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The Major Histocompatibility Complex Class II Promoter-Binding Protein RFX (NF-X) Is a Methylated DNA-Binding Protein

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A mammalian protein called RFX or NF-X binds to the X box (or X1 box) in the promoters of a number of major histocompatibility (MHC) class II genes. In this study, RFX was shown to have the same DNA-binding specificity as methylated DNA-binding protein (MDBP), and its own cDNA was found to contain a binding site for MDBP in the leader region. MDBP is a ubiquitous mammalian protein that binds to certain DNA sequences preferentially when they are CpG methylated and to other related sequences, like the X box, irrespective of DNA methylation. MDBP from HeLa and Raji cells formed DNA-protein complexes with X-box oligonucleotides that coelectrophoresed with those containing standard MDBP sites. Furthermore, MDBP and X-box oligonucleotides cross-competed for the formation of these DNA-protein complexes. DNA-protein complexes obtained with MDBP sites displayed the same partial supershifting with an antiserum directed to the N terminus of RFX seen for complexes containing an X-box oligonucleotide. Also, the in vitro-transcribed-translated product of a recombinant RFX cDNA bound specifically to MDBP ligands and displayed the DNA methylation-dependent binding of MDBP. RFX therefore contains MDBP activity and thereby also EF-C, EP, and MIF activities that are indistinguishable from MDBP and that bind to methylation-independent sites in the transcriptional enhancers of polyomavirus and hepatitis B virus and to an intron of c-*myc*.

Methylated DNA-binding protein (MDBP) is a sequence-specific DNA-binding protein that is ubiquitous in mammalian cells and binds preferentially to certain DNA sequences when they are CpG methylated (10, 16, 29, 34, 41). It recognizes DNA sequences that are related to a 14-bp consensus sequence, 5′-RT(m^5C/T)RYYA(m^5C/T)RG(m^5C/T)RAY-3′ (m^5C, 5-methylcytosine; R, G or A; Y, C or T). There are two types of binding sites for MDBP, those that do and those that do not require methylation of CpG dinucleotides at the recognition site for appreciable binding. Cytosine methylation-independent sites contain T residues replacing m^5C residues (43). Other groups have independently identified DNA-binding proteins EF-C, EP, site III-binding protein, and MIF that recognize methylation-independent MDBP sites in hepatitis B virus, polyomavirus, and cytomegalovirus (CMV) enhancers and in a c-*myc* intron (3, 12, 17, 22, 38, 39). We have shown these activities to be indistinguishable from MDBP (41, 42, 44).

In this report, we demonstrate that a widely studied protein which binds specifically to the X box (or X1 box [13, 25]) of major histocompatibility (MHC) class II promoters has the same DNA sequence specificity as MDBP. The X box has been implicated in the regulation of expression of MHC class II genes in a number of studies (6, 14, 19, 20, 31, 32, 36). The X-box-binding protein is called RFX or NF-X and has been cloned (recombinant RFX1 [rRFX1] [19, 24–26]). The correspondence that we describe between MDBP and RFX1 indicates that an MDBP cDNA is now available. The isolation of this cDNA had appeared elusive despite the work by many groups on the protein. Our study suggests that MDBP plays a role in the control of transcription of some MHC class II genes, and the existence of an MDBP site at the beginning of the RFX1 (MDBP) cDNA might indicate that it regulates its own gene's transcription.

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MATERIALS AND METHODS

Oligonucleotide duplexes. Oligonucleotide ligands were synthesized and radiolabeled as previously described (43). Oligonucleotides were purified by polyacrylamide gel electrophoresis before or after annealing of complementary strands. In the former case, a fivefold excess of the unlabeled strand was annealed to the labeled strand. For generating unlabeled competing oligonucleotide duplexes, equimolar amounts of the complementary strands were annealed. Table 1 shows the sequences of the oligonucleotides within the 13- or 14-bp MDBP site region. All of the duplexes were blunt ended with the sequences surrounding the MDBP site region the same as those found in their DNA of origin. They contained 21 to 25 bp except for pB site 1, which is a 14-bp duplex with 5′-CTAG overhangs (18). The irrelevant duplex (nonspecific duplex 1) used in a number of experiments was 5′-AAGGTTATGAGAGCATCAGCA-3′ annealed to its complement; this sequence contains three deviations from the MDBP consensus sequence in its central 14 bp and shows negligible binding to MDBP under standard conditions (41).

Nuclear extract preparation, affinity column chromatography, and EMSAs. Nuclear extracts were prepared from HeLa or Raji cells as described previously (8, 41). A partially purified fraction of MDBP was obtained by passing the nuclear extract through a nonspecific DNA affinity column and then subjecting it to chromatography on a column with

TABLE 1. Relative binding of X-box sequences and related oligonucleotide duplexes to MDBP in HeLa nuclear extracts[a]

Sequence	Sequence	Relative binding
14-bp MDBP consensus sequence	R T ^{m}Y R Y Y A ^{m}Y R G ^{m}Y R A Y	
pB site 1; standard for MDBP	A T M G T C A M G G M G A C	100
EIAV site 1; standard for MDBP	G T T G C C - T A G C A A C[b]	180
DRA X-box promoter sequence (DRA site 1)	T T C C C C - T A G C A A C	25
DPA X-box promoter sequence	T T T A C C - C A G C A A C	21
Eα X-box promoter sequence	C T T T C C - T A G C A A C	30
Aα X-box promoter sequence	T G C A G C - T G G C A A C	8
DRB X-box promoter sequence	T C T G A C - C A G C G A C	<5
DRB X-box site, methylated	T C T G A C - C A G M G A C	<5
L30 ribosomal protein promoter site	G T C C C C A T A G C A A C	240
DQA X-box promoter sequence	T T C A G C - T A G T A A C	9
HNF1 site	G T T A A C - T A G T A A C	<2
hLyn (tyrosine kinase) site	G T T G T T A T A G T A A C	260
hDystrophin site 1	T T T C C T - T A G T A A C	10
hDystrophin site 2	G T T A C T C T G G T G A C	20
β-Actin-2 sequence	C C T T T T A T G G T A A T	<2
CMV site 2 (IE1 enhancer)	A T C A C C A T G G T A A T	140
	(A T T A C C A T G G T G A T)	
CMV site 2/minus A	A T T A C C - T G G T G A T	<5

