Methylated DNA-binding protein is present in various mammalian cell types

Prakash C. Supakar, David Weist, Daoling Zhang, Nilufar Inamdar, Xian-Yang Zhang, Rhana Khan, Kenneth C. Ehrlich¹ and Melanie Ehrlich

Department of Biochemistry, Tulane Medical School, New Orleans, LA 70112 and ¹Southern Regional Research Center, US Department of Agriculture, New Orleans, LA 70122, USA

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ABSTRACT

A DNA-binding protein from human placenta, methylated DNA-binding protein (MDBP), binds to certain DNA sequences only when they contain 5-methylcytosine (m⁵C) residues at specific positions. We found a very similar DNA-binding activity in nuclear extracts of rat tissues, calf thymus, human embryonal carcinoma cells, HeLa cells, and mouse LTK cells. Like human placental MDBP, the analogous DNA-binding proteins from the above mammalian cell lines formed a number of different low-electrophoretic-mobility complexes with a 14-bp MDBP-specific oligonucleotide duplex. All of these complexes exhibited the same DNA methylation specificity and DNA sequence specificity. From the extracts of rat and calf tissues, oligonucleotide-protein complexes formed that also had the same specificity as human placental MDBP although they had a higher electrophoretic mobility probably due to digestion by proteases in the nuclear extracts. Although MDBP activity was found in various mammalian cell types, it was not detected in extracts of cultured mosquito cells and so may be associated only with cells with vertebrate-type DNA methylation.

INTRODUCTION

A unique kind of protein that binds to certain DNA sequences containing 5-methylcytosine (m⁵C or M) but not to their unmethylated derivatives has been isolated from human placental nuclei (1). This protein has been termed methylated DNA-binding protein (MDBP). The highest-affinity site thus far identified for MDBP is an in vitro-methylated, m⁵CpG-containing pBR322 sequence, pB site 1, each of whose 14 base-pairs (5'-ATMGTACAMGGMGAT-3') is recognized by MDBP (2-5). Because of the novelty of the DNA methylation-dependence of this sequence-specific DNA-binding protein and the large amount of evidence indicating that tissue-specific differences in vertebrate DNA methylation are involved in controlling the expression of some vertebrate genes (6-13), we have looked for MDBP-like activity in other types of human cells, in rodent cells, and in calf thymus. This analysis involved a gel retardation assay with a 14 base-pair, pB site 1-containing oligonucleotide

as the ligand and a fragment selection assay employing nitrocellulose membrane-retention and electrophoresis of restricted, human DNA methyltransferase-methylated pBR322 DNA. We demonstrate that all of these mammalian cell populations have a DNA-binding protein which specifically recognizes methylated but not unmethylated wild-type pB site 1 just as human placental MDBP does. Furthermore, we show that this protein can bind to a mutated form of pB site 1 that is not methylated at C residues but only if certain of the m⁵CpG sequences at this site are replaced by TpG or TpA sequences.

MATERIALS AND METHODS

Preparation of MDBP fractions from tissues and cell cultures. MDBP was partially purified from a 0.3 M NaCl extract of human placental nuclei by phosphocellulose chromatography and hydroxylapatite chromatography (1). In a number of experiments, the crude nuclear extract was assayed or this extract was first precipitated with ammonium sulfate to 30% saturation and the pellet obtained after centrifugation was dissolved and dialyzed against 20 mM Tris-HCl, 0.1 mM Na-EDTA, 0.2 mM dithiothreitol, 20% glycerol, pH 7.6. This is referred to as the 30% (NH₄)₂SO₄ fraction. Rat spleen, lung, and liver and calf thymus samples were prepared similarly from 15 to 200 g of soft tissue except that, in the cases of rat liver and calf thymus, tissue stored at -70°C immediately after harvesting was used. Pepstatin A (1 µg/ml) and chymostatin (1 µg/ml) were present during extraction of nuclei and phenyl-methylsulfonyl fluoride (0.1 µM) was included in the homogenization buffer.

Also, a 30% (NH₄)₂SO₄ fraction was prepared from each of the following established cell lines: mouse LTK⁻ cells and human HeLa S3, embryonal carcinoma NT2/D1 (14), and CD4⁺ lymphoblastoid H-9 cells. These were propagated under standard conditions in roller bottles or multiple stationary bottles to late exponential phase in media containing 10% fetal bovine serum. Similarly, cultured mosquito cells (*Aedes albopictus*, clone C6/36; ATCC) were grown in stationary bottles but at ambient temperature. Approximately 0.3-4 x 10⁹ cells were harvested, rinsed, and nuclei released by homogenization in a Potter-Elvehjem homogenizer. Nuclear extracts were prepared as for placenta (1) except that filtration steps and the rinsing of the nuclear pellet were omitted.

