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Direct Interaction of the Human Cytomegalovirus IE86 Protein with the *cis* Repression Signal Does Not Preclude TBP from Binding to the TATA Box†

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The human cytomegalovirus major immediate-early gene encodes several protein isoforms which autoregulate the major immediate-early promoter (MIEP). One of these isoforms, the IE86 protein, represses the MIEP through a DNA sequence located between the TATA box and the transcription initiation site, designated the *cis* repression signal (crs). Through mutational analysis, amino acid domains within IE86 responsible for binding the crs element were located at the C terminus. Mutation of the putative zinc finger domain, which precluded IE86 from binding DNA, converted the protein from a repressor of MIEP transcription into an activator. DNase I protection analysis demonstrated that the IE86 footprint overlapped the sequence protected by the TATA-binding protein (TBP). Investigation of whether IE86 was able to displace TBP from DNA revealed that both proteins could bind DNA simultaneously. However, higher concentrations of IE86 were required to obtain protection of the crs element in the presence of prebound TBP. Similarly, higher concentrations of TBP were required to obtain protection in the presence of prebound IE86. These observations indicate that steric hinderance impairs but does not prevent both proteins from binding DNA synchronously.

The gene expression of human cytomegalovirus (HCMV) is temporally regulated by host cell and viral proteins during the infection cycle (9, 20, 59, 64, 65). The immediate-early (IE) genes are the first HCMV genes expressed after infection and produce transcriptional regulatory proteins that control subsequent viral gene expression (4, 11, 51, 53, 56–58, 60). The mechanisms of transcriptional regulation by these proteins are not completely understood but may be mediated by direct binding of DNA or protein-protein interactions with cellular transcription factors.

The predominant IE proteins originate from two contiguous regions of abundant IE expression, historically termed IE region 1 (IE-1/UL123) (5, 55, 57) and IE region 2 (IE-2/UL122) (5, 55, 58). Since the major IE promoter (MIEP) directs the expression of both regions, IE-1 and IE-2 constitute the major IE gene. However, within the IE2 region, an internal late promoter directs the expression of an mRNA encoding a 40-kDa protein (L40) with a reading frame that overlaps those for other proteins encoded in this region (52, 55). Alternative splicing and polyadenylation events generate mRNAs that code for variant isoforms of an IE protein (55, 57, 58). The three predominant IE isoforms are phosphoproteins (15) with apparent molecular masses of 86 kDa (IE86), 72 kDa (IE72), and 55 kDa (IE55). IE72 has the same N terminus as IE86 and IE55, while IE86 and IE55 have identical amino acid sequences except for a 154-amino-acid deletion between amino acid residues 365 and 519 in IE86 (Fig. 1) (55, 57, 58). Functionally, the IE isoforms are all capable of transactivating heterologous promoters (1, 11, 14, 17, 19, 56, 61, 63), while IE72 and IE86 together

demonstrate strong synergistic activity (11, 14, 17, 56, 60, 61, 63). The IE proteins also autoregulate their own promoter (1, 8, 16, 32, 43, 49, 56). While IE86 represses MIEP expression through a DNA sequence known as the *cis* repression signal (crs) element (−14 to +1) (7, 29, 41), IE72 (8, 49) and IE55 (1) transactivate this promoter. Levels of these isoforms within a cell are critical for the activity of the MIEP and may be the ultimate determinant for the viral permissiveness of a cell.

The IE isoform proteins contain amino acid sequences with characteristics similar to those of other transcription factors (2, 24, 34, 35, 47, 62). Some putative motifs include three amphipathic helices at the N terminus of all the isoforms, single zinc finger motifs in IE86 and IE72, a leucine zipper in the IE72 protein, and a leucine-rich region in the IE86 protein (Fig. 1). Each isoform also contains two nuclear localization signals, which have been previously described (42). Sequences within exon 3 and the C terminus of IE86 and IE55 were found to be critical for promoter activation (16, 32, 43, 56). Domains responsible for negative regulation by IE86 were localized to the C terminus (29, 41, 56).

The mechanism of IE86 autorepression of the MIEP is unclear. Other viral models of autorepression have suggested that either protein-protein interactions or direct binding of a *cis*-responsive sequence may mediate this process (18, 22, 36, 44, 45). Recently, the IE86 protein was shown to bind the crs element (25). A truncated portion of this protein closely equivalent to the L40 protein was also able to bind this element and induce repression in cell-free nuclear extracts (30). These observations, in combination with the inability of the crs element to function when placed 5′ to the TATA box (7), suggest that the IE86 protein may physically inhibit formation of the transcription initiation complex at
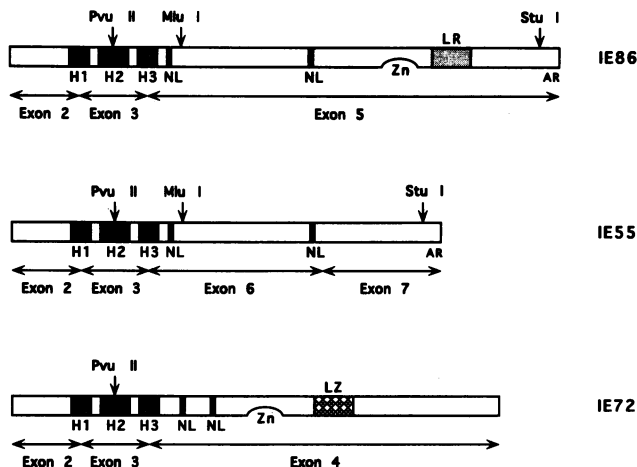
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FIG. 1. Schematic representation of the IE86, IE55, and IE72 proteins of HCMV. The domains shown in the diagram are the three α helices (H1, H2, and H3), zinc fingers (Zn), leucine-rich region (LR), leucine zipper region (LZ), acidic region (AR), and nuclear localization signals (NL). The exons from which each of the domains arise are indicated together with the positions of the restriction enzymes that were used in the construction of the IE86 mutants.

the level of the TATA box-binding protein (TBP) or other basal transcription factors. In these studies, we demonstrate domains within IE86 that are responsible for mediating repression and the effect of this protein on TBP binding of the TATA box.

