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Identification of Binding Sites for the 86-Kilodalton IE2 Protein of Human Cytomegalovirus within an IE2-Responsive Viral Early Promoter

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The 86-kDa IE2 protein (IE86) of human cytomegalovirus (HCMV) can act as both an activator and a repressor of gene expression. The mechanisms for both of these functions are not well defined. It has recently been demonstrated that this protein has sequence-specific DNA binding properties: it interacts directly with a target sequence that is located between the TATA box and the cap site of its own promoter. This sequence, termed the CRS (*cis* repression signal) element, is required for negative autoregulation of the IE1/IE2 enhancer/promoter by IE2. We demonstrate now that binding of this protein to DNA is not confined to this site but occurs also within an early promoter of HCMV that has previously been shown to be strongly IE2 responsive. By DNase I protection analysis using a purified, procaryotically expressed IE2 protein, we could identify three binding sites within the region of −290 to −120 of the UL112 promoter of HCMV. Competition in DNase I protection experiments as well as gel retardation experiments showed that the identified binding sites are specific and have high affinity. Deletion of IE2 binding sites from this promoter reduced the level of transactivation; however, the remaining promoter could still be stimulated about 40-fold. Constructs in which IE2 binding sites were fused directly to the TATA box of the UL112 promoter did not reveal a significant contribution of these sequences to transactivation. However, if an IE2 binding site was reinserted upstream of nucleotide −117 of the UL112 promoter, an increase in transactivation by IE2 was obvious, whereas a mutated sequence could not mediate this effect. This finding suggests that DNA-bound IE2 can contribute to transactivation but seems to require the presence of additional transcription factors. Moreover, a comparison of the detected IE2 binding sites could not detect a strong homology, suggesting that this protein may be able to interact with a broad spectrum of different target sequences.

The genome of human cytomegalovirus (HCMV), a herpesvirus of considerable medical importance in immunosuppressed patients and newborns, comprises 229,354 nucleotides and has the potential to code for more than 200 proteins (4). A few of these proteins have been identified as regulatory molecules that are involved in the coordinate expression of genes during the three distinct phases of the viral replicative cycle, termed immediate early (IE), early, and late (7, 25, 42, 47). The best-characterized regulatory proteins are the 72-kDa IE1 (IE72) and the 86-kDa IE2 (IE86) polypeptides that originate from the major immediate-early gene region of HCMV during initial stages of the replicative cycle (16, 30, 43). The encoding RNAs are transcribed under the control of a strong enhancer/promoter (2, 48) and arise via differential splicing (45). At late times after infection, an additional RNA is transcribed from the IE2 gene region under the control of a true late promoter and encodes a protein of 40 kDa in molecular mass corresponding to the carboxy-terminal part of the 86-kDa IE2 polypeptide (34, 43).

Both IE72 and IE86 are able to positively regulate gene expression independently. This has been demonstrated for several heterologous promoters such as the human immunodeficiency virus long terminal repeat (LTR) or the *hsp70* promoter (1, 12, 14). For IE72, a strong transactivation of the TATA-less DNA polymerase α promoter was found, whereas there was no effect for IE86 (3). This is consistent with the observation that the TATA sequence is required for transactivation by IE86 but not by IE72 (14). For promoters of HCMV, IE86 appears to be more important, as most investigated promoters could be activated independently by IE86 but not by IE72 (21, 27). A cooperative effect of these two polypeptides on some viral early promoters has been reported (27, 44). In addition to its function as a transactivator, IE86 has been shown to negatively autoregulate its own expression; it is able to repress transcription from the major IE enhancer of HCMV (15, 31). This negative regulation is dependent on a sequence element, termed the *cis* repression signal (CRS), that is located between the TATA box and the transcriptional start site of the IE1/IE2 enhancer/promoter (5, 24, 29).

The mechanisms used by IE86 to transregulate gene expression are still not well defined. For transactivation, protein-protein contacts are thought to play a major role, as procaryotically expressed IE86 has been shown to interact with the TATA-binding protein TBP both in solution and when complexed with DNA (11, 14, 20). This correlates with the observation that the TATA sequence is required for IE86-mediated transactivation and suggests that the basal transcription apparatus is one target of IE86 effects (14). In addition to TBP, IE86 is able to interact with itself and a large number of as yet unidentified cellular proteins (6, 11).

The recent observation that procaryotically expressed IE86 is a sequence-specific DNA-binding protein that interacts directly with the CRS element of the IE1/IE2 enhancer/promoter suggested that IE86 can regulate transcription by more than one mechanism (22, 26). However, DNA binding

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appeared to be important for negative regulation but not for transactivation, as no sequences with a significant homology to the CRS element could be identified within IE2-responsive promoters.

In this study, we report on the identification of three binding sites for IE86 within the viral early promoter driving expression of the UL112/UL113 region of HCMV. This promoter has previously been shown to be highly IE86 responsive (21). We show that IE2 binding sites are able to increase the level of IE86-mediated stimulation but are not absolutely required for transactivation. A comparison of the detected IE2 binding sites suggested that this protein can interact with a broad spectrum of sequences. Thus, the multiple mechanisms used by IE86 to transregulate gene expression can even be observed within one single promoter in which several responsive elements, including direct binding sites for IE86, are situated close together.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany) and Eurogentec (Seraing, Belgium). The following oligonucleotides (5′-to-3′ sequences; double-stranded oligonucleotides are indicated by double shills) were used for cloning and PCR, competition in DNase I protection experiments, and gel retardation experiments: CRS, CGTTTAGTGAACCGTCAGAT//CTAGATCT GACGGTTCACTAAACGAGCT; CRS-mut, GCGGCGGT GAACCGTCAGAT//CTAGATCTGACGGTTCACCGC CGCAGCT; EAIE2, AGCTCTCGAGTAGCGTTGCGATT TGCAGTCCGCTCA//AGCTTGAGCGGACTGCAAATCG CAACGCTACTCGAG; EAIE2mut, AGCTCTCGAGTAGC GTTGTAACCCATAGTCCGCTCA//AGCTTGAGCGGAC TATGGGTTACAACGCTACTCGAG; E1, ATAGAAGCT TCGCCACAGAGGTAACAACGTG; E2, TTCAGAGCTC GGCCGTGGAGCGAGTG; E3, ATTGAAGCTTGACAC CGGAGCGGACTGC; E4, ATTGAAGCTTCACCCAGTT ACTTTAAT; and ETATA, CGTCAAGCTTGATGTATAA ATAGAGTC.

