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Functional Interaction between the Human Cytomegalovirus 86-Kilodalton IE2 Protein and the Cellular Transcription Factor CREB

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The 86-kDa IE2 protein (IE86) of human cytomegalovirus (HCMV) has been described as a promiscuous transactivator of viral, as well as cellular, gene expression. Investigation of the mechanism used by IE86 to activate gene expression from the early UL112/113 promoter of HCMV revealed the existence of three binding sites for IE86 located between nucleotides −290 and −120 relative to the transcriptional start site (H. Arlt, D. Lang, S. Gebert, and T. Stamminger, J. Virol. 68:4117–4125, 1994). As shown previously, deletion of these target sites resulted in a reduction of IE86-mediated transactivation by approximately 70%. The remaining promoter, however, could still be stimulated about 40-fold, indicating the presence of an additional responsive element within these sequences. Here, we provide evidence that a binding site for the cellular transcription factor CREB can also act as a target for IE86 transactivation. By DNase I protection analysis, a binding sequence for CREB could be detected between nucleotides −78 and −56 within the respective promoter region. After in vitro mutagenesis of this CREB-binding site within the context of the entire UL112/113 promoter, a marked reduction in transactivation levels was evident. Moreover, when individual CREB-binding sites were positioned upstream of a minimal, TATA box-containing UL112/113 promoter, they were able to confer strong IE86 responsiveness, whereas a mutated sequence did not exert any effect. In far Western blot and pull-down experiments, a direct interaction of IE86 with the cellular transcription factor CREB could be observed. The in vivo relevance of this in vitro interaction was confirmed by using various GAL4 fusion proteins in the presence or absence of IE86 which revealed a strong activation only in the presence of both a GAL4-CREB fusion and IE86. This shows that at least one specific member of the ATF/CREB family of transcription factors is involved in mediating transactivation by the HCMV IE86 protein.

Human cytomegalovirus (HCMV), which belongs to the beta subgroup of herpesviruses, is characterized by its narrow host range and prolonged replicative cycle in tissue culture cells. Gene expression of HCMV, which has been most extensively studied in primary human fibroblasts, occurs, as with other herpesviruses, in a temporally regulated manner (10, 36, 54, 55). The three phases of viral gene expression that have been described were termed immediate early (IE), early, and late. During IE times, several proteins that are required for the activation of promoters of the next temporal class, the early class, are expressed (9, 49, 52). The most important of these proteins in HCMV are the 72-kDa IE1 (IE72, ppUL123) and the 86-kDa IE2 (IE86, ppUL122a) polypeptides that originate from the major IE gene region of HCMV and are transcribed under the control of a complex enhancer-promoter (4, 19, 39, 50, 53).

Both IE72 and IE86 are independent transactivators of heterologous promoters. For instance, IE72 has been shown to be a powerful transactivator of the TATA-less DNA polymerase α promoter (17). In contrast, IE86 requires a TATA-containing promoter in order to be able to transactivate (14). For promoters of HCMV, IE86 appears to be more important, as most investigated promoters could be stimulated independently by IE86 but not by IE72 (24, 34). However, for some viral promoters a cooperative effect of these two polypeptides has been reported (34, 51). In addition to its function as transactivator, IE86 represses transcription from the major IE enhancer-promoter of HCMV and is thereby able to negatively autoregulate its own expression (18, 38). This effect was dependent on a sequence element, termed the *cis* repression signal, that is located between the TATA box and the transcriptional start site of the IE1/2 enhancer-promoter (6, 29, 38).

The mechanism used by IE86 to modulate gene expression seems to involve both protein-protein and protein-DNA contacts (7, 11, 14, 21, 22, 27, 33). While binding of IE86 to numerous cellular and also some viral polypeptides has been demonstrated, most of these interaction partners have not been defined up to now (11). Known interaction partners include the retinoblastoma protein Rb (13, 44), the viral protein pUL84 (45), and factors of the basal transcription machinery such as the TATA-binding protein TBP and the basal transcription factor TFIIB (14, 22, 44). The interaction with factors of the basal transcription apparatus served as an explanation for the TATA dependency of IE86 transactivation and helped to explain how minimal promoter elements are influenced by this protein. However, IE86 transactivation of some HCMV early genes is stimulated significantly by the presence of regulatory sequences upstream of the TATA box, suggesting the importance of additional protein-binding sites (2, 5, 48). Recently, direct DNA-binding sites for IE86 were identified within the early promoter which drives expression from the UL112/113 gene region of HCMV (2, 42). Remarkably, there was only a limited sequence similarity between these interacting sequences, which is probably due to the fact that IE86 is a minor groove-binding protein (28). DNA binding of IE86 appeared to contribute to transactivation of the UL112/113 pro-

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moter, as deletion of direct binding sites resulted in a reduction in the level of IE86-mediated transactivation. The residual promoter fragment, however, could still be stimulated significantly, suggesting that cellular-protein-binding sites within this sequence also play a role (2).

In this paper we show that a binding site for the cellular transcription factor CREB is located within the UL112/113 promoter, which is able to mediate IE86 transactivation. Moreover, we demonstrate that IE86 can interact both in vivo and in vitro with the CREB protein. As several early promoters of HCMV contain ATF/CREB consensus sequences, this may constitute one common target whereby IE86 switches from IE to early gene expression.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). The following oligonucleotides (5′-to-3′ sequences; double-stranded oligonucleotides are indicated by double shills) were used for cloning, in vitro mutagenesis, and competition in DNase I protection experiments: CRE-go, CGAGAAATTGACGTCATGGGTAAG//TCGAGCTCTTTAACTGCAGTA CCCATTCAGCT; CRS-mut, GCGGCGGTGAACCGTCAGAT//CTAGATC TGACGGTTCACCGCCGCAGCT; CRE-smut, CGTCGTGGTGTTGTTTGTG; E1, ATAGAAGCTTCGCCACAGAGGTAACAACGTG; E2, TTCAGAGCT CGGCCGTGGAGCGAGTG; ECRE, AGCTCTCGAGTCGTGACGTTGTT TGTA//AGCTTACAAACAACGTCACGACTCGAG; ECRE-mut, AGCTCT CGAGTCGTGGTGTTGTTTGTA//AGCTTACAAACAACACCACGACTC GAG; N1, AGCGAAGCTTGTCGACCTCGAGCCACCAT; N3, AGCGAA GCTTGTCGACCCGCATCTCCGAGTTG; and cIE2, AGCTGAATTCAAG CTTCCCCACGGCGTAGGC.

