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Repression by a differentiation-specific factor of the human cytomegalovirus enhancer

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ABSTRACT

We detected a novel nuclear protein, MRF, that binds to multiple sites on the modulator which is located upstream of the human cytomegalovirus major immediate early gene enhancer. The expression of MRF is differentiation specific; the DNA binding activity is present in nuclear extracts from undifferentiated Tera-2 and THP-1 cells, but significantly reduced after these cells are induced to differentiate. In undifferentiated cells the enhancer activity is repressed by the modulator and upon differentiation the enhancer becomes active. Competitive binding assays demonstrate that MRF requires the presence of multiple A+T stretches for binding to DNA, rather than binding to a specific DNA sequence. Mutations of these stretches in the modulator reduce the binding activity of MRF, as well as the repressing activity on the enhancer. These results suggest that MRF may act as a repressor of enhancer function. We propose that MRF binds over the entire modulator and exerts repressor activity.

INTRODUCTION

Transcription of the major immediate early (M-IE) gene of human cytomegalovirus (HCMV) in Tera-2 cells, a human embryonal carcinoma cell line, provides a model system for studying cellular factors which regulate gene expression during differentiation. Tera-2 cells can be maintained in an undifferentiated state or can be induced to differentiate by treatment with retinoic acid (1). Undifferentiated Tera-2 cells are not permissive for HCMV replication, but retinoic acid-treated Tera-2 cells are (2,3). The block to viral replication in undifferentiated Tera-2 cells has been demonstrated to be due to repression of M-IE gene expression (4). The M-IE gene product regulates expression of other viral genes (5–8), but M-IE gene expression itself is heavily dependent upon cellular factors (2). Previously described 5′ regulatory elements of the M-IE gene include a very strong enhancer containing repetitive palindromic sequence elements (9,10), a cluster of NF-1 binding sites (11,12) and a far upstream region termed the modulator, comprising nt –750 to –1145 relative to the transcription start site (4,13–16) (see Fig. 1). Nelson *et al.* (4,15) have demonstrated that the modulator plays a crucial role in mediating the cell-specific expression of the M-IE gene observed in undifferentiated and differentiated Tera-2 cells. They found that *in vivo* DNase I hypersensitive and chloracetaldehyde sensitive sites present only in cell types permissive for HCMV M-IE gene expression map to the modulator. They also demonstrated by transient transfection assays that the modulator functions as an up-regulator in permissive cells and as a down-regulator in non-permissive cells. A similar result was reported for transient transfection assays using undifferentiated and differentiated THP-1 cells, where HCMV replication is also differentiation dependent (17). These observations support the role of the modulator of the M-IE gene as a mediator of cell-specific gene expression.

Work by Shelborn *et al.* (18) has identified three cellular factors, termed modulator binding factors (MBF), which recognize portions of a dyad symmetry element in the region from nt –963 to –912. Deletion of the dyad symmetry element increases levels of chloramphenycol acetyltransferase (CAT) activity of reporter plasmids in which the *CAT* gene is placed under the control of the M-IE promoter, enhancer and modulator. MBF-1 is detectable by gel mobility shift assays in nuclear extracts of untreated Tera-2 cells, but its binding activity is decreased in nuclear extracts of Tera-2 cells treated with retinoic acid. On the other hand, MBF-2 and MBF-3 exhibit increased binding activity in Tera-2 cells upon retinoic acid treatment. The same group also reported that YY-1, the ubiquitously expressed zinc finger DNA binding protein, represses M-IE gene expression (19). However, it is clear that factors other than these are required to repress expression of the M-IE gene in undifferentiated Tera-2 cells (20).

In this paper we report the identification of a novel DNA binding protein, termed modulator recognition factor (MRF), that binds to the modulator at multiple sites in a concentration-dependent manner. The DNA binding activity of MRF is significantly reduced in differentiated cells that express the M-IE gene. In undifferentiated cells that express MRF at high levels the HCMV enhancer is silent and expression of the M-IE gene is repressed. However, enhancer activity is restored either by