Plasmid constructions. Cloning reactions were performed by standard procedures. PCR was used to generate specific fragments of the UL112 promoter region. Amplification was performed by using Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) as recommended by the manufacturer. Conditions for amplification were as described previously (39). Plasmid pGJ3, containing a *Bam*HI-*Xba*I subfragment of the UL112 gene region, served as the template in these reactions (32). Primers E1 and E2 (50 pmol of each) were used to amplify a fragment corresponding in sequence to nucleotides −352 to +37 of the UL112 promoter of HCMV (40). After cleavage with *Hin*dIII and *Sac*I, the amplification product was inserted into the luciferase expression vector p19luc or the BlueScribe vector (Stratagene, Heidelberg, Germany), resulting in plasmid pHM142 or pHM143, respectively. A minimal, TATA-box-containing promoter was generated by cloning the amplification product of primer pair ETATA-E2 as a *Hin*dIII-*Sac*I fragment into p19luc, yielding plasmid pETATAluc. The addition of primers E1 and E3 to the amplification reaction resulted in a subfragment of the UL112 promoter comprising nucleotides −352 to −120. This fragment, which contained the IE2 binding sites of the UL112 promoter, was inserted into the *Hin*dIII-cleaved plasmid pETATAluc. Nucleotide sequence analysis using a commercially available T7 polymerase sequencing kit (Pharmacia, Freiburg, Germany) was then performed to select for the proper orientation of the inserted fragment. The resulting plasmid was designated pE(−352/−120)TATAluc. Plasmid pE(−117/+37)luc was created by ligation of the amplification product of the primer pair E4/E2 into the *Hin*dIII-*Sac*I-cleaved expression vector p19luc. The double-stranded oligonucleotides EAIE2 and EAIE2mut were cloned into the *Hin*dIII cleaved vectors pETATAluc (yielding constructs with an IE2 binding site immediately upstream of the UL112 TATA box) and pE(−117/+37)luc (yielding constructs with an IE2 binding site upstream of nucleotide −117 of the UL112 promoter). The copy number and orientation of inserted oligonucleotides were determined by nucleotide sequence analysis of the respective constructs. A 5′ deletion series of the UL112 promoter was made by using a double-stranded deletion kit (Pharmacia) as recommended by the manufacturer. Briefly, plasmid pHM142 was cleaved with *Hin*dIII. After the recessed 3′ ends were filled in with thionucleotides, a second restriction enzyme digestion was performed with *Xho*I, which has a recognition sequence at −156 within the UL112 promoter. Either this site was filled in with Klenow enzyme and the plasmid was religated or, alternatively, a digestion with exonuclease III was performed, blunt ends were created by incubation in the presence of S1 nuclease, and the ends were religated. Nucleotide sequence analysis was then used to determine the exact position of each deletion. Construction of a eucaryotic expression vector containing the cDNA encoding the 86-kDa IE2 protein of HCMV under control of the Rous sarcoma virus (RSV) LTR was performed as follows: plasmid pHM120 which had the IE2 cDNA inserted as a *Hin*dIII-*Eco*RI fragment within the BlueScribe vector, was cleaved with *Hin*dIII (33). The RSV LTR was isolated as a *Hin*dIII fragment from plasmid pIC20HRSV (13) and ligated with the linearized plasmid pHM120. The orientation of the RSV LTR was determined by nucleotide sequence analysis of the respective expression plasmid, designated pHM137.

DNase I protection and gel retardation analysis. For DNase I protection and gel retardation analysis, IE86 was expressed in bacteria as a histidine-tagged protein and purified via metal chelate affinity chromatography exactly as described previously (22). In addition, eucaryotic expression of IE86 as a histidine-tagged protein was accomplished by using the baculovirus system. Details of expression cloning and purification of the eucaryotically expressed protein will be described elsewhere. The *Hin*dIII-*Eco*RI or the *Hin*dIII-*Sac*I fragment of plasmid pHM143 comprising the UL112 promoter region between −352 and +37 was used as a probe in DNase I protection experiments. The fragment was labeled either at the 5′ end of the *Hin*dIII site by using polynucleotide kinase (coding strand) or by filling in with Klenow fragment at the *Hin*dIII site (noncoding strand). Herring sperm DNA (1 μg) was used as a nonspecific competitor DNA. Specific competition was performed by including either 100 ng of the CRS oligonucleotide or 100 ng of the CRS-mut oligonucleotide within the reaction mix. Conditions for incubation of protein with DNA, DNase I digestion, extraction of DNA, and analysis via denaturing polyacrylamide gel electrophoresis (PAGE) were as described previously (22).

For gel retardation assays, the double-stranded oligonucleotides CRS, EAIE2, and EAIE2mut were labeled by using polynucleotide kinase in the presence of [γ-^{32}P]dATP. After purification of the probes via PAGE, purified IE2 protein (100 ng) was incubated for 10 min in a buffer containing 25 mM Tris (pH 8.0), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.01% Nonidet P-40, 9% glycerol, and 1 μg of herring sperm DNA as a nonspecific competitor. The probe (20,000 cpm) was then added, and incubation continued for 10 min at room temperature. Samples were electrophoresed on a 5% (wt/vol) polyacrylamide (50:1) gel containing 0.5× Tris-borate-

EDTA buffer, using 0.5× Tris-borate-EDTA as the running buffer. After electrophoresis, the gels were dried and autoradiographed.