DNase I protection analysis. For DNase I protection experiments, CREB-A was expressed in bacteria as a histidine-tagged protein and purified via metal chelate affinity chromatography as previously described (26, 27). The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 comprising the UL112/113 promoter region between nucleotides −352 and +37 was used as a probe in DNase I protection experiments (2). 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. Conditions for DNase I protection analysis were exactly as described previously, except that 1 μg of poly(dI-dC) · poly(dI-dC) was used as a nonspecific competitor DNA (27). Specific competition was performed by including 100 ng of either the CRE-go or the CRS-mut oligonucleotide within the reaction mix.

Plasmid constructions and site-directed mutagenesis. Construction of the luciferase expression plasmids pHM142 (containing sequences of the UL112/113 promoter between nucleotides −352 and +37) and pETATAluc (also termed pHM214; containing sequences of the UL112/113 promoter between nucleotides −32 and +37) has been described previously (2).

In vitro mutagenesis of the CREB-binding site within the UL112/113 promoter was performed by using a PCR approach according to the method of Landt et al. (25). Briefly, the mutagenic primer CRE-smut and the primer E2 were used in a first PCR together with plasmid pHM142 as the template. After isolation of the respective amplification products via agarose gel electrophoresis, a second PCR was performed with the amplification product and oligonucleotide E1 as primers and plasmid pHM142 as the template. This resulted in a DNA fragment containing UL112/113 promoter sequences between nucleotides −352 and +37 with two nucleotide exchanges at positions −68 and −69. This fragment was then cloned into the luciferase expression vector p19luc as previously described (2). Conditions for the PCR were as described previously (2). Nucleotide sequence analysis was performed in order to confirm the mutations and to exclude any additional sequence variations introduced by the PCR.

The double-stranded oligonucleotides ECRE and ECRE-mut were cloned into the *Hin*dIII-cleaved vector pETATAluc yielding constructs with the CREB-binding site of the UL112/113 promoter immediately upstream of the UL112/113 TATA box. The copy number and orientation of inserted oligonucleotides were determined by nucleotide sequence analysis of the respective plasmids. Construction of the IE86 expression plasmid pHM137 has been described previously (2).

In order to be able to perform in vitro transcription-translation reactions, the cDNA encoding CREB, TBP, or IE72 was subcloned into the BlueScribe vector BS+ (Stratagene, Heidelberg, Germany), resulting in plasmids pBSCREB, pBSTBP, and pBSIE1, respectively.

5′ and 3′ deletion mutants of the IE86 cDNA within the procaryotic expression vector pQE10 (Quiagen, Hilden, Germany) were made by using a double-stranded deletion kit (Pharmacia, Freiburg, Germany) as recommended by the manufacturer. Briefly, plasmid pQE10IE2 was either cleaved with *Bam*HI (for 5′ deletions) or *Hin*dIII (for 3′ deletions) (27). After the recessed 3′ ends were filled in with thionucleotides, a second restriction enzyme digestion was performed with either *Apa*I (for 5′ deletions) or *Ppu*MI (for 3′ deletions). Then, 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. PCR was used to generate specific fragments of the IE86 cDNA. Amplification was performed by using Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) as described previously (2). Plasmid pQE10IE2 served as the template in these reactions. Primers N1 and cIE2 or N3 and cIE2 were used to amplify fragments corresponding in sequence to amino acids (aa) 290 to 548 or 329 to 548 of IE86, respectively. After cleavage with *Hin*dIII and *Sal*I, the amplification products were inserted into the procaryotic expression plasmid pQE10 (Quiagen).

The β-globin reporter constructs OVEC-1 (containing a minimal, TATA-containing β-globin promoter), 5GAL/β (containing five GAL4 binding sites upstream of the β-globin TATA box), OVEC-REF, and the expression plasmid pGAL1-93EV (containing the DNA-binding domain of GAL4 between aa 1 and 93) were kindly provided by M. Gstaiger and W. Schaffner (43, 56). The expression plasmid pGALCREB was constructed by filling in the recessed ends of the *Sal*I-*Hin*dIII fragment of plasmid pBSCREB with Klenow enzyme; this was followed by insertion of the blunt-ended fragment into the *Eco*RV site of pGAL1-93EV. This resulted in an in-frame fusion of the GAL4 DNA-binding domain with the transcription factor CREB. Plasmid pGALUL69 was generated by cloning the *Bam*HI-*Eco*RV fragment of plasmid pHM162 into the *Bam*HI-*Sma*I-cleaved GAL4 expression vector pSG424, which was obtained from M. Ptashne (41, 58). The eukaryotic CREB expression vector pCB6CREB was created by inserting the *Bam*HI-*Hin*dIII fragment of plasmid pQE10CREB into the *Bgl*II-*Hin*dIII-cleaved expression vector pCB6, which was kindly provided by M. F. Stinski (27, 58). In order to express the 72-kDa IE1 and the 86-kDa IE2 proteins of HCMV under control of the HCMV major IE enhancer-promoter, a *Hin*dIII-*Apa*I fragment of plasmid pRR59 was inserted into the corresponding restriction sites of plasmids pHM124 and pHM121, respectively (9, 40). The resulting plasmids were termed pHM135 (IE72 expression plasmid) and pHM134 (IE86 expression plasmid).