Gel retardation assays. Assays were conducted with the following radiolabeled double-stranded oligonucleotides (5) containing the underlined m⁵C residues and the same MDBP-specific pB site 1 sequence:

<u>W17/C17</u>		<u>W9/C9</u>
5'-CTAGATMGTCAMGGMGAT-3'	and	5'-GCTGATMGTCAMGGMGATTTATGCMGCCTMGGMGA-3'
3'-TAGMAGTGMCGMTAGATC-5'		3'-CGACTAGMAGTGMCGMTAAATACGGMGGAGMCGMT-5'.

Also, the 298-bp HinfI DNA fragment from pBR322, which contains pB site 1, was used as a ligand after methylation with human DNA methyltransferase (2, 15). Nonmethylated counterparts were also used namely, W9u/C9u, which has the same sequence as W9/C9 except that all of its cytosine residues are unmethylated, and the mock-methylated 298-bp fragment, which had been incubated with methyltransferase in the absence of S-adenosylmethionine. The oligonucleotides were labeled at the 5' terminus of one strand with [γ - 32 P]ATP in the presence of T4 polynucleotide kinase and then annealed with a five-fold excess of the complementary strand. To perform the assays, MDBP fractions were first incubated at ambient temperature with 500 ng each of unlabeled poly(dI)·poly(dC) and MboI-digested Micrococcus luteus DNA in 10% glycerol, 0.2 mM Na-EDTA, 5 mM MgCl₂, 2 mM dithiothreitol, 80 mM NaCl, 10 mM Tris-HCl (pH 7.6) for 10 min. The radiolabeled ligand was then added, incubation of the 30 μ l sample continued for 15 min, and then the samples were immediately electrophoresed at ambient temperature on a 6% polyacrylamide gel and autoradiographed (16). Electrophoresis was carried out in 22 mM Tris base, 22 mM boric acid, 0.5 mM Na-EDTA except that 25 mM Tris base, 192 mM glycine, 10 mM Na-EDTA was used for Fig. 1A. When additional unlabeled competing DNAs or oligonucleotides were used, they were added at the same time as the poly(dI)·poly(dC) and the M. luteus DNA.

Fragment selection assays and DNase I footprinting. These were performed, as previously described (2), with human DNA methyltransferase-methylated pBR322 DNA digested with HinfI and HindIII. Also, an M13mp19/pBR322 recombinant (MpB-A2) containing pB site 1 was subjected to site-specific mutagenesis and methylation (2, 4) followed by digestion with RsaI. Then, the digested replicative form (RF) DNA was used for fragment selection assays. The fragment selection assay involves incubating a radiolabeled restriction digest of a small DNA molecule with a protein fraction, filtration through a nitrocellulose membrane to obtain membrane-bound DNA·protein complexes, dissociation of DNA from these complexes, and gel electrophoresis of the DNA fragments to assess which had been preferentially bound to protein. DNase I protection experiments (footprinting) were done on the methylated 214-bp, AluI/HinfI fragment of pBR322 methylated with human DNA methyltransferase as previously described (2) except that the samples were filtered through a nitrocellulose membrane after DNase I treatment and the DNA in the membrane-bound fraction was analyzed.

RESULTS

Detection of MDBP activity in nuclear extracts of various types of mammalian cells by the gel retardation assay