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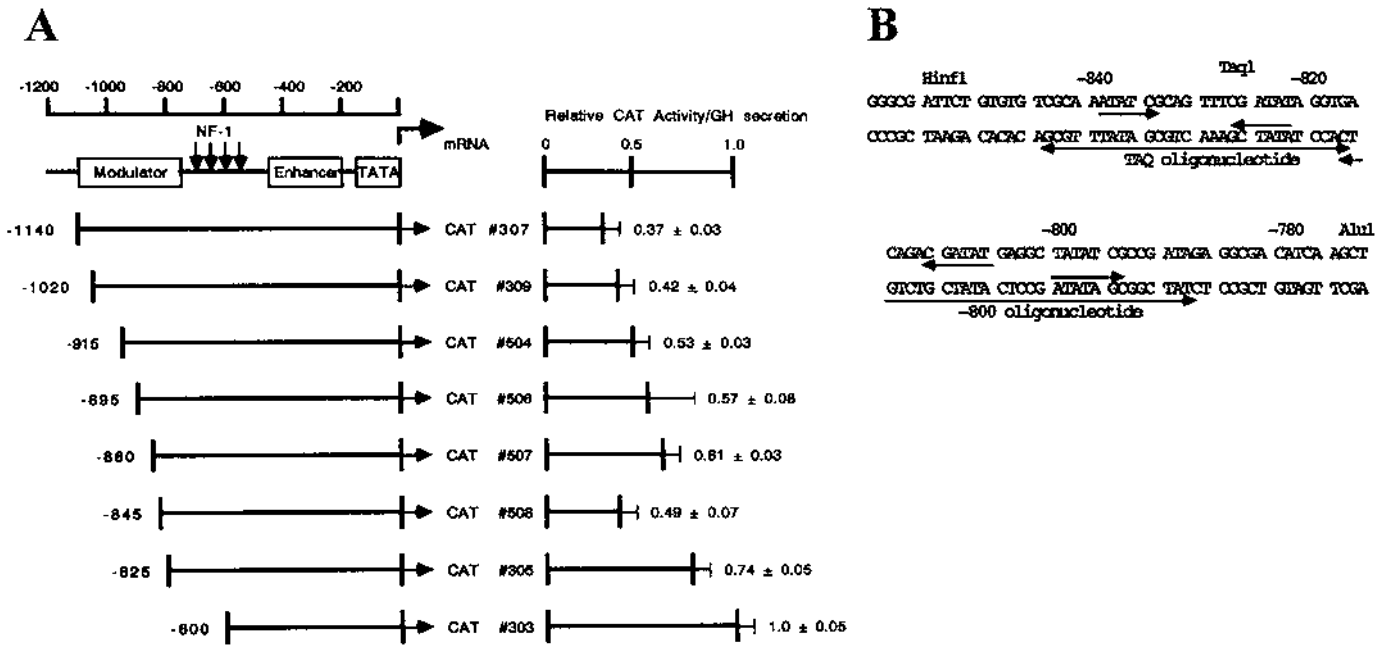

Figure 1. Location of *cis*-acting negative regulatory elements in the modulator region. (**A**) Aliquots of 0.05 µg of the indicated *CAT* expression vectors, 1.0 µg growth hormone expression vector and 4 µg carrier DNA (Bluescript SKII+; Stratagene) were transiently transfected into undifferentiated Tera-2 cells using the calcium phosphate method. The normalized ratios of CAT and hGH activities are plotted versus the 5′ deletion points, numbered relative to the transcription start site. A value of 1.0 represents the activity ratio of the –600 deletion construct (p303CAT). Three independent assays were performed and the numbers indicate the mean± SD of those assays. A schematic presentation of the 5′ controlling region of the *MIE* gene of HCMV linked to the *CAT* gene is shown in the upper left of the figure. (**B**) The DNA sequence around the HA probe is shown. Double arrowhead lines show oligonucleotide probes (TAQ and –800) and single arrowhead lines denote the positions and orientations of the inverted repeats (ATATCG).

deletion or by mutation of the factor binding sites. These results suggest that MRF is a *trans*-acting factor involved in repressing enhancer activity.

MATERIALS AND METHODS

Cell culture

Tera-2 cells (subclone NT2D1; 1) were obtained from P.W. Andrews of the Wistar Institute and grown in DME/high glucose medium containing 10% fetal calf serum. For retinoic acid treatment cells were plated at a density of 10^6 cells/T75 flask in medium containing 10^{-5} M retinoic acid and harvested after retinoic acid treatment for the indicated times. THP-1 cells were purchased from ATCC and grown in RPMI medium containing 10% fetal calf serum. For differentiation cells were seeded at a density of 5×10^5 cells/ml in medium containing 1.6×10^{-7} M TPA and harvested after 3 days (21).

