C− and 10-Decarbamoylmitomycin C−DNA Bonding with Sodium Dithionite. Specified amounts of drugs were added to the ^{32}P-labeled DNA in 25 mM Tris·HCl buffer, pH 7.4, to give the desired final concentration. The solutions were deaerated with argon (15 min), and then freshly prepared, deaerated, aqueous sodium dithionite solution (1 equiv for 0.15 and 0.2 mM MC, 0.66 equiv for 0.3 mM MC, and 1 equiv for 0.45 mM DC-MC) was added in three incremental portions (20 min). The reactions were maintained under argon (1 h) at 0 °C for mitomycin C and 22 °C for 10-decarbamoylmitomycin C.

N-Methyl-7-methoxyaziridinomitosene−DNA Bonding. The MS-NMA was added to the ^{32}P-labeled DNA in 25 mM Tris·HCl buffer, pH 7.4, to a final concentration of 1.5 mM. The solution was incubated at 37 °C for 1.5 h. The unreacted drug was removed by phenol−chloroform extractions.

Kinetic Study of UvrABC Incision of Mitomycin C and N-Methyl-7-methoxyaziridinomitosene−Methylated DNA Adducts. The ^{32}P-labeled DNA fragments were modified with MC or MS-NMA as described above. Seven tubes with equal amounts of drug−modified DNA were prepared. Each tube was treated with the same amount of UvrABC nuclease and incubated at 37 °C for different time periods (0, 5, 10, 20, 30, 60, and 90 min).

Purification of UvrA, UvrB, and UvrC Protein. UvrA, UvrB, and UvrC proteins were isolated from *E. coli* K12 strain CH292 (*recA endA*/F′ *lacI*^{q}) carrying plasmids pUNC45 (*uvrA*), pUNC211 (*uvrB*), and pDR3274 (*uvrC*) (*32*). The methods of purification were the same as described previously (*26*).

UvrABC Nuclease Reactions. The UvrABC nuclease reactions were carried out in a reaction mixture (30 μL) containing 50 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 1 mM dithiothreitol, 60 nM UvrA, 120 nM UvrB, 60 nM UvrC, and substrate DNA (enzyme/DNA molar ratio ≥6). The mixture were incubated at 37 °C (1 h), and the reaction were stopped by phenol−chloroform extractions followed by ethanol precipitation in the presence of aqueous ammonium acetate (2.5 M). The precipitated DNA was recovered by centrifugation and washed with 80% ethanol.

DNA Sequencing, Gel Electrophoresis, and Autoradiography. The 3′-end ^{32}P-labeled DNA fragment was sequenced by the method of Maxam and Gilbert (*33*). The ^{32}P-labeled fragments with or without various enzyme treatments were suspended in sequencing tracking dye (80% v/v deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), heated at 90 °C (3 min), and quenched in an ice bath. The samples were applied to an 8% denaturing sequencing gel in parallel with the Maxam−Gilbert sequencing reactions. After electrophoresis, the gels were exposed to Kodak X-Omat AR film or Fuji RX film with an intensifying screen at −70 °C.

Densitometric Scanning. The intensities of UvrABC nuclease incision bands were determined with a Bio-image Open Windows Version 3 system consisting of a Howtek Scanmaster 3+ and whole band analysis software.

RESULTS

C5 Cytosine Methylation at CpG Sites Enhanced MC−, MS-NMA−, and DC-MC−Guanine Bonding at the Methylated CpG Site. To determine the effects of C5 cytosine methylation on drug−DNA bonding, 188-bp *Stu*I−*Mse*I fragments of CHO APRT exon III gene were isolated, the 3′-end was labeled with ^{32}P, and then the cytosines at CpG sites were methylated with *Sss*I CpG methylase. The extent of methylation was determined by Maxam−Gilbert sequencing reactions (*33*). C5-methylated cytosines are refractory to hydrazine modification; thus the phosphodiester bonds at its 5′ and 3′ sides are resistant to piperidine hydrolysis, and consequently no cytosine ladders are produced (*34*). Results in Figure 2 (lane 2 vs lane 3) show that no C ladders were produced by Maxam−Gilbert sequencing reaction at three CpG sites in the methylated 188-bp DNA fragment; this result demonstrates that under our reaction conditions methylation at these three CpG sites in this DNA fragment was virtually complete.

