Effect of site-specific DNA methylation and mutagenesis on recognition by methylated DNA-binding protein from human placenta

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ABSTRACT

Methylated DNA-binding protein (MDBP) from human placenta is the first protein shown to bind specifically to certain DNA sequences only when they are methylated at cytosine residues. Among the sites recognized by MDBP is pB site 1, a pBR322-derived sequence which has a high affinity for MDBP when methylated at all CpG positions. We have substituted pB site 1 with 5-methylcytosine (m⁵C) residues at one to three of its CpG dinucleotides on one strand by the use of m⁵C-containing oligonucleotides. MDBP binds best when all three CpG dinucleotides in the region 5'-ATCGTCACGGCGAT-3' are methylated. Even more binding is obtained when both strands are methylated. Alteration of various residues in this binding site by oligonucleotide-directed mutagenesis decreased the binding. However, two mutations which increased the dyad symmetry of part of the binding site yielded ligands with a higher affinity for MDBP.

INTRODUCTION

Methylated DNA-binding protein (MDBP) from human placenta binds selectively in a sequence-specific manner to methylated DNA sequences (1,2). The dependence of this binding on the presence of 5-methylcytosine (m⁵C) residues in various ligands has been demonstrated by introducing these residues via nick translation or primer extension in the presence m⁵dCTP, by methylating CpG positions with human DNA methyltransferase (hDMT), or by comparing as ligands a naturally m⁵C-rich DNA (bacteriophage XP12 DNA) with m⁵C-poor DNAs (1,2). In the present study we assess the importance to MDBP binding of m⁵C residues at specified positions of a high-affinity binding site from pBR322, namely, pB site 1, which had been identified by DNase I footprinting (2). To precisely target DNA methylation at pB site 1, we have incorporated into this site synthetic oligonucleotides with m⁵C residues at given positions. We have also utilized oligonucleotide-directed mutagenesis to investigate the consequences on MDBP binding of mutagenizing various residues of pB site 1.

MATERIALS AND METHODS

Preparation of oligonucleotide primers. Oligonucleotides were synthesized using the standard deoxynucleoside phosphoramidites and 5'-methyl-deoxycytidine phosphoramidite and their 5'-termini were phosphorylated as previously described (3). These oligonucleotides had 13-21 nucleotides from part of pBR322's high-affinity MDBP site with m⁵C residues in specific positions (designated M) and/or one base mutated from the normal pBR322 sequence.

The sequences of these oligonucleotides with m⁵C residues and mutated bases underlined are: 8, GCTGATMTGCAMGGMGATTTA; 11, TMGTCACGGMGATTTATG; 12, TMGTCAMGGCGATTTATG; 18, GTCACGGMGATTT; 19, TGATCGTCAMGGCGA; 43, GTCAMGGMGATTTATG; 3, GTCATGGMGATTTATG; 6, GTCAMGAMGATTTATG; 7, GTCAMGTMGATTTATG; 14, TCGTCGMMGGMGATTTATG; 16, TCAMGGMAATTTATGC; 27, CTGATAGTCAMGGMGA; and 28, CTGATTGTCAMGGMGA. They were purified by electrophoresis in a 20% polyacrylamide gel containing 7 M urea and then desalted by reversed-phase chromatography (C-18 Sep-Pak; Millipore).

Formation of partially hemimethylated, non-mutant replicative form DNAs. Single-stranded template DNA was prepared by standard methods (3) from recombinant MpB-A2. This recombinant contains pB site 1, the high-affinity MDBP-binding site from pBR322, in a 298-base HinfI insert in phage M13mpl9 DNA (2). One microgram of phage DNA was annealed to 8 pmol of kinased oligonucleotide in 20 μ l of 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl at 55°C for 30 min and cooled slowly to room temperature. Polymerization in the presence of [α -³²P]dATP was performed and the covalently closed, locally hemimethylated replicative form (RF) DNA was purified as previously described (4) except that the carrier during the concentration step was 30 μ g of tRNA. The specific activity of the purified DNA was $\sim 10^7$ cpm/ μ g.