Pull-down assay and GST fusion proteins. Glutathione *S*-transferase (GST) expression plasmids pGEX-IE1 and pGEX-IE2 were kindly provided by J. Sinclair (14). For purification of GST fusion proteins, protein expression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to an exponentially growing *Escherichia coli* culture harboring the appropriate GST expression vector. After 3 h of induction, cells were harvested, resuspended in phosphate-buffered saline (PBS) containing 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol, and lysed by shock freezing in liquid nitrogen; this was followed by mild sonication on ice. After centrifugation, the supernatant was rocked gently for 15 min at 4°C with 400 μl of glutathione-agarose beads (Pharmacia). The beads were then pelleted, washed three times with PBS and two times with ELB buffer containing 125 mM NaCl, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0), 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 0.5 mM EDTA. After resuspension of the beads in 1 volume of ELB buffer, glycerol was added to a final concentration of 10%. For analysis of bound proteins, the beads were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were visualized by Coomassie blue staining. For pull-down assays, 100 ng of the GST fusion proteins on beads was preincubated for 10 min in 200 μl of ELB buffer containing bovine serum albumin (final concentration, 1 mg/ml). After addition of 1 to 5 μl of in vitro translated test protein which had been generated by using the TNT system (Promega, Heidelberg, Germany), the GST beads were incubated overnight at 4°C. The beads were then washed five times in 1 ml of ELB buffer, pelleted, and boiled in 2× SDS-PAGE sample buffer, and bound proteins were resolved in SDS–12.5% polyacrylamide gels. The gels were fixed and rocked in a fluorograph for 30 min prior to drying and autoradiography.

In vitro labeling of CREB protein. Histidine-tagged CREB protein was coupled to Ni^{2+}-nitrilotriacetic acid agarose (Quiagen), and the beads were washed three times with ELB buffer. Labeling of CREB protein (500 ng) was accomplished by the addition of 40 μCi of [γ-^{32}P]dATP in the presence of 250 U of the catalytic subunit of protein kinase A (Sigma P2645) in a final reaction volume of 200 μl containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 12 mM $MgCl_2$ (35). After 30 min of incubation at room temperature, the kinase reaction was terminated with 1 ml of HMK stop buffer containing 10 mM sodium phosphate, pH 8.0, 10 mM sodium pyrophosphate, 10 mM EDTA, and 1 mg of bovine serum albumin per ml (23). After a short centrifugation at 300 × *g*, the supernatant was removed and the beads were washed three times with HBB buffer (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM KCl, 2 mM dithiothreitol). The ^{32}P-labeled CREB protein was eluted in 50 μl of HBB buffer containing 200 mM imidazole, pH 8.0, and used in far Western blot analyses.

Far Western blotting. Test proteins were resolved on SDS–12.5% PAGE and transferred to nitrocellulose filters in blotting buffer containing 190 mM glycine and 25 mM Tris-HCl. The blots were denatured and renatured exactly as previously described (23). The filters were then blocked in HBB buffer containing 0.05% Nonidet P-40 and 5% dry milk. After a single wash with 50 ml of hybridization buffer consisting of HBB buffer with 0.05% Nonidet P-40 and 1% dry milk, the filters were probed with the radiolabeled CREB protein (5×10^4 cpm/ml) at 4°C overnight. The filters were then washed three times with 50 ml of hybridization buffer, dried, and autoradiographed.

Cell culture, transfections, and luciferase assays. U373 cells were cultured and transfected by using the DEAE-dextran procedure exactly as described previously (2). In cotransfection experiments, 3 μg of luciferase target genes and 7 μg of the cotransfected plasmids were used. Cotransfection was performed with either the IE86 expression plasmid pHM137 or plasmid pIC20HRSV, containing the Rous sarcoma virus long terminal repeat, as a negative control. About 48 h after transfection, cell extracts were prepared and luciferase assays were performed as previously described (2).

For in vivo interaction studies, HeLa cells were transfected by the calcium phosphate coprecipitation procedure with 10 μg of β-globin reporter plasmid, 3 μg of GAL4 fusion plasmid, 3 μg of the transactivator plasmid, and 1.5 μg of the reference plasmid. The reference plasmid used in all transfections was OVEC-REF (56). If no transactivator plasmid was added, the amount of DNA was kept constant by adding 3 μg of the plasmid pBS+ (Stratagene). Each transfection was performed at least three times.

RNA extraction and S1 analysis. About 48 h after transfection, total cellular RNA was harvested as described previously (47a). For S1 nuclease analysis, a single-stranded oligonucleotide probe extending between positions −18 and +75 of the rabbit β-globin gene was labeled by using [γ-^{32}P]dATP and polynucleotide kinase (56). RNA (20 μg) was hybridized with the probe (50,000 cpm) overnight at 30°C in 10 μl of hybridization buffer containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 400 mM NaCl, and 1 mM EDTA. Digestion of single-stranded RNA and/or single-stranded DNA was performed for 1 h at room temperature with 150 U of S1 nuclease in S1 buffer containing 0.25 M NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$, 13 μg of herring sperm DNA per ml, and 10 μg of single-stranded calf thymus DNA per ml. After phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation, samples were analyzed in a 10% polyacrylamide–7.5 M urea gel.

