Transactivation by the Human Cytomegalovirus IE2 86-Kilodalton Protein Requires a Domain That Binds to both the TATA Box-Binding Protein and the Retinoblastoma Protein

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The human cytomegalovirus major immediate-early (IE) proteins play an indispensable role in regulating viral gene expression. One of these gene products, the IE2 86-kDa protein (IE2 86), is a potent activator of both homologous and heterologous promoters and can form a complex with a component of the basal transcription apparatus, the TATA box-binding protein (TBP). In this report, we show that when IE2 86 is expressed as a glutathione *S*-transferase (GST)–IE2 86 fusion protein, there are three independent regions that can interact with TBP and with another important cellular regulatory protein, the retinoblastoma gene product (RB). One of these three regions, as well as a domain at the carboxy terminus, contain consensus sites for casein kinase phosphorylation and negatively regulate binding of in vitro-translated IE2 86 to GST-TBP or GST-RB. The dimerization domain of IE2 86 must be present for the interaction of the in vitro-translated protein with GST-TBP and GST-RB. Analysis of IE2 86 mutants in vivo demonstrates that one of the strong binding regions is required for the protein to function as a transactivator. Our results also indicate that domains other than those that interact with TBP and RB are required for the activation function of this protein.

Temporal and quantitative control of gene expression plays a pivotal role in the life cycle of viruses. As is the case for other herpesviruses, the gene products of human cytomegalovirus (HCMV) may be divided into three major classes: immediate early (IE), early, and late (23, 39). The IE genes are expressed immediately after infection, and their expression is required for the transcription of the early genes. Following viral DNA replication, the late genes are transcribed and expressed.

The HCMV major IE genes are transcribed from two genetic units designated IE1 and IE2 (39). The IE1 region gives rise to a 1.95-kb mRNA that is translated to yield a 72-kDa protein (IE 72) (32). Two mRNA species, a 2.25-kb and a 1.7-kb mRNA, arise by differential splicing of the IE2 region (33). These RNAs encode proteins of 86 and 55 kDa (IE2 86 and IE2 55), respectively. IE2 86 regulates its own expression (10, 27) and is a potent transactivator of HCMV early promoters as well as heterologous promoters (27, 42). IE1 72 enhances the activity of the major IE promoter (3) and augments the activation by IE2 86 (22, 31). The exact role of IE2 55 is not yet known.

In view of the importance of IE2 86 in HCMV transcriptional regulation, it is likely that this protein interacts directly with cellular regulatory and transcription factors, as has been shown for other viral transactivating proteins such as adenovirus E1A (11, 18) and herpes simplex virus VP16 (19, 35). These interactions may be a major part of the mechanism by which IE2 86 can transactivate a variety of heterologous and homologous promoters. In support of this idea, a series of recent studies has shown that IE2 86 can form a complex in vitro and in vivo with a component of the TFIID complex, the TATA

box-binding protein (TBP), as well as with several cellular proteins of unknown identity (2, 5, 8, 12). An additional report has presented evidence that HCMV infection can activate transcription from E2F-dependent promoters and promote the formation of a complex containing E2F, cyclin A, p33^{cdk2}, and the p107 retinoblastoma-related protein (37). Whether IE2 86 binds directly to this complex remains to be determined.

In this report, we have analyzed the interactions of the HCMV IE proteins with TBP and the retinoblastoma protein (RB). Binding experiments were carried out in which in vitro-translated TBP and RB were bound to a set of glutathione *S*-transferase (GST)–IE2 86 (IE86) deletion mutants (forward experiments). In a series of reverse experiments, in vitro-translated IE proteins were bound to GST-RB and GST-TBP. We show that three distinct regions in the GST-IE86 fusion protein mediate interaction with in vitro-translated TBP and RB. In the reverse experiments, however, one of these binding regions is nonfunctional and a second region inhibits binding of IE2 86 to GST-TBP or GST-RB. Binding to GST-TBP and GST-RB in the reverse experiments also requires the dimerization domain of IE2 86 and is negatively affected by a domain at the extreme carboxy terminus. The third binding region, which mediates complex formation in both forward and reverse experiments, is essential for the activation function of IE2 86.