Methylated and unmethylated DNA fragments were subsequently modified with MC, MS-NMA, and DC-MC under conditions that render, on average, fewer than one adduct per DNA fragment (*4*, *5*, *35*−*37*). Although $Na_2S_2O_4$

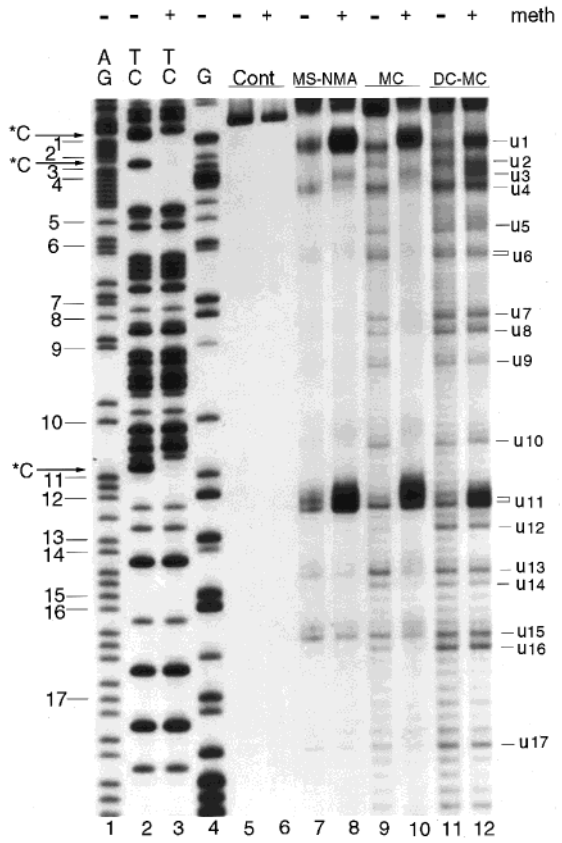

FIGURE 2: Effect of C5 cytosine methylation at CpG sites on UvrABC cutting of MC (0.15 mM), MS-NMA (1.5 mM), and DC-MC (0.45 mM) modified DNA. The 188-bp *Stu*I−*Mse*I fragments of CHO APRT gene were 3′-end ^{32}P-labeled and methylated with CpG methylase, modified with drug, and reacted with UvrABC nuclease as described in text. *C represents methylated cytosines. Guanine residues are labeled 1−17 and the corresponding UvrABC incision bands are labeled U1−U17. Meth indicates methylation; symbols + and − represent with and without designated treatment. G, GA, C, and TC represent Maxam−Gilbert sequencing reactions.

reduction of MC can produce interstrand cross-links, under our reaction conditions MC produces mainly monoadducts (*4*, *5*, *35*−*37*). Similarly, we have observed only monoadducts with MS-NMA under the nonreductive activation conditions (*35*, *36*). DC-MC is a MC derivative that can form only monoadducts (*4*, *5*, *35*−*37*). After drug modification, the DNA fragments were treated with UvrABC under conditions such that the enzyme/DNA molar ratio was greater than or equal to 6.

