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Increasing binding of a transcription factor immediately downstream of the cap site of a cytomegalovirus gene represses expression

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ABSTRACT

A closely related family of ubiquitous DNA binding proteins, called MDBP, binds with high affinity to two 14 base pair (bp) sites within the human cytomegalovirus immediate early gene 1 (CMV IE1) enhancer and with low affinity to one site beginning 5 bp downstream of the CMV IE1 transcription start point (+5 site). Unlike several cap position downstream MDBP sites in mammalian genes, these MDBP sites do not require cytosine methylation for optimal binding. Mutation of one of the enhancer MDBP sites to prevent MDBP recognition modestly increased the function of a neighboring CREB binding site in a transient transfection assay in the context of one promoter construct. A much larger effect on reporter gene expression (a 10-fold reduction) was seen when the low affinity MDBP recognition sequence at position +5 was converted to a high affinity site in a plasmid containing the CMV IE1 promoter upstream of the reporter gene. Evidence that the increased binding of MDBP at the mutant site is largely responsible for the observed results was provided by transfection experiments with this high affinity MDBP +5 site re-mutated to a non-binding site and by *in vitro* transcription assays.

INTRODUCTION

The immediate early gene 1 (CMV IE1) of the human cytomegalovirus encodes the most abundant of the first viral transcripts to be expressed after infection by this ubiquitous and sometimes pathogenic virus (1). Its promoter/enhancer is one of the most potent known and is often used to drive expression of genes in mammalian expression vectors (2). This enhancer contains multiple transcription regulatory elements, including binding sites for proteins in the family of cyclic AMP response element binding proteins (CREB, also known as ATF) and in the NF-κB family of phorbol ester-inducible transcription factors (3–6). Although in expression assays on cultured cells the many transcription regulatory sites in the CMV IE1 enhancer appear to be redundant, these multiple sites may play critical roles during the course of infection or activation of latent virus *in vivo*, because the outcome of virus infection is partly determined by control of expression of this immediate early transcription unit, which is up-regulated early in infection and down-regulated later in the infectious cycle (7,8).

In this study we examined the influence of binding sites for a family of transcription factors called MDBP (9,10) on expression driven by parts of the CMV IE1 enhancer/promoter unit. There are two high affinity, 14 base pair (bp) MDBP sites in the CMV IE1 enhancer, one of which is sandwiched between a CREB site and an NF-κB site, and there is another 13 bp MDBP site beginning 5 bp after the transcription start point (+5 site), although the latter is a low affinity site (11). That these fairly long sites for specific protein binding are all within a 500 bp transcription regulatory region suggests their functional importance. MDBP is a closely related family of ubiquitous mammalian sequence-specific DNA binding proteins composed of homodimers or heterodimers of polypeptides RFX1, RFX2, RFX3 and RFX4, which are more distantly related to a protein recently shown to be necessary for expression of MHC class II genes and to be missing in a subclass of MHC class II deficiency disease (10,12). MDBP (methylated DNA binding protein) was originally isolated on the basis of its much higher affinity for binding to some of its recognition sites when they are methylated at their CpG dinucleotides (13). These methylation-dependent MDBP sites include sequences within the first 50 bp of the human α-galactosidase A gene and several MHC type I genes and within the first 100 bp of the hypoxanthine phosphoribosyl transferase gene, for which DNA methylation has been implicated in down-regulation of expression (9). However, MDBP family members recognize other related sites which contain TpG or TpA dinucleotides in a cytosine methylation-independent manner (14). All of the MDBP sites in the CMV IE1 enhancer/promoter region are methylation-independent binding sites. We demonstrated that the high affinity MDBP sites in the CMV IE1 enhancer do not act as enhansons even when multiple tandem copies are placed upstream of the IE1 promoter or a truncated form of that promoter (11). In the present study we have examined the effects on transcription of converting the low affinity MDBP site in the leader region of the IE1 gene to a high affinity site and have further studied the functionality of one of the high affinity MDBP sites in the IE1 enhancer.

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MATERIALS AND METHODS

Plasmids

Plasmid pCMV^{-97}+5up and pCMV^{-524}+5up were made by replacing the 72 bp *Sst*I–*Xba*I fragment of pCMV^{-97}CAT or pCMV^{-524}CAT (11) containing a wild-type (WT) MDBP site (ME3) at position +5 of the CMV IE1 gene (all positions are given relative to the CMV IE1 transcription start site) with an analogous oligonucleotide duplex containing a sequence mutated from a low affinity MDBP site to a high affinity site (+5up, Fig. 1). To make these mutants, a 72 bp synthetic oligonucleotide duplex with *Xba*I and *Sst*I overhangs and the centrally located mutated MDBP site was inserted into a 4.1 kb fragment from pCMV^{-97}CAT or a 4.5 kb fragment from pCMV^{-524}CAT obtained after digestion with *Xba*I at a unique site and then partial digestion with *Sst*I at the two sites for this enzyme in the plasmid. Plasmid pCMV^{-97}+5down, which was mutated to no longer bind MDBP from the +5 site (Fig. 1B, +5down), was constructed analogously, except that the 72 bp sequence was generated by the polymerase chain reaction (PCR). For PCR, a primer containing this mutant MDBP site deviating at four positions from the MDBP consensus sequence and extending from position –26 to +24 and a second primer from position +83 to +103 of the other strand were used to amplify part of the pCMV^{-97}+5up template DNA. After digestion of the PCR product with *Sst*I and *Xba*I, the purified mutant 72 bp fragment was ligated into the 4.1 kb *Sst*I/*Xba*I-linearized pCMV^{-97}CAT. Plasmids pCMV^{-55}CRE-ME1, pCMV^{-55}CRE-ME1mut, pCMV^{-55}(CRE-ME1)×2, pCMV^{-55}(CRE-ME1mut)×2, pCMV^{-55}(CRE-ME1)×3 and pCMV^{-55}(CRE-ME1mut)×3 were constructed by inserting one to three copies of a 47 bp blunt-ended duplex from position –426 to –472 of the CMV IE1 gene into the *Sma*I site at position –55 of plasmid pCMV^{-55}CAT. For all constructs we selected clones in which the cap site-proximal CRE was in the WT orientation relative to the cap site (Fig. 1). When two copies of these inserts were present, the upstream copy faced in the opposite direction. When three copies were present, the central copy, as well as the promoter-proximal copy, had the WT orientation and the upstream copy faced in the opposite direction. For the ME1mut constructs the MDBP site had been mutated to a non-binding site (Fig. 1B) and the inserts had the same orientation as for the analogous plasmids with WT MDBP sites. Also, pCMV^{-97}CAT was linearized at its *Hin*dIII site at position –97, followed by end-filling for insertion of one copy of the same oligonucleotide; plasmids with the WT orientation were cloned. The inserts and junction sequences of the plasmids were checked by sequencing.