Cell culture, transfections, and luciferase assays. Primary human foreskin fibroblasts were cultured as described previously (39). U373 cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in minimal essential medium (Gibco/BRL, Eggenstein, Germany) supplemented with 5% fetal calf serum. Cells were plated the day before transfection in 100-mm-diameter plastic dishes at 1.2×10^6 cells per dish. Plasmid transfections were done by the DEAE-dextran procedure. Cells were washed two times with phosphate-buffered saline (PBS). The DNA was then added in 5 ml of minimal essential medium without fetal calf serum containing 0.4 mg of DEAE-dextran per ml and 100 mM Tris (pH 7.4). Routinely, 3 μg of luciferase target genes and 7 μg of the cotransfected plasmids were used. Cotransfection was performed with either the IE2 expression plasmid pHM137 or plasmid pIC20HRSV, containing the RSV LTR, as a negative control. After incubation for 2 h at 37°C, the cells were washed with PBS and 10 ml of complete medium was added.

For luciferase assays, cells were harvested about 48 h after transfection in 1 ml of extraction buffer containing 100 mM potassium phosphate (pH 7.8)–1 mM dithiothreitol and were collected by low-speed centrifugation. Extracts were prepared by three cycles of freezing and thawing and were cleared by centrifugation at 10,000 × *g* at 4°C for 5 min. An equal amount of protein extract was mixed with 100 μl of reaction buffer containing 100 mM potassium phosphate (pH 7.8), 15 mM $MgSO_4$, and 5 mM ATP. Luciferase activity was determined by injection of 100 μl of reaction buffer containing 1 mM luciferin (Boehringer, Mannheim, Germany), using a luminometer (Berthold, Wildbad, Germany) as described previously (8). Each transfection was performed at least three times.

RESULTS

IE86 interacts with three binding sites located within the early UL112 promoter of HCMV. IE86 of HCMV is a sequence-specific DNA-binding protein that can interact directly with a sequence located between the TATA box and the transcriptional start site of its own promoter. This sequence, termed CRS, is required for IE86-mediated negative regulation of the IE1/IE2 enhancer/promoter. To investigate whether IE86 is also able to recognize sequences within an IE2-responsive viral early promoter, DNase I protection experiments were performed. The UL112 promoter was chosen as a well-characterized early promoter of HCMV that is strongly activated upon cotransfection by IE2. The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 spanning nucleotides −352 to +37 of the UL112 promoter was labeled at the 5′ end of the upper strand and used as the probe. This DNA fragment was incubated with purified IE2 protein in the presence of herring sperm DNA as a nonspecific competitor. After DNase I digestion of the unprotected phosphodiester backbone of DNA, separation of the resulting fragments via denaturing PAGE, and autoradiography, three strong protections were visible in reactions that contained IE2 protein (Fig. 1A, lanes 3 and 4). These protections were located in the region between −290 and −120, upstream of the UL112 TATA box. To confirm the observed protection pattern of IE2 on the UL112 promoter, the noncoding strand was labeled by filling in at the *Hin*dIII site of the same fragment and was also used in DNase I protection analysis. Again, the IE2 protein interacted with three sites of the noncoding strand located in the region between −290 and −120 (Fig. 1B, lanes 2 and 3). No significant protection could be observed downstream of nucleotide −120. The results of a detailed mapping of the sequences that were protected by IE2 are summarized in Fig. 2. This analysis revealed that nucleotides −288 to −260 (protection ECIE2), −251 to −221 (protection EBIE2), and −151 to −126 (protection EAIE2) were protected on the coding strand. The observed interaction on the noncoding strand covered nucleotides −294 to −266 (protection ECIE2), −248 to −226 (protection EBIE2), and −157 to −137 (protection EAIE2). Sites of DNase I hypersensitivity could be observed in the vicinity of the protected regions.

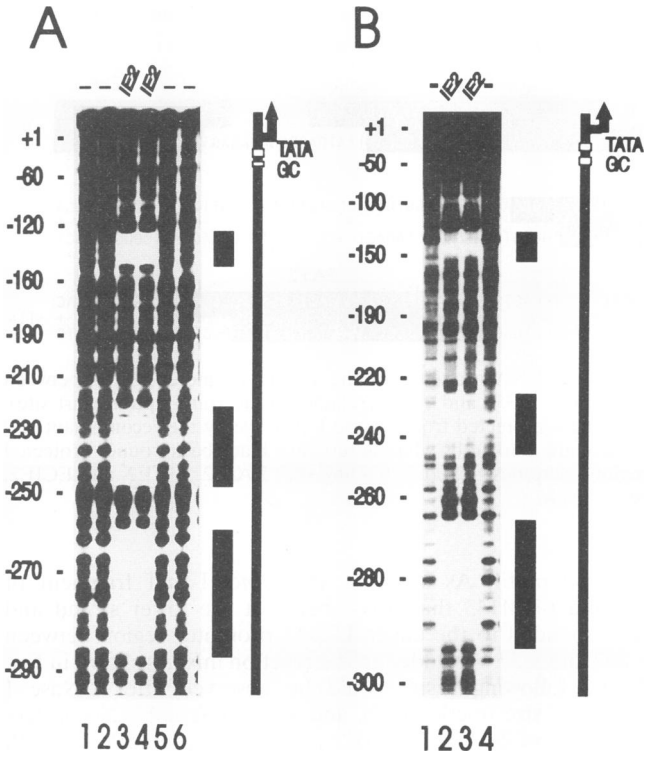

FIG. 1. DNase I protection analysis of the UL112 promoter region with the recombinant 86-kDa IE2 protein of HCMV. (A) The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 (promoter sequences from nucleotides −352 to +37) that was labeled at the 5′ end of the upper strand was used as the probe. Lanes: 1, 2, 5, and 6, no added extract; 3 and 4, 5 μl of affinity-purified IE2 protein added. (B) The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 (promoter sequences from nucleotides −352 to +37) that was labeled at the 3′ end of the lower strand was used as the probe. Lanes: 1 and 4, no added extract; 2 and 3, 5 μl of affinity-purified IE2 protein added. Numbers on the left refer to nucleotide positions of the UL112 promoter. Diagrams of the end-labeled DNA fragments are shown on the right. The arrows indicate the transcriptional start site of the UL112 promoter. TATA, TATA box sequence; GC, potential Sp1 binding site. Filled bars on the right indicate regions that were protected by IE2.