MDBP activity in human placental nuclei fractions was initially determined with a nitrocellulose filter-binding assay using a mixture of ^3H -labeled DNA and ^{32}P -labeled DNA, one highly methylated and the other not (1). Greater sensitivity was provided by the fragment selection assay (2, 3), in which a restriction digest of methylated DNA is incubated with MDBP and the nitrocellulose filter-retained restriction fragments are dissociated from protein and visualized by gel electrophoresis and autoradiography to reveal the enrichment or selection of MDBP-specific DNA fragments (3). However, less interference by other contaminating DNA-binding proteins was seen in the gel retardation assay (5). This involves visualizing specific complexes between a DNA-binding protein and a small, purified restriction fragment or duplex oligonucleotide containing the protein's recognition sequence and showing that excess nonspecific DNA is a poor competitor for the binding reaction unlike an excess of the specific DNA ligand (16-18). The gel retardation assay was previously used on the hydroxylapatite fraction of human placental MDBP to demonstrate DNA sequence-specific complex formation with W17/C17, a 14-bp m^5CpG -containing duplex (5). This oligonucleotide duplex has a high-affinity MDBP site (pB site 1) derived from pBR322 (2, 5). Fig. 1A indicates that the gel retardation assay with this ligand yields a similar set of closely spaced bands of low-mobility oligonucleotide-protein complexes from the crude nuclear extract, the phosphocellulose fraction, and the more enriched hydroxylapatite fraction. However, as the partial purification proceeded, there was a decrease in the amount of the slowest-moving complexes and an increase in somewhat higher-mobility complexes electrophoresing as a smear (Fig. 1A). This suggests partial proteolysis of MDBP by contaminating proteases. Nonetheless, the formation of all the DNA-protein complexes obtained with W17/C17 and human placental MDBP fractions, including the complexes electrophoresing as a smear, was strongly competed by an excess of MDBP-specific ligands and not by non-specific DNA (Fig. 4A). No MDBP-DNA complexes were seen with single-stranded W17 or C17 as the ligand (5; and data not shown).

Almost all of the human placental MDBP activity in the crude nuclear extract precipitated when $(\text{NH}_4)_2\text{SO}_4$ was added to 30% saturation; in contrast, only 10% of the total nuclear protein precipitated under these conditions. Therefore, to look for MDBP activity in other mammalian cell populations,

Fig. 1. Visualization of specific MDBP·oligonucleotide complexes in human and rodent nuclear extracts. MDBP activity was detected by the gel retardation assay with 10 fmol of ^{32}P -labeled W17/C17 as described in *Materials and Methods*. This ligand is methylated and contains pB site 1 in its 14-bp duplex. Except in panel A, gel 2, the uncomplexed oligonucleotide was electrophoresed off the gel to afford better resolution of the complexes. In panel A, gel 1, MDBP activity in the crude nuclear extract (CE) from human placenta, which was obtained by incubating nuclei with a buffer containing 0.3 M NaCl, was compared to that in the phosphocellulose peak fraction (PC) or the subsequent hydroxylapatite fraction (HA). The arrows indicate the three major MDBP·DNA complexes. In panel A, gel 2, electrophoresis on the assay mixture containing the placental crude extract was carried out for a shorter time to show the relative position of MDBP·DNA complexes (C) and free duplex oligonucleotide (F). The band below the free duplex is the single-stranded form of the labeled oligonucleotide. In panel B, the MDBP activity in the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction of a different but analogous preparation of crude nuclear extract from placenta was similarly compared to the analogous fraction from murine LTK⁻ cells (L), human HeLa cells, embryonal carcinoma (EC) cells, or H9 cells, and rat spleen and lung. For panel B, three discrete bands of the same relative intensity and in the same position were seen in the HeLa and LTK⁻ samples in the region of the original autoradiogram that appears as a dark smear in the photograph. Bands at the same position but of different relative intensities were seen in the human placental, EC, and H9 samples in this autoradiogram.

this gel retardation assay with ^{32}P -labeled W17/C17 was applied to the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction of various nuclear extracts. Bands of protein·oligonucleotide complexes were seen in this fraction from mouse LTK⁻ cells, HeLa cells, human embryonal carcinoma (EC) cells, a human lymphoblastoid cell line (H-9), rat spleen and rat lung. The gel electrophoresis profiles of these

Fig. 2. Determination of the specificity of MDBP activity in LTK⁻ cells by the use of various competitor DNAs in the gel retardation assay. Gel retardation assays were performed with the 30% (NH₄)₂SO₄ fraction of LTK⁻ cells as in Fig. 1. The radiolabeled ligands that contained a methylated, pB site 1 were W17/C17, a 14-bp duplex, (panel A) and W9/C9, a 35-bp duplex, (panel B). They were used as described in Materials and Methods. For the indicated sample in panel B, the unmethylated form of W9/C9 (W9u/C9u) was the radiolabeled ligand. In addition to the standard 500 ng each of poly(dI)·poly(dC) and *M. luteus* DNA, the following DNA was included in the pre-incubation mixture as competing DNA (Comp. DNA): 2 pmol of W9u/C9u or W9/C9; an additional 600 ng of *Mbo*I-digested *M. luteus* DNA; 1.5 µg of pBR322 DNA; or 400 ng of native XP12 DNA (panel B) or either native (ds) or denatured (ss) XP12 DNA (panel A). The denatured XP12 DNA probably underwent a small degree of renaturation due to the extraordinarily high T_m (43) of this m⁵C-rich DNA. Arrows in panels A and B indicate the positions of four distinct bands of MDBP-specific complexes that could be seen in the original autoradiogram. The triangles indicate the complexes that do not show the specificity of MDBP.