MATERIALS AND METHODS

Plasmid constructions. Oligonucleotides 5'-TCCTTGGAT CCATGGAGTCCTCTGCCAAG-3' and 5'-TCCCGAATTC GGATCCTTACTGAGACTTGTTCCTC-3' were used to produce a fragment encoding the IE86 gene from plasmid pIE86kd (48, 56) by polymerase chain reaction (PCR). The *Bam*HI sites are underlined, and the ATG and TTA codons are shown in boldface. After this fragment was digested with *Bam*HI, it was cloned into vector pDS56,6His, also digested with *Bam*HI (27). This vector contains six codons encoding histidine residues immediately upstream of the *Bam*HI site. The orientation and nucleotide sequence of the 5' and 3' ends were confirmed by direct sequencing of the plasmid (50). To ensure that any mutations introduced by the *Taq* polymerase were eliminated, the *Apa*I-*Stu*I fragment encoding virtually all of IE86 was subcloned back into this plasmid to generate p86,6His.

To produce the point mutations in the putative zinc finger of IE86, the *Eco*RI-*Hin*dIII fragment from plasmid pRSV86 (1), encoding part of the Rous sarcoma virus long terminal repeat, all of IE86, and the IE2 3' untranslated region, was subcloned into the *Eco*RI and *Hin*dIII sites of M13mp19. Oligonucleotide 5'-GCCAGGTTGGACATGGTGCCCAGT CTAGACCGC-3' (underlined bases are mutated from the wild-type sequence) was used in conjunction with an oligo-nucleotide mutagenesis kit purchased from Amersham (Arlington Heights, Ill.) to mutate cysteines 428 and 434 to serine residues. Following confirmation of the correct clone by sequencing, an *Mlu*I-*Stu*I fragment was subcloned back into the expression plasmids p86,6His and pRSV86 to generate p86,6HismZn and pRSV86mZn, respectively. The C-terminal truncations were generated by digesting p86,6His

with *Hin*dIII and either *Mlu*I (p86,6HisΔC1) or *Stu*I (p86, 6HisΔC2), filling in the ends with Klenow polymerase, and religating the plasmids. The *Hin*dIII site and sequence immediately downstream encodes stop codons in all three reading frames.

The double stranded oligonucleotide 5'-aattgattaaagag gagaaattaactATGcatcaccatcaccatcacgtggatccaagaattcgg-3' contains a *Pml*I site (shown in boldface) and also encodes the sixth histidine residue of the tag. The remaining five histidine codons are situated between the *Pml*I site and the ATG codon (shown in capital letters). The oligonucleotide also contains *Eco*RI- and *Sal*I-compatible ends for cloning into pDS56,6His to generate plasmid pDS56,6His(P). Note that the *Eco*RI site originally present in the vector is not regenerated, but a new *Eco*RI site (underlined) is present in the oligonucleotide. This vector was digested with *Pml*I and *Hin*dIII, and the *Pvu*II-*Hin*dIII fragment from p86,6His was cloned to juxtapose leucine 48 next to the six histidine residues (p86,6HisΔN1). Plasmid p55,6His was constructed by inserting an *Apa*I-*Stu*I fragment from pIE55kd(56) into p86,6His.

The nucleotide sequence encoding human TBP was obtained from pT7hD (54) by PCR with the primers 5'-TACAC GGCACGTGGATCAGAACAACAGCCTGC-3' and 5'-AC CTAGAATTCTTACGTCGTCTTCCTGAA-3'. This PCR product was digested with *Pml*I and *Eco*RI (sites are indicated in bold) and inserted into pDS56,6His(P) previously digested with the same enzymes, generating expression vector phTBP,6His, capable of expressing human TBP with an ATG-(6 His)-Val sequence preceding the Asp at position 2 of hTBP.

Purification of recombinant proteins. *Escherichia coli* strains harboring each of the expression plasmids were grown to an optical density at 550 nm of 0.7 to 0.8 prior to induction with 200 μg of isopropyl-β-D-thiogalactopyrano-side (IPTG) per ml. Cells were harvested after 90 to 120 min of induction and stored at −70°C. Cells were thawed and then lysed in buffer containing 50 mM sodium phosphate (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 1% Tween 20, 1 M NaCl, and 1 mg of lysozyme per ml for 20 min prior to sonication. Following centrifugation at 16,000 rpm in a Sorvall SS34 rotor, the cleared lysate was subjected to Ni^{2+} chelate chromatography (Qiagen, Chatsworth, Calif.) over a column equilibrated in buffer containing 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, and 10% glycerol. After being washed with this buffer, the column was washed in a similar buffer at pH 6.0. A final wash was performed with the latter buffer containing 75 mM imidazol prior to elution with the same buffer containing 500 mM imidazol. Fractions containing each of the proteins were pooled and dialyzed against either 50 mM sodium phosphate (pH 7.8)–250 mM NaCl–1 mM β-mercaptoethanol–30% glycerol or 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 8.0)–0.5 mM EDTA–2 mM MgCl₂–1 mM β-mercap-toethanol–50 mM KCl–30% glycerol for the HCMV IE proteins and TBP, respectively. Purified proteins were ana-lyzed by sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE) followed by Coomassie blue staining; concentrations were then determined by the Bradford method (Bio-Rad, Hercules, Calif.).

Mobility shift assay. The mobility shift assay was per-formed essentially as described previously (13) except that the assay buffer contained 25 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 0.5 mM EDTA, 6.25 mM MgCl₂, 0.2 μg of poly(dG)-poly(dC), 10% glycerol, and cold competitor oligo-nucleotides where indicated, in a total volume of 25 μl. The

IE86 or IE55 protein was incubated for 2 min in assay buffer prior to the addition of 0.5 pmol of probe which had been labeled with Sequenase (USB) and [α-^{32}P]dATP. The incubation was continued for 10 min before loading onto a 4% polyacrylamide gel. Assay mixes which contained monoclonal antibody to IE86 or gp120 were incubated for 2 min following the addition of probe prior to the addition of antibody. Incubation was then continued for a further 10 min. The gel and running buffer contained 6.7 mM Tris-HCl (pH 7.3), 3.3 mM sodium acetate, and 1 mM EDTA. Following electrophoresis, the gel was dried and autoradiographed.