Plasmid construction

A plasmid containing the entire control region of the M-IE gene, derived from HCMV strain AD169, was a gift from Dr H.Pande (Beckman Research Institute, City of Hope). For the location of *cis*-acting suppressor elements plasmids containing the region from –1140 to +74 (p307CAT), –825 to +74 (p305CAT) and –600 to +74 (p303CAT) were constructed by cloning the *Pst*I–*Xma*III, *Taq*I–*Xma*III and *Hinc*II–*Xma*III fragments of the M-IE gene of HCMV into the *Hin*dIII site of pSVO-CAT. Plasmids with 5′ deletions to –915 (p504CAT), –844 (p508CAT) and –800 (p507CAT) were constructed by Bal31 digestion of the p307CAT construct. A *Hin*dIII linker was ligated to the 5′-end of each deletion product and the DNA was cut with *Hin*dIII and *Nsi*I. The *Hin*dIII–*Nsi*I fragment was then cloned into the *Hin*dIII *and Nsi*I sites of p307CAT. The 5′ deletion position of each plasmid was determined by sequencing. These original plasmids were inconvenient for the construction of plasmids necessary for subsequent experiments and therefore all inserts were subcloned into pCAT-Basic (Promega) by digesting with *Hin*dIII, filling in with Klenow enzyme and blunt-end ligating to the filled in *Xba*I site. For convenience the same nomenclature of plasmids was used after subcloning. The HA probe was produced by digestion of p508CAT with *Xba*I and *Alu*I unless otherwise noted. pM_5CAT were produced by mutation of TA to GC at the underlined places in the palindromic sequence ATATCG N_6CGATAT (–839 to –822) of p508CAT using the Transformer mutagenesis kit (Clontech). Two TA→GC mutations were also introduced at the underlined places in another palindromic sequence CGATAT N_6ATATCG (–811 to –794) to give pM_6CAT. DNA fragments used as cold competitors in gel mobility shift assays in Figure 4A were prepared by digestion of these constructs with *Xba*I and *Hae*III restriction enzymes. Mutations accidentally created an *Alu*I site in the right side of the palindromic sequences and thus the *Hae*III site 10 bp downstream of the original *Alu*I site was used to produce the mutated probes.

Preparation of nuclear extracts

Nuclear extract preparation was adapted from the method of Dignam *et al.* (22), except that the homogenization step of

isolated nuclei was eliminated because the yield of MRF was significantly reduced. Washed cell pellets were suspended in 5 vol buffer A, which contained 10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1 mM PMSF. The suspension was incubated on ice for 10 min and centrifuged at 900 *g* for 5 min at 4°C. The pellet was resuspended in 4 vol buffer A, homogenized with a Dounce homogenizer using 20 strokes with a 'B' pestle and then centrifuged at 325 *g* for 10 min. The pellet was resuspended in 0.8 ml buffer A and centrifuged at 325 *g* for 10 min. The supernatant was removed and the pellet was suspended in 4 vol buffer C (20 mM HEPES, pH 7.4, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF and 0.5 mM DTT) and placed on a tube rotator at 4°C for 30 min. The tubes were then centrifuged at 8000 *g* for 30 min at 4°C. The supernatant was removed and dialyzed against buffer D (20 mM HEPES, pH 7.4, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). After dialysis for 5 h the sample was centrifuged at 8000 *g* for 30 min at 4°C and the supernatant aliquoted and stored at –80°C.

Transient transfection

Calcium phosphate transfections were performed as described (23). For the series of nested deletion mutants Tera-2 cells (0.5×10^6) were co-transfected with 0.05 µg *CAT* vector, 1 µg human growth hormone (hGH) expression vector (24) and 4 µg Bluescript SKII+ (Stratagene) plasmid as carrier DNA. Levels of bacterial CAT were assayed by thin layer chromatography after 48 h transfection (23). Secreted human growth hormone was assayed with a radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), following the manufacturer's recommended protocol. For the base mutation studies cells were co-transfected with 0.1 µg *CAT* vector and 10 µg Bluescript. CAT activities were measured by direct scintillation counting (25). THP-1 cells (2×10^6) in 2 ml RPMI without serum were transfected with 20 µl DEAE–dextran (10 mg/ml) containing 3 µg *CAT* vector for 20 min. Cells were collected and resuspended in 4 ml RPMI plus 10% fetal calf serum. After 48 h CAT activities were measured as described above.

Gel mobility shift assay

Up to 10 µl of nuclear extract was added to a tube containing 4 µl H_2O, 2 µl 10× binding buffer (500 mM NaCl, 100 mM Tris, pH 7.5, 10 mM DTT, 10 mM EDTA and 50% glycerol), 1 µg poly(dI-dC), 1 µl 200 mM $MgCl_2$ and 1 ng end-labeled DNA probe. Competitor DNA, if added, was dissolved in 4 µl water. The volume was brought to 20 µl with nuclear extract in buffer D described above. The mixture was incubated at room temperature for 30 min to allow complex formation and was analyzed on a 3.5% polyacrylamide gel using a low ionic strength buffer system (6.7 mM Tris, pH 7.5, 3 mM sodium acetate) at room temperature for 60–90 min at 100 V.

Preparation of DNA competitors

The sequences of the plus strands of the synthetic oligonucleotide duplexes used in gel mobility shift assays are as follows:
TAQ, TCGCAAATATCGCAGTTTCGATATAGGTGA (–845 to –816);
–800, GACAGACGATATGAGGCTATATCGCCGATA (–817 to –788);
MBF-1, GATTTTTGGGCATACGCGATATCTG (–938 to –914);
NF-1, CGCCCTCTCTCGCCCTTGCCAAGTTGGAAG.

