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The Human Cytomegalovirus IE2 86-Kilodalton Protein Interacts with an Early Gene Promoter via Site-Specific DNA Binding and Protein-Protein Associations

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The 86-kDa immediate-early 2 protein (IE2 86) of human cytomegalovirus is a powerful transactivator of homologous and heterologous promoters, including the human cytomegalovirus 1.2-kb RNA early promoter. Two potential mechanisms for gene activation by IE2 86 include interaction with cellular proteins and direct DNA binding. In this report, we show that the 1.2-kb RNA promoter contains a *cis*-acting AP-1 site, critical for its activation by IE2 86 in vivo, and that IE2 86, purified as a glutathione *S*-transferase–IE86 fusion protein, can interact with c-Jun and JunB. Additionally, by coimmunoprecipitation, we document that JunB and IE2 86 do associate in vivo. Further in vitro analysis reveals that Fos proteins are able to associate with glutathione *S*-transferase–IE86 only when present as a Jun-Fos heterodimer. With a set of IE2 86 mutants, we demonstrate that three independent regions of the IE2 86 interact in vitro with c-Jun, two of which are essential for activation of the 1.2-kb RNA promoter in vivo. We also show that IE2 86 can bind directly to this promoter through a sequence located just upstream of the AP-1 site between nucleotides −125 and −97. This discrete domain shares sequence homology with the *cis*-repression signal on the IE gene.

Human cytomegalovirus (HCMV) causes a variety of diseases in newborns and immunocompromised individuals because of its opportunistic nature (1, 26). Similar to other herpesviruses, HCMV expresses its genes in three temporal classes: immediate early (IE), early, and late (8, 16, 25, 40). The transcription of early genes is dependent on the expression of the IE genes, which are transcribed immediately after infection without de novo protein synthesis. Viral DNA replication then follows and leads to the induction of late gene expression.

The HCMV major IE genes are transcribed from two genetic units termed IE1 and IE2 (25, 35, 37, 40, 42). The IE1 region transcribes a 1.95-kb mRNA which encodes a 72-kDa protein (IE1 72) (35), while the IE2 region gives rise via differential splicing to two mRNAs: 2.25-kb and 1.7-kb (36), which encode proteins of 86 and 55 kDa, respectively (designated IE2 86 and IE2 55). Although the exact role of IE2 55 is still unknown, IE2 86 is an important transactivator of HCMV early promoters, as well as heterologous promoters (7, 10, 15, 18, 19, 22, 28, 33, 43). This stimulation by IE2 86 can be enhanced by IE1 72, which also functions as an activator of the major IE promoter (5, 24).

Since IE2 86 can activate various viral and cellular promoters, one mechanism by which this protein may function is by binding a specific target sequence on the promoter. Support for this idea was first provided by the demonstration that IE2 86 can down-regulate its own expression (14, 28), by binding to its own promoter at a *cis*-acting repression element (CRS) near the start site of transcription (4, 6, 20, 21, 23, 27). Recently, we and others have reported that IE2 86, a potent activator of the HCMV 2.2-kb RNA early promoter (19, 33), can bind specific upstream sequences of this promoter which share sequence similarity to the CRS (2, 30).

A second mechanism by which IE2 86 could transactivate a variety of heterologous and homologous promoters is through interaction with cellular regulatory and transcription factors. Evidence for protein-protein interactions was first presented in studies showing that IE2 86 could form a complex with a component of the TFIID complex, the TATA box-binding protein (TBP) (3, 9, 13, 17, 31). Further studies have revealed that IE2 86 also binds to other cellular proteins including TFIIB, SP1, Tef-1, and the retinoblastoma gene product, RB (12, 22, 31, 32).

The focus of this report is on the mechanisms of activation of the promoter for the HCMV 1.2-kb early RNA which is encoded by *Eco*RI fragment O within the terminal repeat bounding the long unique segment of strain AD169. Previously, we demonstrated that stimulation of this promoter by the HCMV infection was dependent on an AP-1 site located 75 bp upstream from the start site of transcription (39). Since preliminary experiments showed that this promoter was also activated by the IE2 86 protein (38), we were interested in determining whether the IE2 86 protein could interact with any members of the AP-1 family of transcription factors. We show here that the AP-1 site is critical for activation by IE2 86 and that both c-Jun and JunB can directly bind IE2 86. Although c-Fos, FosB, Fra-1, and Fra-2 were unable to bind independently to IE2 86, they could be found in the complex with IE2 86 following dimerization with either c-Jun or JunB. Mutational analysis of IE2 86 revealed that three distinct domains of this protein can bind to c-Jun, two of which are important for the in vivo activation of the 1.2-kb RNA promoter. We also found that the IE2 86 protein can bind directly to a sequence located just upstream from the AP-1 site which shows striking similarity to the CRS motif.

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MATERIALS AND METHODS

Molecular cloning. The individual cDNAs coding for the IE1 72-, IE2 86-, and IE2 55-kDa proteins (34) were obtained from R. Stenberg (Eastern Virginia Medical School) and cloned into the glutathione *S*-transferase (GST) fusion vector pGEX-KG (11) and the eukaryotic expression vector pSG5 (Stratagene) as previously described (19). Deletions of the IE2 86 coding region were generated as described by Sommer et al. (31).