In vitro transcription

Run-off transcription was carried out at 30°C for 1 h with a HeLa nuclear extract (Promega; 7 µl, ~50 µg protein/25 µl reaction mixture) and 0.5 µg *Eco*RI-linearized plasmid as the template under the conditions specified by the manufacturer, except that [^{32}P]UTP (5×10^4 d.p.m./pmol final sp. act.) was the source of the radiolabel. The extracted and ethanol-precipitated products were analyzed by polyacrylamide gel electrophoresis in the presence of 7 M urea, followed by autoradiography and quantitation by phosphorimager analysis. The antibody used in one set of experiments was raised against a 19 amino acid peptide from the N-terminus of recombinant RFX1, which is a member of the MDBP family, and an immunoglobulin (IgG) fraction purified as described previously (12). This IgG fraction (2 µl with an A_{280} of 3) was incubated with the HeLa nuclear extract and transcription buffer for 15 min at room temperature before adding the template and nucleoside triphosphates.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were purified and one strand for each duplex was labeled with [γ-^{32}P]ATP as described previously (15) and annealed to a 5-fold excess of the unlabeled complementary oligonucleotide for EMSA. EMSA was conducted as previously described with 1–3 µl nuclear extracts (~3–10 mg protein/ml) from HeLa, Raji or HL-60 cells preincubated with 400 ng poly(dI·dC)·poly(dI·dC) as non-specific competitor for 5 min, followed by a 30 min incubation at room temperature with 20–40 fmol ^{32}P-labeled ligand and then electrophoresis on a 5% polyacrylamide gel (16). Nuclear extracts were prepared as previously described (9), except that the cells, where indicated, had been treated with 5 µM forskolin and 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 1 h or 200 ng/ml PMA for 24 h before harvesting.

Transfection and CAT assay

CV-1 cells were transfected with calcium phosphate co-precipitated DNA using 10 µg test plasmid and 2 µg reference plasmid pSV$_\beta$ DNA (Clontech), as previously described (11). After 18 h the cells were washed with phosphate-buffered saline and overlaid with fresh medium with or without forskolin or PMA at final concentrations of 10 and 0.1 µM, respectively. Cells were harvested after a further 18 h incubation and lysates prepared for chloramphenicol acetyltransferase (CAT) assays, normalizing for β-galactosidase activity from the reference plasmid as described previously (11).

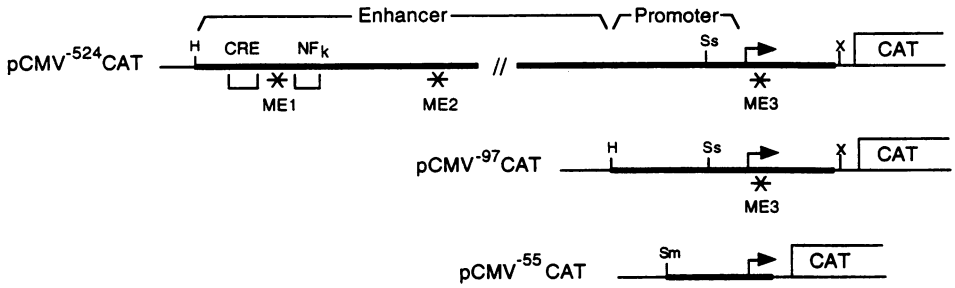
RESULTS

Conversion of the MDBP binding site at position +5 of the IE1 gene of CMV from a low to a high affinity site decreases expression of a downstream reporter gene

The low affinity MDBP site (ME3; Fig. 1), which is located 5 bp after the cap site in the CMV IE1 leader region, was mutated to a high affinity site (+5up) in order to test the effect on gene expression in transient transfection assays. For these assays we used reporter plasmids containing a CAT gene whose expression was driven by either just the CMV IE1 promoter region, positions –97 to +55 (pCMV^{-97}CAT constructs), or that promoter plus the adjacent enhancer region, positions –524 to +55 (pCMV^{-524}CAT constructs; Fig. 1A). This +5up MDBP site exactly matches the MDBP degenerate 14 bp consensus sequence, while the WT site that it replaced differs from the consensus sequence at three positions (Fig. 1B).