[a] The relative binding to MDBP of the indicated radiolabeled oligonucleotide duplexes (40 fmol) was determined by EMSA, under standard conditions, with a nuclear extract from HeLa cells (1 U). The sequence (5′ to 3′) of one strand of the tested oligonucleotide duplex in the region that corresponds to the MDBP recognition site is given, as is the consensus sequence for 14-bp MDBP sites. Abbreviations: R, purine; Y, pyrimidine; ^{m}Y, either T or 5-methylcytosine; M, 5-methylcytosine. Note that the 13-bp MDBP sites are missing a central A residue that is otherwise highly conserved. Only the MDBP recognition site region is shown for the tested sequences. For the DRA X box, the residues shown span positions −112 to −100 relative to the transcription initiation site, and the entire oligonucleonucleotide spanned positions −116 to −92 of the promoter. Sequences for the other duplexes relative to the transcription start point of the genomic sequences are as follows: Eα X box, −97 to −85; Aα X box, −87 to −75; DPA X box, −102 to −90; L30 site, −135 to −122; HNF1 site, −50 to −38; hLyn site, +1088 to +1101; and β-actin-2 site, +766 to +779. The DRB X-box site is from positions −193 to −181 relative to the translation start site. The hLyn site is a sequence within a human *src*-related gene called *lyn*, which is proposed to encode a tyrosine kinase (37). Four to six base pairs of the genomic sequence surrounded the MDBP site region of the oligonucleotide duplex ligands other than pB site 1 (see Materials and Methods). The positions in the tested sites that match the consensus sequence are underlined. The MDBP sites, pB site 1, EIAV site 1, and CMV site 2 were previously described (34, 41, 42) and are included for comparison. The sequences of both strands of CMV site 2 are shown, with the lower strand in parentheses.

[b] When CA appears in place of ^{m}YR in MDBP sites ending in CAAC, the CA dinucleotide is considered a match to the consensus sequence and is underlined because there will be an ^{m}YR in the opposite strand and because such a CAAC sequence at the 3′ end (or GTTG at the 5′ end) of an MDBP site appears to be especially favorable for complex formation with MDBP (41).

an oligonucleotide duplex containing a methylation-independent MDBP site (CMV site 1 [40, 42]). Electrophoretic mobility shift assays (EMSAs) using 4.5 or 5% polyacrylamide gels were conducted with 40 fmol of oligonucleotide duplex as described by Supakar et al. (30). One unit of MDBP complexes 1 fmol of pB site 1 under these conditions. Quantitation of complexes was done by cutting out bands and determining their Cerenkov radiation. For Fig. 6, EMSA was performed as described by Tsang et al. (32) in the binding buffer of Ohlsson et al. (21). Similar results were obtained with this set of conditions and the above-mentioned conditions.

In vitro transcription-translation. The full-length rRFX1 cDNA was obtained by ligation of an RFX1 cDNA encoding the carboxy terminus (rRFX9 [26]) to a 5′ fragment of this RFX1 cDNA. All but the first 154 amino acids of the 979-residue-long rRFX1 are encoded by rRFX9. The 5′ fragment was obtained by the polymerase chain reaction performed with an internal primer and a primer corresponding to the 5′ end of the full-length RFX1 cDNA. DNA sequencing confirmed the correct sequence of this polymerase chain reaction product. The full-length cDNA was cloned into the *Eco*RI site of a plasmid vector, pGEM (Promega), lacking the start codon near the cloning site so that the first ATG of the RFX1 cDNA insert would encode translation initiation. The structure of the rRFX1 DNA was checked by restriction mapping.

Plasmid DNA was linearized and transcribed in vitro by using SP6 RNA polymerase. After phenol-chloroform extraction, the in vitro transcripts (1 μg) were used in a 50-μl reaction mixture for translation by a rabbit reticulocyte lysate (Promega) in a 1-h reaction at 30°C under standard conditions.

Supershifting of MDBP (RFX) complexes with antibody. Anti-RFX1 antibody was raised against a 19-amino-acid peptide (residues 3 to 21) at the amino terminus of rRFX1. For the supershift experiments, 2 μl of a purified immunoglobulin G fraction (purified on a Protein A column [Pierce]) with an A_{280} of 3 was added to the binding reaction at the start of the 10-min preincubation for EMSA. An immunoglobulin G fraction of the same optical density was prepared from preimmune serum and used for the control reaction.