Cell culture and transfection assays. Transfections were performed by the calcium phosphate precipitation method on monolayers of cells which were approximately 80% confluent. In each case, the total amount of DNA was kept constant by the addition of Bluescript II. No effect on the reporter plasmid was observed when it was cotransfected with just Bluescript II. Cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (38). Briefly, for each assay, 100 μg of protein was assayed for CAT activity by measuring the amount of [^{3}H]acetyl coenzyme A label transferred to chloramphenicol. The reaction mix contained 25 mM Tris-HCl (pH 7.8), 3 mM chloramphenicol, 100 μM cold acetyl coenzyme A, and 0.2 μCi (5 μM) of [^{3}H]acetyl coenzyme A. Following addition of the protein extract, 3 ml of Econofluor 2 (New England Nuclear, Boston, Mass.) was added. At increasing time intervals, the reaction was monitored by scintillation counting, and the linear range was determined, at which point the fold induction was calculated. Each experiment was repeated at least three times, from which an average and standard deviation were determined.

DNase I protection analysis. The DNase I protection analysis was performed essentially as described previously (12). Briefly, the assay buffer contained 25 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 0.5 mM EDTA, 6.25 mM MgCl$_2$, 0.2 μg of poly(dG)-poly(DC), 10% glycerol, and cold competitor oligonucleotides where indicated, in a total volume of 25 μl. Recombinant IE86, TBP, or bovine serum albumin (BSA) was incubated for 2 min in assay buffer prior to the addition of probe which had been labeled with [α-^{32}P]ATP on either the top- or bottom-strand oligonucleotide before annealing with the complementary cold oligonucleotide. After a further 10-min incubation, 1 U of DNase I was added, and the incubation was continued for 90 s, at which time the reaction was stopped with the addition of 100 μl of 200 mM NaCl–1% SDS–20 mM EDTA–50 μg of tRNA per ml–2.5 μg of proteinase K per ml. Following a further 10-min incubation at 37°C, the samples were extracted with phenol and chloroform before being loaded on a 12% denaturing polyacrylamide gel. In the experiments with both IE86 and TBP, either IE86 or TBP was allowed to bind the probe for 5 min prior to the addition of the remaining protein, at which time the incubation was continued for 10 min before addition of the DNase I. Subsequently, the gel was dried and the protection patterns were visualized by autoradiography.

Oligonucleotides. The following oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia LKB): −35 to +4 MIEP, (top) 5′-AGCTTGAGGTCTATATAAGCA GAGCTCGTTTAGTGAACCGTCAG-3′ and (bottom) 5′-GATCCTGACGGTTCACTAAACGAGCTCTGCTTATAT AGACCTCA-3′; −35 to +18 MIEP, (top) 5′-AGCTTGAGG TCTATATAAGCAGAGCTCGTTTAGTGAACCGTCA GATCGCCTGGAGACG-3′ and (bottom) 5′-GATCCGTC TCCAGGCGATGTACGGTTCACTAAACGAGCTCTGC

TTATATAGACCTCA-3′; −35 to +4 (m2crs) MIEP, (top) 5′-AGCTTGAGGTCTATATAAGCAGAGCTCAAAGGC CTGTACGTCAG-3′ and (bottom) 5′-GATCCTGACGTAC AGGCCTTTGAGCTCTGCTTATATAGACCTCA-3′; −35 to +18 (mcrs) MIEP, (top) 5′-AGCTTGAGGTCTATATAA GCAGAGCTGCGGCGTGTCCACGTCAGATCGCCTG GAGACG-3′ and (bottom) 5′-GATCCGTCTCCAGGCGAT CTGACGTGGACACGCCGCAGCTCTGCTTATATAGA CCTCA-3′; −35 to +18 (m3crs) MIEP, (top) 5′-AGCTTGA GGTCTATATAAGCAGAGCTCGTCTAGTGAACCGT CAGATCGCCTGGAGACG-3′ and (bottom) 5′-GATCCGT CTCCAGGCGATGTACGGTTCACTAGACGAGCTCT GCTTATATAGACCTCA-3′; and the USF binding site oligonucleotide, (top) 5′-AGCTTAGCATTTCATCACATGG CCCGAGA-3′ and (bottom) 5′-TCGTAAAGTAGTGTACC GGGCTCTTCGA-3′. The oligonucleotides were subjected to denaturing preparative PAGE with 12% polyacrylamide. The oligonucleotides were visualized by low-power UV (210 nm) shadowing on photocopying paper, and full-length product was excised from the gel and electroeluted into 1× TBE (90 mM Tris-borate and 2 mM EDTA) buffer.

RESULTS

Binding of IE86 to the crs element. To examine the ability of IE86 to bind to the crs element, the gene encoding IE86 was expressed in *E. coli* under the control of an IPTG-inducible promoter. To facilitate purification, a short linker sequence encoding six histidine residues was cloned onto the 5′ end of the IE86 cDNA (Fig. 2B). Under nondenaturing conditions, IE86 was purified to approximately 90% homogeneity by nickel chelate chromatography (Fig. 2A, lane 1). A similar methodology using denaturing conditions has been described previously (25). To identify the domain responsible for contacting DNA, a variety of mutant IE86 proteins were constructed (Fig. 2B) and purified (Fig. 2A, lanes 2 to 5). In addition, the gene encoding IE55 was expressed and purified to a similar homogeneity from *E. coli* (data not shown). Initially, binding of IE86 to a labeled probe whose sequence corresponded to that of the MIEP from −35 to +4 was examined in a mobility shift assay (Fig. 3A, lane 5). Although highly pure IE86 protein was used, a monoclonal antibody that recognized an epitope encoded within exon 2/3 was used to supershift the IE86 nucleoprotein complex to show that IE86 was indeed part of this complex (Fig. 3A, lane 7). A supershift was not observed with a monoclonal antibody directed against the gp120 envelope protein of human immunodeficiency virus (Fig. 3A, lane 6). The probe was not shifted with either antibody in the absence of IE86 (data not shown). In addition, a second probe whose sequence corresponded to that of the MIEP from −35 to +18 was also used in some of the mobility shift experiments. Binding of IE86 to this probe was identical to that of the previous probe (Fig. 3B, lane 2).