TAQ–800 corresponds to nucleotides –845 to –788. Concatenated oligonucleotide duplexes (a mixture of dimer to octamer) were prepared by self-ligation of kinased oligonucleotide duplexes with T4 DNA ligase. After phenol extraction and ethanol precipitation the ligation reaction mixtures were analyzed on polyacrylamide gels by electrophoresis and used as competitors without further purification. Dimer oligonucleotides were separated by gel electrophoresis, extracted from the gel by electroelution and cloned into the Bluescript plasmid. TAQ dimer was cloned into the blunt-ended *Hin*dIII site such that only the tandem repeat of the dimer regenerated a *Hin*dIII site. Both the –800 dimer and TAQ–800 were cloned into blunt-ended *Hin*dIII and *Hin*cII sites. All DNA sequences were confirmed by sequencing. The DNA fragments from the modulator were prepared by digestion with appropriate restriction enzymes. Bluescript SKII+ was digested with *Hae*III to obtain two DNA fragments of 102 (509–611) and 79 bp (661–740). All these DNA fragments were used as competitors for gel mobility shift assays after gel purification.

RESULTS

Location of a negative regulatory element (–845 to –825)

Previous work indicated that the modulator is responsible for repression of HCMV M-IE gene transcription in undifferentiated Tera-2 cells (13,15,16). With the intention of locating negative regulatory elements in the modulator, we constructed a series of nested deletion mutants in the modulator that is linked to the enhancer and promoter of the M-IE gene fused to the bacterial *CAT* gene. We co-transfected these reporter constructs with a human growth hormone (hGH) expression vector into undifferentiated Tera-2 cells using the calcium phosphate method and the levels of transiently expressed CAT enzyme and secreted hGH were measured. The ratio of CAT activity to secreted hGH for each construct was plotted versus the 5′ deletion positions (Fig. 1A). The relative CAT activities of the constructs gradually increased as the modulator sequence was deleted, except for deletion of the nucleotides between positions –860 and –845. Deletion of the 20 nt between positions –845 and –825 resulted in a significant increase in relative CAT activity. These results suggest that there are several *cis*-acting negative regulatory elements in the modulator. The increase in expression seen upon deletion of the 20 nt from –845 to –825 led us to concentrate our initial work on a putative negative regulatory element that might be present near or within this sequence. However, it should be noted that the observed increase in transcription might be caused by losing an activating element or creating an artificial repressing element at the new junction in the –845 construct. The increase in CAT activity due to the deletion from –845 to –825 was roughly equal to the decrease due to deletion from –860 to –845.

Detection of a nuclear factor recognizing the negative regulatory element

In order to identify DNA binding factors that recognize the negative regulatory element we labeled the HA probe (–845 to –774 bp; Fig. 1B) and used it for gel mobility shift assays with nuclear extracts from undifferentiated and retinoic acid-treated Tera-2 cells. With the nuclear extract from untreated Tera-2 cells a slow moving DNA–protein complex was detected (Fig. 2, left panel, lane 2). Formation of the complex was significantly reduced with the nuclear extracts prepared from Tera-2 cells after

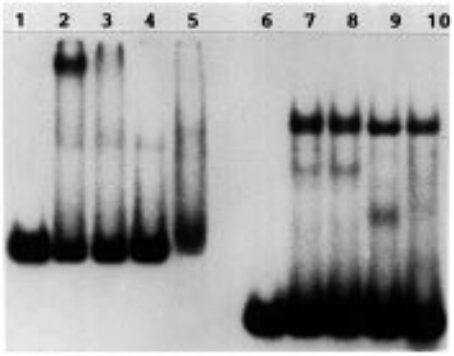

Figure 2. DNA binding assays of the HA and NF-1 probes with nuclear extracts from untreated and retinoic acid-treated Tera-2 cells. The conditions for gel mobility shift assays are described in Materials and Methods. The end-labeled probes (1 ng) were incubated in the presence of poly(dI-dC) (1 μg) with nuclear extracts (2 μg) from untreated Tera-2 cells (lanes 2 and 7) or from Tera-2 cells treated with retinoic acid for 2 (lanes 3 and 8), 5 (lanes 4 and 9) or 7 days (lanes 5 and 10). Lanes 1 and 6 are the free probes for MRF and NF-1 respectively.