To demonstrate the effect of C5 cytosine methylation at CpG sites on drug−guanine adduct formation, nearly equal ^{32}P counts of drug-modified methylated and unmethylated DNA fragments after treatment with UvrABC were separated by gel electrophoresis. A typical result is shown in Figure 2, and the corresponding histograms are presented in Figure 3. There were three important findings: first, C5 cytosine methylation at the three CpG sites enhanced drug−DNA bonding at all three CpG sites. This enhancement was most prominent for MS-NMA, followed by MC and then DC-MC. Second, large differences in the extent of drug−DNA bonding were observed among the three methylated CpG sites; two were extensively modified (CC*GG and TC*GA) and one was weakly modified (GC*GA). This finding suggests that neighboring sequences affect drug−DNA bonding at a methylated CpG site. Third, compared with unmethylated DNA, UvrABC incisions were greatly reduced at guanines located in most non-CpG sites in methylated DNA that had been modified with MC. DNA methylation has a mixed effect on MS-NMA bonding; while drug bonding at G4 and G13 positions was reduced, no significant effect was observed at the G15 position. Methylation of DNA, however, did not change the extent of UvrABC incisions at non-CpG sites for DNA modified with DC-MC. These results suggest that the effects of C5 cytosine methylation at CpG sites on the DNA structure were not necessarily limited to the CpG sites and that the nature of such methylation-related changes in drug−DNA interaction depended on the structure of the drug.

We have previously shown that the intensity of UvrABC incisions at different sequences is proportional to the concentrations of MC used for DNA modification (*4*, *26*). The reduced MC and MS-MNA bonding at non-CpG sites in the methylated DNA fragment may be due to the fact that C5 cytosine methylation simultaneously increases drug affinity toward methylated CpG sites and decreases drug affinity toward nearby non-CpG sequences. If this is the case, a qualitatively different relationship between the extent of drug−DNA adduction and drug concentrations for CpG sites and for non-CpG sites in methylated and unmethylated DNA fragments is expected. To test this possibility, methylated and unmethylated DNA fragments were modified with increased concentrations of MC. Results in Figure 4 show that the degree of drug−DNA bonding at both CpG and non-CpG sites in unmethylated DNA fragments was proportional to MC concentration; however, the relationship between the drug−DNA bonding and the MC concentrations in methylated DNA was more complicated. We found that drug−DNA bonding at two CpG sites, U11 and U1, reached a plateau at 0.15 mM MC, while at one CpG site (U3) and all non-CpG sites in the methylated DNA fragment, bonding increased from 0.15 mM to 0.2 mM MC and then plateaued (Figure 4B). Correspondingly, in the unmethylated DNA, the extent of drug modification at G sites increased from 0.15 to 0.3 mM MC. Furthermore, the degree of MC−DNA bonding at most non-CpG sites in the methylated DNA fragments was lower than their counterparts in unmethylated DNA fragments. Collectively, these results suggest that C5 cytosine methylation at CpG sites not only enhanced drug modification at these sites but also reduced the MC affinity toward nearby non-CpG sequences.

Kinetics of UvrABC Incisions on Drug−DNA Adducts Formed in Methylated and Unmethylated DNA Were Identical. There are two possible explanations for our observation that C5 cytosine methylation changed the extent of UvrABC incision at drug−DNA bonding sites: methylation may have affected drug−DNA adduct formation, or DNA methylation may have affected the affinity of UvrABC toward drug−DNA adducts. It is worth noting that, under our reaction conditions, UvrABC incision was irreversible because no DNA polymerases and ligases were present. If the observed enhancement of UvrABC cutting at methylated CpG sites was due to the enzymes' having a higher cutting efficiency toward the drug adducts formed at methylated CpG sites, compared with those formed at other sequences, then the kinetics of UvrABC incision for methylated CpG and other sequences should be different. To test this, we examined the time course of UvrABC cutting at different sites in MC- and