Isolation of site-specific mutant viral DNA. Heteroduplex RF was prepared as described above except that the primer for synthesis was one of the mutant oligonucleotides (primers 3, 6, 7, 14, 16, 27, or 28). The purified, covalently closed heteroduplex RF (5-10 ng) was transfected into DNA mismatch repair-deficient Escherichia coli ES553 (mutL) provided by E. Siegel (5); this host was used in order to increase the frequency of mutant phage (manuscript in preparation). Transfection was performed as described by Kramer et al. (6) except that fresh YT medium was added for overnight incubation. The resulting phage supernatant with a titer of $\sim 10^5$ phage particles per ml was plated on JM101 cells. Single plaques were screened for mutant MpB-A2 DNA by determining the sequence through the MDBP binding region of the recombinant phage DNA by the dideoxy sequencing method (7) with the "sequencing primer" (M13 penta-

decamer; New England Biolabs). The screened mutants had only the single expected mutation in the MDBP binding site. Single-stranded mutant DNA was isolated from plaque-purified mutant phage (3).

Formation of partially hemimethylated, homoduplex mutant replicative form DNAs. Mutant viral DNA template (1 μ g) was annealed to 8 pmol of primer which contained m⁵C residues as well as the corresponding mutant base (primer 3, 6, 7, 14, 16, 27, or 28). The annealed primer-template with its homoduplex mutant pB site 1 was replicated in vitro and relaxed, covalently closed RF (RF IV) molecules (4) were purified and concentrated as described above.

Analysis of the relative extent of binding by MDBP. Partially hemimethylated wild-type or homoduplex mutant RF IV was digested with RsaI followed by extraction with organic solvents and precipitation with ethanol. The radio-labelled restricted DNA (20 ng) was incubated with MDBP (the DEAE-cellulose fraction; 1,2) in the presence of 100 ng of MboI-digested M. luteus DNA under standard conditions for 15 min (2). The DNA-protein complex was passed through a nitrocellulose filter and MDBP•DNA complexes were eluted, dissociated, and subjected to electrophoresis on a 5% polyacrylamide gel and then to autoradiography as previously described (2). After autoradiography, the relative retention by MDBP of the 1026-bp DNA fragment containing pB site 1 from different mutant or wild-type RF preparations was quantitated by excising the 1026-bp band from the gel and measuring its Cerenkov radiation. Each MDBP binding experiment included DNA fragments from RF molecules synthesized on an MpB-A2 template with oligonucleotide 8 as the primer and also RF synthesized with a primer complementary to the M13mp19 vector (the above-mentioned sequencing primer). The former RF, which was hemimethylated at three positions of pB site 1, served as a reference for comparison of the differently substituted RF molecules. The latter RF, which was nonmethylated, was used to determine the background of nonspecific binding to be subtracted from the sample values.

RESULTS

The effect of site-specific DNA methylation on the binding of MDBP

We previously studied the binding of MDBP to hemimethylated pBR322-M13mp19 recombinants MpB-A2 and MpB-A3. Each of these has a different orientation of the pB site 1-containing pBR322 insert. That study indicated that replacement of all the C residues on one or the other strand of pB site 1 allows similar preferential binding by MDBP (2). There are three m⁵CpG dinucleotides in one strand of the hDMT-methylated pB site 1 sequence protect-

Table 1. Binding of MDBP to the wild-type pB site 1 substituted with m⁵C residues at specific positions in one strand.

Number of primer	Part of the binding sequence in the methylated strand ^a	Relative retention by MDBP ^b (%)
0 ^c	A T C G T C A C G G C G A T	0
8	A T <u>M</u> G T C A <u>M</u> G G <u>M</u> G A T	100
18	A T C G T C A C G G <u>M</u> G A T	<5
19	A T C G T C A <u>M</u> G G C G A T	<5
43	A T C G T C A <u>M</u> G G <u>M</u> G A T	16
11	A T <u>M</u> G T C A C G G <u>M</u> G A T	<5
12	A T <u>M</u> G T C A <u>M</u> G G C G A T	34

^aMethylation in one strand of pB site 1 in the pBR322-M13mpl9 recombinant MpB-A2 (2) was obtained by the use of m⁵C-containing synthetic oligonucleotide primers for synthesis of RF molecules. These RF molecules were prepared, purified, and restricted and MLBP assays were performed as described in Materials and Methods. Only part of the MDBP binding site is shown. The m⁵C residues are underlined.