In vitro-translated proteins. In vitro transcription-translation reactions were carried out with the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Vectors containing the coding region for JunB (p465.20), Fra-1 (RSV-Fra1), and Fra-2 (RSV-Fra2) were provided by M. Karin (University of California, San Diego), and those for c-Jun (pGEM-Jun), c-Fos (T7 Fos), and FosB (SK-FosB) were a gift from I. Verma (Salk Institute). For binding studies involving Jun-Fos interaction, c-Jun and JunB were each cotranslated with c-Fos and Fra-1 and radiolabeled with [^{35}S]methionine. However, because Fra-2, FosB, c-Jun, and JunB have similar molecular weights, it was necessary to use a different strategy for experiments involving these latter Jun-Fos heterodimers. The protocol we developed involved adding FosB or Fra-2 protein, already labeled with [^{35}S]methionine in a separate TNT reaction, to a TNT mixture (lacking [^{35}S]methionine) containing c-Jun or JunB cDNA. In this way, the resulting unlabeled Jun protein could bind to the labeled Fos protein before Jun homodimers formed. We verified that the Jun protein did not become labeled in these reactions with a control experiment using c-Fos and JunB, which could easily be separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. When ^{35}S-labeled c-Fos (made in a separate TNT reaction) was added to a TNT reaction (lacking [^{35}S]methionine) with c-*jun* cDNA and the resulting protein products were subjected to SDS-PAGE, only the c-Fos protein was radiolabeled (data not shown).

Binding assays. Expression and purification of GST fusion proteins were carried out as formerly described (19). Briefly, the immobilized GST fusion proteins were resuspended in NETN buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% Nonidet P-40, 100 mM NaCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium metabisulfite) and then in vitro-translated ^{35}S-labeled protein was added. For competition assays, the labeled proteins were first mixed and then added to a saturable amount of GST-IE86. Following incubation for 1 h at room temperature (RT) with constant rotation, the samples were divided into two portions, one of which was not treated further and represents the input fraction. The complexes in the remaining portion were pelleted at 14,000 × *g* and washed five times in NETN buffer, resuspended in SDS-PAGE sample buffer, and boiled for 5 min. Proteins were resolved on SDS-polyacrylamide gels and exposed by fluorography on Kodak X-Omat film. The gels were quantitated with a Molecular Dynamics PhosphorImager.

Transient expression assays. Human U373MG astrocytoma-glioblastoma cells (a gift from R. LaFemina, Merck, Sharpe and Dohme), which are fully permissive for the HCMV infection, were grown as previously described (19). For the IE2 86 deletion analysis, flasks containing 1.5×10^6 cells were transfected with 4 μg of the HCMV 1.2-kb RNA early promoter-chloramphenicol acetyltransferase (CAT) construct p456-OCAT (39) and 2 μg of the IE constructs contained in pSG5. For the analysis of *cis*-acting regulatory sequence on the 1.2-kb RNA promoter, 2.5×10^6 cells were transfected with 4 μg of pSGIE86 and 5 μg of the CAT constructs containing various deletions of this promoter (39). DEAE transfection, harvesting, and CAT assays were carried out as previously described (19). Duplicate flasks were transfected for some experimental conditions, and all conditions were repeated independently at least three times.

DNA labeling for binding assays. Plasmids used, containing the full length and various truncations of the 1.2-kb RNA promoter, have been described previously (39), with the exception of p197-OCAT, which was created in the same manner as p328-OCAT. It should be noted that p162-OCAT has been renamed p163-OCAT to account for an additional upstream base pair which was missed previously. Purified fragments of the 1.2-kb RNA promoter cleaved with *Sst*I and *Hin*dIII were radiolabeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dXTP (dXTP = all dinucleoside triphosphates are labeled [ICN Radiochemicals]).

DNA binding assays. For each DNA fragment tested, approximately 6.5 μg of GST-IE 86 immobilized on glutathione agarose beads (19) was incubated in binding buffer with constant rotation for 30 min at RT with 50,000 cpm of the indicated ^{32}P-labeled DNA fragment and 20 μg of dAdT. The DNA binding buffer contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 300 mM KCl, 20% glycerol, 0.1% Nonidet P-40, 10 μM $ZnSO_4$, 0.2 mM EDTA, 1 mM dithiothreitol, and protease inhibitors as previously noted (30). After incubation, the beads and supernatant were counted in a scintillation counter and represent total counts per minute. The supernatant was then removed, and the beads were washed twice with binding buffer and counted; this represents bound counts per minute. Results of specific binding are expressed as percent bound counts per minute divided by total counts per minute. All of the constructs were tested in at least three independent experiments with GST control reactions. The CRS oligonucleotide has been previously described (30).

DNase I footprinting. To generate the probe representing the 1.2-kb RNA promoter, the p241-OCAT plasmid (39) was cut with *Hin*dIII, treated with calf intestinal phosphatase, end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and then cut with *Sst*I and purified from a 5% polyacrylamide gel. This probe was then used in the above DNA binding assay after which the DNA-protein complexes were eluted from the beads as formerly described (30). The DNA-protein complexes were then digested with DNase I and further treated as previously specified (29).

Coimmunoprecipitation and immunoblotting. COS cells were maintained in medium as previously described and transfected by the DEAE-dextran method (41), with 3 μg of pSGIE86 and 7.5 μg of the plasmid coding for JunB (SRα3-3XHA-TB, provided by M. Karin). For coimmunoprecipitations, 3×10^6 transfected COS cells were lysed by sonication on ice in NETN (0.1% Nonidet P-40, 1 mM EDTA, 20 mM Tris [pH 8.0], 50 mM NaCl) containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, and 1 mM benzamidine. Lysates were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C. Immunoprecipitations were carried out for 4 h at 4°C with 4 μg of polyclonal rabbit anti-JunB antibody (Ab) (Santa Cruz Biotechnology), 4 μg of rabbit anti-mouse Ab (Cappel), or 12 μg of rabbit anti-IE2 Ab, IE2.4 1218, a gift from J. Nelson (Oregon Health Science University) (15). Immune complexes were immobilized with protein A-Sepharose beads (Pharmacia) and washed four times with NETN. Precipitated proteins were separated by SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore). Immunoblotting was performed by standard procedures, and proteins were detected with primary Abs anti-JunB and monoclonal mouse anti-IE72/IE86 CH160 (a gift from L. Pereira, University of California, San Francisco), horseradish peroxidase-linked secondary Abs (GIBCO and Amersham), and enhanced chemiluminescence reagents (Amersham).