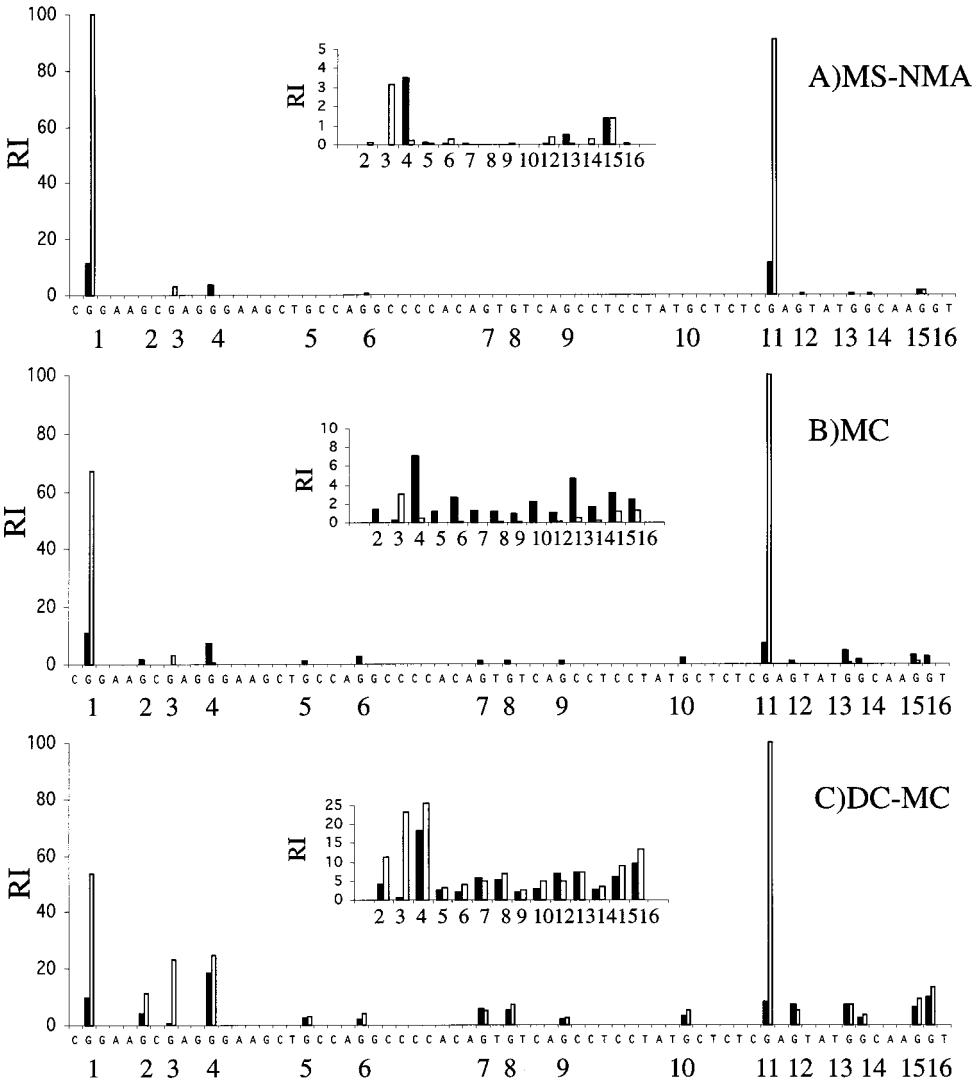

FIGURE 3: Quantification of the effect of C5 cytosine methylation at CpG sites on UvrABC cutting on MS-NMA (A), MC (B), and DC-MC (C) modified DNA. The intensities of drug-induced UvrABC incision bands in Figure 2 were quantified. Open bars: methylated DNA; solid bars, unmethylated DNA. The guanines are numbered the same as shown in Figure 2. RI, relative intensity. For clarity, expanded scales were used for each inset. The numbers on the *x*-axis of the insets represent the G numbers.