As expected, the mutant 72 bp oligonucleotide duplex sequence used for construction of the IE1 leader region containing the +5up consensus MDBP site yielded characteristic MDBP–DNA complexes in EMSA, whose formation was competed for by MDBP-specific duplexes and not by non-specific duplexes (Fig. 2A). These were the only specific protein–DNA complexes formed by the radiolabeled 72 bp duplex spanning CMV IE1 sequences from positions –14 to +55 (plus 3 bp of downstream vector sequence; Fig. 2A, lanes 1 and 5). The extent of MDBP–DNA complex formation by the +5up MDBP site as part of a 22 bp duplex was compared with that of the analogous WT duplex under the standard assay conditions described in Materials and Methods. The WT site

A



B

MDBP consensus RTmRYYAmRGmRAY (m, m^{5}C or T; Y, C or T; R, G or A)

	+5	+17
WT ME3	ATCGCC-TGGAGAC	
+5up	gTtGCCaTaGtGAC	
+5down	cTaGCgATcGTGAC	

	-472	-443	-426
CRE-ME1	CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCA		
CRE-ME1mut	CCCGCCCATTGACGTCAATAATGACGTATGTTCCCAgctgtgaGCCA		
	--CREB--	-----MDBP-----	

CRE	CTAGCGGCTGACGTCATCAAGCTAG	
pBm	CTAGATMGCCAMGGMGATCTAG	

	-428	-411
NFκ	CCAATAGGGACTTTCCAT	
	-447	-411
ME1-NFκ	GTATGTTCCCATAGTAACGCCAATAGGGACTTTCCAT	
	-----MDBP-----	--NF-κB---

Figure 1. Plasmids and DNA sequences containing CMV IE1 transcription regulatory sequences that were used in transfection assays or EMSA. (**A**) Schematic representation of the reporter plasmids that were used for transfection and that contain parts of the CMV IE1 5′ region. The CMV IE1 gene 5′ sequences are indicated by a heavy line. Plasmid pCMV^{-524}CAT contains the IE1 enhancer, promoter and leader region from positions –524 to +55 (all positions are relative to the transcription start site of the gene, which is indicated by an arrow) fused to the CAT reporter gene in pUC12. Plasmids pCMV^{-97}CAT and pCMV^{-55}CAT are similar, except they contain only the IE1 promoter region from –97 to +55 or –55 to +7, respectively. ME1, ME2 and ME3 are three MDBP sites in this region and CRE and NF$_k$ are the CREB and NF-κB sites neighboring ME1. The sequences and positions of ME1 and ME3 are shown in (B). Restriction sites used for generation of mutant plasmids or insertion of oligonucleotide duplexes are indicated as: H, *Hin*dIII; Ss, *Sst*I; X, *Xba*I; Sm, *Sma*I. (**B**) The sequences and positions of oligonucleotides used for EMSA. WT ME3, the wild-type low affinity MDBP site, which begins at position +5 of the IE1 gene. +5up and +5down are mutant sites with the bases altered (lower case letters) to make this a high affinity MDBP site (+5up) or a non-binding site (+5down). The MDBP, CREB and NF-κB binding sites are underlined. CRE-ME1, a 43 bp duplex with neighboring WT MDBP and CREB sites; CRE-ME1mut, the analogous duplex with a mutant MDBP site that differs from the consensus sequence at seven positions (lower case letters). NFκ, an 18 bp sequence containing an NF-κB site; ME1-NFκ, a 37 bp sequence containing the same NF-κB site and the neighboring MDBP site, ME1. CRE, a 25 bp duplex containing a consensus CREB site; pBm, a 22 bp duplex containing a standard methylation-dependent MDBP site. The consensus sequence for MDBP binding is also shown, but it should be noted that the central A is, in effect, deleted from several high affinity MDBP sites, as well as from ME3.

bound <5% as much MDBP as did the +5up site, which, in turn, gave about the same amount of MDBP–DNA complex as did a standard high affinity MDBP ligand, pBm (Fig. 2B). The specificity of the WT +5 site for MDBP was previously demonstrated by oligonucleotide competition experiments using nuclear extracts or partially purified MDBP (11). The MDBP binding activity of the *Xba*I–*Sst*I fragments containing the +5up site from four independent clones of pCMV^{-97}+5up and two of pCMV^{-524}+5up was also verified by EMSA (data not shown). The pCMV^{-97}+5down mutant, which was made by further mutation of the pCMV^{-97}+5up construct, was shown by EMSA to have a mutant +5 site which did not detectably bind to MDBP (Figs 1B and 2B and data not shown).

Reporter gene expression from these CMV IE1–CAT chimeras was compared with CAT expression from the analogous WT constructs upon transient transfection. With pCMV^{-97} constructs