^bBinding of the 1026-bp RsaI fragment from MpB-A2 RF containing pB site 1 methylated at defined CpG positions was measured relative to that of the analogous fragment containing three CpG dinucleotides supplied by primer 8. The extent of binding by the nonmethylated 1026-bp RsaI fragment obtained from RF primed with oligonucleotide 0 was subtracted as background. Its binding was no greater than that of the nonmethylated vector fragments.

^cOligonucleotide 0 refers to the commercially available pentadecamer sequencing primer which primes within the M13mpl9 vector sequences. The sequences of the other oligonucleotides, all of which were complementary to pB site 1, are given in Materials and Methods.

ed against DNase I cleavage by MDBP (2), namely, 5'-CTGATMGTCAMGMGATTATG-3' (M represents m⁵C). In order to determine the individual role of these three underlined, methylated CpG dinucleotides (numbered CpG-1,2, and 3 from left to right) in the strand shown above, site-specific methylated oligonucleotides were synthesized. These were homologous to part of pB site 1 and served as primers for the formation of MpB-A2 RF molecules. Primers 19 and 18 contain only one m⁵CpG at position CpG-2 or CpG-3, respectively. Primers 11, 12 and 43 contain two m⁵CpG sites in three different combinations (Table 1) and primer 8 has these three CpG nucleotides replaced by m⁵CpG. Use of these

Table 2. Binding of MDBP to mutated, hemimethylated forms of pB site 1^a.

Number of primer	Part of the binding sequence in the methylated strand	Relative retention by MDBP (%)
	Position number	
	1 5 10 14	
43 ^b	A T C G T C A <u>M</u> G G <u>M</u> G A T	16
6	A T C G T C A <u>M</u> G <u>A</u> <u>M</u> G A T	46
7	A T C G T C A <u>M</u> G <u>T</u> <u>M</u> G A T	<5
16	A T C G T C A <u>M</u> G G <u>M</u> <u>A</u> A T	<5
27	A T <u>A</u> G T C A <u>M</u> G G <u>M</u> G A T	<5
28	A T <u>T</u> G T C A <u>M</u> G G <u>M</u> G A T	15
8 ^b	A T <u>M</u> G T C A <u>M</u> G G <u>M</u> G A T	100
14	A T C G T C <u>G</u> <u>M</u> G G <u>M</u> G A T	<5
3	A T C G T C A <u>T</u> G G <u>M</u> G A T	202

^aThe use of the mutant oligonucleotide primers to obtain mutant viral DNA is described in Materials and Methods. Each of the mutant oligonucleotides was also used to prime synthesis of RF molecules from the analogous purified mutant viral DNA. The resulting homoduplex mutant, hemimethylated RF molecules were purified, restricted, and used to quantitate MDBP binding relative to that of the non-mutant RF containing primer 8 as described in the legend to Table 1. The number designations of the mutant primers in this table are also used to denote the corresponding mutant pB site 1. The m⁵C residues are underlined and the mutant bases overlined. Only part of the MDBP binding site is shown. Note that mutants 3, 6, and 14 have more dyad symmetry around the center of this 14-bp sequence than does the non-mutant DNA.

^bThe non-mutant RFs with incorporated primer 43 or 8 were included in these experiments for comparison to mutant RFs.

primers for RF synthesis yielded molecules specifically methylated in the above strand, the minus strand of MpB-A2 at pB site 1. An earlier study (3) and a comparison of the results from many of the RF preparations in this study (Tables 1 & 2) indicate that the intact synthetic primers were efficiently incorporated into the RF IV molecules.