2 days of retinoic acid treatment (lane 3) and barely detectable after 5 days (lane 4). On the other hand, complex formation of the oligonucleotide probe with an NF-1 recognition sequence was not significantly affected by retinoic acid treatment (Fig. 2, right panel). To examine whether formation of the slow moving DNA–protein complex is specific to the HA probe we carried out competitive gel mobility shift assays. Complex formation was extinguished by competition with an excess amount of the non-radioactive HA probe (10-fold), suggesting that binding of the nuclear factor(s) to the probe was specific (Fig. 3B, lanes 3 and 4). We refer to this DNA binding factor as the modulator recognition factor (MRF), to distinguish it from the modulator binding factors (MBF) described by Shelbourn. The latter recognizes sequences within the dyad symmetry element (–963 to –912 bp; 18). Occasionally we observed formation of other shifted bands with higher mobility than the slow moving complex in gel mobility shift assays. However, formation of those bands was not inhibited in the presence of cold HA probe and, therefore, the complexes may be formed by non-specific factors. Sinclair *et al.* demonstrated by transient transfection assays that the monocytic cell line THP-1 is non-permissive for HCMV replication due to a block in expression of the M-IE gene promoter (17). Differentiation of THP-1 cells into macrophages lifts this block and permits M-IE gene expression. To investigate whether binding activity of MRF to the HA probe is present in THP-1 cells and controlled by differentiation of THP-1 cells we carried out gel mobility shift assays with nuclear extracts from untreated and TPA-treated THP-1 cells. Formation of a DNA–protein complex similar to the MRF–HA complex was observed with untreated THP-1 cells and significantly reduced with the differentiated cells (Fig. 4A, lanes 6 and 7) in a similar manner to that described above for Tera-2 cells.

Binding sites of MRF

In order to identify the DNA sequence recognized by MRF we carried out methylation interference and DNase I footprinting assays with the HA probe, but failed to determine the recognition sequence. Methylation interference assays with the crude nuclear extract from Tera-2 cells did not identify any guanine bases at

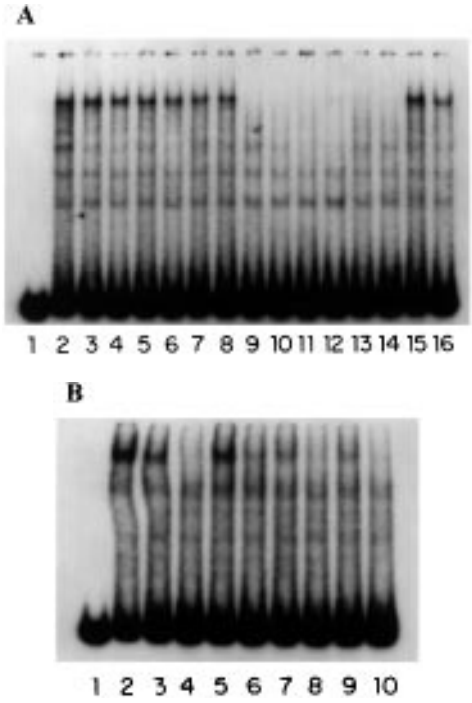

Figure 3. Competition of various oligonucleotides with the HA probe for binding to MRF. The radioactive HA probe was incubated with nuclear extract (2 μg) from undifferentiated Tera-2 cells in the presence of various ligated and unligated oligonucleotide probes. (**A**) The reaction mixtures included labeled HA probe only (lane 1), probe plus nuclear extract only (lane 2) or probe plus extract in the presence of various unlabeled competitor oligonucleotides (lanes 3–16). Unligated oligonucleotides (lanes 3–8) or ligated oligonucleotides (lanes 9–16) were used as competitors. The competitors were TAQ (lanes 3–4 and 9–10), –800 (lanes 5–6 and 11–12), MBF-1 (lanes 7–8 and 13–14) and NF-1 (lanes 15–16). The competitors were present at a 10-fold excess (odd numbered lanes) or 100-fold excess (even numbered lanes). (**B**) The reaction mixtures included labeled HA probe only (lane 1), probe plus nuclear extract only (lane 2) or probe, nuclear extract and various competitors (lanes 3–10). The competitors were HA (lanes 3–4), TAQ dimer (lanes 5–6), –800 dimer (lanes 7–8) and TAQ–800 (lanes 9–10). The competitors were equimolar (odd numbered lanes) or at a 10-fold excess (even numbered lanes) with respect to the labeled probe.

which methylation significantly disturbed DNA–MRF complex formation (data not shown). DNase I footprinting assays in the presence of nuclear extracts from Tera-2 cells gave unusual results. Instead of distinct footprints we observed simultaneously enhanced and suppressed DNase I digestion over the entire HA probe in the presence of the nuclear extract (data not shown).

Examination of the DNA sequence of the HA fragment revealed the presence of one inverted repeat motif, 5′-ATATCG N_6 CGATAT-3′ at nucleotide positions –839 to –822 (Fig. 1B) and the same inverted repeat in the opposite orientation at positions –811 to –794. Thus we synthesized two oligonucleotides (TAQ and –800) which contained the respective inverted repeat motif (Fig. 1B) and investigated whether either or both of the oligonucleotides were involved in binding of MRF. We performed gel mobility shift assays, using the HA fragment as a labeled probe and the double-stranded oligonucleotides as cold competitors (Fig. 3A). The results showed that these single oligonucleotides could not inhibit formation of the specific high molecular weight complex, even when present at 100-fold molar excess (lanes 3–6). However, the complex disappeared completely