complexes from HeLa and LTK⁻ cells were almost identical whether W17/C17 (Fig. 1B) or the closely related, methylated pB site 1-containing 35-bp W9/C9 (data not shown) was used. They were also similar to those of human placenta except that more of the ligand was seen in somewhat lower-molecular-weight complexes in the placental sample (Fig. 1B), which may be due to more degradation in the tissue-derived sample than in the HeLa and LTK⁻ cell samples. The human EC and H-9 samples gave bands of protein·oligonucleotide complexes with similar mobilities but different relative intensities (Fig. 1B). In contrast, the complexes formed from the corresponding rat spleen and

lung samples had a considerably higher electrophoretic mobility (Fig. 1B). Similar results were obtained with an analogous calf thymus $(\text{NH}_4)_2\text{SO}_4$ fraction (data not shown).

We first examined whether the protein in the complexes observed in the reaction mixtures containing the LTK⁻ cell extract had the same methylation specificity as does human placental MDBP. The formation of all of these W17/C17-containing complexes was competed with a 200-fold molecular excess of the unlabeled, MDBP-specific, CpG-methylated 35-bp W9/C9 (Fig. 2A). In contrast, the analogous unmethylated duplex (W9u/C9u) gave no detectable competition under the same conditions (Fig. 2A). Similarly, native m⁵C-rich phage XP12 DNA, a very good ligand for human placental MDBP (1), was a very effective competitor of complex formation with the murine extract but unmethylated pBR322 and denatured XP12 DNA were not (Fig. 2A). Low-mobility complexes with the methylation specificity of human placental MDBP were also seen when radiolabeled W9/C9 was used as the ligand instead of radiolabeled W17/C17 but not when the unmethylated, radiolabeled analog, W9u/C9u, was used (Fig. 2B). However, with the radiolabeled methylated 35-bp W9/C9, several additional higher-mobility bands were observed (Fig. 2B). These bands were also seen when unmethylated W9u/C9u was the radiolabeled ligand and their formation was not competed specifically by methylated double-stranded DNA (Fig. 2B and data not shown). Complexes that were competed similarly by MDBP-specific ligands and the analogous unmethylated ligands or single-stranded DNA were never seen with radiolabeled W17/C17 (Fig. 2A). We conclude that these higher-mobility bands obtained with W9/C9 as the radiolabeled ligand contain proteins other than MDBP. The similarity of human placental MDBP and the corresponding murine activity was also confirmed by gel retardation assays revealing low-mobility complexes with a 298-bp fragment of pBR322 containing pB site 1 that was methylated with human DNA methyltransferase but not with the mock-methylated DNA fragment (data not shown). Also, gel retardation assays with analogous rat or bovine tissue extracts and radiolabeled pB site 1-containing ligands in the presence of excess unlabeled specific or non-specific competitor DNAs gave results similar to those seen for the LTK⁻ extracts except that the complexes had a higher electrophoretic mobility (Fig. 1B and data not shown).

MDBP in mammalian nuclear extracts and phosphocellulose chromatography fractions analyzed by the fragment selection assay

The fragment selection assay is useful for indicating the sequence-specificity of a DNA-binding protein by demonstrating its preferential retention of one restriction fragment compared to that of others in a digest

Fig. 3. Determination of the specificity of MDBP activity from various mammalian cell populations by the fragment selection assay. An end-labeled, *HinfI/HindIII* digest of CpG-methylated pBR322 DNA was used as the ligand in fragment selection assays (see Materials and Methods) on the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction of various nuclear extracts or phosphocellulose chromatography fractions derived from these extracts. For comparison to the other lanes, the first two lanes in panel A contained aliquots of the *HinfI/HindIII* digest that had been neither incubated with MDBP fractions nor filtered through nitrocellulose. In the other lanes of panel A, human placental, EC cell, LTK⁻ cell, and rat spleen $(\text{NH}_4)_2\text{SO}_4$ fractions were used. In panel B, phosphocellulose chromatography fractions (1), fractions 13-15 eluting at ~ 0.12 to 0.22 M KCl, were used. The 298-bp, MDBP-specific DNA fragment containing pB site 1 is noted as well as a 603-bp fragment that contains a lower-affinity MDBP recognition site (pB site 2). The extra bands near the top of the gel in the samples containing the human placenta or LTK⁻ fractions are probably residual complexes of DNA-binding proteins and radiolabeled fragments which were not dissociated by detergent treatment and phenol extraction.