The binding to MDBP of these variously methylated forms of pB site 1 was studied using RsaI digests of MpB-A2 RF DNA. RsaI releases the hemimethylated pB site 1 sequences in a 1026-bp fragment containing vector as well as pBR322 insert sequences (2). As shown in Fig. 1, when the RsaI digest of the primer

Fig. 1. Autoradiograph of DNA fragments retained by MDBP. Hemimethylated wild-type or homoduplex mutant RF DNA was prepared, digested with *RsaI*, and analyzed for which fragments were selectively retained as described in Materials and Methods. For this experiment ~ 1 unit of MDBP (2) was used. Lane 1, input *RsaI*-digested mutant 27 RF DNA (unincubated and unfiltered). Lanes labeled 14, 27, 28, 6, 7 & 18, MDBP-bound *RsaI* fragments from mutant RF DNA prepared with the correspondingly numbered primer (Table 2). Lanes labeled 8, 43, & 0, the analogous fragments from *RsaI* digests of wild-type 2 RF synthesized by using primer 8, 43, and or the sequencing primer, respectively (Table 1).

8-extended RF was used as a ligand for MDBP, only one fragment, the 1026-bp fragment, was preferentially retained by MDBP. As expected, the retention of the 1026-bp fragment containing three $m^5\text{CpG}$ dinucleotides in the minus strand was dependent upon DNA methylation (Fig. 1 & Table 1, the 1026-bp fragment containing primer 0 compared to that with primer 8). Methylation of only CpG-2 (using primer 19) or CpG-3 (using primer 18) in the minus strand was not sufficient to elicit any detectable MDBP-binding activity. When two $m^5\text{CpG}$ dinucleotides were in the minus strand the strength of binding depended upon the position of methylation (Table 1). Methylation of only CpG-1 and CpG-3 (primer 11) did not allow recognition of pB site 1 by MDBP but methylation at CpG-1 and CpG-2 (primer 12) gave a moderate-affinity ligand for MDBP. When CpG-2 and CpG-3 were methylated (primer 43), pB site 1 had an affinity for

MDBP intermediate to those of the other two doubly hemimethylated ligands.

The binding of pB site 1 methylated on both strands by human DNA methyltransferase (8) was compared to that of these hemimethylated molecules. MpB-A2 RF DNA having ~60% of its CpG dinucleotides methylated was digested by HinfI and end-labeled. The relative retention of this pB site 1-containing fragment by MDBP was twice that of the analogous fragment containing hemimethylated pB site 1 prepared by incorporation of primer 8 (with three m⁵CpG dinucleotides).

The effect of site-specific mutagenesis of a DNA ligand on the binding of MDBP

In order to evaluate the importance of the sequences neighboring the recognized m⁵CpG positions, oligonucleotides homologous to part of pB site 1 but with single base changes were synthesized. They were used for priming synthesis of MpB-A2 RF followed by isolation of mutant viral DNA as described in Materials and Methods. The binding of mutated forms of pB site 1 to MDBP was studied using RsaI digests of homoduplex mutant RF DNA synthesized from the mutant template DNA and the corresponding mutant primer. All of the mutant primers were methylated at positions CpG-2 and CpG-3 except for primer 3, which contained only a methylated CpG-3 (Table 2). As shown in Fig. 1 and Table 2, the following mutations in pB site 1 abolished detectable preferential binding to MDBP: G•C → T•A at position 10 (see Table 2) of mutant 7; G•C → A•T at position 12 of mutant 16; A•T → G•C at position 7 of mutant 14; and C•G → A•T on position 3 of mutant 27. Although a C•G → A•T change in the latter mutant resulted in the loss of specific binding, a C•G → T•A change at the same position in mutant 28 did not affect the binding (Fig. 1; Table 2, primers 43, 27, & 28). An even greater difference between mutants at the same position is seen in comparison of mutants 7 and 6 (Table 2). In mutant 7 a G•C → T•A transversion abolished detectable MDBP binding, whereas in mutant 6 a transition to A•T at the same position approximately tripled the specific binding activity (Fig. 1; Table 2, primers 43, 7, & 6). A dramatic increase in binding activity was seen when the C•G base pair at position 8 was changed to T•A in mutant 3 (Fig. 1, Table 2).