RESULTS

An AP-1 site is critical for activation of the 1.2-kb RNA promoter by IE2 86. Preliminary studies in our laboratory revealed that IE2 86 activates the 1.2-kb RNA promoter, although viral infection produces 5- to 10-fold higher relative CAT activity values compared with IE2 86 alone (38, 39). Since we demonstrated earlier that the AP-1 site is important for transactivation of the 1.2-kb RNA promoter by the viral infection (39), we examined the role of the AP-1 site in activation by IE2 86. Using transient transfection assays with the 1.2-kb RNA promoter-CAT reporter construct, p456-OCAT, and the expression vector for IE2 86, pSGIE86, we documented that the full-length promoter was strongly activated by IE2 86 (Fig. 1). To confirm that IE2 86 was responsible for the promoter activation, we performed control transfections with the p456-OCAT plasmid alone. Only background levels of CAT activity were detected (see bottom of Fig. 1). When the promoter with a double mutation in the AP-1 site, p456-OCAT/AP-1dm, was used in these assays, activation by IE2 86 was negligible, indicating that the AP-1 site is critical for activation of the 1.2-kb RNA promoter by IE2 86. To further delineate other regions necessary for activation by IE2 86, additional 5′-deletion mutants of the 1.2-kb RNA promoter were analyzed in transient transfection assays. Activation by IE2 86 was moderately affected when the promoter was deleted to −83, but when the AP-1 site was removed, activation dropped 10-fold compared with the full-length promoter. Activation was then reduced to background levels when there was further 5′ deletion of the region downstream of the AP-1 site (−68 to −41). This latter reduction in promoter activity may be due to a putative *ets* binding domain that lies in this region. However, because we have not yet documented by mutational analysis whether this site has in vivo significance in the context of the full promoter, and because mutation of the AP-1 site reduced activation by IE2 86 to background levels, we have focused in this report on the AP-1 site.

c-Jun and JunB, but none of the Fos family proteins, bind IE2 86 independently. Having shown that IE2 86 activates the 1.2-kb RNA promoter through its AP-1 site, we characterized the ability of various Jun and Fos proteins to bind independently to IE2 86. In this first series of experiments, IE fusion proteins containing GST were immobilized on glutathione agarose beads and then incubated with [^{35}S]methionine-labeled in vitro-synthesized proteins. Following incubation, the protein

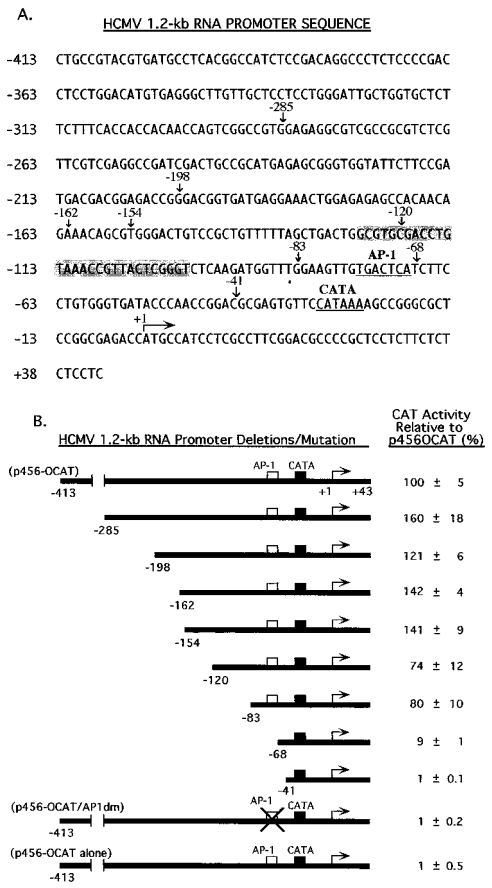

FIG. 1. Sequence and deletional analysis of the HCMV 1.2-kb RNA early promoter. (A) DNA sequence of the 1.2-kb RNA promoter region present in p456-OCAT. The nucleotide numbering relative to the RNA start site is shown on the left. The start site is indicated as +1. The 5′ endpoints of the deletion mutants are indicated by vertical arrows above the sequence. The site marked AP-1 binds proteins of this complex, and the CATA site is suspected to function as the binding site for the general transcription factor TFIID. The domain with similarity to the CRS motif (−120 to −107) is included within the shaded IE2 86 binding region. (B) Schematic representation of the deletion constructs and the *cis* sites present in each. The double mutation in the AP-1 site, p456-OCAT/AP1dm, has the AP-1 site changed from TGACTCA to TTACTTA. CAT activity of the AP-1 double mutant and promoter deletion plasmids is shown relative to that of p456-OCAT (defined as 100%). Activity of the 1.2-kb RNA promoter in the absence of IE2 86 is shown as p456-OCAT alone. All reactions were run with amounts of extract such that the values of CAT activity were in the linear range. Promoter-CAT plasmids were cotransfected with pSGIE86 into U373MG cells by the DEAE-dextran method.