RESULTS

MDBP binds to the X box of the MHC class II DRA promoter. MDBP that had been purified from HeLa nuclear extracts on a DNA affinity column containing one of the MDBP sites from the human CMV IE1 enhancer (42) was tested in EMSA for binding to a ^{32}P-labeled oligonucleotide duplex containing the X box (or X1 box) of the DRA promoter (DRA site 1; Table 1). This site matches the 14-bp consensus sequence of MDBP at 10 positions (Table 1). The resulting DNA-protein complexes of very low mobility coelectrophoresed with those containing a methylation-independent MDBP site from equine infectious anemia virus (EIAV site 1; Table 1 and Fig. 1, lanes 1 and 5). Excess unlabeled EIAV site 1 or pB site 1, a methylation-dependent

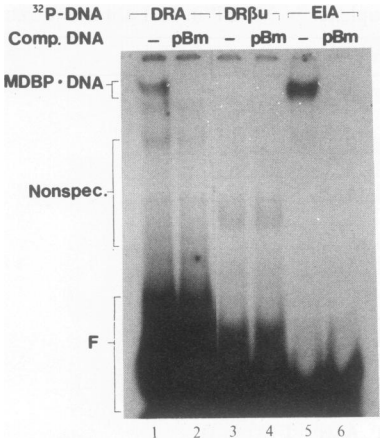

FIG. 1. Formation of complexes between affinity-purified MDBP and the X-box sequence from the DRA promoter as monitored by EMSA. MDBP (about 1.5 U) partially purified approximately 500-fold on a DNA affinity column (see Materials and Methods) was used for EMSA with 40 fmol of the DRA site 1 duplex (DRA), the unmethylated DRB X-box-containing duplex (DRβu), or a methylation-independent MDBP site, EIAV site 1 (EIA; Table 1), as the radiolabeled ligand (specific activities, approximately 770, 220, and 400 cpm/fmol for DRA site 1, EIAV site 1, and DRB site 1, respectively). Where indicated, 1 pmol of unlabeled pB site 1 (pBm; Table 1), a methylation-dependent MDBP ligand, was added as a specific competitor of MDBP-DNA complex formation. In parallel experiments, irrelevant oligonucleotide duplexes gave no decrease in the intensity of the signal from MDBP-DNA complexes (data not shown). The positions of MDBP-DNA complexes, nonspecific protein-DNA complexes (Nonspec.), and free ligand (F) are indicated.

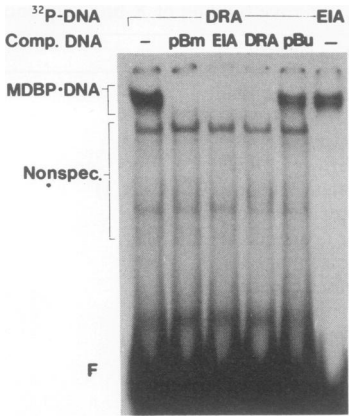

FIG. 2. Formation of MDBP-type complexes in EMSA with crude Raji nuclear extracts and the X-box sequence from the DRA promoter. EMSA was performed and the figure is labeled as described in the legend to Fig. 1 except that a crude nuclear extract (approximately 1 μg of protein and 1 U of MDBP activity) from Raji cells was used instead of affinity-purified MDBP from HeLa cells. The radiolabeled ligand was DRA site 1 or the MDBP-specific EIAV site 1 as in Fig. 1, and their specific activities were approximately 1,290 and 360 cpm/fmol, respectively. Where indicated, 2 pmol of unlabeled pB site 1 (pBm), EIAV site 1, DRA site 1, or unmethylated pB site 1 (pBu) was present as an oligonucleotide duplex competitor.

MDBP site (pBm), competed with the DRA site 1 duplex for formation of these low-mobility complexes, while a nonspecific oligonucleotide duplex gave no competition (Fig. 1 and data not shown). Similar results were obtained with MDBP activity in crude nuclear extracts from HeLa and Raji cells, a B-lymphoblastoid cell line (Fig. 2). The specific complexes that formed appeared as two or three clustered bands in EMSA. Previously, we noted that the number of bands and their mobilities varied with the source of mammalian cells and the degree of purification of the MDBP (with more smearing and a higher mobility sometimes evident upon increased purification) but that all of these bands displayed similar DNA sequence specificity (29, 41).

MDBP from crude nuclear extracts or from the DNA affinity chromatography fraction bound to DRA site 1 with a lower affinity than to the MDBP-specific EIAV site 1 or pB site 1. For example, 2 pmol of unlabeled EIAV site 1 or pB site 1 abolished almost all of the binding to 40 fmol of DRA site 1, while 2 pmol of unlabeled DRA site 1 lowered complex formation between MDBP and 40 fmol of ^{32}P-labeled EIAV site 1 by only 57% (Fig. 2 and 3). However, in very large amounts (5 to 15 pmol), this oligonucleotide duplex could abolish most of the complex formation between pB site 1 and MDBP in HeLa or Raji nuclear extracts, while a nonspecific ligand gave only minimal competition (Table 2). This cross-competition between pB site 1 and DRA site 1 indicates that most of the proteins in these nuclear extracts that recognize pB site 1 also recognize DRA site 1. However, there appeared to be a minor fraction of MDBP-pB site 1 complexes whose formation was much more resistant to competition by excess DRA site 1 (Table 2).

We also used the unmethylated form of pB site 1 (pBu) as a competitor for complex formation with DRA site 1. Two picomoles of pBu hardly affected complex formation between EIAV site 1 and MDBP, whereas it decreased complex formation with DRA site 1 by 58% (Fig. 3). In contrast, 2 pmol of the methylated analog (pB site 1) competed for almost all of the binding to EIAV site 1 as well as to DRA site 1. pBu is approximately a 10- to 50-fold-poorer ligand for MDBP than the methylated analog (pB site 1) is under standard conditions for EMSA (11, 18, 29) (data not shown).