RESULTS

The cellular transcription factor CREB binds at the UL112/113 promoter between nucleotides −78 and −56. In previous studies investigating the mechanism used by the IE86 protein of HCMV to transactivate viral gene expression, we identified three binding sites for IE86 within the early UL112/113 promoter of HCMV (2). After deletion of these binding sites, however, the residual promoter could still be transactivated significantly, suggesting the existence of at least one additional responsive element. Within the residual promoter fragment, spanning nucleotides −117 to +37 relative to the UL112/113 transcription start site, a sequence motif with homology to a binding site for CREB/ATF factors could be detected. In order to see whether CREB could bind to this sequence, DNase I protection experiments were performed. The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 comprising the UL112/113 promoter region between nucleotides −352 and +37 was labeled either at the coding or noncoding strand and used as a probe. The labeled DNA fragments were incubated with procaryotically expressed and purified CREB protein in the presence of poly(dI-dC) · poly(dI-dC) as a nonspecific competitor DNA and digested with DNase I. After separation of the resulting fragments via denaturing PAGE and autoradiography, a strong protection on both the coding and noncoding strands could be observed in reaction mixtures that contained CREB protein (Fig. 1A, lanes 2 and 3, and B, lane 2). This protection was located between nucleotides −77 and −56 on the coding strand and between nucleotides −78 and −57 on the noncoding strand of the UL112/113 promoter covering the CREB/ATF-binding site homology (Fig. 1C and D). To analyze whether the binding of CREB to the site located within the UL112/113 promoter is sequence specific, binding competition was investigated in DNase I protection experiments. CREB protein was preincubated either with 100 ng of an oligonucleotide corresponding in sequence to the CRE of the gonadotropin gene (CREg) or with an unrelated oligonucleotide (CRSm). As a probe, the *Hin*dIII-*Eco*RI fragment of plasmid pHM143 that was labeled at the lower strand was added to the reaction mix. After DNase I digestion, size fractionation, and autoradiography, no protection was visible when CREB protein was preincubated with the gonadotropin gene CRE (Fig. 2, CREg). The nonspecific oligonucleotide did not compete for binding of CREB to the UL112/113 promoter (Fig. 2, CRSm). Thus, CREB interacts in a sequence-specific manner with the identified binding site of the UL112/113 promoter.

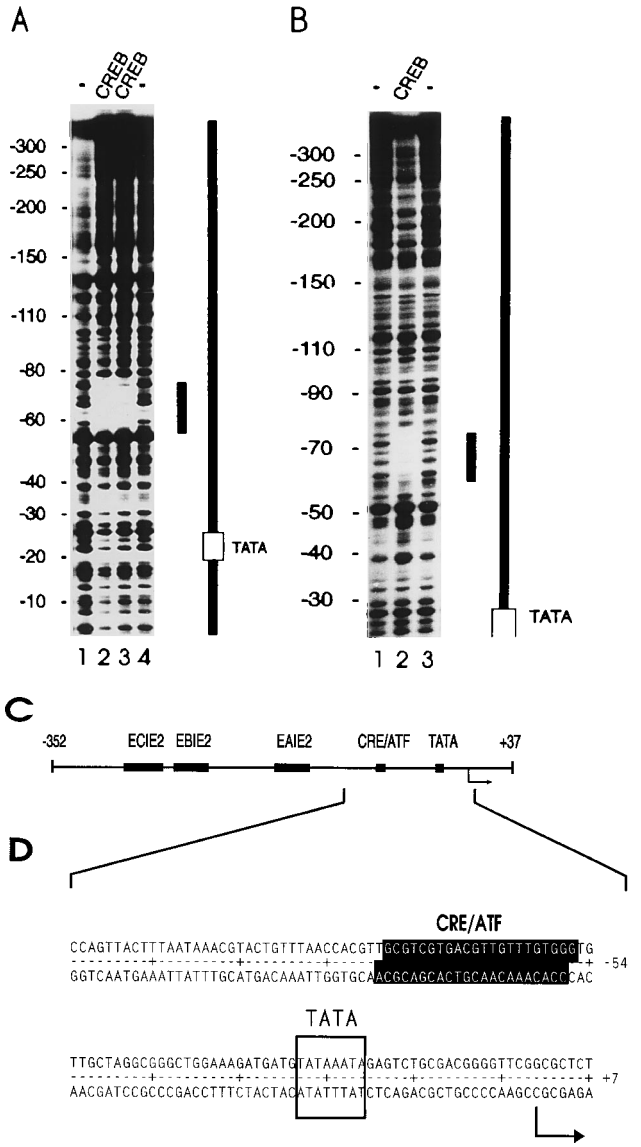

FIG. 1. DNase I protection analysis of the UL112/113 promoter region with the recombinant CREB protein. (A) The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 (promoter sequences from nucleotides −352 to +37) that was labeled at the 5′ end of the upper strand was used as the probe. Lanes: 1 and 4, no added extract; 2 and 3, 5 μl of affinity-purified CREB 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 3, no added extract; 2, 5 μl of affinity-purified CREB added. (A and B) Numbers on the left refer to nucleotide positions of the UL112/113 promoter. Diagrams of the end-labeled DNA fragments are shown on the right. TATA, TATA box sequence. Filled bars on the right indicate regions that were protected by CREB. (C) Diagram of the UL112/113 promoter region between nucleotides −352 and +37. Relative positions of IE86-binding sites (EAIE2, EBIE2, and ECIE2), the identified CREB-binding site, and the TATA sequence are indicated by filled bars. (D) DNA sequence of the UL112/113 promoter region between nucleotides −113 and +7 (relative to the transcription start site). Sequences protected from DNase I cleavage by the recombinant CREB protein are marked by white letters on a black background. The TATA sequence is indicated by a box. The arrow indicates the transcriptional start site.

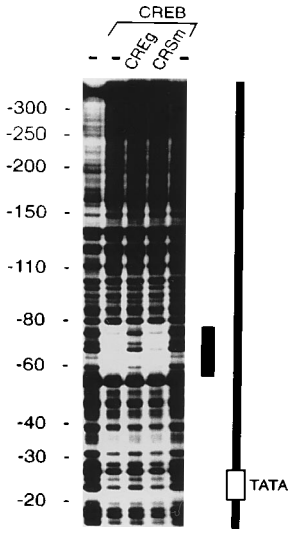

FIG. 2. Effects of specific competitor DNAs on the binding of recombinant CREB protein to the UL112/113 promoter in DNase I footprinting experiments. The *Hin*dIII-*Eco*RI fragment of plasmid pHM143 (promoter sequences from nucleotides −352 to +37) that was labeled on the lower strand was used as the probe. −, no competitor DNA was added; CREg, competition with 100 ng of the CRE element of the gonadotropin gene; CRSm, competition with 100 ng of an unrelated oligonucleotide. Lanes enclosed by a bracket contained 5 μl of affinity-purified CREB protein. Numbers on the left refer to nucleotide positions of the UL112 promoter. A diagram of the end-labeled DNA fragment and the protected region is shown on the right (for details, see the legend to Fig. 1).