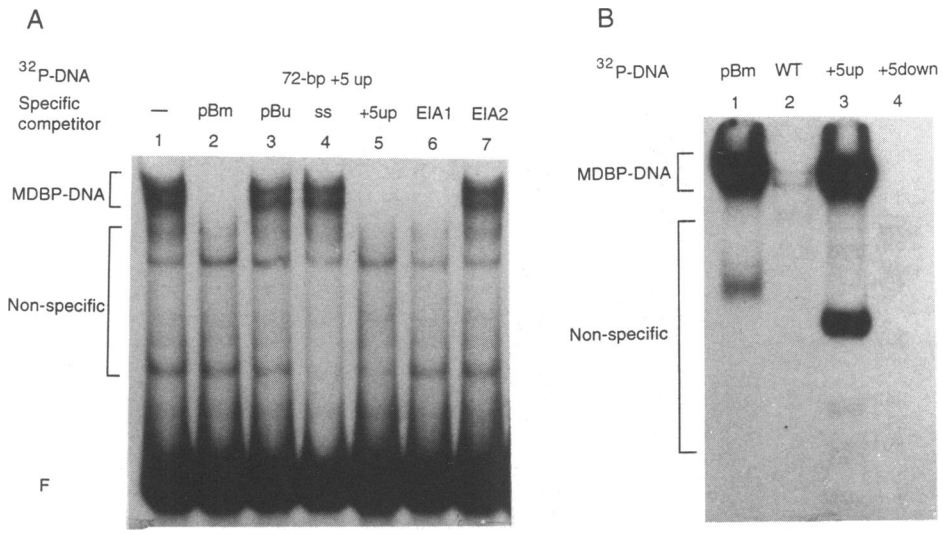


Figure 2. High affinity binding of the +5up mutant MDBP site in the CMV IE1 leader region. (**A**) The 72 bp oligonucleotide duplex used for converting the WT +5 CMV IE1 site from a low affinity MDBP site (WT) to a high affinity site (+5up; Fig. 1B) was checked for MDBP binding by EMSA. HeLa nuclear extract was incubated with 20 fmol radiolabeled duplex after preincubation with 1 pmol of the indicated oligonucleotide competitor. Lane 1, no competitor; lane 2, a standard methylation-dependent ligand for MDBP (Fig. 1B); lane 3, the unmethylated form of the latter duplex; lane 4, one of the two strands of the 72 bp duplex; lane 5, the 72 bp duplex used to mutate the +5 site from a low affinity site (WT) to a high affinity site (+5up) for MDBP; lane 6, a 21 bp duplex containing a methylation-independent MDBP site from the equine infectious anemia virus long terminal repeat (EIAV site 1; 9); lane 7, a non-specific 21 bp duplex from the same long terminal repeat (EIAV site 2; 9). The MDBP–DNA complexes, non-specific complexes (not competed for by excess unlabeled standard MDBP ligands nor by the 72 bp duplex added as an unlabeled competitor) and free ligand (F) visualized in the autoradiogram are indicated. (**B**) The affinity of 22 bp duplexes containing the WT or +5up site or the +5 site mutated to a non-binding site (+5down) was compared by EMSA as in (A) using the ^{32}P-labeled sequences shown in Figure 1B (except that an additional 4 bp from the IE1 leader region were present on each side of the +5 site-containing oligonucleotides). pBm was included as a standard high affinity MDBP site. The free DNA was electrophoresed off the gel.

mutation of the MDBP site at position +5 from a low affinity to a high affinity site resulted in an ~10-fold reduction in CAT expression upon transfection into CV-1 cells (Table 1, pCMV^{-97}+5up versus pCMV^{-97}CAT) as well as in Raji cells (data not shown). Reduced CAT expression was also observed, although to a lesser extent, in a comparison of the same +5up site with the WT sequence in the analogous pCMV^{-524} plasmid (Table 1, pCMV^{-524}+5up versus pCMV^{-524}CAT). Mutation of the +5 site in the pCMV^{-97}+5up construct to the non-binding sequence in pCMV^{-97}+5down restored CAT expression ~4–5-fold (Table 1). Independently isolated clones for a given recombinant plasmid gave essentially identical results in these transfection experiments.

The decrease in reporter gene expression upon increasing the affinity of the +5 site for MDBP is also seen during transcription *in vitro*

Run-off transcription of the CAT reporter gene linearized at an *Eco*RI site was performed with a HeLa nuclear extract and the above reporter constructs containing the CMV IE1 promoter/enhancer. The pCMV^{-524}+5up mutant with its high affinity MDBP site at position +5 gave ~60% less specific transcript (~320 nt product) than did the analogous plasmid containing the WT, low affinity MDBP site at the same position (Fig. 3, lanes 4–9 versus 1–3). This decrease in transcript formation was observed with two independent clones of the pCMV^{-524}+5up mutant (Table 2). When the templates were linearized with *Pvu*II, rather than *Eco*RI, a run-off RNA product of ~210 nt was obtained, as expected, and less of this transcript was observed from the +5up plasmid than from the analogous WT plasmid (data not shown). When the +5up MDBP site was compared with the WT site in the context of the pCMV^{-97}CAT vector containing only the IE1 promoter without the IE1 enhancer and its two naturally high affinity MDBP sites, a decrease in specific transcript formation was again seen (Table 2).

Table 1. Down-regulation of CAT expression by conversion of a low affinity MDBP site to a high affinity site at position +5 in the CMV IE1 leader region

Constructs		Relative CAT activity[a]
pCMV^{-97}CAT		1
pCMV^{-97}+5up	clone 1	0.08 ± 0.01 (2)
	clone 2	0.19 ± 0.06 (2)
	clone 3	0.11 ± 0.01 (3)
	clone 4	0.11 ± 0.04 (5)
pCMV^{-97}+5down	clone 1	0.41 ± 0.03 (2)
	clone 2	0.51 ± 0.03 (2)
pCMV^{-524}CAT		1[b]
pCMV^{-524}+5up	clone 1	0.34 ± 0.09 (3)
	clone 2	0.32 (1)

[a]The mean CAT activity in transiently transfected CV-1 cells was normalized for β-galactosidase activity to correct for transfection efficiency. The CAT activity from cells transfected with a given plasmid relative to that from cells transfected with the analogous WT construct is given ± SD. The sequences of the +5up and +5down MDBP site mutations and of the WT low affinity MDBP site are shown in Figure 1B. The different clones were independent isolates of the same recombinant plasmid. The number of experiments is given in parentheses. Duplicate or triplicate plates were used in each experiment.

[b]The relative activity of the WT pCMV^{-524}CAT to that of the WT pCMV^{-97}CAT was ~8–10.