of a plasmid or viral DNA. Because placental MDBP has a high affinity for one fragment (298 bp) in *HinfI/HindIII* digests of human DNA methyltransferase-methylated pBR322 DNA, the fragment selection assay performed on *HinfI/HindIII* digests of methylated versus unmethylated pBR322 can be highly diagnostic of MDBP activity. Indeed, as seen in Fig. 3A, incubation of the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction of nuclear extracts of human EC cells, mouse LTK⁻ cells, and rat spleen as well as of human placenta gave a large extent of enrichment of the 298-bp, pB site 1-containing fragment, which was dependent on its methylation. Similar methylation-dependent selection of the 298-bp fragment by analogous extracts of rat lung, rat liver, human H-9 cells, HeLa cells, and calf thymus was observed (Table 1 and data not shown). In assays with these extracts, the 298-bp fragment was the only one that

Table 1. MDBP activity in nuclear extracts from different animal tissues and cells

Tissue or cell type	Wt. of tissue or No. of cells	Units of activity ^a	Amount of protein ^b (mg)	Sp. activity (units/mg)	Binding Ratio ^c XP12 [³ H]DNA + <i>M. luteus</i> [³ P]DNA	
					In absence of competitor DNA	In presence of <i>M. luteus</i> DNA
Human placenta	560 g	563	13	44	1.2	3.6
Human EC cells	0.3 x 10 ⁹ cells	6	0.25	24	3.0	9.6
HeLa	1.3 x 10 ⁹ cells	24	1.2	20	-	-
LTK ⁻ cells	3.9 x 10 ⁹ cells	53	2.4	22	1.4	5.3
Rat spleen	15 g	34	0.46	74	1.4	5.2
Rat lung	30 g	16	0.35	46	1.0	3.3
Rat liver	201 g	9	2.61	3	-	-

^aThe activity in the fraction of nuclear extract that precipitated with 30% saturation $(\text{NH}_4)_2\text{SO}_4$ was determined by the fragment selection assay with ³²P-labeled *HinfI/HindIII*-digested, methylated pBR322. This ammonium sulfate fraction contains most of the activity of the crude nuclear extract. One unit of activity is defined as the amount of MDBP that binds to 1 fmol of the 298-bp *HinfI* fragment of methylated pBR322 in 15 min at 24° C (3, 5).

^bThe amount of protein that precipitated with 30% saturation $(\text{NH}_4)_2\text{SO}_4$ was assayed with the method of Bradford (42) with bovine serum albumin as a standard.

^cThe ratio of binding XP12 [³H]DNA and *M. luteus* [³²P]DNA in a double-label experiment was determined in a nitrocellulose filter-binding assay (1) in the absence of unlabeled DNA or in the presence of 800 ng of unlabeled *M. luteus* DNA to depress binding of non-specific DNA-binding proteins to the radiolabeled DNA.

showed reproducibly much more binding when methylated. The fragment selection assay revealed somewhat similar specific activities of MDBP in these extracts except for rat liver, which probably had unusually low activity because of the instability of its MDBP activity upon storage, in contrast to the stability of the other rodent samples. The specificity of the MDBP activity was also checked by comparing binding to naturally m⁵C-rich XP12 DNA and to *M. luteus* DNA in a double-label nitrocellulose filter-binding assay (1,3). In the presence of sufficient unlabeled *M. luteus* DNA as a non-specific competitor (3), preferential binding to XP12 DNA was observed in all of the tested mammalian extracts (Table 1).

When the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction of LTK⁻ or rat spleen extracts was subjected to phosphocellulose chromatography, the MDBP activity peaked at approximately 0.12-0.22 M KCl as does the human placental MDBP (1). There was much less interference from non-specific DNA-binding proteins in these fractions than in the 30% $(\text{NH}_4)_2\text{SO}_4$ fraction (Fig. 3). Similar results were obtained upon phosphocellulose chromatography of the rat spleen extract (Fig. 3B). In fraction 13 from both extracts, some DNA methylation-dependent selection of the 603-bp *HinfI/HindIII* fragment of pBR322 was also seen