The identified CREB-binding site is involved in IE86-mediated transactivation of the UL112/113 promoter. To investigate the functional importance of the identified CREB-binding site, in vitro mutagenesis was performed within the context of the entire UL112/113 promoter as contained within the luciferase reporter plasmid pHM142 (Fig. 3). This resulted in the exchange of two nucleotides within the CRE consensus sequence which abolished binding of CREB in DNase I protection experiments (data not shown). Permissive U373 cells were then cotransfected with either the wild-type luciferase plasmid pHM142 or the mutated plasmid pHM223 together with an IE86 expression plasmid. Fig. 3 summarizes the results of three independent cotransfection experiments that were performed for each plasmid. It shows that mutation of the CRE sequence leads to a significant reduction in IE86-mediated transactivation of the UL112/113 promoter (Fig. 3, bars 1 to 4). To further corroborate this, oligonucleotides which corresponded in sequence to either the genuine or the mutated CRE of the UL112/113 promoter were synthesized. These oligonucleotides were cloned as single copies and as multimers upstream of the UL112/113 TATA box. The resulting constructs were then used in cotransfection experiments with the IE86 expression vector (Fig. 3, bars 5 to 14). As reported earlier, the UL112/113 TATA box alone was sufficient to mediate a low level of transactivation (Fig. 3, bars 5 and 6) (2); however, after insertion of the genuine CRE-element (Fig. 3A, ECRE) upstream of the TATA box, there was a significantly higher level of transactivation compared with that for the TATA-containing plasmid pHM214 (Fig. 3, bars 7 and 8). This could not be observed if plasmid pHM254 containing the mutated CRE (Fig. 3, ECREm) was used (Fig. 3, bars 9 and 10). A trimer of the CRE was nearly as effective in mediating transactivation as the entire UL112/113 promoter, whereas multimerization of the mutated CRE did not result in a higher activity (Fig. 3, bars 11 to 14). This shows that the CREB-binding site within the UL112/113 promoter is an important target for the IE86 transactivator protein.

IE86 interacts directly with the cellular transcription factor CREB in vitro. After we had detected that the CREB-binding site of the UL112/113 promoter contributes significantly to transactivation, we were interested in elucidating the responsible mechanism. As protein-protein interactions are increasingly recognized as a means by which viral and cellular regu-

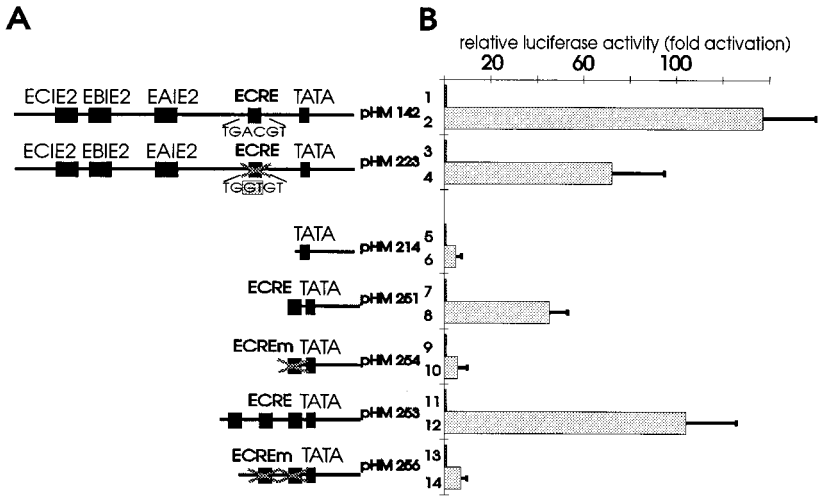

FIG. 3. Effects of various luciferase expression constructs containing either the genuine or mutated CREB-binding site of the UL112/113 promoter in mediating transactivation by IE86. (A) The UL112/113 promoter sequences contained within each luciferase expression plasmid are indicated on the left. Relative positions of IE86-binding sites (EAIE2, EBIE2, and ECIE2), genuine and mutated CREB-binding sites (ECRE and ECREm, respectively), and the TATA sequence are indicated by filled bars. Names of the luciferase constructs are on the right. (B) Schematic diagram of activation values obtained after cotransfection of various luciferase expression 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 the 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 pHM223; 5 and 6, plasmid pHM214; 7 and 8, plasmid pHM251; 9 and 10, plasmid pHM254; 11 and 12, plasmid pHM253; 13 and 14, plasmid pHM256. For bars 1, 3, 5, 7, 9, 11, and 13, cotransfection was performed with plasmid pIC20HRSV. For bars 2, 4, 6, 8, 10, 12, and 14, cotransfection was performed with the IE86 expression plasmid pHM137. Results are from at least three independent experiments; standard deviations are indicated by error bars.

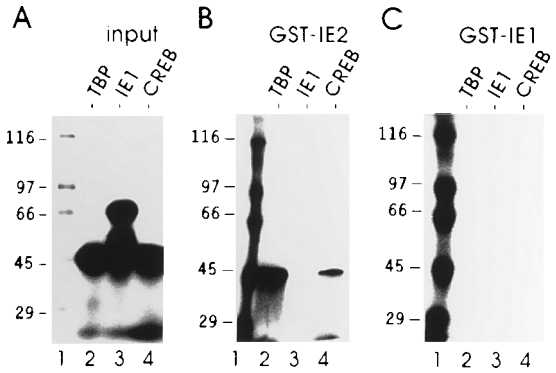

FIG. 4. IE86 physically interacts with CREB in a pull-down assay. In vitro translated ^{35}S-labeled TBP (lanes 2), IE1 (lanes 3), and CREB (lanes 4) proteins were used for pull-down assays. (A) SDS-PAGE of input proteins. (B) SDS-PAGE of proteins after incubation with a GST-IE2 fusion protein. (C) SDS-PAGE of proteins after incubation with a GST-IE1 fusion protein. Molecular mass markers are shown in lanes 1 and refer to proteins of 116, 97, 66, 45, and 29 kDa, as indicated on the left of each panel.