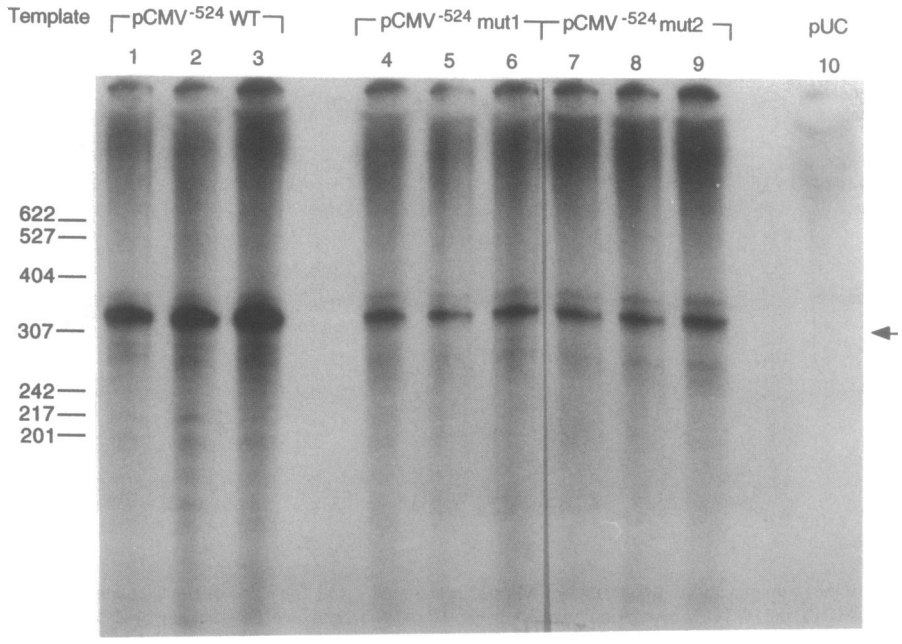


Figure 3. *In vitro* transcription driven by a WT CMV IE1 enhancer/promoter versus by the same enhancer/promoter containing a high affinity MDBP site at position +5. Triplicate run-off transcription reactions were performed as described in Materials and Methods with the WT pCMV^{-524}CAT template (lanes 1–3) containing its low affinity MDBP site or the analogous template mutated so that it contains a high affinity MDBP site (+5up; Fig. 1) at position +5 (lanes 4–9) after linearization of the plasmid with *Eco*RI. Template DNA was prepared from two independent clones (lanes 4–6 versus lanes 7–9) containing the same +5up mutation. The position of the expected transcript (~320 nt) is indicated by an arrow.

Table 2. Transcription *in vitro* from the WT CMV IE1 promoter/leader region or from the mutant region with a high affinity MDBP site at position +5[a]

Plasmid vector	Relative transcription (%)		
	WT	+5up clone 1	+5up clone 2
pCMV^{-524}CAT	100	37 ± 4 (6)	40 ± 2 (3)
pCMV^{-524}CAT + MDBP1 IgG	100	91 ± 16 (3)	84 ± 9 (2)
pCMV^{-97}CAT	100	40 ± 8 (3)	45 ± 10 (2)

[a]The mean relative amount of the specific run-off transcript product (~320 nt) from *Eco*RI-linearized plasmids is given ± SD and the indicated number of experiments performed is in parentheses. Samples in each experiment were performed in duplicate or triplicate. In the experiments labeled pCMV^{-524} + MDBP IgG, HeLa nuclear extract was preincubated with an IgG fraction of a polyclonal antibody to an N-terminal peptide from recombinant MDBP/RFX1 before being used for *in vitro* transcription.

We tested the effect of adding an antibody preparation directed against the N-terminal portion of a member of the MDBP family, namely RFX1 (12), to the transcription mixture containing the nuclear extract. This batch of antibody was previously shown to supershift ~50% of the MDBP–DNA complexes, specifically the slower moving RFX1-containing complexes, generated upon incubation of HeLa nuclear extract with a standard MDBP ligand (pBm) (12). Multiple forms of MDBP consisting of heterodimers or homodimers of polypeptides RFX1–4 are responsible for the appearance of several clustered bands in EMSA, the exact pattern of the bands depending upon the cell type used to prepare the nuclear extracts (9,10). The IgG fraction of MDBP/RFX1 antiserum was preincubated with the HeLa nuclear extract and, within a given experiment in parallel reactions, the following templates were used: WT pCMV^{-524} DNA and the analogous, identical mutant clones +5up clone 1 and +5up clone 2 (Fig. 1). In five experiments using different batches of template the DNA construct with the high affinity (+5up) MDBP site was consistently almost as good a template for *in vitro* transcription in the presence of this MDBP/RFX1-specific antibody preparation as the analogous WT construct with its low affinity MDBP site (Table 2). In the same experiments this mutant duplex was a several fold poorer template than the WT construct in the absence of this antibody preparation. Therefore, a component of the antibody preparation, most probably the antibody which supershifts MDBP/RFX1-containing complexes, changed the relative template activity specifically in response to the mutations in the DNA that converted the low affinity MDBP site to a high affinity site.

Inhibition of transient reporter gene expression by an MDBP site adjacent to a CREB site in the CMV IE1 enhancer