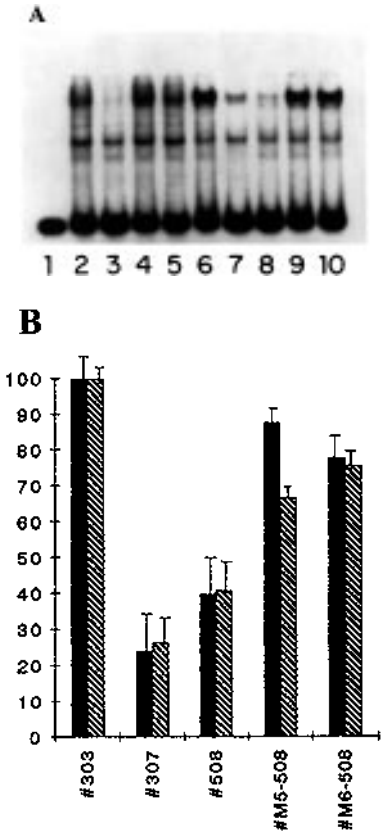

Figure 4. Correlation between the DNA binding and repression activity of HA mutants. (**A**) *In vitro* DNA binding activity of MRF to the HA probe carrying mutated sequences. The gel mobility shift assays of the radioactive HA probe with nuclear extracts from undifferentiated Tera-2 cells were carried out in the presence of the non-radioactive mutated HA probes. The reaction mixtures included labeled HA probe only (lane 1), probe plus Tera-2 nuclear extract (lane 2) or probe, extract and various competitors (lanes 3–5). The competitors were HA (lane 3), M5 (lane 4) and M6 (lane 5). The same competition assay was carried out with THP-1 nuclear extract, probe plus extract (lane 6) or probe, extract and the competitors HA (lane 8), M5 (lane 9) and M6 (lane 10). Lane 7 shows the gel mobility shift assay of the HA probe with nuclear extract (2µg) from TPA-treated THP-1 cells. The competitors were present at a 10-fold excess. The radioactivity of the HA–MRF complex (top band) in each lane was measured with an Ambis 400 (Scanalytical, Billerica, MA). (**B**) Repressing activities of mutated expression vectors. Transient transfection assays of the expression plasmids carrying the above mutations were performed in undifferentiated Tera-2 and THP-1 cells respectively. Numbers indicate the average of three independent assays. A value of 100 represents the mean ± SD activity of the –600 deletion construct (pCAT303). The dark bars represent Tera-2 cells and shaded bars represent THP-2 cells respectively.

in the presence of a 10-fold excess of the concatenated TAQ and –800 oligonucleotides (lanes 9–12), but not in the presence of concatenated NF-1 (26,27) oligonucleotide (lanes 15–16). The finding that concatenated probes competed more efficiently than monomer probes suggests that formation of the high molecular weight DNA–protein complex requires the presence of the two juxtaposed oligonucleotides (TAQ and –800) and that each oligonucleotide plays a similar role in complex formation. In order to investigate this, tandemly repeated dimers of TAQ and –800 oligonucleotides (TAQ_2 and -800_2) and TAQ–800 oligonucleotide were prepared and examined for their ability to inhibit formation of the HA–MRF complex. As shown in Figure 3B, both the homodimers (lanes 5–8) and the TAQ–800 oligonucleotide (lanes 9 and 10) abolished formation of the complex. We conclude that the two juxtaposed oligonucleotides are required for binding to MRF.

Correlation of DNA–MRF complex formation with MRF repressor activity

The above result suggests that ATATCG could be a part of the recognition sequence for MRF, since inspection of the oligonucleotide sequences did not reveal any homology other than ATATCG tandem repeats. However, our methylation interference experiments suggested that guanine bases do not play a significant role in formation of the HA–MRF complex (not shown). Therefore, we speculated that four short A+T stretches (4–6 bp) with the common ATAT sequence in the tandem repeats may play an important role in MRF binding.

In order to test this speculation and examine whether the ability to form a complex with MRF correlates with repressor activity we produced two mutants of the plasmid p508CAT, which carries 845 nt of the 5′ regulatory sequence of the M-IE gene (Fig. 1A). In mutant M5 both of the inverted repeats ATATCG in the TAQ sequence were mutated to A*GC*TCG. M6 incorporated both of the mutations in the inverted repeats of the –800 oligonucleotides. Base modified HA fragments were prepared from these mutated plasmids as described in Materials and Methods and used as cold competitors in gel mobility shift assays with the labeled wild-type HA probe (Fig. 4A). Radioactivity of the residual HA–MRF complex in each lane showed that the wild-type probe competed better (lane 3, Tera-2 cells and lane 8, THP-1 cells) than the two mutants (lanes 4–5, Tera-2 cells and lanes 9–10, THP-1 cells). This result suggests that the A+T stretches in the HA probe play an important role in MRF binding. In order to investigate the effect of the mutations on repressing activity we carried out transient transfection assays in uninduced Tera-2 and THP-1 cells with plasmids carrying these mutations (Fig. 4B). We observed that the plasmid carrying the full-length modulator (p307CAT) had ~25% of the CAT activity of the plasmid carrying only the enhancer (p303CAT). As shown above (Fig. 1A), deletion of the nucleotides from –845 to –820 produced a sharp decrease in repressing activity. The mutations in the ATATCG inverted repeats in this region produced a comparable decrease in repressing activity: both p508-M5CAT (mutations in TAQ) and p508-M6CAT (mutations in –800) showed a consistent 2-fold increase in CAT activity compared with p508CAT in both Tera-2 and THP-1 cells. Therefore, mutations in the TAQ and –800 sequences that disrupt binding of MRF also decrease the *cis*-repressing activity of the modulator. This suggests that MRF is a *trans*-acting regulatory factor that represses the M-IE enhancer activity.