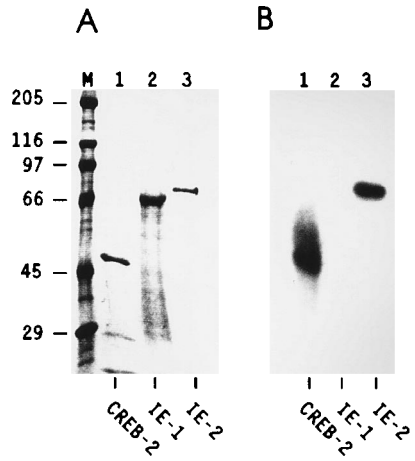

FIG. 5. CREB and IE86 interact directly in a far Western blot. (A) Affinity-purified CREB, IE1, and IE2 proteins were separated by SDS–12.5% PAGE. (B) After it was blotted onto a nitrocellulose membrane, the filter was incubated with ^{32}P-labeled CREB protein and washed, and then radioactively labeled bands were identified by autoradiography. Lanes 1, CREB protein; 2, IE1 protein; 3, IE2 protein. The molecular mass markers shown in lane M refer to proteins of 205, 116, 97, 66, 45, and 29 kDa.

latory proteins stimulate gene expression, we asked whether IE86 could interact directly with the CREB transcription factor. To answer this, GST fusion pull-down assays were performed. Transcription factor CREB, the TATA-binding protein TBP, and the HCMV transactivator IE72 (IE1) were expressed and radiolabeled in reticulocyte lysates (Fig. 4A). These proteins were then incubated with either a bacterially expressed GST-IE2 or a GST-IE1 fusion in a pull-down assay. As previously reported, IE86 was able to interact strongly with the TATA-binding protein TBP (Fig. 4B, lane 2) (14). In addition, we could also observe an interaction between IE86 and CREB, which was not as strong as the interaction of IE86 with TBP but could easily and reproducibly be detected (Fig. 4B, lane 4). In contrast, no binding could be observed when the radiolabeled IE72 protein was used together with GST-IE86 or when the GST-IE1 protein was incubated together with the three radiolabeled proteins (Fig. 4B, lane 3, and C). This argues against a nonspecific interaction of IE86 with CREB.

As proteins present in reticulocyte lysates might influence the detected interaction between CREB and IE86, we wanted to confirm our result by performing a far Western blot experiment. Procaryotically expressed and purified CREB, IE72, and IE86 proteins were separated by SDS-PAGE (Fig. 5A), blotted onto nitrocellulose, and probed with bacterially expressed CREB protein that had been labeled with ^{32}P by using protein kinase A. As expected, CREB gave a strong signal with the blotted CREB protein, due to dimerization via the leucine zipper domain (Fig. 5B, lane 1). There was also a strong signal corresponding to the procaryotically expressed IE86 protein, whereas IE72 did not react at all. This finally shows that IE86 can interact directly with CREB in in vitro binding assays.

To delineate the regions of IE86 involved in CREB interactions, a set of N- and C-terminal deletion mutants was constructed within the procaryotic expression vector pQE10. After purification of procaryotically expressed IE86 proteins, a far Western blot analysis, using radiolabeled CREB as a probe, was done (Fig. 6). As shown in Fig. 6B, lanes 1 to 4, deletion of N-terminal amino acids led to a gradual reduction in CREB binding; however, a mutant comprising aa 293 to 579 was still able to interact weakly. Deletion of 39 aa from the carboxy terminus did not result in a detectable loss of binding affinity (Fig. 6B, lane 6); a further deletion of 130 aa, however, weakened the interaction drastically (Fig. 6B, lane 7). A mutant comprising aa 290 to 548 of IE86 still showed a detectable interaction, whereas a further N-terminal deletion resulted in a total loss of binding (Fig. 6B, lanes 8 and 9, and C, lanes 8 and 9). Thus, a minimal binding domain seems to be located between aa 290 and 410 of IE86; however, N- and C-terminal sequences appear to contribute significantly to the affinity for binding. Alternatively, as described for the interaction of IE86 with TBP and Rb, additional independent binding domains may be located within the respective regions (44).

IE86 interacts with CREB in vivo. Although the in vitro interaction experiments strongly argued for a direct contact between IE86 and CREB proteins, we sought to confirm this within the context of a cell. In order to circumvent the problem of endogenous CREB protein, a CREB cDNA was fused to the DNA-binding domain of the *Saccharomyces cerevisiae* factor GAL4. This approach has been previously used to demonstrate an in vivo interaction between E1A and specific ATF factors (30). HeLa cells were then cotransfected with various GAL4 fusions either in the absence or in the presence of an IE86 expression vector or an IE72 expression vector, which served as a negative control. For the reporter, we used the β-globin gene with or without five GAL4 binding sites upstream of a minimal, TATA box-containing β-globin promoter (43). In addition, a reference plasmid was transfected, which gives rise to a truncated β-globin transcript and allows for normalization of transfection variations (56). After isolation of RNA and quantitative S1 nuclease analysis, the result shown in Fig. 7 could be observed. Cotransfection of the unfused GAL4 DNA-binding domain with the IE86 expression vector resulted in a low level of activation which, however, was not dependent on the presence of GAL4 binding sites within the reporter gene (Fig. 7A, lanes 3 and 4, and B, lanes 3 and 4). This effect is most probably due to IE86-mediated activation of the β-globin promoter via interactions with factors of the basal transcription machinery. When a vector encoding a GAL4-CREB fusion was used in combination with IE86, a very strong signal could be detected after S1 nuclease analysis which was not present when GAL4-CREB was transfected alone (Fig. 7A, lanes 6 and 7). This was dependent on the presence of GAL4 binding sites, as there was only weak activation in the experiment with the reporter gene without GAL4 binding sites (Fig. 7B, lanes 6 and 7). This activation was specific, as it could not be observed