IE86 interacts in a sequence-specific manner at the UL112 promoter. To analyze whether the binding of IE86 to the sites located within the UL112 promoter is sequence specific, binding competition was investigated in DNase I protection experiments. In analogy to experiments performed at the CRS element (22), an oligonucleotide that corresponded in sequence either to the intact CRS element of the IE1/IE2 enhancer/promoter (CRS) or to a mutated form of this motif (CRS-mut) was used. IE2 protein was preincubated with either 100 ng of the intact CRS oligonucleotide or 100 ng of the

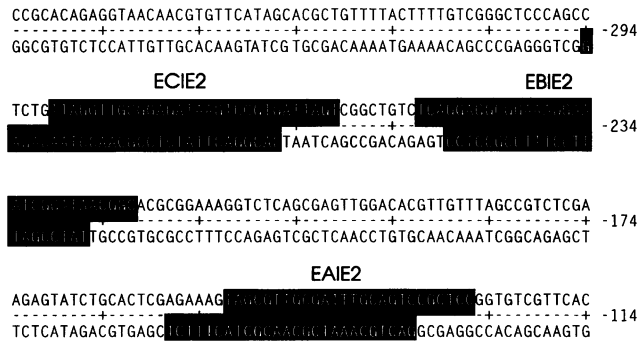

FIG. 2. DNA sequence of the UL112 promoter region between nucleotides −353 and −114 (relative to the transcription start site). Sequences protected from DNase I cleavage by the recombinant IE2 protein are marked by white letters on a black background. Protected regions were designated as binding sites EAIE2, EBIE2, and ECIE2, respectively.

mutated motif. As a probe, the *Hin*dIII-*Sac*I fragment of plasmid pHM143 that was labeled at the lower strand and corresponded to the entire UL112 promoter region between −352 and +37 was added to the reaction mix. As shown in Fig. 3, the following result could be observed after DNase I digestion, size fractionation, and autoradiography. No protection was visible when the IE2 protein was preincubated with

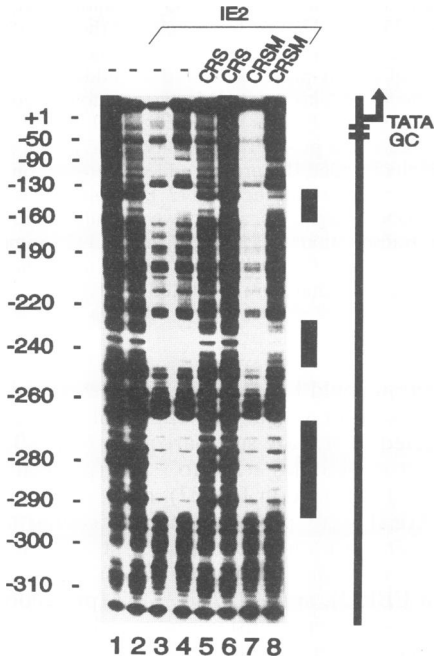

FIG. 3. Effects of specific competitor DNAs on the binding of recombinant IE2 protein to the UL112 promoter in DNase I footprinting experiments. The *Hin*dIII-*Sac*I fragment of plasmid pHM143 (promoter sequences from −352 to +37) that was labeled on the lower strand was used as the probe. Lanes: 1 and 2, no protein was added; 3 to 8, recombinant IE-2 protein was added; 5 and 6, competition with 100 ng of the CRS oligonucleotide; 7 and 8, competition with 100 ng of the CRS-mut oligonucleotide. Numbers on the left refer to nucleotide positions of the UL112 promoter. Diagrams of the end-labeled DNA fragment and protected regions are shown on the right (for details, see Fig. 1).

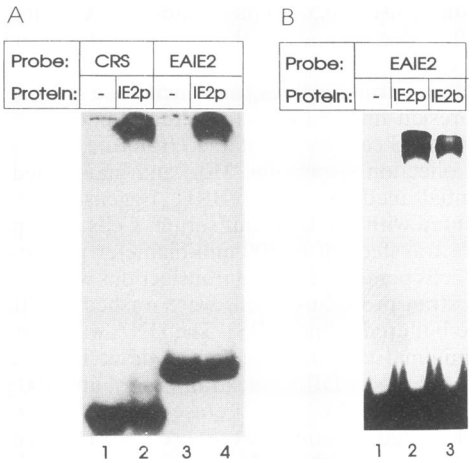

FIG. 4. Comparison of the relative affinities of IE86 to the CRS element of the IE1/IE2 enhancer/promoter and the EAIE2 binding site of the UL112 promoter. Either the CRS oligonucleotide (CRS) or the EAIE2 oligonucleotide (EAIE2) was used as the probe in gel retardation analyses. The oligonucleotides were incubated either without protein (panel A, lanes 1 and 3; panel B, lane 1) or with the procaryotically expressed IE86 protein (panel A, lanes 2 and 4; panel B, lane 2) or the eucaryotically expressed IE86 protein (panel B, lane 3), and the reaction mixtures were separated via nondenaturing PAGE. After autoradiography, retarded complexes of about equal intensities could be observed for both the CRS and EAIE2 probes in reactions that contained the IE2 protein. IE2p, procaryotically expressed IE2 protein; IE2b, eucaryotically expressed IE2 protein.

the intact CRS oligonucleotide (Fig. 3, lanes 5 and 6), demonstrating an efficient competition of this sequence for IE2 protein. The protection pattern observed after incubation in the presence of the mutated CRS oligonucleotide corresponded to the pattern obtained in the absence of any competitor (Fig. 3, lanes 7 and 8). Thus, IE86 interacts in a sequence-specific manner with the identified binding sites of the UL112 promoter.