MS-NMA-modified methylated DNA. The results are shown in Figures 5 and 6. These results demonstrate that UvrABC incision at all drug−DNA bonding sites was a function of incubation time and appeared to plateau after 60 min of incubation. We have found that under our UvrABC reaction conditions the enzymes were not only excessive compared to DNA substrates but also active in cutting additional DNA substrates after 60 min of incubation (data not shown). These results suggest that the plateau of UvrABC cutting resulted from the exhaustion of DNA substrates rather than enzymes. Six well-resolved bands were quantified, and as shown in Figure 6, the kinetics of UvrABC incision at all six sites, including three methylated and three non-CpG sites, were virtually identical. Previously, using the same approach we have found that the kinetics of UvrABC incision at CpG sites and other sequences in MS-NMA-modified unmethylated DNA are identical (*36*). On the basis of these results we concluded that, under our reaction conditions, UvrABC had the same affinity toward drug−DNA adducts formed at methylated or at unmethylated CpG sites, as well as at other sequences, and therefore, the degree of UvrABC incision represented the extent of drug−DNA adduct formation.

DISCUSSION

Using gel electrophoresis and HPLC to determine MC adduction, Millard and Beachy (*38*) and Johnson et al. (*39*) reported that methylation at CpG sites enhances MC alkylation 1.4−2.4-fold. While this finding provides valuable information on drug−DNA interactions, it also raises two interesting questions. First, are the effects of methylation at CpG sites on drug−DNA bonding limited to just the methylated CpG sites? Second, to what extent is the methylation-mediated enhancement of drug−DNA bonding at CpG sites affected by neighboring sequences? These answers are important in understanding the antitumor activity of MC and its biological effects because 90% of cytosine methylation in mammalian cells occurs at CpG sites, and CpG sequences are involved in expression regulation of many genes (*15*, *16*, *23*, *25*).

In this study, we address these two questions by using the UvrABC nuclease incision method to quantify drug−DNA monoadduct formation at the sequence level. We have shown that, under proper reaction conditions, UvrABC nuclease can incise MC− and MS-NMA−DNA mono-