For three of the mutant primers, the effect of the mutation in a heteroduplex pB site 1 was determined. Heteroduplex DNA recognition sites for restriction endonucleases may or may not be recognized by the enzyme depending on the nature of the mismatch, its exact position, and the enzyme involved (3). In this experiment, wild-type rather than mutant MpB-A2 viral DNA was used as a template to synthesize heteroduplex mutant RF with the m⁵C-containing mutant oligonucleotides as primers. The 1026-bp fragment in the RsaI

digest from the purified RF, therefore, contained one mismatch as well as one or two m^5C residues (Table 2) at pB site 1. Despite the presence of a T·G mismatch at position 8 of pB site 1 in the heteroduplex containing primer 3 (Table 2), autoradiography revealed strong preferential binding of the 1026-bp fragment (data not shown). At pB site 1, this heteroduplex has one m^5CpG dinucleotide (at CpG-3) and a mismatch-containing TpG dinucleotide (substituting for CpG-2). Therefore, as in the case of the corresponding homoduplex mutant (Table 2), the C $\rightarrow$ T transition mutation next to a single m^5CpG converted this substituted pB site 1 into a high-affinity MDBP site. In contrast, a heteroduplex mutant pB site 1 with a G $\rightarrow$ A transition and two m^5CpGs (primer 6; Table 2), did not exhibit any selective binding to MDBP (data not shown) although the analogous homoduplex mutant did (Fig. 1 and Table 2). Lastly, a heteroduplex mutant pB site 1 was synthesized with primer 7 (Table 2), which contained two m^5CpG dinucleotides and a G $\rightarrow$ T transversion at position 10 (Table 2). It showed no selective binding either in the heteroduplex mutant state (data not shown) nor in the homoduplex form (Fig. 1 and Table 2). Furthermore, when all C residues of the minus strand of MpB-A2 outside the primer region were substituted with m^5C by incorporation of m^5dCTP instead of dCTP in the RF polymerization mixture, still no selective binding of pB site 1 was observed. In this experiment, the extensively hemimethylated RF was cleaved with TaqI instead of RsaI before use as a ligand so that the high degree of hemimethylation would not inhibit restriction (9).

DISCUSSION

The sequence-specific recognition of m^5CpG dinucleotide residues by MDBP was demonstrated in this study using synthetic oligonucleotides for site-specific DNA methylation and mutagenesis at pB site 1. This site, which is derived from pBR322, was previously shown to be recognized specifically by MDBP when it was methylated at all of its CpG dinucleotides on both strands or when methylated at all C residues on one or the other strand (2). In the case of the bifilarly CpG-methylated DNA, this site was localized by DNase I footprinting (2). Preliminary to the present study, this site was cloned in an M13mpl9 vector yielding recombinant MpB-A2 (2). This allowed insertion of m^5C -containing oligonucleotides into one strand by primer extension on viral DNA as a template.

By using these chemically synthesized oligonucleotides as primers, methylation was precisely targetted to 1-3 CpG positions on one strand of pB site 1 (Table 1). Methylation at only one of these positions (resulting in

one hemimethylated dinucleotide base pair) allowed little or no detectable binding by MDBP (Table 1). Methylation at two of the CpG sites on one strand gave a ligand with negligible to moderate affinity for MDBP depending on which two of the three sites were methylated (Table 1). Methylation at all three of these sites of this same DNA strand resulted in much better binding by MDBP (Table 1). The ability of MDBP to bind to pB site 1 is further improved when the DNA is bifilarly methylated at most CpG sites in a reaction catalyzed (2, 8) by human DNA methyltransferase (see Results). As expected, when all C residues outside pB site 1 on the minus strand of the RF were methylated by using mutant oligonucleotide 7 as a primer and m⁵dCTP rather than dCTP in the polymerization mixture, no MDBP binding was observed. Therefore, MDBP specifically recognizes methylation at pB site 1 and binds better to this site the more highly methylated it is at CpG sites.