IE86 binds with high affinity to the EAIE2 site of the UL112 promoter. Although the results of competition analysis could demonstrate the specificity of the observed interaction, one might still argue that there are substantial differences in affinity between the IE2 binding site at the CRS element and those within the UL112 promoter. To clarify this, gel retardation experiments were performed. An oligonucleotide corresponding in sequence to the CRS element of the IE1/IE2 enhancer/promoter or the EAIE2 binding site of the UL112 promoter was used as the probe. Both probes were incubated with affinity-purified IE2 protein in the presence of herring sperm DNA as a nonspecific competitor. After fractionation of the reaction mixtures on a nondenaturing polyacrylamide gel and autoradiography, retarded complexes of about equal intensity could be observed for the CRS and EAIE2 probes (Fig. 4A, lanes 2 and 4). Titration of the amount of IE2 protein in DNase I protection experiments using the UL112 promoter as probe did not reveal gross differences in the affinities of IE2 to their respective target sequences within this promoter (data not shown). This observation rules out major differences between the affinities of IE2 to the CRS element and to the sites of the UL112 promoter. In addition, eucaryotic IE86, protein, expressed in insect cells by using the baculovirus system, was used in gel retardation experiments. As shown in Fig. 4B, lane 3, there was efficient binding of eucaryotic IE86 to the EAIE2 site of the UL112 promoter, which excludes the

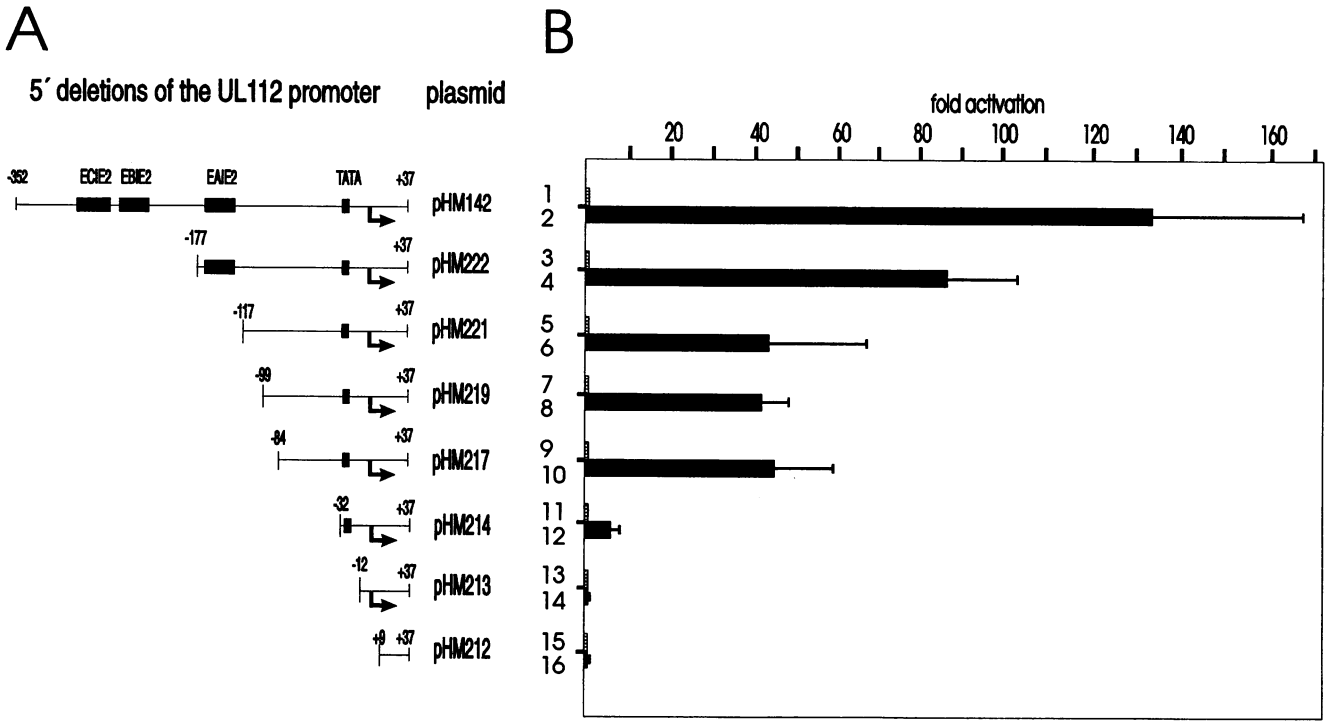

FIG. 5. Transactivation of 5′ deletion constructs of the UL112 promoter by IE86 in U373 cells. (A) 5′ deletion constructs of the UL112 promoter. The UL112 promoter sequences contained within each luciferase expression plasmid are indicated. Relative positions of IE2 binding sites (EAIE2, EBIE2, and ECIE2) and the TATA sequence are shown as filled bars. Names of the 5′ deletion plasmids are on the right. (B) Schematic diagram of activation values obtained after cotransfection of various 5′ deletion constructs with either the vector pIC20HRSV as a negative control (stippled bars) or the IE86 expression vector pHM137 (filled bars). Shown is the relative luciferase activity expressed as fold increase relative to the activity of the respective construct in the absence of IE86. Bars: 1 and 2, plasmid pHM142; 3 and 4, plasmid pHM222; 5 and 6, plasmid pHM221; 7 and 8, plasmid pHM219; 9 and 10, plasmid pHM217; 11 and 12, plasmid pHM214; 13 and 14, plasmid pHM213; 15 and 16, plasmid pHM212. In bars 1, 3, 5, 7, 9, 11, 13, and 15 cotransfection was performed with plasmid pIC20HRSV. In bars 2, 4, 6, 8, 10, 12, 14, and 16 cotransfection was performed with the IE86 expression plasmid pHM137. Results are from at least four independent experiments; standard deviations are indicated by bars.

possibility that any contaminating protein from *Escherichia coli* cells is responsible for the observed interaction of IE86 with DNA.