although no fragments other than the 298-bp and 603-bp showed significant increases in binding upon methylation (Fig. 3B). This fragment contains a site (pB site 2) that exhibits considerable homology to pB site 1 and is recognized by MDBP but with a lower affinity (3, 5). Binding to pB site 2 has been seen only in chromatography fractions of human placental MDBP (3) and not in crude extracts (Fig. 3). Also, the ratio of binding of MDBP to pB site 2 compared to pB site 1 is higher in hydroxylapatite and DEAE-cellulose peak fractions than in the less pure phosphocellulose fractions although binding to pB site 1 always predominates (P. Supakar and R. Wang, unpubl. results). The peak phosphocellulose fractions from both LTK⁻ cells and rat spleen extracts were used for DNase I footprinting of pB site 1 (2). Although only a small amount of MDBP activity was available in these fractions, weak footprints (data not shown) were seen in the same pB site 1 region protected by human placental MDBP (2).

Examination of mosquito nuclear extracts for MDBP

Mosquito cells were examined for the presence of MDBP because they exemplify higher eukaryotic cells which, in contrast to all examined vertebrate cells (19), probably contain very little or no m⁵C in their genomes (20, 21). With W17/C17 as a ligand, a nuclear extract from cultured mosquito cells gave a prominent, high-mobility band in a gel retardation assay, which did not show sequence-specific binding in competition experiments and appears to prefer single-stranded oligonucleotide (Fig. 4A) unlike human, rodent, and bovine MDBP.

When the mosquito extract was incubated with the MDBP-specific 35-bp W9/C9 instead of the 14-bp W17/C17, many different oligonucleotide-protein bands were observed in the gel retardation assay (Fig. 4B). One of these, the major low-mobility band (Fig. 4B), was competed somewhat better with a 200-fold molecular excess of MDBP-specific, methylated ligands than with their unmethylated, nonspecific counterparts; however, the differences were much less than those observed for human MDBP (Fig. 4B) or rodent MDBP (Fig. 2). Also, unlike the situation for these mammalian proteins, the mosquito protein in this complex, was competed by single-stranded W9 slightly better than by double-stranded W9u/C9u (Fig. 4B). Furthermore, an excess of poly(dG-m⁵dC)·poly(dG-m⁵dC) competed well for formation of this complex involving a mosquito protein but not for formation of the human MDBP·W17/C17 complex (data not shown).

We also tested an m⁵C-containing duplex that is not a ligand for MDBP,
 5'-CTAGAMGGMGATTATGC-3'
 W1/C1, whose sequence is 3'-TCMCGMTAAATACGGATC-5'. This duplex did form a

Fig. 4. Search for MDBP activity in mosquito cell extracts. Gel retardation assays were performed on a crude nuclear extract of cultured mosquito cells or on the analogous extract of human placenta using W17/C17 as the radio-labeled ligand in panel A and W9/C9 in panel B. The indicated competitors (Comp. DNA) were present in the pre-incubation mixture as in Fig. 2. In two samples of panel B, 6 pmol of unlabeled oligonucleotide duplex competitor (W9u/C9u and W9/C9) was used instead of the standard 2 pmol. Also, 800 ng each of XP12 DNA and of *M. luteus* DNA was used as the additional competitor DNA for the indicated samples in panel B. The triangle in panel B denotes the mosquito protein·oligonucleotide complex whose formation was only weakly competed by W9/C9 and XP12 DNA.

low-mobility complex similar to that described above with the mosquito extract but only if the 14-bp duplex was first self-ligated to give larger duplexes (data not shown). In contrast, human MDBP does not bind to W1/C1 even after ligation. Therefore, although the protein in this complex binds more readily to certain methylated ligands than to their non-methylated counterparts, this preference is much weaker than that of MDBP and does not have MDBP's sequence-specificity.

Use of MpG + TpG mutants of pB site 1 as ligands.

We previously showed that unmethylated pB site 1 containing a single C → T mutation at its third CpG dinucleotide in the upper strand (MpB-A4, Table 2) does not specifically bind to MDBP (5) and that an m⁵C → T transition at the first CpG dinucleotide of this site (MpB-A28, Table 2) greatly decreases binding (4). Using human placental MDBP, we tested the effect of a C → T mutation at the nearby second CpG dinucleotide in this

Table 2. Binding of MDBP to the hemimethylated or unmethylated form of pB site 1 with C + T mutations