Multiple binding sites for MRF are present in the modulator region

The results of transient transfection assays using nested deletion mutants suggested that there are several *cis*-acting negative elements within the modulator (Fig. 1A). In order to investigate whether MRF binds to other DNA fragments in the modulator we conducted competitive gel mobility shift assays. We prepared several restriction fragments from the modulator and used them as non-radioactive competitors for binding of the HA probe to MRF in Tera-2 nuclear extracts. As shown in Figure 5, all the modulator-derived fragments, including *Pst*I–*Cla*I (–1137 to

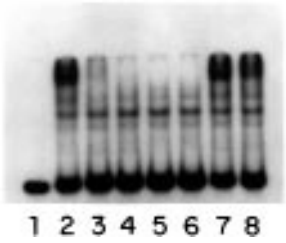

Figure 5. DNA fragments from the modulator compete with the HA probe for DNA–protein complex formation. Gel mobility shift assays using the radioactive HA probe and nuclear extracts from undifferentiated Tera-2 cells were performed in the presence of an excess of the cold DNA fragments prepared by digestion of the modulator region and the plasmid DNA (Bluescript SKII+) with appropriate restriction enzymes. The reaction mixtures included labeled HA probe only (lane 1), probe plus nuclear extract (lane 2) or probe plus extract in the presence of various competitors (lanes 3–8). The competitors were HA (lane 3), *Pst*I–*Cla*I fragment (lane 4), *Cla*I–*Hin*fI fragment (lane 5), *Hin*fI–*Hin*cII fragment (lane 6) and *Hae*III fragments of the plasmid (lanes 7–8). Competitors were present at a 10-fold excess.

–1020, lane 4), *Cla*I–*Hin*fI (–1020 to –856, lane 5), *Hin*fI–*Hin*cII (–856 to –599, lane 6), inhibited binding as efficiently as unlabeled HA itself (lane 3). Two DNA fragments from the Bluescript SKII+ plasmid did not, however, compete as well (lanes 7–8). These results suggest that MRF could bind to the modulator at multiple sites, which may be necessary for full repression of M-IE gene transcription. All of the modulator fragments are A+T rich (*Pst*I–*Cla*I, 60% A+T; *Cla*I–*Hin*fI, 55%; *Hin*fI–*Hin*cII, 66%), but the Bluescript fragments are not (45 and 41%). The modulator fragments all have uninterrupted stretches of A+T and some, but not all, have the ATAT sequence; the Bluescript fragments have neither of these sequences. These results are consistent with our belief that A+T stretches are required for MRF binding.

DISCUSSION

The results of transient transfection assays by Nelson *et al.* indicated that the modulator of the M-IE gene represses its expression in cells that are non-permissive for HCMV (15). Shelbourn *et al.* have demonstrated that deletion of the imperfect dyad symmetry element within the modulator (–963 to –912) releases some repressor activity in undifferentiated Tera-2 cells (18). However, it was apparent from further deletion analysis that sites other than the dyad symmetry element repress expression of the M-IE gene in undifferentiated Tera-2 cells (20,28). Our transient transfection assays using a series of deletion mutants also suggest that there are several repressor elements in the region (Fig. 1A). We have focused our initial attention on a *cis*-acting element within the HA probe (nt –845 to –774), since we consistently observed an increase in CAT activity upon deletion of the sequence.