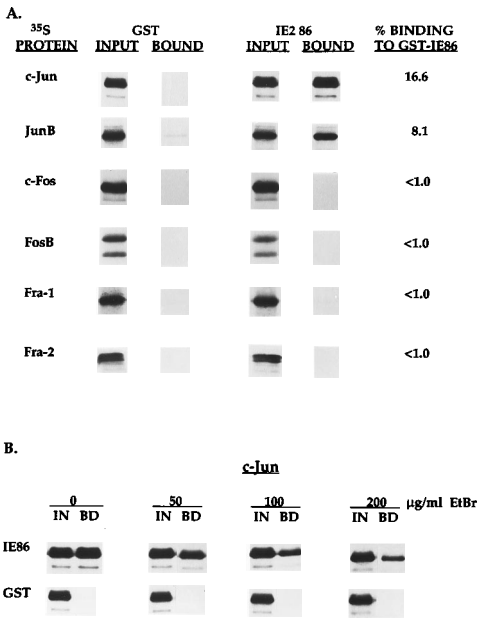

FIG. 2. Binding assays of Jun and Fos for IE2 86. (A) AP-1 proteins were independently labeled in vitro with [^{35}S]methionine and added to GST or GST-IE86 fusion protein immobilized on glutathione-agarose beads. Following incubation for 1 h at RT, an aliquot, representing 5 to 20% of the initial total protein, was removed and represents the input fraction. The remaining beads were split into duplicate aliquots, each representing 40% of the initial total protein, and were washed in NETN. The proteins in the input and bound fractions were resolved by SDS-PAGE, and the gels were quantitated with a Molecular Dynamics PhosphorImager. The percent binding to GST-IE86 represents the amount of protein remaining bound after washing compared with the total protein added initially. (B) Immobilized GST or GST-IE86 fusion proteins were incubated on ice in the presence of the indicated concentration of EtBr for 30 min. ^{35}S-labeled c-Jun was added to the beads, incubated for 1 h at RT, washed in NETN containing the appropriate amount of EtBr, and then resolved and quantitated as described above. IN, input; BD, bound.

complexes were washed extensively, and the bound material was analyzed by SDS-PAGE. These assays showed that c-Jun and JunB, but none of the Fos proteins, were able to bind independently to IE2 86 (Fig. 2A). Relative to our previous studies on the interaction of IE2 86 with TBP (31), it appears that c-Jun binds to IE2 86 with a slightly lower affinity. As a negative control, each ^{35}S-labeled protein was incubated with GST protein alone. None of these proteins bound to the control GST (Fig. 2A) or to IE1 72 (data not shown). To eliminate the possibility that contaminating DNA from the bacterial lysates was mediating the interaction between the Jun proteins and IE2 86, we repeated the binding assays in the presence of ethidium bromide (EtBr) (Fig. 2B). In the presence of 50 μg of EtBr per ml, there was less than a twofold decrease in binding of c-Jun to GST-IE86, and c-Jun continued to bind IE2 86 even in the presence of 200 μg of EtBr per ml. Thus, it appears that the c-Jun association with IE2 86 is not DNA dependent, although DNA may facilitate this interaction to a small degree.

Coimmunoprecipitation of IE2 86 and JunB. On the basis of the above experiments, which showed that Jun proteins can bind IE2 86 in vitro, we proceeded to determine whether this interaction also could be formed in vivo. For these experiments, COS cells were transfected with expression vectors for JunB, IE2 86, both, or neither (mock). Lysates were incubated with anti-JunB Ab, anti-IE2 86 Ab (1218), or rabbit anti-mouse Ab (RαM), as a control for nonspecific binding to rabbit Ab. The immunoprecipitated proteins were separated by SDS-PAGE and then analyzed by Western blot (immunoblot) with Abs to both JunB and IE2 86 (Fig. 3). Lane 5 shows the total lysate from cells cotransfected with both vectors as a control for protein expression. The results demonstrated that anti-IE2 86 Ab (1218) but not anti-JunB Ab could specifically immunoprecipitate IE2 86 from lysates of cells transfected with the expression vector for IE2 86 alone (compare lanes 1 and 2). In contrast, with lysates from cells cotransfected with the expression vectors for both JunB and IE2 86, the anti-JunB Ab was able to specifically immunoprecipitate IE2 86 (lane 4). It should be noted that JunB migrates to approximately the same position as the denatured immunoglobulin G (IgG) and consequently is obscured by the IgG in lane 4. Also, the apparent lower mobility of IE2 86 in lanes 2 and 4 is due to the large amount of IgG in those samples. As a key negative control, we showed that the rabbit anti-mouse Ab (RαM) did not immu-

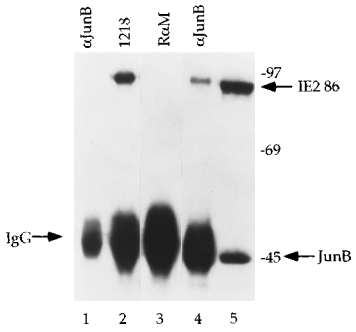

FIG. 3. Coimmunoprecipitation of JunB and IE2 86. Lanes 1 and 2 represent lysates, from COS cells transfected with pSGIE86, subjected to immunoprecipitation with anti-JunB (lane 1) or anti-IE2 IE2.4 1218 Ab (lane 2). Lanes 3 and 4 represent lysates from cells cotransfected with the expression vectors for IE2 86 and JunB, which were immunoprecipitated with rabbit anti-mouse (lane 3) or anti-JunB (lane 4) Ab. Lane 5 is cell extract from cotransfected cells to confirm the protein expression of IE2 86 and JunB. JunB runs at approximately 45 kDa, which overlaps with the size of the denatured IgG. Consequently, JunB is obscured by the IgG in lane 4. Proteins were resolved by SDS-PAGE and analyzed by simultaneous anti-IE86 and anti-JunB immunoblotting. Lanes 1 and 2 are from one blot, while lanes 3, 4, and 5 are from a second blot. Therefore, lane 2 is present as a positive control for the presence of IE2 86 in the lysate used for lane 1.

noprecipitate IE2 86 from lysates of the cells cotransfected with both expression vectors (lane 3), thus documenting that the coimmunoprecipitation of IE2 86 by anti-JunB Ab is not due to nonspecific binding of IE2 86 to rabbit Ab. The fact that the anti-JunB Ab did not independently associate with IE2 86 but did immunoprecipitate IE2 86 in the presence of JunB indicates that JunB is able to form a complex with IE2 86 in vivo.