MDBP complexes with X-box sequences from several MHC class II promoters. We compared the ability of X-box-containing regions of different MHC class II gene promoters to bind to MDBP. Using the putative X-box-containing oligonucleotide duplexes from the human DPA and murine Eα promoters, we observed DNA-protein complexes that coelectrophoresed with the analogous DRA complexes and formed in similar amounts (Table 1). However, only low yields of these MDBP-type complexes were observed with two other X boxes, those from the human DQA and murine Aα promoters, and negligible amounts of MDBP-type complex formed with one of the X-box regions, namely, that from the human DRB promoter (Fig. 4 and Table 1). Furthermore, the Aα X box was a weak competitor of DRA site 1 for complex formation with MDBP and gave negligible competition with EIAV site 1, which is a high-affinity MDBP site (Fig. 3). The DRB X box was the only one containing a CpG dinucleotide. Therefore, given MDBP's preferential binding to certain DNA sequences only when they are CpG methylated, we tested the DRB X box in both CpG-methylated and unmethylated forms. With both forms of the DRB promoter site, complex formation was only barely, and not consistently, demonstrable (Table 1, Fig. 1, and data not shown). The relative amounts of X-box- and MDBP-type complexes which formed from the other MHC class II X boxes, namely, those with the Eα, DRA, DPA, DQA, and Aα promoters and HeLa nuclear extracts, were approxi-

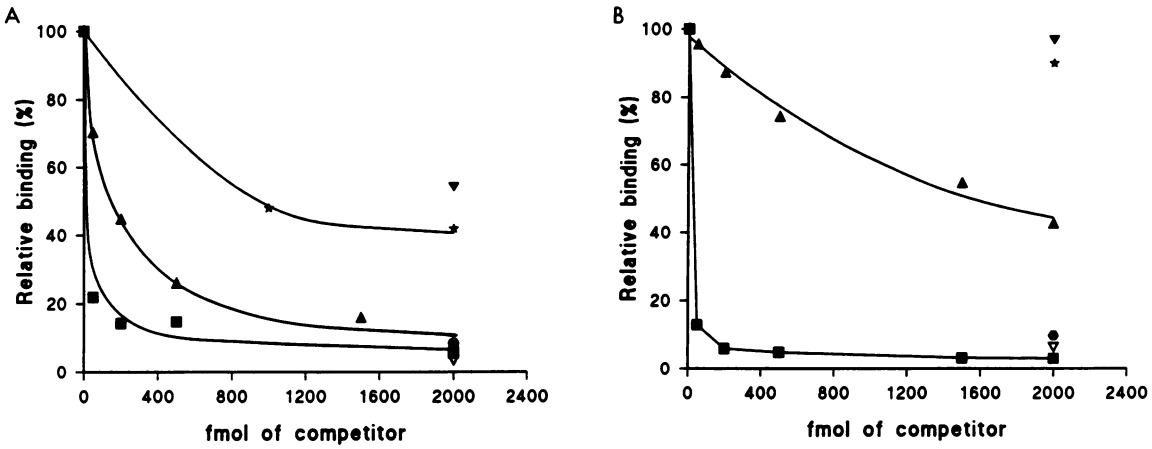

FIG. 3. Competition by different amounts of oligonucleotide duplexes for formation of MDBP-DNA complexes between MDBP activity in crude HeLa nuclear extracts and either ^{32}P-labeled DRA site 1 (A) or EIAV site 1 (B). The indicated amounts of unlabeled EIAV site 1 (■), DRA site 1 (▲), pB site 1 (●), unmethylated pB site 1 (★), Aα X-box promoter site (▼), and L30 promoter site (▽) were added to the reaction mixtures containing 40 fmol of radiolabeled DRA site 1 or EIAV site 1 and about 1.5 U of MDBP. Both pB site 1 and the L30 duplexes were used only at the 2-pmol level, and their datum points were clustered with those for 2 pmol of EIAV site 1. The Aα site and, in panel B, the unmethylated pB site 1 were also used only at the 2-pmol level. The average extent of complex formation from duplicate samples with radiolabeled duplex in the presence of the competitor relative to that in the absence of the competitor was determined by EMSA as described in Materials and Methods.

mately 1:0.8:0.7:0.3:0.3, similar to what was reported previously for RFX (NF-X) (19, 26).

MDBP recognizes other DNA sequences that are related to the MHC class II X boxes. MDBP sites are generally partially palindromic (41). However, because the left halves of the MDBP sites of the DRA, DQA, and Aα promoters show a match in only two or three of seven positions to the MDBP consensus sequence, it was possible that these sites were binding to MDBP (with low to moderate affinity) only by virtue of their perfect match in their right halves to this consensus sequence. Alternatively, the partial homology of the left half-site may suffice for direct contact with MDBP or may critically affect the local DNA conformation (2, 15). To examine the importance of these left half-sites, we first tested as an MDBP ligand a sequence from the mouse ribosomal protein L30 promoter region (Table 1) (35) which has five of the same six consecutive pyrimidine residues that are in the left half of DRA site 1 and also has the identical consensus right half-site. We showed that the L30 site is a very high affinity MDBP site (Fig. 5 and Table 1), and Safrany and Perry (26a) have analogously demonstrated that it is recognized by RFX. Therefore, the left half-site of the DRA X box apparently favors recognition by MDBP despite its only partial match to the MDBP consensus sequence. However, a G at the first position, as in the L30 site, is probably much better for binding than the pyrimidine residue at that position in the DRA X box (41).

TABLE 2. Competition of large excesses of unlabeled oligonucleotide duplexes for binding to MDBP[a]

Competing ligand	% Competition for:					
	HeLa extract			Raji extract		
	5 pmol	10 pmol	15 pmol	5 pmol	10 pmol	15 pmol
EIAV site 1	100	100	100	97	97	97
pB site 1	97	98	99	95	96	97
DRA site 1 (X box)	77	82	87	69	79	87
Nonspecific duplex 1	16	22	20	11	17	33

[a] The relative ability of the indicated amount of unlabeled oligonucleotide duplex to compete with 20 fmol of ^{32}P-labeled pB site 1, a standard methylation-dependent MDBP site, for binding to MDBP in HeLa or Raji cell nuclear extracts was determined by EMSA. The sequences of pB site 1, EIAV site 1 (a standard methylation-independent MDBP site), the X-box promoter site of the DRA gene, and nonspecific duplex 1 are given in Table 1 and Materials and Methods.