To show the specificity of binding, various competition experiments were performed with a 50-fold excess of a variety of different unlabeled probes. Competition was initially performed with a cold excess of the −35/+4 and −35/+18 MIEP probes. Under these conditions, no nucleoprotein complexes were observed between either the shorter or the longer labeled probe and IE86 (Fig. 3A, lane 8, and Fig. 3B, lane 3, respectively). By contrast, competition with excess probe containing a series of clustered point mutations in the crs element, −35/+18 (mcrs) MIEP, that had previously been shown to eliminate repression of MIEP transcription by IE86 in vivo (1, 7), did not abrogate the formation of
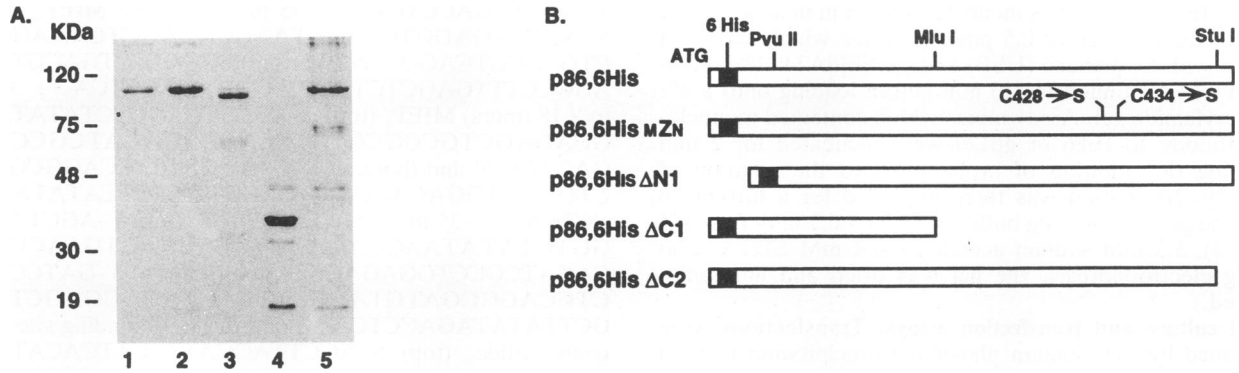
A.

B.

FIG. 2. Wild-type and mutant forms of IE86. (A) Coomassie-stained gel showing wild-type and mutant IE86 proteins following dialysis of the pooled fractions after nickel chelate chromatography. Lane 1, 250 ng of wild type IE86; lanes 2 to 5 correspond to the mutant forms IE86 mZn, IE86ΔN1, IE86ΔC1, and IE86ΔC2, respectively. (B) Schematic diagrams of the wild-type and mutant IE86 proteins. The position of the first ATG codon (encoding methionine) preceding the six-histidine tag (shaded box) is shown. The restriction sites in the IE86 cDNA used in the construction of the mutant proteins are indicated together with the residues in IE86 that were changed to destroy the putative zinc finger.

the nucleoprotein complex between the shorter or longer labeled MIEP probes (Fig. 3A, lane 9, and Fig. 3B, lane 4, respectively). In addition, an excess of another mutation in the crs element, in the probe designated m2crs, which had the following changes in the context of the shorter probe, CTCGTTTAGTGAACCG to CTCAAAGGCCTGTACG (the underlined nucleotides were mutated), did not affect the binding of the longer MIEP probe to IE86 (Fig. 3B, lane 5). An excess of a cold probe whose sequence corresponded to the binding site of USF did not compete for the binding of the shorter or longer labeled probe to IE86 (Fig. 3A, lane 10, and Fig. 3B, lane 6, respectively).

To delineate the domain in IE86 responsible for the binding of IE86 to DNA, various mutant proteins were

examined. A deletion in the N terminus of IE86 (IE86ΔN1) did not affect the ability of the protein to bind DNA (Fig. 3A, lane 4, and Fig. 3B, lane 9). Moreover, a protein expressing the N terminus alone, mutant IE86ΔC1, was ineffective at binding probe (Fig. 3A, lane 2). The C-terminal half of the IE86 protein contains a number of putative domains, including a zinc finger, a leucine-rich region, and an acidic region (37, 56, 58). The IE55 isoform, which is identical to the IE86 protein except for a 154-amino-acid deletion between amino acid residues 365 and 519 of IE86, has previously been shown to be unable to repress the MIEP in vivo (1). The domains absent in the IE55 protein include the putative zinc finger and the leucine-rich region but not the acidic region at the extreme C terminus (Fig. 1). Therefore, we hypothesized
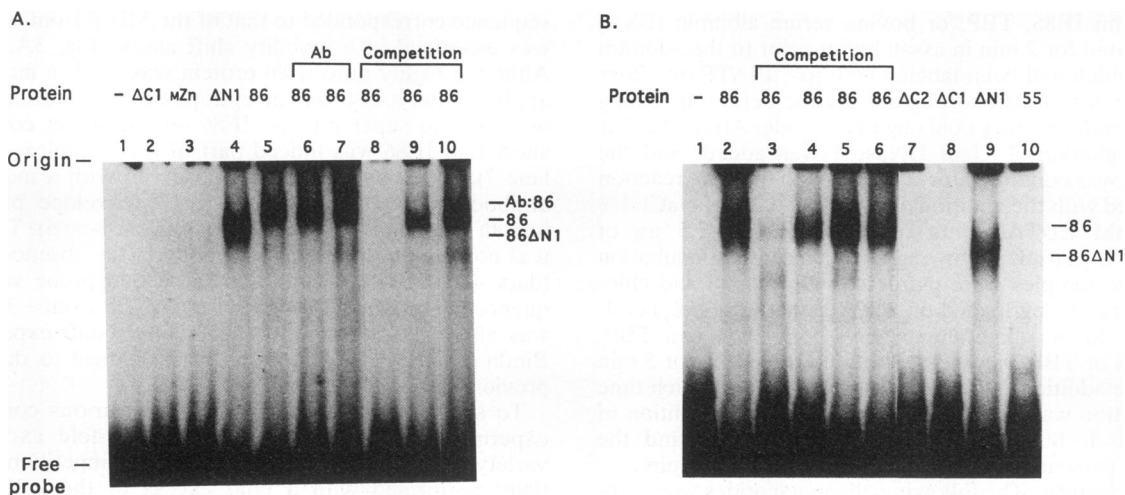
A.

B.