Because the IE1 gene region contains two MDBP sites in its enhancer, in addition to the MDBP site at position +5, we tested one of the two IE1 enhancer high affinity MDBP sites in conjunction with its neighboring CREB/ATF site (Fig. 1, CRE) to determine whether it might act synergistically with the latter to stimulate gene expression when introduced 5′ to the IE1 promoter. One to three copies of a 47 bp duplex containing the adjacent CREB and MDBP sites in the native sequence context or with its MDBP site mutated to no longer bind MDBP (CRE-ME1 or CRE-ME1mut; Fig. 1B) were inserted upstream of a truncated form of the CMV IE1 promoter. This promoter was used to drive CAT reporter gene expression in plasmid pCMV^{-55}CAT in transient transfection experiments. In forskolin-treated cells the construct with the single copy of the WT insert, with its high affinity MDBP site, gave ~45% of the CAT activity

seen with the insert containing the mutated MDBP site (Table 3, pCMV^{-55}CRE-ME1 versus pCMV^{-55}CRE-ME1mut). This lower extent of reporter gene expression obtained from the plasmid with the WT insert versus that from plasmid with the mutant insert (containing a mutated site no longer able to bind MDBP) depended upon treatment with forskolin, on having one rather than two or three copies of the CRE-ME1 insert and on having the truncated, rather than full-length promoter (in the pCMV^{-97}CAT) vector driving reporter gene expression (Table 3). The pCMV^{-97}CAT vector, unlike the pCMV^{-55}CAT vector, contains one sequence related to a CREB site, as well as two putative Sp1 sites and one putative CCAAT box, as part of the full-length promoter sequence (7,17). When the promoter activity was stronger due to the presence of two or three CRE sites, instead of one or the full-length promoter (in the pCMV^{-97}CAT vector) and rather than the truncated promoter (in the pCMV^{-55}CAT vector), interference with CREB/ATF function at this promoter by MDBP binding may have been overshadowed by having more copies of positively acting promoter elements (17).

Table 3. Effect of the presence of an MDBP site next to a CREB site inserted upstream of minimal CMV IE1 promoter driving CAT expression

Constructs	Relative CAT activity[a]	
	No stimulation	Stimulated by forskolin
pCMV^{-55}CAT	1	1[b]
pCMV^{-55}CRE-ME1	2.4 ± 0.1 (2)	9.9 ± 2.6 (2)
pCMV^{-55}CRE-ME1mut	2.9 ± 0.1 (2)	21.8 ± 2.8 (2)
pCMV^{-55}(CRE-ME1)×2	4.0 ± 0.8 (2)	20.1 ± 0.4 (2)
pCMV^{-55}(CRE-ME1mut)×2	3.1 ± 0.1 (2)	23.8 ± 3.6 (2)
pCMV^{-55}(CRE-ME1)×3	ND	31.0 (1)
pCMV^{-55}(CRE-ME1mut)×3	ND	27.0 (1)
pCMV^{-97}CAT	1[c]	1[d]
pCMV^{-97}CRE-ME1	1.5 (1)	2.0 ± 0.2 (2)
pCMV^{-97}CRE-ME1mut	ND	1.9 (1)

[a]The mean CAT activity is expressed from duplicate plates of CV-1 transfectants as indicated in Table 1. The numbers of independent experiments is shown are parentheses. CAT reporter constructs contained just the IE1 minimal promoter (pCMV^{-55}CAT or pCMV^{-97}CAT) or one to three copies of an insert with an additional IE1 enhancer-derived CREB site (CRE) and a WT (ME1) or mutant (ME1mut) MDBP site immediately upstream of the minimal promoter. The mutant site differs from the MDBP consensus sequence at seven positions (Fig. 1B).

[b]The relative CAT activity from forskolin-treated transfectants containing this construct was ~2.2-fold higher than that in analogous transfectants not treated with forskolin.

[c]The CAT activity of this –97 construct was ~15 times higher than that of the –55 construct.

[d]The relative CAT activity of cells transfected with this construct in the presence of forskolin was ~7-fold higher than that in the absence of forskolin.

Binding of proteins to adjacent MDBP and CREB or NF-κB sites in the CMV IE1 enhancer

Oligonucleotide duplexes containing the CRE next to the WT MDBP site (CRE-ME1) or next to the MDBP site mutated at seven positions (CRE-ME1mut; Fig. 1B) were used as ligands for EMSA with nuclear extracts from forskolin- and PMA-treated HeLa cells or PMA-treated or uninduced HL-60 cells. The extent of formation of MDBP–DNA complexes was comparable with that of CREB–DNA complexes (Fig. 4, lane 3 and Fig. 5, lanes 5 and 8). The specificity of these complexes was determined by co-electrophoresis with complexes containing standard MDBP or CREB ligands (Fig. 4, lanes 1–3), by competition with specific versus non-specific oligonucleotide competitors (Fig. 4, lanes 3–7 and Fig. 5, lanes 5–10) and by using the CRE-ME1mut duplex as a ligand (Fig. 4, lanes 8–12). We also examined interaction of the HL-60 nuclear extracts with an oligonucleotide duplex containing the same MDBP recognition sequence and the NF-κB site, which is the downstream neighbor of the MDBP site in the enhancer (ME1-NFκ, Fig. 1). NF-κB-type complexes formed, but in considerably lower amounts than MDBP-type complexes (Fig. 5, lanes 1–4). There was no indication of the formation of very low mobility complexes which could represent ternary complexes between these MDBP family proteins, the DNA ligands and either CREB-type or NF-κB-type proteins (Figs 4 and 5). Upon transient transfection into PMA-treated CV-1 cells the ratio of CAT activity from a construct containing a single copy of the WT ME1-NFκ insert immediately upstream of the truncated IE1 promoter in the pCMV^{-55}CAT vector compared with that obtained from the analogous plasmid having one copy of the WT NFκ insert at the same position and in the same orientation was 2.2.