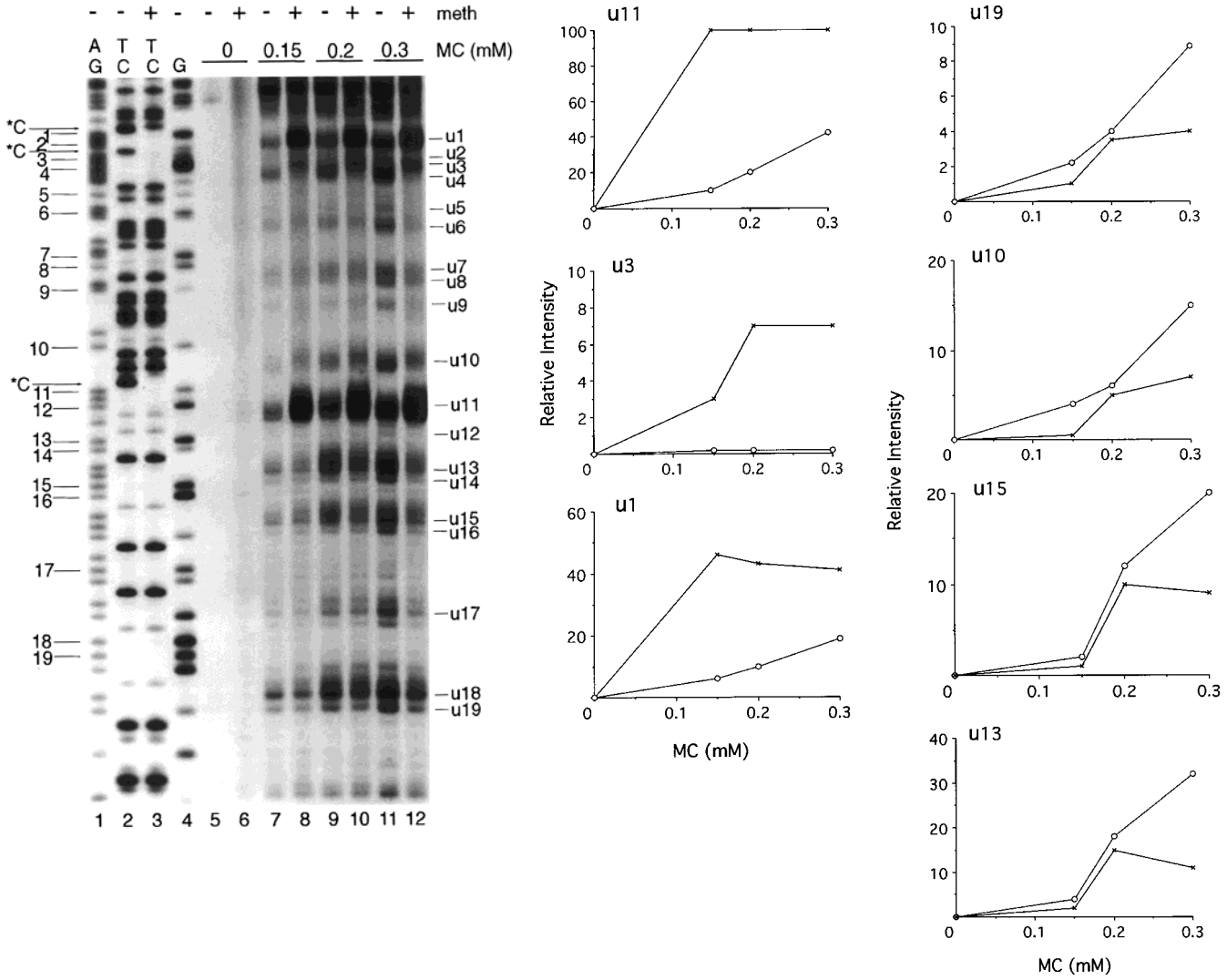

FIGURE 4: C5 cytosine methylation at CpG sites enhances MC-DNA bonding at CpG sites but reduces MC-DNA bonding at non-CpG sites. Methylated and unmethylated 188-bp *Stu*I−*Mse*I fragments of CHO APRT gene were modified with different MC concentrations and the extent of drug−DNA adduct formation was determined by UvrABC. Symbols are the same as described in Figure 2. A typical autoradiograph is shown in the left panel. The relationship between drug−DNA formation and MC concentrations at three CpG sites (U11, U3, and U1) and four non-CpG sites (U19, U10, U15, and U13) is presented in the right panel. (○) Unmethylated DNA; (×) methylated DNA.

adducts without sequence preference in both methylated and unmethylated DNA, using incision kinetics analysis (Figure 6). These results allow us to use the UvrABC incision data to determine the extent of adduct formation at different sequences.

Among the three CpG sites examined we have found that MC bonding and MS-NMA bonding at two methylated CpG sites (CGG, U1, and CGA, U11) are enhanced at least 7−14-fold. We are unable to calculate the exact methylation-enhanced drug−DNA bonding at these two methylated CpG sites due to the fact that MC−DNA bonding reactions plateau even at the lowest drug concentrations used for DNA modification. We also are unable to calculate the precise extent of methylation-enhanced drug−DNA bonding at the third CpG site (CGA, U3) due to negligible drug bonding in unmethylated DNA fragment, but on the basis of visual inspection we estimated that the enhancement at this site exceeds 10-fold (Figure 4). Overall, the degree of this methylation-dependent enhancement of drug−DNA bonding for these sites was significantly higher than that reported by Millard and Beachy (*38*) and Johnson et al. (*39*). It appears that the extent of enhancement is sequence-dependent. Interestingly, we also found that drug bonding at non-CpG sites is lower in methylated than in unmethylated DNA fragments. This effect may provide a partial explanation for why less than a 3-fold enhancement of MC-DNA bonding by CpG methylation was observed by Millard and Beachy (*38*) and Johnson et al. (*39*) because they quantified the total drug−DNA bonding. On the other hand, we quantified the effects only at the nucleotide level. No reduction of drug−DNA bonding at non-CpG sites was observed with DC-MC in methylated DNA, and it appears that drug−DNA bonding at methylated CpG sites for DC-MC was significantly lower than for MC and MS-NMA. Together, these results suggest that (1) C5 cytosine methylation at CpG sites greatly enhances sequence selectivity of MC−, MS-NMA−, and DC-MC−DNA bonding and (2) the DNA structure changes induced by C5 cytosine methylation at CpG sites hinder the interaction of MC and MS-NMA with surrounding nonCpG sites. It is worth noting that, using a 10-mer oligonucleotide containg a single CpG site, Heinemann and Hahn (*40*) demonstrated that the effect of C5 cytosine methylation on