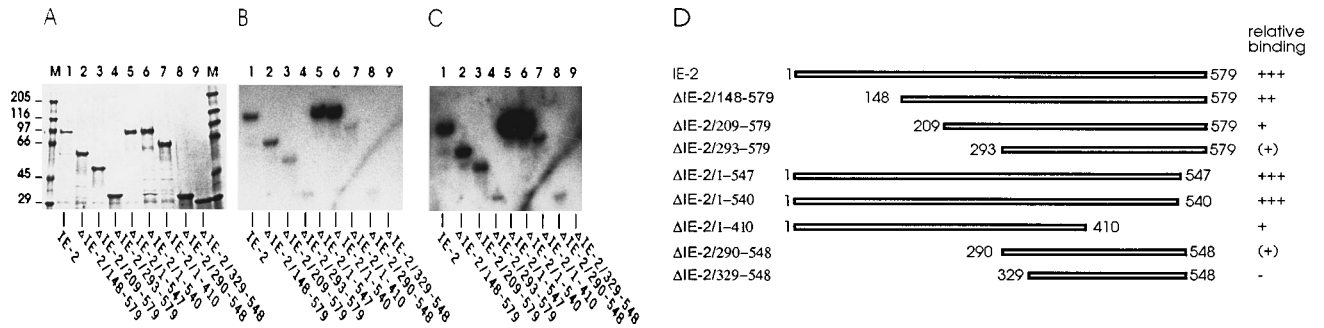

FIG. 6. Delineation of a minimal CREB-binding domain within IE86. (A) Affinity-purified IE86 proteins (full-length IE86 and various N- and/or C-terminal deletion mutants) were separated by SDS–12.5% PAGE and visualized by Coomassie blue staining. (B) An identical gel was blotted onto a nitrocellulose membrane, and the filter was incubated with ^{32}P-labeled CREB protein, washed, and then autoradiographed. (C) Longer exposure of panel B. Lanes 1, full-length IE86 protein; lanes 2 to 9 contain the indicated IE86 deletion mutants. The molecular mass markers shown in lanes M refer to proteins of 205, 116, 97, 66, 45, and 29 kDa. (D) Schematic drawing of regions contained within each IE86 deletion mutant together with the observed relative CREB binding affinity. Numbering indicates the positions of these regions in the amino acid sequence of IE86.

when a CREB expression vector was used instead of a GAL4-CREB expression vector and when IE72 was cotransfected instead of IE86 (Fig. 7A, lanes 8 to 10). In addition, an unrelated GAL4 fusion protein (GALUL69) was also not able to mediate a comparable effect (Fig. 7A, lanes 11 to 13). On the basis of these results, we conclude that the HCMV IE86 protein is able to interact with the cellular transcription factor CREB in vivo.

DISCUSSION

The 86-kDa IE2 protein (IE86, pUL122a) of HCMV is the most important transactivator of viral early promoters. In this study, we investigate the mechanism used by IE86 to activate early gene expression. We chose the early promoter driving expression from the UL112/113 gene region of HCMV as a target (48). This gene region, which encodes four phosphoproteins, is required for transient complementation of HCMV *ori*Lyt-dependent DNA replication, suggesting an important function in either the regulation of viral DNA replication or gene expression (37, 59).

The UL112/113 promoter has previously been shown to be highly responsive upon cotransfection of IE86 expression plasmids and also upon the addition of purified IE86 protein to in vitro transcription systems (24). In recent studies, direct DNA-binding sites for IE86 could be detected within this promoter, suggesting one possible mechanism for transactivation (2, 42). However, it turned out that deletion of these binding sites diminished transactivation but did not abrogate it; a promoter without direct DNA-binding sites could still be transactivated significantly (2). This indicated that, in addition, cellular transcription factor-binding sites are required for efficient stimulation of this promoter by IE86.

In an effort to localize *cis*-acting elements within the UL112/113 regulatory region, a binding site for the cellular transcription factor CREB was identified by performing DNase I protection experiments. This site was located between nucleotides −78 and −56 upstream of the TATA box in a part of the promoter which was required for high IE86 responsiveness in deletion analyses (2, 42). Site-directed mutagenesis of this sequence resulted in a marked reduction in the level of transactivation, showing that this element is of importance. Moreover, an isolated CREB motif could confer strong IE86 responsiveness upon a minimal UL112/113 promoter, stressing the relevance of this sequence element. However, we assume that additional *cis*-acting sites within the sequence between nucleotides −117 and −32 of the UL112/113 promoter are involved in mediating an IE86 response, as a deletion of IE2-binding sites from the construct containing the mutated CREB motif did not totally abrogate transactivation (46). Nevertheless, this clearly shows that, in addition to the basal transcription apparatus, defined binding sites for cellular transcription factors are necessary for a high level of IE86 transactivation. This is consistent with a previous publication by Lukac and colleagues showing that multiple interactions with the promoter complex including factors binding to upstream regulatory sites are crucial for strong IE86-mediated promoter stimulation (31). However, in contrast to their findings, we could observe that the TATA box alone was sufficient for a low-level response to IE86, which confirms earlier observations by Hagemeier et al. (14). In addition, we find that an ATF/CREB site can confer strong IE86 transactivation, whereas Lukac et al. reported only about threefold effects for this *cis*-acting element. This maybe due to the fact that they used nonpermissive CV1 cells for cotransfection experiments, whereas we used permissive U373 cells. In agreement with this, transactivation levels of the UL112/113 promoter have been shown to be highly dependent on the cell type used for transfection experiments (9, 24).