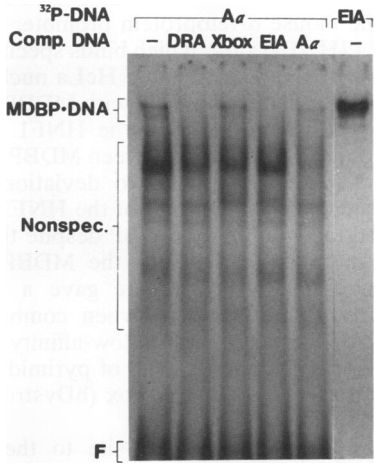

FIG. 4. Formation of MDBP-type complexes with the Aα X-box sequence and MDBP activity in a HeLa nuclear extract. EMSA was conducted and the figure is labeled as described in the legend to Fig. 1, except that the source of MDBP activity was crude nuclear extract from HeLa cells (approximately 1.5 U) and the radiolabeled oligonucleotide duplex ligand was the Aα X-box promoter sequence (Table 1) or, as a standard, the MDBP-specific EIAV site 1. Two picomoles of the indicated unlabeled oligonucleotide duplex was present as a competitor. In addition to several of the duplexes shown in Table 1, a truncated X-box sequence (Xbox) duplex was used. This oligonucleotide duplex (5′-CCCTAGCAACAGATGCG-3′) is missing the first 3 bp of the MDBP recognition site region (Table 1), although those residues had generally not been considered part of the X box in past studies.

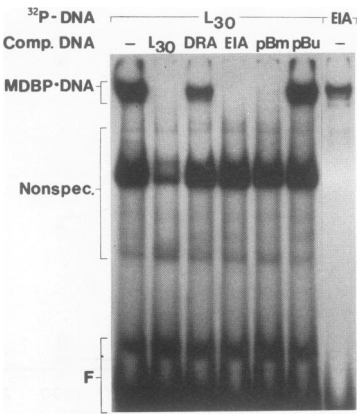

FIG. 5. Complex formation between MDBP and a sequence in the promoter region of the mouse L30 ribosomal protein gene. HeLa nuclear extract (about 1.5 U) was incubated for EMSA with 40 fmol of a ^{32}P-labeled oligonucleotide duplex containing the L30 ribosomal protein promoter site shown in Table 1 with or without 2 pmol of oligonucleotide competitors as described in the legend to Fig. 1. The unlabeled competitors were the L30 site, DRA site 1, EIAV site 1, and methylated and unmethylated pB site 1 (pBm and pBu, respectively; Table 1). Also in the gel was radiolabeled EIAV site 1 incubated with the same extract. Formation of complexes with the radiolabeled L30 promoter site duplex was strongly competed for by 2 pmol of high-affinity MDBP ligands, such as EIAV site 1, but much less efficiently by DRA site 1, just as DRA site 1 is an inefficient competitor of formation of complexes between MDBP and pB site 1 or EIAV site 1 (Table 2 and Fig. 3). Labeling is as described in the legend to Fig. 1.

Second, we determined whether MDBP recognized a DNA sequence having the same "good" right half-site as the DQA X box (Table 1) but a different left half-site. This DNA sequence in the mouse α-fetoprotein promoter (hepatocyte nuclear factor 1 [HNF1] site), which binds specifically to the HNF1 (7), formed complexes with a HeLa nuclear extract, but none of them coelectrophoresed with MDBP-DNA complexes (Table 1). Also, 2 pmol of the HNF1 site did not compete for complex formation between MDBP and 40 fmol of DRA site 1. Therefore, the two deviations from the consensus sequence in the left half of the HNF1 site apparently precluded recognition by MDBP despite the complete homology of the right half-site to the MDBP consensus sequence. This same right half-site gave a high-affinity MDBP site (hLyn site; Table 1) when combined with a consensus left half-site and gave a low-affinity MDBP site with a left half-site containing a run of pyrimidines slightly different from that in the DRA X box (hDystrophin site 1; Table 1).

That a match of only one half-site to the consensus sequence does not suffice for binding and that the DNA sequence context within the recognition site rather than just the base pair-by-base pair match to the MDBP consensus sequence is important was confirmed by a comparison of the extents of complex formation with MDBP by a β-actin gene sequence, β-actin-2, and a CMV enhancer site for MDBP, CMV site 2 (Table 1). CMV site 2 has the central A of the consensus sequence that is generally highly conserved among MDBP sites, although this A residue is missing from the 13-bp X-box sites and EIAV site 1 and replaced in hDystrophin site 2 (Table 1) (41). With a synthetic variant of CMV site 2 (CMV site 2/minus A), we showed that deletion of this central A can abrogate complex formation with MDBP in certain DNA sequence contexts. In contrast, MDBP displays a high affinity for EIAV site 1 even though that sequence has the equivalent deletion of the central A residue. It should be noted that all 11 sites that completely correspond to the 14-bp consensus sequence for MDBP binding and that were tested for complex formation with MDBP have a moderately high to very high affinity for this protein (41–43; unpublished data).

Last, an oligonucleotide from the X-box region of the DRA promoter that was a truncated version of the DRA site 1 duplex from positions −109 to −93, rather than from positions −116 to −92, was also tested for complex formation with MDBP because it should be a poor ligand since it is missing the first three positions of the left half of the MDBP site region (the sequence TTC as shown in Table 1). The first 3 bp of the MDBP site region of DRA site 1 had not commonly been thought to be part of the RFX-binding site. When this −109 to −93 duplex was previously tested, at a very high concentration, for binding to RFX in a competition experiment, it was shown to compete for binding (32). Also in our study, when a very large amount (15 pmol) of this truncated DRA site 1 were used as a competitor, it abolished about 80% of the specific complex formation with 40 fmol of ^{32}P-labeled DRA site 1 whereas a nonspecific duplex gave only minimal competition. However, 40 fmol of this radiolabeled truncated DRA site 1 formed no detectable MDBP complexes with crude nuclear extracts from HeLa cells (even upon prolonged exposure of the autoradiogram from EMSA), indicating that, as expected, it is a very poor MDBP site because of its incomplete left half-site (see also Fig. 4).