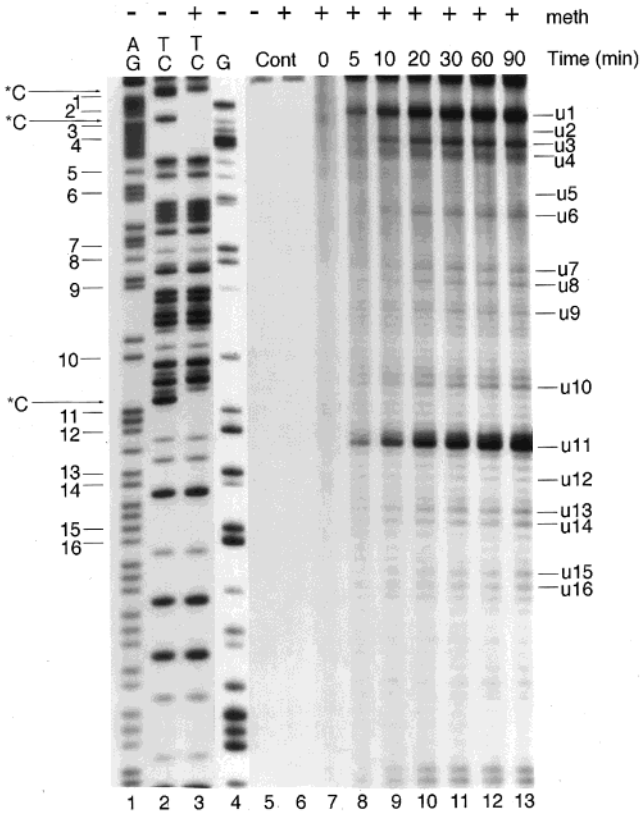

FIGURE 5: Time course of UvrABC cutting on CpG-methylated 188-bp *Stu*I−*Mse*I fragments of CHO APRT gene modified with MC. The 3′-end-labeled fragments were modified with MC (0.2 mM) and reacted with UvrABC for different time periods. The symbols and numberings are the same as in Figure 2. Similar results were obtained for MS-NMA (1.5 mM) modified DNA fragments (data not shown).

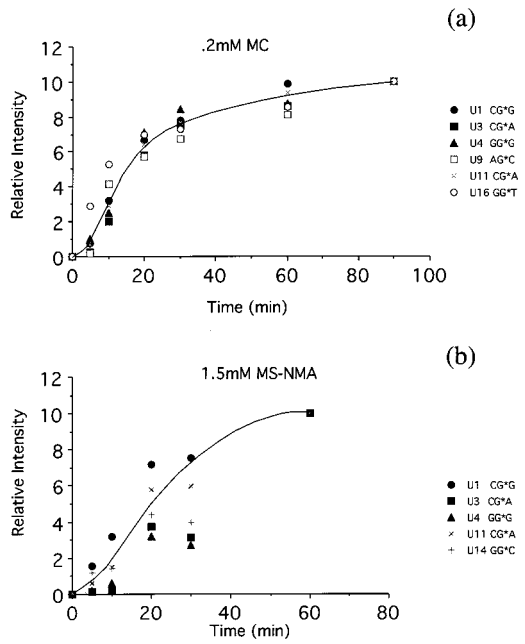

FIGURE 6: Kinetics of UvrABC cutting on CpG-methylated 188-bp *Stu*I−*Mse*I fragments of CHO APRT gene modified with MC (a) and MS-NMA (b). The intensities of drug-induced UvrABC incision bands in Figure 5 were quantified. Results of three CpG sites and three non-CpG sites were presented.