The CRE consensus sequence as contained within the UL112/113 promoter can act as a target for several cellular proteins. The ATF/CREB family of transcription factors recognizing this motif comprises a large number of polypeptides which all have nearly indistinguishable DNA binding properties but differ in their transcriptional effects (15, 16). For instance, the CREB protein is regulated by the cyclic AMP (cAMP)-dependent protein kinase PKA, which phosphorylates CREB at a single phosphoacceptor site and thereby activates this factor, whereas ATF2 has been implicated in mediating transcriptional activation by the adenovirus E1A protein (12, 30). Up to now, we have been able to demonstrate a direct interaction with one distinct member of this transcription factor family, termed CREB-A or ΔCREB (3, 60). This factor arises via alternative splicing of the CREB gene transcript and lacks a 14-aa sequence, the so-called α-region, which appears to be critical for transcriptional activation by PKA in certain cell types (60). Although CREB-A is 10-fold less active after PKA phosphorylation, it is about 5-fold more abundant than CREB protein, suggesting an important function in transcription regulation (60). Interaction of CREB-A with IE86 was demonstrated by both pull-down assays and far Western blot analysis. Most importantly, however, we were able to confirm the results of in vitro interaction studies by using a GAL4-

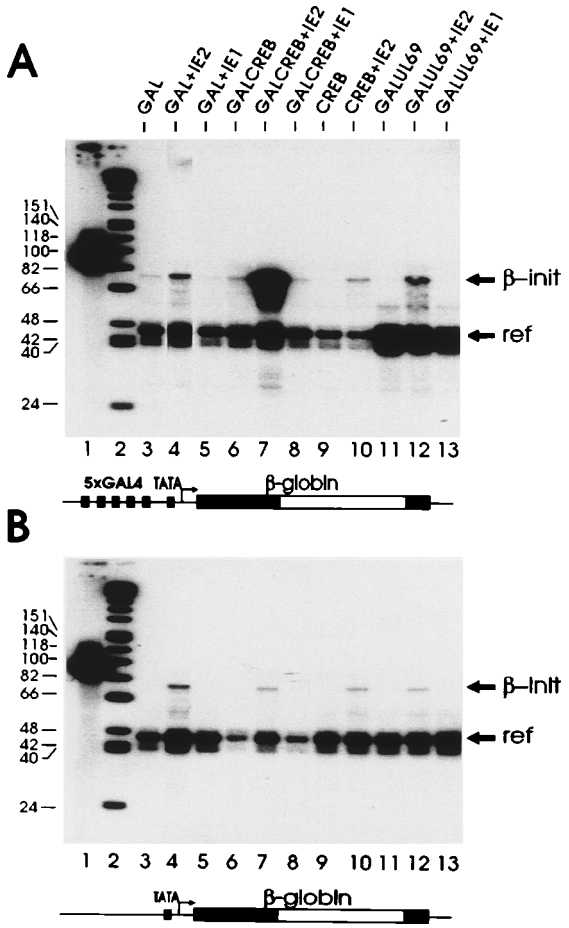

FIG. 7. CREB interacts with IE86 in vivo. S1 nuclease analyses of cotransfection experiments with various GAL4 fusions in either the absence or the presence of IE72 or IE86 expression plasmids. (A) A β-globin gene containing five GAL4 binding sites upstream of the β-globin TATA box was used as a reporter in cotransfection experiments. (B) A β-globin gene without GAL4 binding sites was used as a reporter in cotransfection experiments. β-init, the correctly initiated RNA from the reporter genes; ref, the reference signal from OVEC-REF, which was cotransfected as a control for transfection variation. Lanes 1, S1 nuclease probe; 2, molecular mass markers (sizes [in nucleotides] are indicated on the left), 3 to 5, transfection with a vector expressing the GAL4 DNA-binding domain; 6 to 8, transfection with a vector expressing a GAL4-CREB fusion protein; 9 and 10, transfection with a CREB expression vector; 11 to 13, transfection with a vector expressing a GAL4-UL69 fusion protein. In lanes 3, 6, 9, and 11, cotransfection was performed with the cloning vector pBS+ as a negative control. In lanes 4, 7, 10, and 12, cotransfection was performed with the IE86 expression vector pHM134. In lanes 5, 8, and 13, cotransfection was performed with the IE72 expression vector pHM135.

CREB fusion protein, proving that there is also a direct contact between CREB-A and IE86 in vivo. This favors a model whereby the activation domain of IE86 is positioned near the TATA box via tethering to promoter-bound CREB. Whether other members of the ATF/CREB family also associate with IE86 remains to be determined. However, it is tentative to speculate that the interaction might be specific for only distinct members of this family. As the expression pattern of ATF/CREB factors differs between cell types, this could explain why transactivation levels of the UL112/113 promoter vary depending on the cell type used for cotransfection experiments (9, 24).

Thus, IE86 appears to use at least one specific ATF/CREB factor in order to mediate transactivation of an HCMV early promoter. As several other early promoters of HCMV such as the recently described UL94, UL95, and UL98 regulatory regions contain consensus sequences for ATF/CREB factors, one might hypothesize that this *cis* element is one common target whereby IE86 switches from IE to early gene expression (57). In addition to consensus sequences within early promoters, HCMV contains cAMP response elements within the major IE enhancer-promoter which are functional and mediate a strong cAMP stimulation of enhancer activities in certain cell types (20, 47). Thus, HCMV appears to make extensive use of integral parts of the cAMP pathway which, however, is not specific for this virus. For instance, the adenoviral E1A protein specifically interacts with the ATF2 factor and has recently been shown to contact the adaptor protein CBP, which is associated with and coactivates the transcription factor CREB (1, 8, 30, 32). The latter interaction results in an E1A-mediated repression of cAMP-dependent transcription and may thereby modulate the growth-inhibitory and differentiation-inducing effects of cAMP (1, 32). In light of these observations, we think that it will be of great biological importance to further elucidate interactions of the HCMV IE86 protein with this family of transcriptional regulators.

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