Deletion analysis of the UL112 promoter suggests a functional importance of IE2 binding sites. To investigate the functional importance of the detected binding sites for IE86, a series of 5′ deletions of the UL112 promoter was constructed and tested in cotransfection experiments of permissive U373 cells (Fig. 5). Cotransfection was performed either with plasmid pHM137, containing the IE2 cDNA under control of the RSV LTR, or with plasmid pIC20HRSV, containing the RSV LTR as a negative control for promoter competition. Figure 5B summarizes the results of at least four independent cotransfection experiments that were performed for each deletion plasmid. Identical results were obtained when primary human foreskin fibroblasts were used for transfection (data not shown). Deletion of sequences between nucleotides −352 and −117 of the UL112 promoter containing the identified IE2 binding sites resulted in a reduction in the level of transactivation by about 70% (Fig. 5B, bars 1, 2, 5, and 6). Most importantly, after removal of nucleotides between −177 and −117 comprising precisely the EAIE2 site of the UL112 promoter, a significantly lower transactivation by IE2 could be observed. This finding suggests that the EAIE2 binding site contributes to transactivation. The remaining promoter, however, could still be stimulated about 40-fold, indicating that transactivation by IE2 is not dependent on the presence of IE2 binding sites. As demonstrated earlier, the TATA box itself was sufficient to mediate a low level of transactivation; however, after deletion of TATA box sequences, IE2 responsiveness of the promoter was lost entirely. When a construct containing sequences between −84 and +37 was tested in cotransfection experiments, a significantly higher level of transactivation could be detected compared with the TATA-containing plasmid pHM214. This finding indicates that an additional positive element that is important for IE2 transactivation is located between −84 and −32 upstream of the TATA motif. Thus, the UL112 promoter appears to contain several responsive elements that contribute to IE2-mediated stimulation. The observed reduction in transactivation after deletion of IE2 target sequences suggests that binding sites for this protein may constitute one class of responsive elements.

An IE2 binding site cannot mediate IE2 responsiveness when positioned immediately upstream of the UL112 TATA box. We wanted then to test whether the low level of transactivation observed with the TATA-containing construct pHM214 could be augmented when IE2 binding sites are located immediately upstream. For this reason, the IE2 binding domain of the UL112 promoter between nucleotides −352 and −120 was amplified by PCR and cloned upstream of the UL112 TATA box. In addition, as cellular factors might also bind to this domain, an oligonucleotide corresponding in

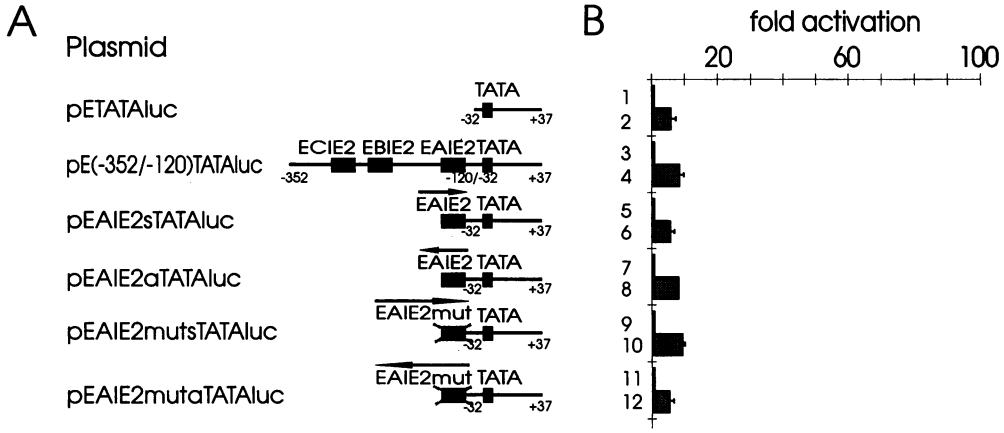

FIG. 6. Effects of various luciferase expression constructs containing IE2 binding sites of the UL112 promoter immediately upstream of the UL112 TATA box in mediating transactivation by IE86. (A) Luciferase expression constructs that were used in transfection experiments of U373 cells. The UL112 promoter sequence contained within each construct is indicated. Relative positions of genuine (EAIE2, EBIE2, and ECIE2) or mutated (EAIE2mut) IE2 binding sites and the TATA sequence are shown as filled bars. Arrows indicate the orientation of cloned motifs. Names of the plasmids are on the left. (B) Schematic diagram of activation values obtained after cotransfection of various UL112 promoter constructs with either the vector pIC20HRSV as a negative control (filled bars) or the IE86 expression vector pHM137 (stippled bars). Shown is the relative luciferase activity expressed as fold activation relative to the activity of the respective construct in the absence of IE86. Bars: 1 and 2, pETATAluc; 3 and 4, pE(−352/−120)TATAluc; 5 and 6, pEAIE2sTATAluc; 7 and 8, pEAIE2aTATAluc; 9 and 10, pEAIE2mutsTATAluc; 11 and 12, pEAIE2mutaTATAluc. In bars 1, 3, 5, 7, 9, and 11 cotransfection was performed with plasmid pIC20HRSV. In bars 2, 4, 6, 8, 10, and 12, cotransfection was performed with the IE86 expression plasmid pHM137. Results are from at least two independent experiments; when more than three experiments were performed, standard deviations are indicated as bars.

sequence to the EAIE2 site of the UL112 promoter was inserted in both orientations upstream of the UL112 TATA box. As a negative control, an oligonucleotide that contained mutations within the EAIE2 binding sequence was used (Fig. 6A). These constructs were then cotransfected with either the IE2 expression plasmid pHM137 or the control vector pIC20HRSV. Surprisingly, however, neither the entire IE2 binding domain comprising all three IE2 interaction sites nor the EAIE2 oligonucleotide was able to increase IE2-mediated transactivation significantly compared with the TATA-box-containing vector that was used for cloning or the constructs containing the mutated EAIE2 site (Fig. 6B). Thus, we were not able to detect any significant effect of IE2 binding sites in combination with a minimal, TATA-box-containing promoter.