FIG. 3. Mobility shift assay showing the direct binding of IE86 to the crs element. Where included, the total IE (wild-type or mutant) protein concentration was 250 nM. (A) The −35/+4 MIEP oligonucleotide fragment (0.5 pmol) was used as a probe in lanes 1 to 10. Additions: lane 2, IE86ΔC1 (ΔC1); lane 3, IE86 mZn (mZn); lane 4, IE86ΔN1 (ΔN1); lanes 5 to 10, IE86 wild type (86). Lanes 6 and 7 also contained monoclonal antibody (40 μg/ml) to IE86 and gp120 (from human immunodeficiency virus), respectively. The shifted (86 and 86ΔN1) and supershifted (Ab:86) complexes are indicated. Competition analysis was performed in lanes 8 to 10 by the inclusion of a 50-fold excess of cold −35/+4 MIEP, −35/+18 (mcrs) MIEP, or USF-binding site oligonucleotide, respectively. (B) The −35/+18 MIEP oligonucleotide fragment (0.5 pmol) was used as a probe in lanes 1 to 10. Additions: lanes 2 to 6, IE86; lane 7, IE86ΔC2 (ΔC2); lane 8, IE86ΔC1 (ΔC1); lane 9, IE86ΔN1 (ΔN1); lane 10, IE55. For the competition analysis, lanes 3 to 6 also contained a 50-fold excess of cold −35/+18 MIEP, −35/+18 (mcrs) MIEP, −35/+18 (m2crs) MIEP, or USF-binding site oligonucleotide, respectively. The shifted (86 and 86ΔN1) complexes are indicated.
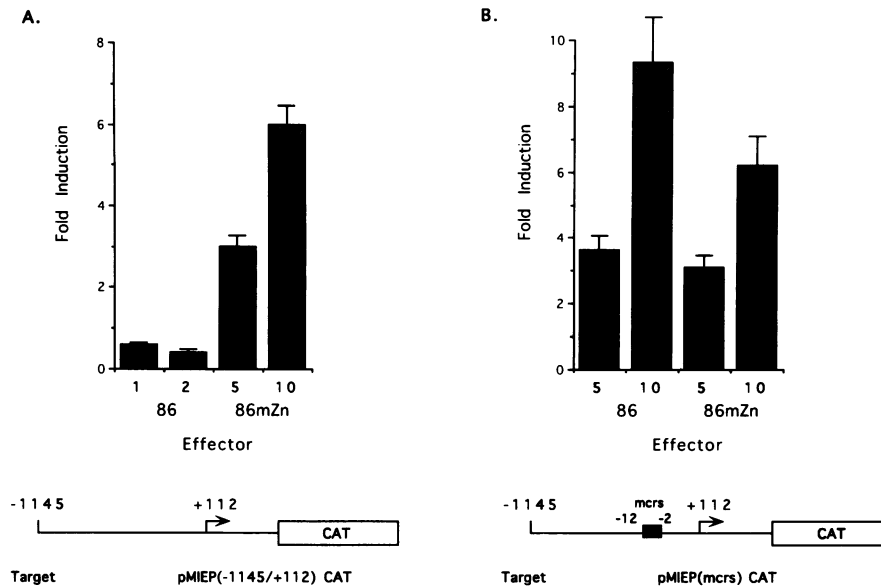
FIG. 4. Activation and repression of the MIEP by wild-type and mutant forms of IE86. The fold induction produced by either IE86 or IE86 mZn compared with basal promoter levels is shown. Permissive U373-MG cells were transfected with 1 μg of pMIEP(−1145/+112)CAT (A) or pMIEP(mcrs)CAT (B). The quantity (in micrograms) of the effector plasmids RSV86 and RSV86 mZn is indicated. The results were calculated in the linear range of the assay after 20 to 30% of the substrate had been converted and are the averages of at least three independent experiments; the error bars indicate the standard deviation. Schematics of the reporter plasmids pMIEP(−1145/+112)CAT and pMIEP (mcrs)CAT are also shown.

that the putative zinc finger might be responsible for mediating the binding of IE86 to DNA, since similar structures have been shown to be critical for the binding of other transcription factors (2, 21, 35) as well as hormones (31) to DNA. Therefore, mutations were made in the putative zinc finger of IE86 which changed the cysteine residues at positions 428 and 434 to serine residues. Nucleoprotein complex formation was not detected with the IE86 mZn protein in the gel mobility shift assay (Fig. 3A, lane 3).

Therefore, the putative zinc finger is important for DNA binding. Examination of the literature, however, revealed that deletion of the acidic C-terminal domain of IE86 from amino acids 542 to 579 eliminated the ability of IE86 to negatively regulate the MIEP in vivo (43). To determine whether this mutation abrogated the ability of IE86 to bind DNA, we analyzed the IE86ΔC2 mutant (Fig. 2) in a mobility shift assay. Nucleoprotein complex formation was not detected with this mutant protein and the crs probe (Fig. 3B, lane 7). From these data, the region of IE86 responsible for contacting DNA would appear to be more extensive and not restricted to the putative zinc finger domain. Alternatively, the C-terminal domain could be solely responsible for mediating the binding of IE86 to DNA, and disruption of such an important structural component as a zinc finger may have caused more extensive perturbations in the overall configuration of the protein that would contribute to its nonfunctionality. Therefore, the naturally occurring IE55 isoform of the IE86 protein described above, which lacks the putative zinc finger but has an intact C-terminal acidic region, was examined for its ability to bind DNA. As shown in Fig. 3B, lane 10, no nucleoprotein complex formation was observed between IE55 and probe. Therefore, the C-terminal acidic domain cannot bind DNA in the absence of the putative zinc finger and/or possibly the leucine-rich domain. Thus, the inability of IE55 to repress transcription from the MIEP in

vivo (1) is probably a direct result of the protein's inability to bind DNA.