Binding of the in vitro-transcribed-translated product of recombinant RFX1 cDNA to methylation-dependent and methylation-independent MDBP-specific oligonucleotides. Using a cDNA library from a human B-cell line and an X-box oligonucleotide as a recognition site probe, Reith et al. cloned rRFX1 (25). rRFX1 binds the X box in a sequence-specific manner, and antibodies against this recombinant protein retard the mobility of RFX-DNA complexes formed with nuclear extracts from a B-cell line (25, 26).

In this study, specific DNA-protein complexes were formed between the in vitro-transcribed-translated product of RFX1 cDNA and the MDBP-specific ligand pB site 1 or EIAV site 1 as well as between this recombinant RFX1 and DRA site 1. Binding was much weaker to the unmethylated form of radiolabeled pB site 1 than to the methylated form, as seen by the intensities of the ^{32}P-labeled DNA-protein complexes formed under analogous conditions (Fig. 6). Furthermore, a 50-fold excess of the unlabeled DRA site 1 was a much more effective competitor of complex formation with the unmethylated form of radiolabeled pB site 1 than with the methylated form of this site, just as would be predicted from the higher affinity of methylated pB site 1 than of DRA site 1 for native MDBP and the much lower affinity of the unmethylated form of pB site 1 (Fig. 6). EIAV site 1 formed low-mobility complexes with the recombinant protein that coelectrophoresed with those of pB site 1, while an irrelevant oligonucleotide duplex did not.

A 50-fold excess of unlabeled DRA site 1 partially competed for the formation of complexes between 20 fmol of radiolabeled EIAV site 1 and the recombinant protein just as it did with pB site 1 (Fig. 6). This was also seen with the native protein and either of these ligands (Fig. 3 and Table 2). The same excess of unlabeled DRA site 1 almost abolished formation of analogous radiolabeled low-mobility complexes with 20 fmol of ^{32}P-labeled DRA site 1 and the recombinant protein (Fig. 6). However, a large amount of

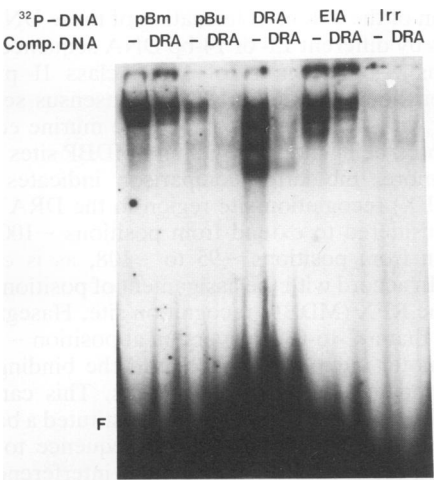

FIG. 6. Complex formation between recombinant RFX1 and MDBP ligands. EMSA was performed with the in vitro-transcribed-translated product of RFX1 cDNA and 20 fmol of ^{32}P-labeled pB site 1 (pBm), unmethylated pB site 1 (pBu), DRA site 1 (DRA), EIAV site 1 (EIA; Table 1), or an irrelevant oligonucleotide (Irr; see Materials and Methods). Where indicated, 2 pmol of unlabeled DRA site 1 was added as a specific competitor. The free ligand (F) is seen at the bottom of the autoradiogram.

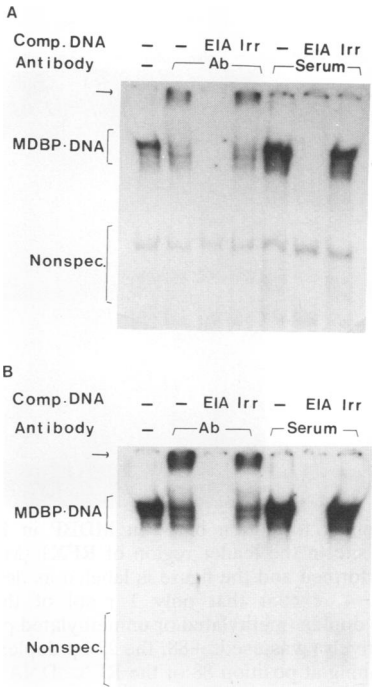

FIG. 7. Binding of a recombinant RFX1-specific antibody to MDBP in HeLa nuclear extracts. Either an antibody to a synthetic peptide containing residues 3 to 21 from the amino terminus of recombinant RFX1 (Ab) or preimmune serum (Serum) was added to the preincubation mixture for EMSA as described in Materials and Methods. The ligand was 50 fmol of ^{32}P-labeled DRA site 1 (Table 1) (A) or pB site 1 (B). Where indicated, 1 pmol of unlabeled EIAV site 1 or an irrelevant duplex (Irr) was the competing oligonucleotide. MDBP-DNA complexes are labeled, and the ternary complex containing antibody in addition is indicated by an arrow and seen in the second and fourth lanes in both panels. In this experiment, to obtain better resolution of MDBP-DNA complexes, the free ligand was run off the gel.