Fos proteins associate with IE2 86 via a Jun-Fos heterodimer. Since c-Jun and JunB can bind IE2 86 directly, we tested whether a Jun-Fos heterodimeric complex could bind IE2 86 without disrupting the Jun-Fos interaction. For these experiments, c-Jun or JunB was cotranslated in vitro with c-Fos or Fra-1 in the presence of [^{35}S]methionine to yield a total of four different reactions, each containing a different ^{35}S-labeled heterodimer. Four additional heterodimers were formed in a different fashion because their similar molecular weights would prevent distinguishing Jun and Fos binding to IE2 86 if both Fos and Jun proteins were labeled. As described in Materials and Methods, we added ^{35}S-labeled FosB or Fra-2 to an unlabeled in vitro transcription-translation mix containing either c-Jun or JunB cDNA. In this way, four different Jun-Fos heterodimers in which only the Fos protein is labeled are created. Each heterodimer was independently incubated with GST-IE86 as described in Materials and Methods. Figure 4 shows that the Fos protein remains bound to the IE2 86 complex for all eight of the heterodimers tested. Moreover, for the first four heterodimers for which the Jun component was also labeled, it is clear that Jun also remains bound to the IE2 86 complex. None of the heterodimers bound to the GST control or to IE1 72 (data not shown). Since the Fos proteins cannot independently bind IE2 86, their association with IE2 86 is most likely due to their dimerization with c-Jun or JunB.

Binding of c-Jun to IE2 86 mutants. To determine which regions of IE2 86 are vital for c-Jun interaction, various mutants of GST-IE86 containing either internal deletions, carboxy-terminal truncation, or both were tested with in vitro-translated c-Jun in the same type of binding experiment as that described above. Figure 5 shows that the interaction of c-Jun involves the same three regions of IE2 86 that we previously showed to be required for TBP and RB association (31). These three regions, which span amino acids (aa) 85 to 364, are depicted in the figure as differentially shaded boxes. To completely abolish c-Jun binding, all three regions (aa 85 to 135, 136 to 290, and 291 to 364), as in mutants IE86TM85 and IE86ΔMN, need to be removed. On the other hand, the carboxy-terminal region of GST-IE86 is not necessary for c-Jun interaction since a deletion up to aa 364, IE86TM364, did not negatively affect c-Jun binding. In addition, the IE2 86 mutants containing only one of the three regions, as with IE86TM138, IE86ΔHS/XN, and IE86ΔMX, were still able to complex with c-Jun, thus indicating that each of these three regions can independently bind c-Jun. Nevertheless, the ability of each region to bind c-Jun does vary. The region spanning aa 291 to 364 is the strongest binding domain and maintains binding levels that are 85% compared with wild-type IE2 86. The other two regions, aa 136 to 290 and 85 to 135, are weaker binding domains since their bindings are reduced 2.3- and 5-fold, respectively, relative to full-length IE2 86.

c-Jun-binding regions of the IE2 86 are involved in transcriptional regulation of the 1.2-kb RNA promoter. To determine the role of the different domains of IE2 86, which bind c-Jun, in the transactivation of the 1.2-kb RNA promoter, transient transfection assays were performed with the IE2 86 deletion mutants and with the HCMV 1.2-kb RNA early promoter-CAT construct, p456-OCAT, as the reporter plasmid. As shown in Fig. 5, the domain of strong c-Jun binding contained within aa 290 to 368 is essential for transactivation of

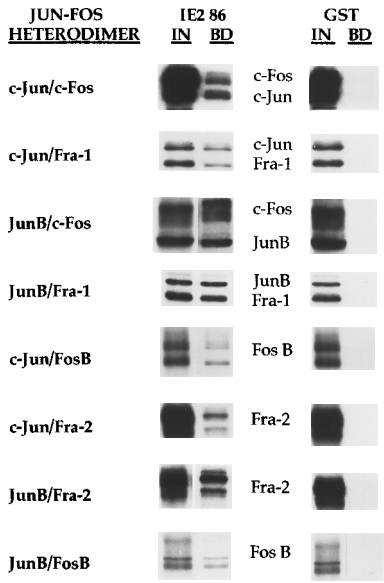

FIG. 4. Jun-Fos heterodimer binding to IE2 86. Each labeled heterodimer was subjected to the same type of binding assay as described for the binding of Jun to GST-IE86 and GST. The first four heterodimers shown were made by cotranslating the Jun and Fos proteins together so that both proteins were ^{35}S-labeled (c-Jun/c-Fos, c-Jun/Fra-1, JunB/c-Fos, and JunB/Fra-1). The last four heterodimers, because of their similar molecular weights, were made by adding already ^{35}S-labeled Fos protein to an unlabeled in vitro transcription-translation mix containing either c-Jun or JunB cDNA. Therefore, these four heterodimers, c-Jun/FosB, JunB/FosB, c-Jun/Fra-2, and JunB/Fra-2, have only the Fos protein labeled as indicated to the right of the input (IN) and bound (BD) lanes. Following incubation for 1 h at RT, an aliquot, representing 5 to 20% of the initial total protein, was removed and represents the input fraction. The remaining beads were split into duplicate aliquots, each representing 40% of the initial total protein, and were washed in NETN. It should be noted that there was some degradation of the FosB protein in the JunB/FosB heterodimer shown in this figure.