Gel mobility shift assays demonstrated that the HA probe binds specifically to a DNA binding factor (MRF) that is present in undifferentiated Tera-2 and THP-1 cells but substantially reduced in differentiated cells. Unfortunately, all attempts to identify the DNA recognition sequence by methylation interference and DNase I footprinting assays have failed. From the results of gel mobility shift (Fig. 3A and B) and methylation interference assays we speculated that A+T stretches in ATATCG repeats are involved in binding of the HA probe to MRF. In order to test this speculation various mutations in the A+T stretches present in the repeats were produced. Competition experiments showed that HA fragments with TA→GC mutations in the A*TA*TCG repeats exhibit decreased MRF binding activity and thus support our speculation that A+T stretches with the ATAT sequence in the repeats are required for binding to MRF (Fig. 4A). The finding that formation of the HA–MRF complex is specifically inhibited in the presence of DNA fragments from the modulator (Fig. 5) indicates that binding sites for MRF are located throughout the modulator. Examination of the sequences of these DNA fragments revealed that the *Cla*I–*Hin*fI fragment has four ATATCG repeats, the *Pst*I–*Cla*I fragment has one and the *Hin*fI–*Hin*cII fragment has none. These facts suggest that juxtaposed palindromes containing the ATATCG repeats, present only in the HA probe, are not absolutely required for recognition by MRF. The competing DNA fragments have numerous contiguous A+T stretches, some of which carry the ATAT sequence, and they are relatively A+T rich (53–66%). On the other hand, the non-competitive DNA fragments from the plasmid have fewer A+T stretches and no ATAT sequences and are not A+T rich overall (41–45%). Our preliminary data of carbethoxylation interference assays (29) using nuclear extract from THP-1 cells and the HA probe demonstrated that A+T stretches are major contact sites for binding to MRF (not shown). A+T-rich regions are known to adopt unusual DNA conformations, such as single-stranded (30) and bent (31). Therefore, we propose that specificity of binding is related to A+T stretches in DNA which may have certain structural features, but not to a specific DNA sequence, and that these features could be adopted preferentially by multiple ATAT sequences.

Plasmids (p508-M5 and p508-M6) carrying mutations in the ATAT sequences of HA have lower MRF binding activities and higher CAT activities than the wild-type plasmid (Fig. 4A and B). MRF is highly abundant in undifferentiated cells such as Tera-2 and THP-1 and is reduced significantly upon differentiation (Figs 2 and 4A). In undifferentiated cells enhancer activity is repressed by the modulator located upstream of the enhancer and the repressing activity falls off with progressive deletion from the 5′-end of the modulator (Fig. 1A). In differentiated cells the repressing activity is released and enhancer activity is restored (15,17; our unpublished results) with the decreasing amount of MRF (Figs 2 and 4A). We conclude that MRF is a transcription factor involved in repression of the enhancer activity in undifferentiated Tera-2 and THP-1 cells. *In vivo* footprinting assays of the M-IE gene showed no DNase I hypersensitive sites in the modulator in undifferentiated Tera-2 cells. Upon differentiation DNase I hypersensitive sites are induced in the modulator. In contrast, DNase I hypersensitive sites in the enhancer are constitutive (4). These assays indicate that differentiation of the cellular host drastically alters the structure of viral chromatin in the modulator, but not in the enhancer. Taking these results together we propose that MRF binds over the entire modulator and exerts a repressing activity which is dependent on the amount of MRF bound. It is a very interesting problem to examine whether a mutated virus carrying deletion of the modulator expresses the M-IE gene in undifferentiated cells (non-permissive for wild-type HCMV replication). These experiments are in progress in other laboratories (private communications from Drs Mocarski and Stinski).

Recently numerous YY-1 binding sites have been identified in the modulator (19; our unpublished results) and co-transfection assays show that YY-1 is a *trans*-acting repressor of the M-IE gene in Tera-2 cells (19). YY-1 is a ubiquitously expressed zinc finger DNA binding protein which may function as a positive or negative regulator of transcription, depending upon the context of the binding site (32–35). The HA probe does not appear to have a YY-1 recognition site and formation of the HA–MRF complex is not inhibited in the presence of oligonucleotides carrying either single or multiple YY binding sites (not shown). We conclude that MRF is not identical to YY1. It might be possible that YY-1 cooperates with MRF to exert the repressing activity. The monomer of the 3′-half of the dyad symmetry sequence (18) which binds to MBF-1 does not compete with formation of the HA–MRF complex (Fig. 3A, lanes 7–8), but the concatenated oligonucleotide does (lanes 13–14). We speculate that concatenation of the oligonucleotide fortuitously creates a MRF binding site, since the oligonucleotide carries two A+T stretches, ATTTTT and ATAT (see the sequence in Materials and Methods). Thus we believe that MRF is a novel DNA binding protein that negatively regulates transcription of the M-IE gene. In order to characterize the protein and study mechanisms involved in the transcriptional regulation and interactions with DNA we are in the process of cloning and purifying MRF.

NOTE ADDED IN PROOF

During the time this manuscript was being considered for publication Thrower and co-workers (Thrower *et al.*, 1996, *J. Virol.*, **70**, 91–100) reported that a repeated sequence of TATCG or TGTCG found in the modulator and in the R1 region of the US3 promoter down-regulates transcription from either the US3 promoter or a heterologous promoter. In contrast to our results, the repressor binding to the repeated sequence was found in both undifferentiated and differentiated cells. They propose that the DNA binding activity is controlled by phosphorylation.

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