higher-mobility complexes that were similarly competed for also formed with this ligand (Fig. 6). In contrast, with radiolabeled nonspecific oligonucleotides and recombinant RFX1 or with the above-mentioned specific oligonucleotides and the rabbit reticulocyte lysate alone, none of these complexes formed. The origin of the higher-mobility complexes with DRA site 1 is uncertain. Much smaller amounts of these complexes were seen when pB site 1 and EIAV site 1 were the ligands. Those apparently smaller complexes might be due to the formation of truncated forms of rRFX1 during the in vitro transcription-translation reaction as a result of proteolysis or the use of alternative translation initiation sites, as would be consistent with the cDNA sequence. Alternatively, specific complexes of different electrophoretic mobilities could represent two-subunit and one-subunit complexes (26, 27). Moreover, we have previously shown that a partial proteolysis product of MDBP can have large differences from native MDBP in relative affinities for certain MDBP sites and that these fragmented proteins can form both one-subunit-type and two-subunit-type complexes (30). The relative amounts of these complexes vary with the MDBP ligand. In our study, most noteworthy is that the low-mobility doublet band of complexes formed between rRFX1 and DRA site 1 coelectrophoresed with the analogous complexes containing the MDBP ligands pB site 1 and EIAV site 1.

Antibodies to rRFX1 supershift MDBP-DNA complexes. A polyclonal antibody was raised to a synthetic peptide representing 19 amino acids at the amino terminus of the in vitro-transcribed-translated product of rRFX1. In these assays, the three slowest-moving complexes that formed upon incubation of DRA site 1 with HeLa nuclear extract coelectrophoresed with those of pB site 1 (Fig. 7). When the antibody was present during the incubation of the nuclear extract with DRA site 1, most of the slowest-moving complex, which was the most abundant of the complexes, supershifted (Fig. 7A). Although formation of all three of these low-mobility complexes with HeLa or Raji nuclear extracts in the absence of antibody was competed for specifically by EIAV site 1, only the upper band was supershifted (Fig. 7) even when additional antibody was added. With either pB site 1 or EIAV site 1 as a ligand, again only the top band was supershifted with the specific antibody (Fig. 7B and data not shown). Preimmune serum did not supershift any of the complexes. With Raji nuclear extracts, similar results were obtained. This supershifting of only the top band could be due to differences localized to the amino-terminal region of the protein in these three bands. The DNA-binding domains of the protein in these bands should be identical or very similar because when one ligand is compared with another, no difference in DNA-binding specificities over a wide range of sequences is seen for the protein in these bands (41, 43, 44) (Fig. 7).

A high-affinity methylation-independent MDBP site in the leader region of RFX1 cDNA. We noticed a possible binding site for MDBP which begins 88 bp after the first residue of the presumably full-length RFX1 (MDBP) cDNA (26). This site (RFX+88) is as follows: 5′-GTTGGCATGGCAAC-3′. The bases that match the 14-bp MDBP consensus sequence, including the CAAC sequence at the 3′ end and the complementary GTTG at the 5′ end, which are highly favorable for MDBP binding (Table 1) (41), are underlined. This site was tested in EMSA as part of a 22-bp duplex bordered by the

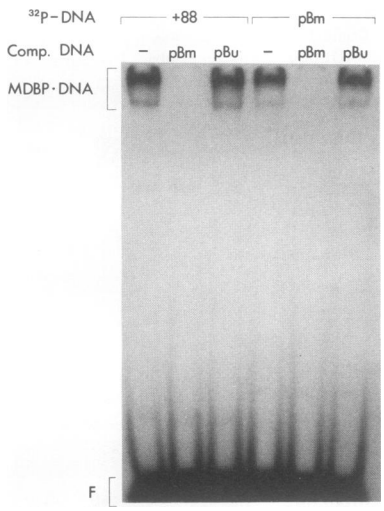

FIG. 8. Complex formation between MDBP in HeLa nuclear extracts and a site in the leader region of RFX1 (MDBP) cDNA. EMSA was performed and the figure is labeled as described in the legend to Fig. 4, except that only 1 pmol of the competitor oligonucleotide duplex (methylated or unmethylated pB site 1; pBm or pBu, respectively) was used. +88, the 22-bp duplex containing a sequence beginning at position 88 of the RFX cDNA.

same 4 bp adjacent to it in the cDNA. With HeLa or Raji nuclear extracts, it formed complexes that coelectrophoresed with those of the methylation-dependent MDBP standard, pB site 1 (Fig. 8 and data not shown). These ligands cross-competed with one another, as did the MDBP-specific EIAV site 1 and the RFX+88 site, while unmethylated pB site 1 and irrelevant oligonucleotide duplexes gave no appreciable competition for complex formation with the RFX+88 site (Fig. 8 and data not shown). When a DNA-affinity column fraction of MDBP was used for EMSA, the RFX+88 site and pB site 1 complexes again coelectrophoresed (data not shown). The RFX+88 site has a high affinity for MDBP. The relative binding of this site to pB site 1 was 1.3 when 40 fmol of these oligonucleotide duplexes was tested in EMSA under standard conditions with 1 U of MDBP.

DISCUSSION

The so-called X (or X1) box in the promoter of the human MHC class II DRA gene is the binding site for a ubiquitous mammalian sequence-specific DNA-binding protein called RFX, NF-X, NF-Xc, or RFX1 (4, 19, 23, 24, 27). We have investigated the relationship of RFX to MDBP, a ubiquitous mammalian DNA-binding protein or closely related group of proteins displaying DNA methylation specificity as well as DNA sequence specificity. We demonstrated that RFX is MDBP by a number of criteria. Their DNA-protein complexes formed exactly coelectrophoresing clustered bands (Fig. 1 and 2), their DNA ligands showed cross-competition (Fig. 3 and Table 2), their DNA-protein complexes displayed the same partial supershifting in EMSAs with an antiserum to an N-terminal peptide from rRFX1 (Fig. 7), and an rRFX bound specifically to MDBP ligands and showed methylation-dependent binding to a ligand with three CpG dinucleotides per strand (Fig. 6). In addition, both MDBP and RFX have a high molecular mass (~2×10^5 to 3×10^5 kDa as a dimer [references 19, 27, and 30 and unpublished data]). A comparison of the extent of formation of these DNA-protein complexes by different 13- or 14-bp DNA sequences, including various X-box regions of MHC class II promoters, versus their homology to an MDBP consensus sequence is consistent with the DRA X box and the murine equivalent, the Eα X box, being moderate-affinity MDBP sites (Table 1).