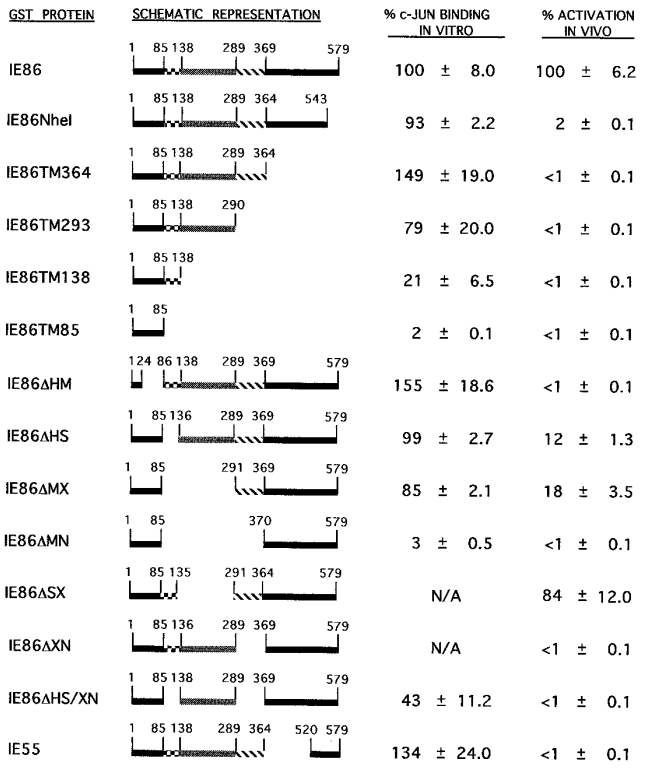

FIG. 5. Deletion analysis of the IE2 86 protein. ^{35}S-labeled c-Jun was added to the indicated immobilized GST-IE86 fusion proteins in the binding assay as described in Materials and Methods. The percent c-Jun binding in vitro is shown relative to that of full-length IE2 86 (defined as 100%). The wild-type IE2 86 and the indicated deletion mutants are illustrated with numbers representing the position of important amino acid residues. The three regions involved in c-Jun binding are shown as differentially shaded bars, and N/A indicates that the particular IE2 86 mutant was not tested. For percent in vivo activation of the 1.2-kb RNA promoter, values are relative to that of full-length IE2 86 (defined as 100%). U373MG cells were cotransfected with the indicated IE protein and the p456-OCAT vector which contains the 1.2-kb RNA promoter driving the expression of the CAT gene.

the 1.2-kb RNA promoter since specific deletion of this region, IE86ΔXN, decreased activation to background levels. In addition, the domain which served as a weak binding domain in vitro, aa 85 to 135, has a significant role in vivo as evidenced by an eightfold drop in activation when this region is deleted (IE86ΔHS). As shown previously by our laboratory (31) and repeated with the above experiment as a control for plasmid integrity (data not shown), IE86ΔHS was able to fully transactivate another HCMV early promoter, the 2.2-kb RNA promoter, thereby demonstrating that the aa 85 to 135 region is specifically required for the transactivation of the 1.2-kb RNA promoter. The third binding domain (aa 136 to 290) proved to have little effect on the ability of IE2 86 to transactivate the 1.2-kb RNA promoter since the deletion of this region (IE86ΔSX) caused only a small drop in activation levels. Furthermore, when this region was removed in addition to the weak binding region (IE86ΔMX), activation levels did not further decrease but remained similar to those with just the weak binding region removed (IE86ΔHS). Besides the c-Jun binding domains, the amino terminus (IE86ΔHM) and carboxy terminus (IE86NheI and IE55) are also necessary for transactivation because removal of either of these regions reduces activation to background levels.

IE2 86 binds to a specific site in the 1.2-kb RNA promoter. A second possible mechanism by which IE2 86 may be regulating the expression of the 1.2-kb RNA promoter is through direct binding to the DNA. We and others have shown previously that IE2 86 binds to discrete domains on the 2.2-kb RNA promoter (2, 30) which share sequence homology with the 14-nucleotide (nt) CRS element (CGTTTAGTGAACCG) located near the cap site of the major IE promoter (4, 6, 20, 21, 23, 27). Analysis of the DNA sequence of the 1.2-kb RNA promoter revealed several sites which had loose similarity to the 14-nt CRS motif and were framed by CG residues at both ends. However, only one of those sites, starting at −120 nt relative to the transcription start site, was significantly AT rich internal to the CG ends and closely matched the other IE2 86 DNA binding sites (Fig. 1A). Therefore, we tested whether IE2 86 could bind to the 1.2-kb RNA promoter via any of the sites with similarity to the CRS motif. The in vitro experiment involved incubating immobilized GST-IE86 with various fragment lengths of the 1.2-kb RNA promoter end labeled with ^{32}P. Specific binding of IE2 86 to the promoter was evaluated by retention of the DNA on the immobilized protein after incubation and washing. Figure 6 shows that IE2 86 bound the varying fragments of the 1.2-kb RNA promoter until the minimal site for binding, starting at −120, was eliminated. Further 5′-end deletions after this region, −83, −68, and −41, reduced binding to background levels. The binding of IE2 86 to −120 is specific for that domain and is not just randomly associating with DNA binding sites for major transactivators, since IE2 86 failed to bind the −83 fragment, which still contains the AP-1 binding site. Additionally, these assays were done in the presence of dAdT as a nonspecific competitor which had no effect on binding and with a GST control reaction in which all labeled fragments were negative for binding (data not shown). The protein-DNA-binding assays were also performed with dIdC and a CRS oligonucleotide as competitors. Both dIdC and the CRS oligonucleotide effectively blocked the binding of GST-IE86 to all the fragments (data not shown).

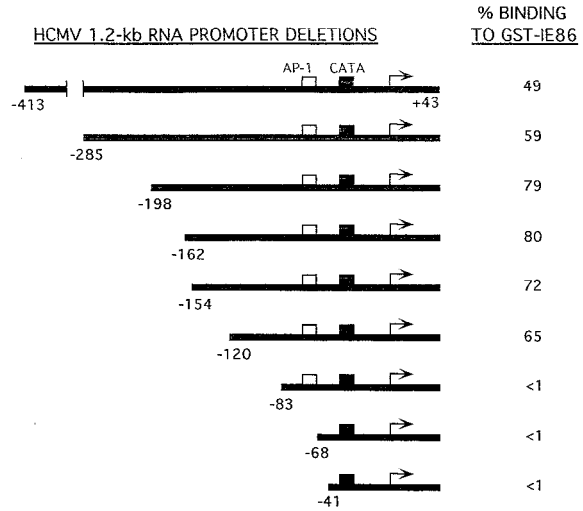

FIG. 6. IE2 86 binding to the 1.2-kb RNA promoter. Immobilized GST-IE86 was incubated at RT for 30 min with the indicated fragment length of the 1.2-kb RNA promoter end labeled with ^{32}P. The percent binding to GST-IE86 was evaluated by retention of the labeled DNA on the immobilized protein after incubation and washing, compared with total label added. Nucleotide numbers relative to the transcription start site are shown on the left side of each fragment drawing, and the AP-1 and CATA sites are indicated in the fragments containing them.