DNA structure is limited to the base pair of the methylated site. A 188-mer DNA fragment containing three CpG sites was used in this study; it remains unclear whether the particular DNA sequence and/or the multiple CpG sites in this DNA fragment result in the unusual drug−DNA interaction. We found that methylation at CpG sites affects interactions of MC and MS-NMA with non-CpG sites but does not affect DC-MC bonding at such sites. There are two possible explanations for these results. First, because DNA modification is less efficient for reductive DC-MC than for reductive MC, a higher concentration of DC-MC (0.45 mM) than MC (0.15 mM) was used for DNA modification. It is possible that modifications are saturated at both CpG and non-CpG sites at this concentration of DC-MC. Second, the C10 carbamoyl group of MC may reduce the affinity or the interaction of the drug with the C5-methylated CpG-induced DNA structure.

The mechanisms of how methylated DNA enhances drug−DNA bonding remain to be elucidated. Johnson et al. (*39*) have suggested that the nucleophilicity of the 2-amino group of the guanine increases as a result of pairing with methylated cytosine residues and this increase in nucleophilicity may enhance its interaction with electrophilic compounds. However, we have found that C5 cytosine methylation at CpG sites enhances guanine alkylation, not only at the exocylic amino group but also at C8 and N7 positions (*41*). The enhancement of alkylation at the N7 and C8 positions is unlikely to be due to a change of nucleophilicity resulting from base pairing with methylated cytosine. It has been shown that C5 cytosine methylation induces DNA structural changes, which include a cruciform extrusion from a double-helix structure, an increase in the helical pitch, and the conversion of DNA from the B to Z form (*42*, *43*). In addition, C5 cytosine methylation increases the hydrophobicity of its surrounding region by extending the methyl groups into the major groove of B-DNA (*44*). We suggest that these multiple effects induced by C5 cytosine methylation may result in methylated CpG sites being structurally more available for alkylation and guanines at non-CpG sequences being less available for alkylation than their unmethylated counterparts. Although MC does not bind to DNA, it has been shown that 2,7-diaminomitosene (2,7-DAM), a major product of the reducting activation of MC, intercalates in helix DNA; furthermore, this intercalation is modestly enhanced in methylated CpG DNA (*45*). If this drug intercalation is a prelude for drug−DNA bonding, then methylation-enhanced drug−DNA intercalation may increase drug−DNA covalent binding.

Cytosine methylation can influence chromatin structure (*46*, *47*), transcription factor binding (*48*, *49*), and deacetylation of histone proteins, and through these effects it ultimately plays a key role in governing gene expression (*44*) and differentiation (*49*−*51*) in mammalian cells. Of the estimated 45 000 CpG islands within mammalian DNA, most are associated with genes (*52*); the methylation pattern and status (hypo- or hypermethylation) of genomic DNA may lead to a methylation mosaic within a given cell type (*53*). It is conceivable that the methylation mosaic of a cell may in turn determine the drug sensitivity of this cell. If this is the case, then the particular methylation mosaic of a cancer cell may determine its susceptibility to MC treatment or to any drug whose DNA bonding efficiency is influenced by cytosine methylation.

In conclusion, our results demonstrate that C5 methylation leads to a significant change in sequence selectivity of MC-DNA modification that greatly enhances MC-DNA bonding at methylated CpG sites but reduces bonding at non-CpG sites. The results presented in this study, as well as other studies examining alkylating carcinogen−DNA interactions (*41*), clearly show that electrophilic reactive intermediates prefer methylated CpG over unmethylated CpG and guanine at other sequences. This study provides information about the MC−DNA interaction and may contribute to the future investigation of the role of epigenetics in cancer chemotherapy.

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