Template	Number of primer ^b	The binding sequence in the methylated strand ^a				Relative retention by MDBP (%)
		Position number				
		1	5	10	14	
MpB-A2	8	A	T	<u>M</u>	G T C A <u>M</u> G G <u>M</u> G A T	100
MpB-A28	28	A	T	<u>T</u>	G T C A <u>M</u> G G <u>M</u> G A T	15
MpB-A2	43	A	T	C	G T C A <u>M</u> G G <u>M</u> G A T	16
MpB-A2	0	A	T	C	G T C A C G G C G A T	0
MpB-A4	0	A	T	C	G T C A C G G <u>T</u> G A T	<5
MpB-A3	0	A	T	C	G T C A <u>T</u> G G C G A T	48
MpB-A74	0	A	T	C	G T C A <u>T</u> <u>A</u> G C G A T	34
MpB-A75	75	A	T	C	G T C A <u>T</u> G G <u>T</u> G A T	88
MpB-A3	3	A	T	C	G T C A <u>T</u> G G <u>M</u> G A T	144

^a RF M13 recombinant DNAs were synthesized *in vitro* and contained an unmethylated or a hemimethylated, wild-type (MpB-A2) or mutant (MpB-A3, 74, or 75) pB site 1 in a 238-bp pBR322 insert (2, 4). The 5' to 3' sequence of pB site 1 is given for the strand which was locally methylated at this site. 5-Methylcytosine residues are underlined and mutant bases overlined. These RF DNAs were prepared by extending a primer complementary to part of pB site 1 and annealed to the appropriate template as previously described (4). Site-specific hemimethylation of pB site 1 was obtained by using ¹⁴C-containing primers. The relative retention of the resulting pB site 1 variants compared to that of wild-type triply methylated pB site 1 was determined by the fragment selection assay on *Ksa*I-digested DNA (4, 5). The data for MpB-A28 with primer 28, MpB-A2 with primer 43, and MpB-A4 with primer 0 were previously described (4, 5) and are included for comparison.

^b Oligonucleotide 0 refers to the commercially available pentadecamer sequencing primer which was used to obtain unmethylated pB site 1 sequences in the resulting RF (4). The primers were previously described (4) except for primer 75 whose sequence is as follows: 5'-TCATGCTGATTATG-3'.

strand in an M13 recombinant RF DNA containing pB site 1 (Table 2). Surprisingly, that single C + T mutation did convert pB site 1 into a methylation-independent binding site (MpB-A3 with primer 0, Table 2). MDBP binding was increased when the third CpG (position 11) in this C + T mutant was methylated (MpB-A3 with primer 3, Table 3) or, to a lesser extent, when an additional C + T mutation was introduced at this third CpG site (MpB-A75, Table 2). No further increase in binding was seen when the second CpG site is doubly mutated to a TpA site (MpB-A74, Table 2). The above studies involved site-specific methylation of only one strand. When unmethylated mutant MpB-A3 with a single C + T mutation (Table 2) was subjected to bifilar methylation catalyzed by human DNA methyltransferase, there was a nine-fold increase in human placental MDBP binding compared to the mock-methylated mutant (data not shown). A mutant pB site 1 with M + T transitions at all three positions of the upper strand was bound specifically by the 30%

(NH₄)₂SO₄ fractions of mouse LTK⁻ cells, rat lung, and calf thymus as well as by the analogous fraction of human placenta (unpubl. results).

DISCUSSION

As described in this report, an MDBP-like activity similar to that of human placenta (1-4) is present in various cultured human cells (HeLa, embryonal carcinoma, and H-9 lymphoblastoid cells), in rodent cells and tissues (murine LTK⁻ cells, rat lung, spleen, and liver), and in calf thymus. This sequence-specific DNA-binding activity was detected using a high-affinity binding site originally identified in human DNA methyltransferase-methylated pBR322 DNA (2), namely, pB site 1 (Table 2). This human DNA methyltransferase methylated only CpG sites (2), the predominant methylated dinucleotide sequence of vertebrate DNA (19). The mammalian extracts examined in the present study bind specifically to the 14-bp oligonucleotide duplex W17/C17, which contains pB site 1 with its three m⁵CpG dinucleotide pairs. Human placental MDBP recognizes all 14 bp of pB site 1 (3-5). MDBP activity in each of the studied mammalian nuclear extracts forms a number of different complexes with W17/C17 (Fig. 1A). Formation of all of these complexes was competed by oligonucleotide duplexes containing methylated pB site 1 or by naturally m⁵C-rich phage XP12 DNA (34 mol% m⁵C; 22, 23) but not by nonspecific DNAs or an oligonucleotide duplex with unmethylated pB site 1 (Figs. 2 and 4A). The electrophoretic mobilities of these complexes of W17/C17 and MDBP from mouse cells and various human cells were similar (Fig. 1B). The analogous complexes from rat tissue and calf thymus samples had a higher electrophoretic mobility (Fig. 1B and data not shown) as will be discussed below.