Furthermore, the latter comparison indicates that the MDBP (RFX) recognition site region in the DRA promoter can be considered to extend from positions −100 to −112 rather than from positions −95 to −108, as is commonly assumed. In accord with the assignment of positions −100 to −112 as the RFX (MDBP) recognition site, Hasegawa et al. (14) found that a C-to-G transversion at position −109 of the DRA promoter inexplicably increased the binding of RFX from fractionated Raji nuclear extracts. This can now be explained because that transversion substituted a base which deviates from the MDBP consensus sequence to one that matches it (Table 1). Dimethyl sulfate interference assays have probed which G residues in the DRA X box interact with rRFX1 or native RFX (25). A comparison of the results obtained from those studies with the results from analogous studies of MDBP interacting with various cognate DNA sites (42, 43) is consistent with positions −100 to −112 of the DRA promoter being the RFX (MDBP) recognition site. In addition, the relative affinity of different X boxes for MDBP is similar to that reported for RFX (19, 26). Although both RFX and MDBP may represent groups of closely related proteins rather than single protein species, the MDBP (RFX) proteins in all clustered bands of DNA-protein complexes that we detect in EMSA have the same DNA sequence specificity and methylation specificity. Our results lead to the conclusion that the main protein in the RFX and MDBP groups is the same.

MDBP was initially isolated as a methylation-specific DNA-binding protein from human placental muclear extracts (16), and RFX was identified as a protein that binds to the X box of the MHC class II genes (25). We had previously shown that MDBP activity is indistinguishable from protein activities variously called EF-C, EP, or MIF (41, 44) and that MDBP binds to certain 13- or 14-bp sequences irrespective of C methylation because of the presence of T residues replacing m^5C residues in those sites (43). Just as there was no previous indication in the literature from the many groups studying this MHC class II promoter-binding activity (4, 5, 13, 23, 27, 36) that RFX can bind to DNA sequences in a cytosine methylation-dependent mode, so it had been apparently unanticipated by the different laboratories studying EF-C (EP) or MIF (3, 17, 22, 38) that these activities could differentiate between methylated and unmethylated DNA sequences. However, coelectrophoresis and cross-competition experiments by Kouskoff et al. (19) with a polyomavirus enhancer site for EF-C and the Eα X-box site for NF-X (Table 1) showed EF-C and NF-X to be indistinguishable. We had demonstrated that an oligonucleotide duplex containing this polyomavirus site, which has the same 13-bp MDBP recognition sequence as does EIAV site 1 (Table 1) but different surrounding sequences, is one of two sites in the polyomavirus enhancer B to bind specifically to MDBP (41). Hence, an indirect argument for the correspondence of RFX and MDBP via EF-C can be made, in addition to the several direct lines of evidence presented in this study.

The physiological significance of the appreciable binding of MDBP (RFX) to the X boxes of several of the tested MHC class II promoters remains to be determined. Our understanding of the role of this site is complicated by the appearance of more than one band of MDBP-DNA com-

plexes upon EMSA, the report that more than one type of cloned cDNA from human B cells encodes proteins that can bind to the X box (24), and the binding of other proteins to an adjacent sequence called the X2 box (1, 13, 23). Nonetheless, much evidence indicates that the X box in the MHC class II human DRA and murine Eα promoters helps positively regulate transcription of the DRA gene. Sequential deletion analysis, clustered point mutagenesis, and substitution mutagenesis of the X box of the DRA promoter revealed a role for this site in promoter function (19, 28, 31, 32). A 25-bp sequence containing the X box gave large increases in reporter gene expression when placed in front of a thymidine kinase promoter driving this expression (31). However, methylation-independent MDBP sites, even from viral enhancers, function poorly, singly or in tandem, as enhansons when taken away from their surrounding DNA sequences despite the demonstration of the importance of at least one viral enhancer MDBP site (in a hepatitis B virus enhancer) for enhancer activity (9, 22, 41, 42).

We propose that in the appropriate DNA sequence context, MDBP also functions as a negative regulator of transcription. We previously found methylation-dependent MDBP sites of moderate affinity at approximate positions +49 and +30 relative to the transcription start sites of the α-galactosidase A gene and three MHC class I genes (HLA-A2, -A3, and -A-25), respectively, and two low-affinity methylation-dependent MDBP sites about 85 and 110 bp downstream of the major cap sites in the human hypoxanthine phosphoribosyltransferase gene (41). These might down-modulate transcription in view of the evidence from various studies that the expression of these genes is negatively controlled by DNA methylation (references in reference 41) and our recent finding that increasing the affinity for MDBP of a methylation-independent site 5 bp after the starting point of the CMV IE1 transcription unit (42) can down-regulate gene expression (unpublished data). Like the first of the hypoxanthine phosphoribosyltransferase sites and the α-galactosidase A and HLA-A2 sites, the MDBP site that we found within the MDBP (RFX1) cDNA (Fig. 8) straddles the very beginning of the open reading frame. This methylation-independent MDBP site, which matches the MDBP consensus sequence at 13 of 14 positions, might play a role in autoregulating its gene's transcription, possibly by down-modulating synthesis when MDBP levels are too high.

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