In vivo activation but not repression by mutant IE86. As described above, IE86 mZn was unable to bind the probe in a mobility shift assay. To examine whether this was the result of a specific knockout of the putative zinc finger and not of a general disruption of the structure of the protein, we tested the ability of this mutant to function in vivo in a cell line permissive for HCMV infection. All of the effector plasmids shown in Fig. 4 produced proteins without the six-histidine tag at their N terminus. Expression of the same proteins with six-histidine tags yielded no difference in their ability to transactivate or repress transcription (data not shown). In the first experiment, shown in Fig. 4A, a construct containing the MIEP, including the upstream enhancer and modulator sequences driving the expression of the CAT gene, was used as the target reporter plasmid to detect transactivation or repressive functions of the HCMV IE86 protein. IE86 specifically repressed transcription of the MIEP(−1145/+112)CAT reporter plasmid by 0.4-fold (Fig. 4A). Cotransfection of more IE86 did not result in further repression (data not shown). Greater repression was observed when IE86 was transfected in the presence of IE72, which increased the basal level of promoter activity (data not shown). In contrast, the IE86 protein containing the knockout of the putative zinc finger was not only unable to repress transcription but stimulated it by approximately sixfold (Fig. 4A).

In the second experiment, shown in Fig. 4B, the function of wild-type and mutant IE86 proteins was examined with the construct containing the mutated crs element [pMIEP (mcrs)CAT]. The wild-type and zinc finger mutant IE86 proteins stimulated this reporter construct to a similar degree (Fig. 4B). In this experiment, the fold induction observed for both proteins was similar to that obtained with the
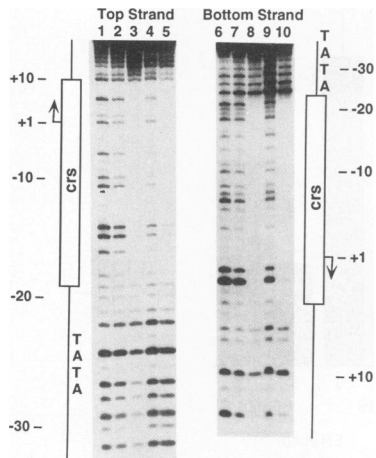
FIG. 5. DNase I protection of the crs element by IE86. Lanes 1 to 5 and 6 to 10 show DNase I protection of the −35/+18 MIEP probe (0.5 pmol) labeled on the top and bottom strands, respectively. In addition, lanes 1 and 5 contain 250 nM BSA as a control protein, lanes 2 and 7 contain 50 nM IE86, and lanes 3, 4, 5, 8, 9, and 10 contain 250 nM IE86. Lanes 4 and 9 and lanes 5 and 10 also contain a 50-fold excess of cold −35/+18 MIEP and −35/+4 (m2crs) MIEP oligonucleotides, respectively. The positions of the nucleotides, indicated by Maxam and Gilbert sequencing of the labeled probes, are shown relative to the transcription start site (arrow). The protected crs element (open box) and position of the TATA box are indicated.

MIEP(−1145/+112)CAT reporter plasmid when stimulated with IE86 mZn (Fig. 4, compare A and B). The IE86 protein has previously been shown to require both its N terminus and an intact C-terminal acidic domain to be competent to transactivate both homologous and heterologous promoters (16, 43, 56). Since the IE86 mZn protein functions identically to the wild-type IE86 protein in its ability to transactivate, we conclude that mutation of the putative zinc finger did not significantly affect other regions of the protein and was limited to this domain.

IE86 and TBP can bind DNA simultaneously. Since IE86 binds to a site overlapping the TATA box, the binding of IE86 to the crs element may block TBP binding or even displace bound protein from the TATA box. Therefore, we determined whether the binding of IE86 and TBP to DNA was mutually exclusive. First, the binding of IE86 to the crs element in the absence of TBP was examined. Each strand of the −35/+18 MIEP probe was individually labeled and used in the DNase I protection experiments shown in Fig. 5. In lanes 2 and 3 (top strand) and lanes 7 and 8 (bottom strand), increasing concentrations of IE86 were used. The concentration required to give complete protection was approximately 250 nM (Fig. 5, lanes 3 and 8), similar to that used in the mobility shift assay and approximately 10-fold over that of the probe. A similar concentration of BSA added as control gave no protection (Fig. 5, lanes 1 and 6). To show that this protection was specific, competition analyses were performed with a 50-fold excess of cold probe (Fig. 5, lanes 4 and 9) and −35/+4 m2crs MIEP probe as a nonspecific competitor (Fig. 5, lanes 5 and 10). The specific probe competed for binding to IE86, since it eliminated the protection of the labeled probes, whereas protection persisted in the presence of the m2crs nonspecific probe. The extent of protection observed on the top strand was between nucle-

otide positions −18 and +10 and on the bottom strand was between positions −24 and +3.

Since the footprint of IE86 overlaps the TATA box, especially on the bottom strand, we hypothesized that IE86 binding might prevent the interaction of TBP with the TATA box. To test this hypothesis, we first investigated the binding of TBP alone to the −35/+18 MIEP probe. We observed two areas of protection with this probe between −31 and −15, corresponding to protection of the TATA box, and between −20 and −5 (data not shown). Closer examination of the latter revealed the sequence TTTAGT between −12 and −7. This sequence was sufficiently similar to a TATA box to behave as a cryptic binding site for TBP. Since this sequence constitutes a large portion of the crs element, this probe could not be used to test the hypothesis that IE86 might be able to abrogate the binding of TBP to the TATA box. Therefore, a mutation in this sequence was introduced which changed the T at position −11 to a C (TTTAGT to TCTAGT) in the context of the −35/+18 MIEP probe (to generate −35/+18 m3crs MIEP), eliminating this cryptic site. Since this substitution was in the crs element, we first determined whether the binding of IE86 to the −35/+18 m3crs MIEP probe was different from that of the wild-type probe. These data are shown in Fig. 6A. Identical protection of this probe was observed with the same concentration of IE86 that yielded complete protection of the wild-type probe (Fig. 6A, lanes 3 and 7). The probe was not protected in the presence of BSA (Fig. 6A, lanes 1 and 5) or when a 50-fold excess of cold probe was added (Fig. 6A, lanes 4 and 8), indicating that the protection was specific.

Incubation of the −35/+18 m3crs probe with 50 nM TBP followed by DNase I digestion provided approximately 15 to 20 bp of protection around the TATA box (Fig. 6B, lanes 2 and 6). The result of increasing the concentration of IE86 following incubation of the probe with 50 nM TBP is shown in Fig. 6B, lanes 3 and 4 (top strand) and lanes 7 and 8 (bottom strand). For complete protection of the top strand in the presence of TBP, at least 500 nM IE86 was routinely required. The bottom strand normally required 250 nM IE86 for complete protection of the crs element while maintaining TBP protection over the TATA box. However, in some experiments, 500 nM of IE86 was required for complete protection of the crs element in the presence of TBP (data not shown). In a reciprocal experiment, increasing concentrations of TBP were added to the probe following incubation with IE86. Under these conditions, both sites could be protected simultaneously, although higher concentrations of TBP were required to show protection of the TATA box in the presence of prebound IE86 (data not shown). In addition, we also observed in these experiments and in a mobility shift assay (unpublished observations) that IE86 stabilized the binding of TBP to DNA.