In addition, all of the above samples bind preferentially to the pB site 1-containing 298-bp fragment in a HinfI/HindIII digest of pBR322 DNA but only if that DNA had been methylated at CpG dinucleotide sites (Fig. 3). Further evidence for the rodent cells having human placental-type MDBP activity is that the LTK⁻ and rat spleen activities were shown to elute from a phosphocellulose column at the same salt concentration as human placental MDBP and to specifically protect the pB site 1 region (2) in a CpG-methylated pBR322 fragment against DNase I (data not shown). In contrast to the mammalian extracts, cultured mosquito cell extracts did not display an activity with a strongly methylation-dependent and sequence-dependent binding to CpG-methylated pB site 1 (Fig. 4). However, a DNA-binding activity was seen in mosquito cell extracts with a weak preference for methylated DNA

sequences (Fig. 4B), which is probably physiologically irrelevant because mosquito cells naturally contain relatively unmethylated DNA (20, 21). Similarly, methylation in vitro of the internal C of GGCC sequences in simian virus 40's T-antigen recognition site increased binding of the T-antigen almost two-fold (24) although there is no evidence that this non-CpG methylation of SV40 DNA, which is normally completely unmethylated (25), is of any direct physiological significance.

The complexes of W17/C17 and MDBP in rat tissue and calf thymus extracts had a higher electrophoretic mobility than those of mouse LTK⁻ cells or human cells although their methylation specificity and sequence specificity were indistinguishable (Figs. 1B and 3 and data not shown). We propose that this is due to limited proteolysis during preparation or storage of the extracts without much loss of DNA sequence-specific binding, as suggested for an oviduct transcription factor (26). In support of this explanation, we found that human MDBP, like a murine CCAAT box-binding protein (27), retained considerable specific DNA-binding activity after partial proteolysis (unpublished observations). Also, human placental MDBP·DNA complexes increased somewhat in electrophoretic mobility during partial purification (Fig. 1A). This may be due to limited degradation of MDBP by contaminating proteases without elimination of the specific binding activity.

Many eukaryotic sequence-specific DNA-binding proteins appear to function in regulating gene expression (28-33). This regulation can involve repressor binding sites as well as transcription activator sequences (34-36). Sequence-specific DNA-binding proteins from mammalian cells may also be involved in other functions, such as DNA replication (37) or recombination (38) or even in multiple functions (39). Elucidation of the function of MDBP awaits isolation and characterization of mammalian MDBP sites. However, studies of prokaryotic MDBP sites indicate that MDBP should recognize certain mammalian DNA sites only when they are methylated at CpG dinucleotides. In pBR322 DNA and M13mp8 replicative form DNA, a total of six MDBP sites were found and all of these required 5-methylation of C residues for any detectable specific binding to MDBP (3, 5). Of all of these sites, pB site 1, has the highest affinity for MDBP and was studied in the most detail (2-5). The more of its six CpG dinucleotides that are methylated in the two strands of this 14-bp site, the higher its affinity for MDBP (4). We have shown here that conversion of the middle MpG in the upper strand of pB site 1 to a TpG generated an MDBP site in the absence of cytosine methylation (Table 2). However, methylation of the other CpG's of this

mutant pB site 1 greatly increased binding (Table 2 and Results). The effects of methylation versus mutation to TpG are not the same at the three CpG's on the upper strand of pB site 1; for example, only at the second CpG did a CpG to TpG transition confer methylation-independent binding (Table 2). At some locations in pB site 1, MDBP prefers m⁵C and at others T; a combination of C → T transitions and C methylation at CpG dinucleotides of pB site 1 gave maximal binding.

In this study, we show that whatever functions MDBP performs, they are not species-specific as in the case of certain enhancer-binding proteins (40). Also, MDBP was found in a wide variety of cell types (Fig. 1B) including pluripotent embryonal carcinoma cells capable of differentiation in vitro (14, 41). However, MDBP may bind differentially to certain of its recognition sites in a cell-specific fashion that parallels the tissue-specific methylation pattern of those sequences. Other of its in vivo-binding sites may contain T residues instead of m⁵C residues at critical positions or may be methylated in all tissues and thus provide constitutive MDBP sites.

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