An IE2 binding site augments transactivation when positioned upstream of nucleotide −117 of the UL112 promoter. The results of deletion analysis suggested a functional importance of IE2 binding sites for IE2-mediated transactivation. However, an isolated IE2 binding site upstream of the UL112 TATA box had no effect on the level of stimulation. Therefore, although the deletion of sequences between −177 and −117 of the UL112 promoter removed precisely the EAIE2 binding site, we cannot exclude the possibility that interaction sites for cellular proteins involved in transactivation were also affected by this deletion. Otherwise, DNA-bound IE2 protein may require the presence of cellular factors interacting with sequences between −117 and −32 of the UL112 promoter in order to be able to stimulate gene expression. To clarify this, additional luciferase expression plasmids were constructed. Plasmid pE(−117/+37)luc contained sequences between nucleotides −117 and +37 of the UL112 promoter and a unique *Hin*dIII site upstream of −117 (Fig. 7A). Either the intact EAIE2 oligonucleotide or a mutated sequence was then inserted upstream of nucleotide −117 (Fig. 7A). After cotransfection of these constructs with the IE2 expression vector and quantitation of the resulting luciferase activities, a strong augmentation of transactivation in the presence of the intact IE2 binding site (EAIE2) was obvious (Fig. 7B, bars 1 to 4). The mutated motif (EAIE2mut) was not able to mediate this effect (Fig. 7B, bars 5 and 6). As tested in gel retardation experiments, the mutations within the EAIE2 binding site abolished binding of IE2 to this sequence (Fig. 8). Thus, an IE2 binding site has a strong effect when it is localized upstream of nucleotides −117 of the UL112 promoter. This finding suggests that cellular factors interacting with sequences between −117 and −32 of the UL112 promoter are necessary for DNA-bound IE2 in order to mediate transactivation.

DISCUSSION

The 86-kDa IE2 (IE86) protein of HCMV is a multifunctional molecule that can regulate gene expression both positively and negatively (15, 16, 27, 31, 44). It appears to be the most important protein for triggering lytic cycle gene expression of HCMV, as its transactivating function is required for the stimulation of viral early promoters (21, 27, 44). This is also implicated by the strong conservation of IE2 between different betaherpesviruses, which cannot be observed for the IE1 polypeptide (6).

IE86 not only exerts two different functions but apparently uses at least two different mechanisms to mediate these effects. Interactions with cellular proteins are thought to play a major role in the positive action of IE2 that is observed on both homologous and heterologous promoters (3, 11, 14). While the identities of most cellular interaction partners remain to be determined, IE86 has already been demonstrated to contact specifically the TATA-binding protein TBP (11, 14, 20). Negative regulation of gene expression is mediated by a direct contact of IE86 with DNA (6, 19, 22, 26). This is specific for the enhancer/promoter that drives transcription of the IE1/IE2 gene region within HCMV and depends on the presence of a sequence element, termed CRS, located between the TATA box and the transcription start site of this promoter (5, 24, 29).

In this study, we show that DNA binding of IE86 can also be

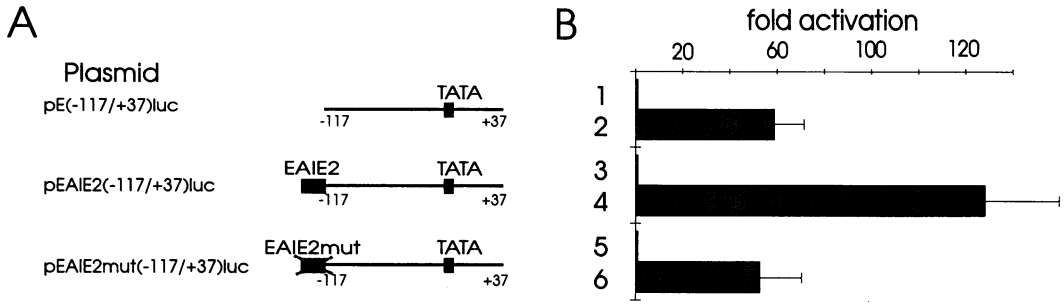

FIG. 7. Effects of luciferase expression constructs containing IE2 binding sites of the UL112 promoter upstream of nucleotide −117 of the UL112 promoter in mediating transactivation by IE86. (A) Luciferase expression constructs that were used in transfection experiments of U373 cells. The UL112 promoter sequence contained within each construct is indicated. Relative positions of genuine (EAIE2) and mutated (EAIE2mut) IE2 binding sites and the TATA sequence are shown as filled bars. Names of the plasmids are on the left. (B) Schematic diagram of activation values obtained after cotransfection of various UL112 promoter constructs with either the vector pIC20HRSV as a negative control (filled bars) or the IE86 expression vector pHM137 (stippled bars). Shown is the relative luciferase activity expressed as fold activation relative to the activity of the respective construct in the absence of IE86. Bars: 1 and 2, pE(−117/+37)luc; 3 and 4, pEAIE2(−117/+37)luc; 5 and 6, pEAIE2mut(−117/+37)luc. In bars 1, 3, and 5, cotransfection was performed with plasmid pIC20HRSV. In bars 2, 4, and 6, cotransfection was performed with the IE86 expression plasmid pHM137. Results are from five independent experiments; standard deviations are indicated as bars.

detected within an early promoter of HCMV that is strongly activated both upon cotransfection with IE86 expression vectors and after addition of purified IE86 to an in vitro transcription system (21). Three binding sites could be detected in the region between nucleotides −290 and −120 of the UL112 promoter of HCMV, which drives expression of several abundant early RNAs, termed the 2.2-kb class of early transcripts (40, 41). The identified binding sites were found to be specific and had an affinity comparable to that of the CRS element. Thus, the 86-kDa IE2 protein is able to recognize additional target sequences which differ from the CRS element. Each of these novel binding sites was unique within the HCMV genome, as no identical sequences could be detected during computer searches. Remarkably, a comparison of the binding sites did not reveal a substantial similarity (Fig. 9). When the protected regions of the UL112 promoter were aligned and compared with the CRS element, the only nucleotides that were common to all four sequences were two copies of the dinucleotide CG, separated by 10 not well conserved nucleotides (Fig. 9). However, it appears unlikely that the sequence $CG(N)_{10}CG$ is the only requirement for IE86 to bind DNA, as it would occur more than 3,000 times within the entire HCMV genome. A second determinant of binding might be a stretch of A/T-rich sequences flanking the CG dinucleotides internally (23). Clearly, additional experiments are necessary to derive a consensus sequence that will allow the prediction of IE2 binding sites within the HCMV genome. The present data, however, suggest that the IE2 protein has a rather loose sequence requirement for binding to DNA and may therefore interact with a broad spectrum of different targets. This is also suggested by the earlier finding that IE2 is able to interact with the nonspecific competitor DNA poly(dI-dC) (22).