To show that the simultaneous protection of both the TATA box and the crs element was specific, we repeated the experiment in Fig. 6B with the putative zinc finger mutant of IE86, which was shown above to be unable to bind DNA and yet was fully competent to act as a transcriptional activator in vivo (Fig. 4A). Shown in Fig. 7, lanes 2 and 6, is the protection pattern obtained in the presence of TBP alone. Addition of IE86 mZn resulted in no significant protection of the crs element even at a concentration of 1 μM (Fig. 7, lanes 4 and 8). In addition, inclusion of the IE86 zinc mutant was frequently observed to enhance the binding of TBP, especially at lower concentrations of TBP (data not shown). Thus, IE86 and TBP are clearly able to bind to their respective sequence elements simultaneously, although
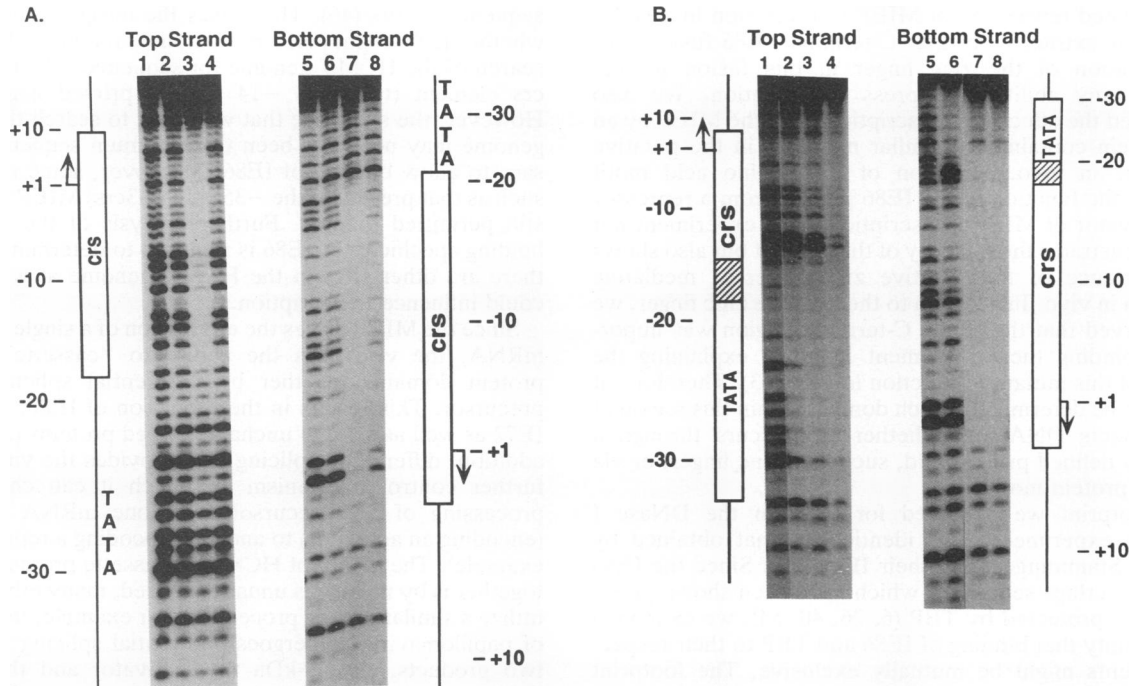
FIG. 6. DNase I protection analysis of the crs element and the TATA box by IE86 and TBP simultaneously. Lanes 1 to 4 and 5 to 8 show DNase I protection of the −35/+18 (m3crs) MIEP probe (0.5 pmol) labeled on the top and bottom strands, respectively. Lanes 1 and 5 are controls showing protection in the presence of 250 nM BSA. (A) Lanes 2 and 6 contain 50 nM IE86. Lanes 3, 4, 7, and 8 contain 250 nM IE86. In addition, a 50-fold excess of cold −35/+18 (m3crs) MIEP oligonucleotide was included in lanes 4 and 8. (B) Lanes 2 to 4 and 6 to 8 contain 50 nM TBP. In addition, lanes 3 and 7 contain 250 nM IE86 and lanes 4 and 8 contain 500 nM IE86 wild-type protein. The positions of the nucleotides, indicated by Maxam and Gilbert sequencing of the labeled probes, are shown relative to the transcription start site (arrow). The protected crs and TATA box elements (open boxes) are indicated. The shaded boxes represent the overlap in IE86 and TBP protection patterns (when footprinted separately).

higher concentrations of IE86 or TBP were required for synchronous binding.

DISCUSSION

We have identified funcitonal domains of the IE86 protein of HCMV, which acts as a repressor of transcription by binding to a specific sequence, termed the *cis* repression signal (crs), located just downstream of the TATA box. We found that IE86 could not displace the binding of human TBP to the TATA box. Moreover, IE86 and TBP could bind their respective elements simultaneously, although higher concentrations of IE86 were required, suggesting that there might be steric constraints disrupting but not preventing both proteins from binding DNA synchronously.