To further define the sequence on the 1.2-kb RNA promoter which binds to IE2 86, we performed DNase I protection analysis with the −198 fragment of the promoter as the probe. Figure 7 shows that GST-IE86 strongly protects the region from nt −125 to −97. Some protection is also seen for the region from nt −62 to −37. However, since the −83 fragment showed no binding to IE2 86, the weak protection of the region from nt −62 to −37 may be due either to changes in the secondary structure of the DNA or to some IE2 86 protein binding to that site as a direct result of upstream IE2 86 binding at −125 to −97.

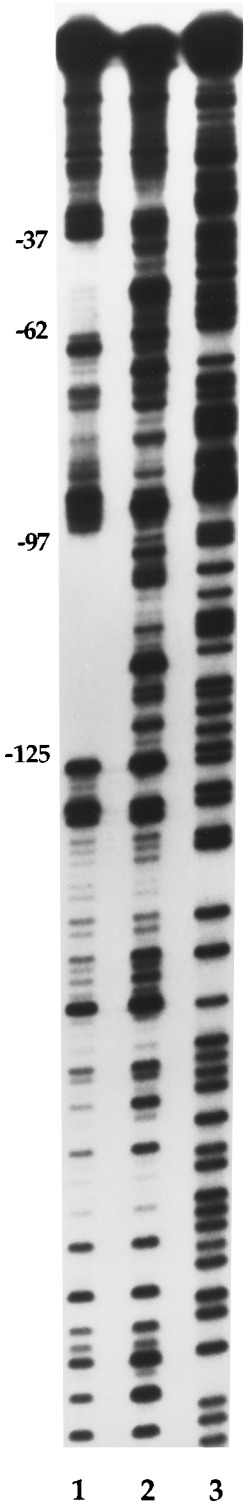

FIG. 7. DNase I protection analysis of IE2 86. The 1.2-kb RNA promoter DNA was bound to immobilized GST-IE86 fusion protein, eluted, and treated with DNase I as described in Materials and Methods. Lane 1 shows the DNA probe in the presence of IE2 86, lane 2 shows the DNA probe in the absence of IE2 86, and lane 3 represents the G+A reference ladder. Nucleotides bounding the regions of protection are shown on the left.

DISCUSSION

Using transient expression assays, we and others have demonstrated that HCMV early promoters as well as heterologous viral and cellular promoters can be activated by the HCMV IE2 86 protein (7, 10, 15, 18, 19, 22, 28, 33, 43). In addition, this protein can also repress its own promoter (14, 28). Our primary focus involves examining the mechanism(s) by which the IE2 86 functions. Can it interact directly with DNA, or does it regulate gene expression by associating with host cellular factors? Recent reports from several laboratories document that the IE2 86 has the potential to employ both types of mechanisms. The IE2 86 protein has been shown to interact with components of the basal transcription complex, TBP and TFIIB, as well as SP1, Tef-1, and the product of the retinoblastoma gene, RB (3, 9, 12, 13, 17, 22, 31, 32). IE2 86 binds to a region between the TATA box and the cap site of its own promoter, presumably as a means of down-regulating its own expression (4, 6, 20, 21, 23, 27). Furthermore, Arlt et al. and our laboratory have recently found that the IE2 86 protein can bind to a region upstream of the TATA box of the HCMV 2.2-kb RNA early promoter (2, 30).

In this study, we have examined the mechanisms by which IE2 86 activates the 1.2-kb RNA early promoter. Previously, we used a series of 5′ deletion constructs, gel retardation analysis, and site-directed mutagenesis to demonstrate the prominent role of the AP-1 binding site for the induction of this promoter at early times in the HCMV infection (39). Using these same mutants, we now show that the AP-1 site is essential for activation by IE2 86 in transient expression assays. On the basis of these results and studies which showed that IE2 86 could interact with other regulatory proteins, we examined whether the association of IE2 86 with members of the AP-1 family could be one mechanism of regulating the 1.2-kb RNA promoter.

For our binding studies, we used the GST-IE86 fusion protein which previously has been shown to be fully functional in in vitro transcription assays (19). The results of these experiments revealed that both c-Jun and JunB were able to associate with IE2 86 but that none of the Fos proteins, c-Fos, FosB, Fra-1, and Fra-2, could independently bind to IE2 86. However, all of the Fos proteins were able to associate with IE2 86 when they were present as a Jun-Fos heterodimer. This binding is not mediated by the GST portion of the GST-IE86 fusion protein since GST alone does not bind any of the proteins tested in these experiments. Additionally, the fact that the addition of EtBr to the binding assay does not significantly affect the observed binding indicates that the binding is not due to the presence of contaminating DNA.

To examine whether the IE2 86-Jun interaction could be maintained in vivo, we coimmunoprecipitated JunB with IE2 86 by using cell lysates from transfected COS cells. Anti-JunB Ab immunoprecipitated IE2 86 only in the presence of JunB, demonstrating that IE2 86 was not associating with this Ab

nonspecifically and that there was an association of JunB and IE2 86 in vivo. As an additional control, we showed that rabbit anti-mouse Ab was unable to immunoprecipitate IE2 86 when coexpressed with JunB. Thus, nonspecific interaction of the complex with rabbit Ab itself is not responsible for the coimmunoprecipitation results with the rabbit anti-JunB Ab.