The above mutant had a transversion mutation which converted the 5'-MGGMG-3' sequence to 5'-MGTMG-3' at positions 8-12 (Table 2) in the complementary strand of pB site 1 in recombinant MpB-A2. That the homoduplex mutant site (containing primer 7; Fig. 1 & Table 2) showed no detectable MDBP-specific binding whereas the identically methylated wild-type site (containing primer 43; Fig. 1 & Table 2) gave clearly demonstrable binding indicates the importance of this pentanucleotide sequence for the MDBP binding. Moreover, as discussed below, a transition mutation at the same position had a very different effect. This pentanucleotide contains two m⁵CpG dinucleotides whose m⁵C residues can be introduced in vitro or in vivo by a human DNA methyltransferase recognizing hemimethylated or unmethylated CpG sites (8,10,11). Therefore, the G residues of these CpG dinucleotides might have been necessary only for this enzymatically catalyzed methylation (2). However, mutagenesis at the 3' G residue of this pentanucleotide to give a site containing 5'-MGGMA-3' revealed that at least one of these G residues is recognized directly by MDBP (mutant 16; Table 2). It should be noted that we are using locally hemimethylated DNA for this analysis and that the effects of these mutations might not be as large in pB site 1 bifilarly methylated at all of its CpG dinucleotides.

That MDBP interacts with residues other than m⁵C was also seen at the A residue 5' to this pentanucleotide. A transition at this position abolished detectable MDBP binding in a homoduplex mutant with the sequence 5'-GMGGMG-3' at positions 7-12 in pB site 1 (mutant 14; Fig. 1 and Table 2). The importance of the A residue at position 7 of the minus strand of MpB-A2 might be partly due to an A residue on this strand specifying a T residue on the

opposite strand. As we previously postulated, T residues, which have a 5-methyl group identical to that of m⁵C, might be specifically recognized by MDBP at certain positions in the DNA ligand (2).

However, T and m⁵C residues do not behave equivalently at different positions. We compared as ligands for MDBP three forms of pB site 1 having m⁵C residues at positions 8 and 11 and one of the following dinucleotide base pairs at positions 3 and 4 shown in Table 2: CpG TpG, or MpG (in RF molecules containing primers 43, 28, and 8). The first two forms of pB site 1 showed virtually the same extent of MDBP binding, which was much less than that of the third form (Table 2). This result suggests that MDBP may make important contacts with both the 5-methyl group and the N⁴-amino group at residue 3. In contrast, replacement of the ^{MpG}_{GpC} with ^{TpG}_{ApC} at positions 8 and 9 (mutant 3; Table 2) had a surprising effect on MDBP binding. There was an increase of ~13 fold as a result of the m⁵C → T substitution in otherwise identically hemimethylated pB site 1 sequences. Therefore, a 5'-TGGMG-3' pentanucleotide functions better than a 5'-MGGMG-3' at pB site 1. This suggests that MDBP does not recognize the amino group of m⁵C at position 8 but rather forms a favorable contact with the 4-keto group of T (or with the A on the opposite strand) as well as interacting with the methyl group of m⁵C or T at this position.

The results of these studies on oligonucleotide-directed mutagenesis of this MDBP-specific site from pBR322 suggest that its sequence (in the fully CpG-methylated form) is close to an optimal ligand sequence. This conclusion is consistent with the hyphenated dyad symmetry observed in the 14-bp portion of pB site 1 depicted in Table 2. In mutant 3, the MpG → TpG transition increased the dyad symmetry of pB site 1 and, as indicated above, gave much higher affinity binding to MDBP. Also, in mutant 6, a G → A conversion increased the dyad symmetry of this 14-bp sequence and gave a better MDBP recognition site. It appears very likely that we will find high-affinity MDBP binding sites in the human genome, which contain CpG dinucleotides (in the methylated form) and dyad symmetry similar to those of pB site 1.

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