Binding of IE86 to DNA has also been recently described by Lang and Stamminger (25), who used a similarly modified IE86 protein purified under denaturing conditions. Macias and Stinski (30) also demonstrated binding to the crs element but used a C-terminal fragment (amino acids 289 to 579) fused to the maltose-binding protein. This deletion mutant, closely corresponding to the L40 protein, demonstrated that the DNA-binding domain was restricted to the C terminus, in agreement with the work presented in this study. However, these investigators could not address any effect that the N terminus of IE86 might have had on binding. This aspect is especially important, since isoforms of IE86 in addition to L40, such as the L55 protein (distinct from IE55), which encompass mainly this domain (42, 52, 55), may possess alternative functions. Macias and Stinski also reconstituted
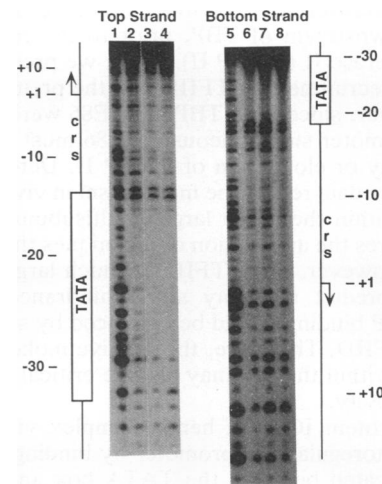
FIG. 7. DNase I protection of the TATA box by TBP but not the crs element by IE86 mZn. Lanes 1 to 4 and 5 to 8 show DNase I protection of the −35/+18 (m3crs) MIEP probe (0.5 pmol) labeled on the top and bottom strands, respectively. Lanes 1 and 5 are controls showing protection in the presence of 500 nM BSA. Lanes 2 to 4 and 6 to 8 contain 50 nM TBP. In addition, lanes 3 and 7 contain 500 nM IE86 mZn and lanes 4 and 8 contain 1,000 nM IE86 mZn mutant protein. The positions of the nucleotides, indicated by Maxam and Gilbert sequencing of the labeled probes, are shown relative to the transcription start site (arrow). The open boxes show the protection provided by TBP. The position of the crs element is also indicated.

IE86-mediated repression of MIEP transcription in a HeLa cell nuclear extract with their C-terminal IE86 fusion protein. Mutation of the zinc finger in this fusion protein destroyed any ability to repress transcription. We also investigated the effect on transcription from the MIEP by an IE86 protein containing a similar mutation in the putative zinc finger in vivo. Mutation of this amino acid motif converted the function of the IE86 protein from a repressor to an activator of MIEP transcription. This experiment not only demonstrates the integrity of this mutant but also shows the importance of the putative zinc finger in mediating repression in vivo. In addition to the putative zinc finger, we also observed that the acidic C-terminal region was important for binding the crs element in vitro, explaining the inability of this mutant to function in vivo (43). Therefore, it remains to be determined which domain within this region of IE86 contacts DNA and whether this occurs through a previously defined protein fold, such as a zinc finger, or via a distinct protein motif.

The footprint we observed for IE86 in the DNase I protection experiments was identical to that obtained by Lang and Stamminger with their IE86 (25). Since the IE86 footprint overlaps sequences which have been shown previously to be protected by TBP (6, 26, 40, 54), we examined the possibility that binding of IE86 and TBP to their respective elements might be mutually exclusive. The footprint obtained for TBP alone was similar to that described previously (6, 26, 40, 54). Complete protection of the probe by IE86 or TBP frequently required a higher concentration of protein if either TBP or IE86, respectively, had been bound first. Thus, TBP and IE86 could impair but not prevent the binding of the other protein to DNA. Binding of TBP, the DNA-contacting domain of the general transcription factor IID (TFIID), to the TATA box is the first step in the formation of a competent preinitiation complex on class II promoters. This interaction serves as a nucleation site for other basal transcription factors, including TFIIB, which interacts downstream of TBP, prior to the recruitment of RNA polymerase II (RNAP II). Thus, we predict that IE86 inhibits the recruitment of TFIIB into the preinitiation complex. However, since both TBP and IE86 were observed to bind the promoter simultaneously, IE86 must abrogate further assembly or elongation of RNAP II. Determination of whether these data reflect the mechanism in vivo where TBP is present within the much larger multisubunit complex of TFIID requires the application of techniques that are not yet available. However, since TFIID is much larger than TBP, we would predict that any steric hindrance from IE86 affecting TBP binding would be enhanced by substitution of TBP with TFIID. Therefore, the relative molarities of IE86 and TFIID within the cell may also be critical determinants of MIEP activity.

The IE protein ICP4 of herpes simplex virus has been shown to autoregulate its promoter by binding to a specific sequence located between the TATA box and the mRNA cap site, as well as transactivate early promoters (22, 45). Therefore, the function of IE86 closely parallels that of ICP4. In addition, the papillomavirus E2 gene product and the adenovirus E1a protein have also been shown to both transactivate and repress transcription (3, 18, 28, 33). In contrast to IE86, the E2 product, although requiring a specific sequence element, will function in cis in an orientation- and position-independent manner. Interestingly, the E2 gene product, in addition to negatively autoregulating expression of its own promoter (18), has been shown to repress transcription of the E6 and E7 gene products through similar

sequence motifs (46). This raises the intriguing question of whether IE86 might perform a parallel function in HCMV. A search of the HCMV genome for sequences identical to the crs element (taken as −14 to +1) proved negative (7). However, the sequence that was used to search the HCMV genome may not have been the minimum sequence necessary to allow binding of IE86. Moreover, some mutations, such as that present in the −35/+18 (m3crs) MIEP sequence, still permitted binding. Further analysis of the sequence-binding specificity of IE86 is required to determine whether there are other sites in the HCMV genome at which IE86 could influence transcription.

Since the MIEP drives the expression of a single precursor mRNA, the virus has the ability to "cassette" various protein domains together by differential splicing of the precursor. This results in the formation of IE86, IE55, and IE72 as well as several uncharacterized proteins (55, 56). In addition, differential splicing also provides the virus with a further control mechanism by which it can channel the processing of the precursor from one mRNA transcript (encoding an activator) to another (encoding a repressor, for example). The ability of HCMV to cassette protein domains together is by no means unusual; indeed, many other viruses utilize a similar editing procedure. For example, the E2 gene of papillomavirus undergoes differential splicing to encode two products, the 48-kDa transactivator and the 28-kDa repressor (23, 33). Similarly, the E1a protein exists in two isoforms as a result of differential splicing of its precursor mRNA (39). In the longer 13s form, the activation domain is dominant over the domain responsible for mediating repression, whereas the shorter 12s form lacks part of the N-terminal domain responsible for mediating activation and therefore functions as a repressor. Interestingly, this is in contrast to IE86, in which the repressive domain is dominant over the activation domain. The results presented herein thus demonstrate some of the piquant features of the major IE proteins, especially IE86, and further emphasize their importance in regulating the transcription of the HCMV genome.

ACKNOWLEDGMENTS

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