DISCUSSION

There is a high affinity transcription factor binding site in the hepatitis B virus (HBV) enhancer 1 which is recognized by a single related family of proteins variously called MDBP, EP, EF-C and RFX (12,18–21). This site is critical for enhancer activity, and overexpression of a member of the MDBP family (RFX1 homodimers) was shown to increase enhancer function (18–20). However, this site, like the CMV IE1 enhancer MDBP sites, gives little or no stimulation of transcription when concatemerized and placed upstream of a minimal promoter (11,20). In contrast to the decreased enhancer activity resulting from down-mutating the HBV enhancer MDBP site, there is little effect on reporter gene expression in transient transfection assays upon deletion of considerable portions of the CMV IE1 enhancer, including its two high affinity MDBP sites (7,22). This is apparently due to redundancy of the CMV IE1 enhancer's many transcription factor binding sites, but these assays on cultured cells might miss effects on gene expression that may occur during *in vivo* propagation or activation of CMV. These effects could be down-modulation of transcription, e.g. late in infection (7,8), as well as up-regulation. Recently an MDBP site downstream of the human transforming growth factor gene was shown to interfere with the ability of AP-1/CREB proteins to stimulate transcription from neighboring sequences (23).

We found that multimerization of one of the MDBP sites from the CMV IE1 enhancer along with its nearby CREB site from this enhancer (Fig. 1) still did not lead to stimulation of transcription of a downstream reporter gene (Table 3). Furthermore, the presence of this MDBP site in addition to the adjacent CREB site or an adjacent NF-κB site did not result in the formation of ternary complexes between DNA, MDBP and CREB-type or NF-κB-type proteins (Figs 4 and 5). It is not known how this MDBP site functions in the context of the entire IE1 promoter/enhancer containing two other MDBP sites, however, the multiplicity of these sites in a 0.5 kb region that controls early viral expression suggests that they are relevant to the life cycle of CMV.

Mutation of one of the other MDBP sites in this region, the +5 site in the untranslated leader, had a major effect on reporter gene expression in the context of the –97 IE1 promoter (Fig. 1A).

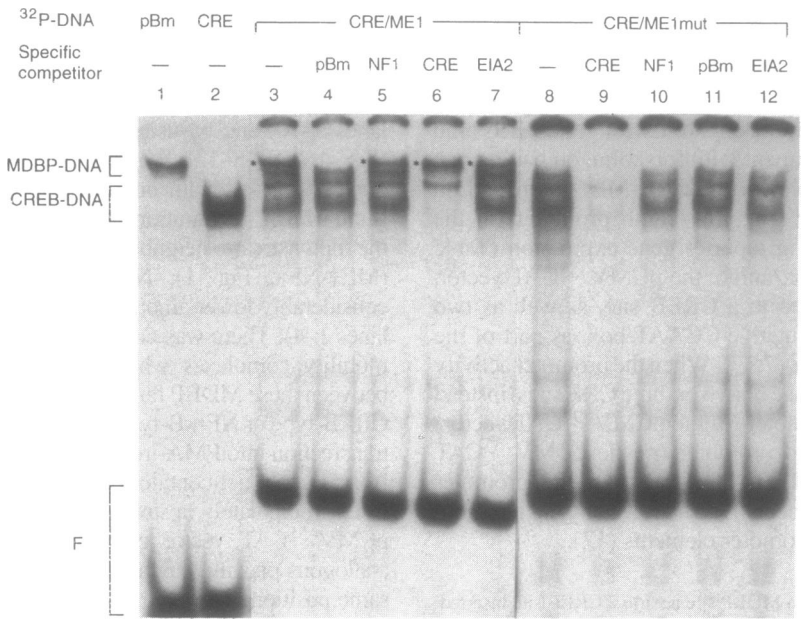


Figure 4. Formation of MDBP-type versus CREB-type complexes with an oligonucleotide duplex from the CMV IE1 enhancer containing an MDBP site and a CREB site. EMSA was conducted with nuclear extracts from HeLa cells treated with forskolin and PMA as described in Materials and Methods. The radiolabeled ligands were: MDBP-specific pBm (lane 1); CREB-specific CRE (lane 2); CRE-ME1, the CMV IE1 enhancer-derived duplex containing the neighboring MDBP and CREB sites (lanes 3–7); CRE-ME1mut, the analogous oligonucleotide with a down-mutated MDBP (lanes 8–12; Fig. 1B). The dots alongside lanes 3 and 5–7 denote the specific MDBP–DNA complexes formed with the CRE-ME1 ligand and not with the CRE-ME1mut ligand. The indicated unlabeled oligonucleotide duplex competitor (see Fig. 1B and the legend to Fig. 2) was preincubated with the nuclear extract. The sequence of one strand of the NF1 competitor (an irrelevant duplex) is as follows: 5′-AATTCCTTATTTTGGATT-GAAGCCAATATGATAATGAGG-3′. The positions of the complexes specifically competed for by an MDBP ligand (MDBP–DNA) and of the major complexes competed for by a CREB ligand (CREB–DNA) are indicated. A minor protein–DNA band immediately beneath the MDBP–DNA complexes was also competed for by the CREB ligand and so probably represents one of the CREB/ATF-type complexes with this ligand. This band was not reduced in intensity upon addition of an MDBP ligand (lane 4) and migrates faster than the MDBP–DNA complexes, so it does not represent a ternary complex with MDBP and CREB protein plus DNA.

When this low affinity (WT) site was converted to a high affinity (+5up) site, there was an ~10-fold decrease in CAT expression in transient transfection experiments (Table 1). Even when the entire IE1 promoter and strong IE1 enhancer region was present, a 3-fold decrease in CAT activity was obtained with the +5up MDBP site rather than the WT MDBP site at this cap downstream position. With the –97 promoter constructs mutation of the +5up site to a sequence that did not bind MDBP (+5down) resulted in an increase of 4–5-fold in CAT activity. That the +5down site, which deviated at 6 out of 14 bp from the WT site, supported reporter gene expression 4–5-fold better than the +5up consensus MDBP site strongly argues that the +5up mutation, which created the high affinity MDBP site, affected reporter gene expression mostly by increasing binding of a repressor protein and not by disrupting a positively acting transcription factor interaction. If the 10-fold decrease in reporter gene expression upon conversion of the low affinity MDBP site to a high affinity site were due to loss of a positive interaction, introducing six essentially random mutations in a 14 bp region should not have restored most of the lost activity. That not all of the lost activity was recovered might indicate that this WT leader region also acts positively to control IE1 expression or that addition of an extra residue to this sequence in order to generate a high affinity MDBP site was deleterious (Fig. 1B).