Deletion analysis of the UL112 promoter confirmed the proposed concept that early promoters of HCMV may contain several IE2-responsive elements (40, 49). One of these elements is the TATA box; however, the levels of induction mediated by the TATA sequence alone were low compared with the entire promoter. As an additional responsive element, we identify binding sites for IE2; this is suggested by the reduction of IE2-mediated transactivation after deletion of IE2 binding sites and by the positive effect observed after

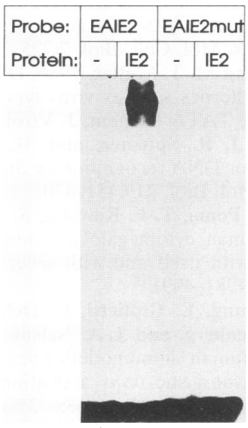

FIG. 8. Gel retardation analysis of the EAIE2 and mutated EAIE2 motifs, using procaryotically expressed IE86 protein. Either the EAIE2 oligonucleotide (EAIE2) or the mutated EAIE2 oligonucleotide (EAIE2mut) was used as the probe in gel retardation analyses. The oligonucleotides were incubated either without protein (−) or with the procaryotically expressed IE86 protein (IE2), and the reaction mixtures were separated via native PAGE. After autoradiography, retarded complexes could be observed only with the EAIE2 probe in the presence of IE86 protein.

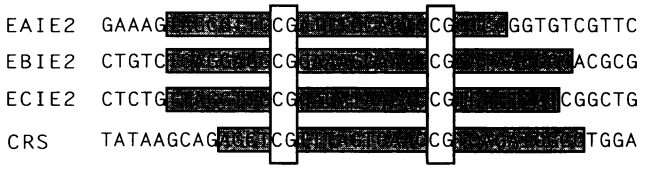

FIG. 9. Alignment of the IE2 binding sites of the UL112 promoter and the CRS element of the IE1/IE2 enhancer promoter of HCMV. Nucleotides on stippled backgrounds correspond to protected regions of the UL112 promoter that were observed in DNase I protection experiments. Boxed sequences indicate nucleotide positions that are conserved in all four sequences. EAIE2, EAIE2 binding site of the UL112 promoter; EBIE2, EBIE2 binding site of the UL112 promoter; ECIE2, ECIE2 binding site of the UL112 promoter; CRS, CRS element of the IE1/IE2 enhancer/promoter.

reinsertion of an IE2 target site upstream of nucleotide −117 of the UL112 promoter. Interestingly, however, there was no effect on IE2 transactivation when binding sites were located immediately upstream of the TATA box. There are at least two potential explanations for that observation. First, DNA-bound IE2 protein may require an exact phasing in order to be able to interact with the basal transcription apparatus; the constructs used might not meet this criteria. However, as both orientations of the EAIE2 oligonucleotide failed to mediate a response, this seems unlikely. Most probably, DNA-bound IE2 requires an additional cellular transcription factor which binds in the region from −117 to −32 in order to mediate transactivation. Binding sites for IE2 would then resemble the subtype B of enhansons, which is not able to activate gene expression by itself but must be combined with a heterologous enhanson to exert a stimulating effect. This has, for example, been shown for the GT-I enhanson of the simian virus 40 enhancer (for a review, see reference 17).

Alternatively, as the sequence between −117 and −32 of the UL112 promoter, which did not contain a high-affinity IE2 binding site, was also IE2 responsive, upstream IE2 binding sites might serve as a sink for IE2 protein and thus help to increase the local density of IE2 protein on the promoter. This could affect the probability of an interaction with cellular factors binding to downstream regions. Identification of the cellular interaction partners of IE2 will be required to resolve this question.

Cellular proteins which might bind to the segment between −117 and −32 of the UL112 promoter include members of the ATF/CREB and E2F families of transcription factors. Potential target sites for these polypeptides could be detected in the respective regions (40, 50). Whether one of these factors plays a role in IE2 transactivation remains to be established. However, given the proposed similarity of IE2 with E1A of adenovirus, which is underscored by the fact that IE2 can efficiently complement adenovirus E1A mutants (38), one could speculate on a direct or indirect interaction of IE2 with at least one of these proteins. Nevertheless, there are also fundamental differences between E1A of adenovirus and IE2 of HCMV. These include the ability of IE2 to dimerize and to regulate gene expression by both protein-protein and protein-DNA contacts. In fact, this bimodal mechanism used by IE2 resembles much more the mechanism used by the ICP4 protein of herpes simplex virus. Both proteins stimulate minimal promoters containing only TATA boxes, which involve an interaction with factors of the basal transcription machinery (14, 18, 37); both polypeptides negatively autoregulate gene expression by binding to a sequence located between the TATA box and the transcriptional start site of their own promoters (9, 22, 26, 35); moreover, binding of ICP4 to early promoter sequences has also been described; however, in analogy to the results presented here, DNA binding sites are not absolutely necessary for transactivation (36, 46). In addition, the sequence requirements for specific ICP4 DNA binding are known to be highly degenerate, again resembling the IE2 protein (10, 28). Thus, this emphasizes our earlier proposal that alpha- and betaherpesviruses may have evolved common mechanisms used by their IE proteins to regulate gene expression, although the amino acid sequences of the respective proteins are divergent (22).

In conclusion, we were able to define novel binding sites for the 86-kDa IE2 protein of HCMV that contribute to but are not absolutely required for transactivation of a viral early promoter. The limited similarity of these binding sites suggests that IE2 may have a loose sequence requirement for interacting with DNA. Thus, a thorough investigation of the exact binding characteristics of IE2 will be necessary in order to be able to predict additional promoters that are a target for either positive or negative regulation by IE2 proteins.

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