To define which regions within IE2 86 were responsible for binding c-Jun, a series of carboxy-terminal and internal deletion mutants of IE2 86 were used in a binding assay with c-Jun. The interaction of IE2 86 with c-Jun is mediated by the same three regions that we previously reported to be important for TBP and RB binding (31), and each of the three regions (aa 85 to 135, 136 to 290, and 291 to 364) can independently bind c-Jun in vitro. The functional significance of these regions for activation of the 1.2-kb RNA promoter was then tested in transient expression assays. We previously reported that the region on IE2 86 from aa 291 to 364 is essential for the transcriptional activation of the 2.2-kb RNA promoter (31), and likewise this region was also found to be necessary for stimulation of the 1.2-kb RNA promoter. Additionally, the region from aa 136 to 290, which was a strong Jun-binding region in vitro, was not by itself essential for activation of the 2.2-kb (31) or 1.2-kb RNA promoter in vivo. However, when the region from aa 85 to 135 was removed (IE86ΔHS), the activation of the 1.2-kb RNA promoter dropped precipitously, quite unlike the 2.2-kb RNA promoter, whose activation was unaffected by the removal of this region (31). The 1.2-kb RNA promoter differs from the 2.2-kb RNA promoter in that it has an AP-1 site which is critical for its activation. Consequently, the differential activation obtained with the IE86ΔHS mutant may be due to preferential binding of AP-1-activating proteins, such as c-Jun, to this region of IE2 86 under in vivo conditions when multiple proteins may be vying for binding to IE2 86. However, domains other than those that interact with c-Jun are required for activation of the 1.2-kb RNA promoter, because deletions near the amino or carboxy terminus of IE2 86 also destroy its stimulatory function.

In view of the evidence that IE2 86 can bind to specific DNA sequences on its own promoter as well as the promoter for another HCMV early gene (2, 4, 6, 20, 21, 23, 27, 30), we also explored whether IE2 86 may be regulating the gene expression of the 1.2-kb RNA promoter via direct interaction with the promoter DNA. Deletion analysis showed that GST-IE86 bound the promoter DNA until a region upstream of the AP-1 site was deleted, with the fragment containing −120 nt upstream of the transcription start site remaining bound to IE2 86 and fragment −83, which still contains the AP-1 site, and further deletions thereafter unable to bind. These results demonstrated that a specific sequence framed by nt −120 and −84 was minimally required for binding to IE2 86. Control experiments with the GST protein alone ruled out the possibility that the IE2 86 interaction with DNA was due to the GST portion of the fusion protein. In addition, the promoter DNA failed to bind GST-IE1 72 fusion protein, which shares 85 aa at its amino terminus with IE2 86.

On the basis of previous results, it seemed likely that IE2 86 was binding to the 1.2-kb RNA promoter DNA at a site that shared sequence homology with the CRS element and the binding sites that had been identified on the 2.2-kb RNA promoter. The major binding regions of the 2.2-kb RNA promoter and the CRS element all have invariant CG residues at both ends of a consensus 14-nt sequence that is AT rich internally (2, 20, 30). Although the 1.2-kb RNA promoter contains several 14-nt sequences framed by CG residues, only one, which starts at nt −120, has internal homology to the other IE2 86 binding sites. Confirmation of the binding of IE2 86 to this domain on the 1.2-kb RNA promoter was provided by DNase I footprint analysis. We found that the IE2 86 protein strongly protected the region located between nt −125 and −97. The length of the protected sequence, which includes a region 4 to 5 nt upstream and 10 to 12 nt downstream of the critical 14-nt domain, correlates well with results previously obtained with the 2.2-kb RNA promoter and the CRS element (2, 20, 30). Interestingly, in the transient expression assays, we found a twofold drop in activation by IE2 86 when we deleted the region of the promoter between −154 and −120, which contains part of this larger sequence protected by the IE2 86 protein as determined by DNase I footprinting. We also noted a weak protection site from nt −62 to −37 which shares loose homology to the CRS motif, albeit missing one of the critical CG ends. However, since the −83 promoter fragment failed to bind to GST-IE86 in vitro, it is possible that the strong upstream binding of IE2 86 (−125 to −97) is responsible for this weak protection either by affecting the secondary structure of the DNA or by promoting some binding of IE2 86 to the −62 to −37 region. The functional importance of this weakly protected region remains to be determined. By 5′ deletion analysis, we did note a further drop in activation of the promoter by IE2 86 in transient expression assays when deletion of the promoter was extended from −68 to −41. However, an answer to the question of whether the putative weak IE2 86 binding site or a potential *ets* protein binding site, which also is in this region, has in vivo significance in the context of the entire promoter will require further mutational analysis.

In support of the hypothesis that IE2 86 may activate HCMV early promoters via two mechanisms, protein association and promoter binding, there appear to be both common and unique domains on the protein for these two functions. Previously, we showed that the binding of IE2 86 to the 2.2-kb RNA promoter required the entire carboxy-terminal half of the protein (aa 290 to 579) (30), which is the same region necessary for binding to the CRS on the IE promoter (6). This domain overlaps with one of the three regions of IE2 86 (aa 291 to 364), which binds c-Jun, as well as TBP and RB (31), and is important for activation of HCMV early promoters in transient expression assays. A key question is how the functions of these DNA and protein binding sites are coordinated in vivo. Perhaps the presence of multiple binding domains allows IE2 86 to interact simultaneously with DNA and with one or more proteins. The binding of IE2 86 to a site on the promoter proximal to a transcription factor binding site may remodel the DNA template and enhance the probability of attracting the factor to its cognate site. With three protein binding domains, IE2 86 also has the potential of forming multimeric complexes whereby several regulatory proteins are brought into close contact with one another. In this way, IE2 86 may also serve as a link between proteins which bind to distant sites on the DNA.

In summary, we have shown that IE2 86 can bind JunB and c-Jun in vitro, and we have characterized the regions of IE2 86 that are involved in these interactions. The IE2 86-kDa protein can also associate with JunB in vivo. Furthermore, we have defined the regions of IE2 86 necessary for activation of the 1.2-kb RNA promoter and the sequence within this promoter where IE2 86 directly binds. We conclude that IE2 86 has the potential to activate HCMV early gene expression via protein-protein associations as well as direct interaction with the promoter.

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