In vitro transcription analysis supports the conclusion that the substitution of a high affinity MDBP site for the WT low affinity site at position +5 inhibited transcription driven by the CMV IE1 promoter and that the increased binding of MDBP was largely responsible for this. Increasing the affinity of the +5 site for MDBP decreased formation of the specific run-off product *in vitro* from pCMV^{-524}CAT and pCMV^{-97}CAT constructs (Table 2). That the relative extent of repression due to the MDBP site mutation in these two reporter constructs differed *in vitro* and *in vivo* (Table 1 versus Table 2) is not surprising, given the differences between these systems. Addition of an antibody to the N-terminal portion of one of the polypeptides in the MDBP family (RFX1) alleviated the repressive effect of the +5up mutant site on transcription *in vitro* (Table 2). Because the N-terminal regions of RFX1, RFX2 and RFX3, the three fully sequenced subunits in the homodimeric or heterodimeric MDBP family, are very different (10), this result suggests that RFX1, a major subunit of MDBP in HeLa cells (12,18), is involved in the down-regulation of expression that we observed *in vitro* from the high affinity MDBP site introduced at position +5.

The binding of some sequence-specific DNA binding proteins to the cap site region or immediately downstream promotes transcription (see, for example 24,25). Although we do not yet know how high affinity MDBP binding interferes with transcription at the mutant high affinity +5 site of the CMV IE1 gene, there are two possibly relevant findings. MDBP proteins form extremely long-lived complexes with high affinity binding sites (up to 1.5 h), although with weak binding sites the complexes are much more short lived (26). Perhaps the long-lived nature of such high affinity complexes is important in impeding the formation, movement or disassembly of the transcription initiation machinery at the transcription initiation site. In this regard, it is possible that

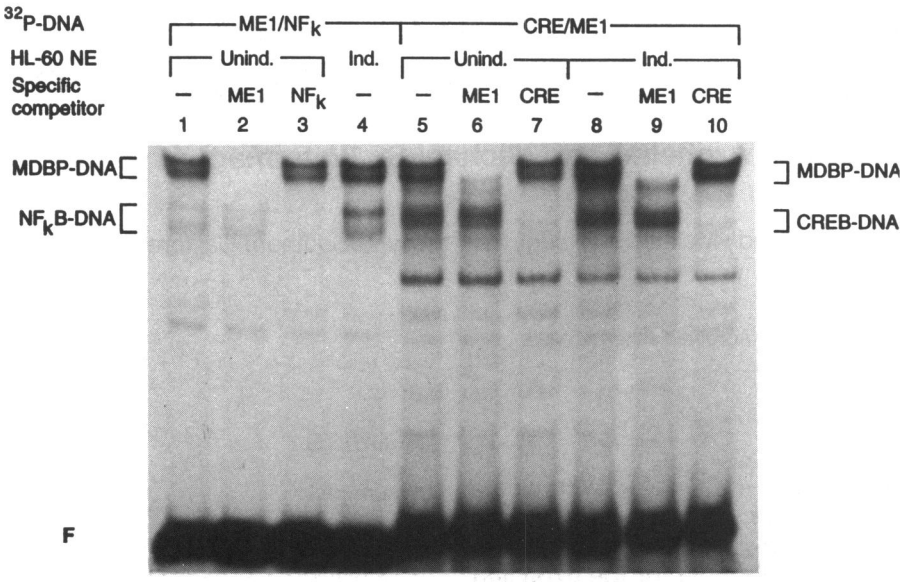


Figure 5. Formation of MDBP-type versus NF-κB-type and CREB-type complexes with oligonucleotide duplexes from the CMV IE1 enhancer containing the corresponding sites. EMSA was performed as for Figure 4 except that the nuclear extract was from uninduced HL-60 cells (lanes 1–3 and 5–7) or PMA-induced HL-60 cells (lanes 4 and 8–10). The radiolabeled ligands were ME1-NFκ (lanes 1–4) or CRE-ME1 (lanes 5–10). The unlabeled competitors (1 pmol) were a 22 bp duplex containing the MDBP site ME1 (Fig. 1B) or the NFκ and CRE sites as indicated. The MDBP–DNA, NF-κB–DNA and CREB–DNA complexes and free DNA are indicated. The other complexes seen are probably non-specific.

the corresponding WT low affinity MDBP site, which should form much more short-lived complexes with MDBP, may not be detrimental to transcription and might even aid transcription initiation. Lastly, we have recently found that MDBP/RFX1 interacts strongly with the TFIID-associated factor TAF110 in the yeast two-hybrid assay (unpublished data). Unlike other TAF110-interactive transcription factors, such as the transcription stimulatory factor Sp1 (27), MDBP family members might impede transcription when tethered to a TAF110-type protein from a high affinity MDBP site downstream of the cap position. Whatever the physiological significance of the low affinity WT MDBP site at position +5, the down-regulation of gene expression upon its conversion to a high affinity, methylation-independent MDBP site may help elucidate the function of methylation-dependent MDBP sites which have already been demonstrated in the leader regions of several genes believed to be down-regulated by DNA methylation (9), as well as of the methylation-independent MDBP site in the leader region of the apparently full-length cDNA encoding the RFX1 subunit of the MDBP/RFX family (12).

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