MATERIALS AND METHODS

Molecular cloning. The individual cDNAs corresponding to IE1 72, IE2 86, and IE2 55 (31) were obtained from R. Stenberg (Eastern Virginia Medical School) and were cloned into the GST fusion vector pGEX-KG (6) and the eukaryotic expression vector pSG5 (Stratagene) as previously described (14). Deletions of the IE2 86 coding region were generated by one of the following methods: (i) the DNA was digested with restriction endonucleases and religated, removing a portion of the internal coding region; (ii) an *NheI* linker, which contains

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stop codons in all three reading frames, was inserted at a convenient restriction endonuclease site; or (iii) an internal portion of the protein coding region was deleted by oligonucleotide-directed in vitro mutagenesis, using the Muta-Gene M13 kit (Bio-Rad). Deletions were verified by restriction enzyme analysis and/or DNA sequencing. The pGEX-IID vector was constructed by removing the coding region for TBP from the vector pGST-IID (obtained from A. Berk, University of California, Los Angeles) as a *Bam*HI fragment and inserting it into pGEX-KG. The pGEM-RB/*As*eI vector was constructed by removing the coding region for the truncated RB (deleted for the 384 amino-terminal amino acids [aa]) as a *Bam*HI fragment from pGSTRB-*As*eI/End (obtained from J. Y. J. Wang, University of California, San Diego) and inserting it into pGEM-3Z.

In vitro-translated proteins. In vitro transcription-translation reactions were carried out by using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Vectors containing the coding region for TBP (pTMhIID), RB (pBS-RB), and CREB (cyclic AMP-responsive element-binding protein (pT7-CREB) were obtained from A. Berk (University of California, Los Angeles), J. Y. J. Wang (University of California, San Diego), and I. Verma (Salk Institute), respectively. The in vitro-translated IE proteins were obtained from constructs in which the IE coding regions were cloned into the pSG5 vector.

Binding assays. Expression and purification of GST fusion proteins were carried out as previously described (14) except that the GST fusion protein-bead complexes were resuspended in NETN buffer (20 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0], 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium metabisulfate, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml) containing 100 mM NaCl, and the in vitro-translated protein was added. Following incubation for 1 h at room temperature 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 $12,000 \times g$ and washed five times in 1 ml of NETN buffer containing 100 mM NaCl, resuspended in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (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 (R. LaFemina, Merck, Sharpe and Dohme) were transfected with the HCMV early promoter-chloramphenicol acetyltransferase (CAT) construct p729-CAT (reporter) (30) and the IE constructs contained in pSG5 (effector). Transfection, harvesting, and CAT assays were carried out as previously described (14).

RESULTS

Specificity of binding. The data presented in this report are based on a series of binding experiments measuring protein-protein interactions. In these experiments, fusion proteins containing GST at their amino termini were expressed in bacteria, bound to glutathione-agarose beads, and then incubated in the presence of [35 S]methionine-labeled, in vitro-translated proteins. The complexes formed were then washed extensively, and bound proteins were visualized following SDS-PAGE and fluorography.

In the first set of experiments, we documented the specificity of the interactions of in vitro-translated TBP and RB with the

FIG. 1. Specificity of the interactions with the IE2 proteins. (A to C) The TBP, full-length and truncated RB, and CREB proteins were translated in vitro in the presence of [35 S]methionine. The radiolabeled protein was incubated with GST or GST-IE fusion protein immobilized on glutathione-agarose beads. Following incubation, an aliquot was removed and the remaining beads were washed extensively. The proteins in the input (IN) and bound (BD) fractions were resolved by SDS-PAGE. (D) GST or GST-IE86 fusion protein was bound to glutathione-agarose beads. The beads were then incubated in the presence of the indicated concentration of EtBr on ice for 30 min, after which radiolabeled TBP was added as described above.

IE fusion proteins. Figures 1A and B show that the full-length TBP and RB interact with IE2 86 and IE2 55 but not with IE1 72. This result is in agreement with the results of Hagemeyer et al. (8), who observed the specific interaction of TBP with IE2 86. To rule out the possible contribution of the GST portion of the fusion protein in binding, we showed that the GST protein alone does not bind TBP or RB. A truncated form of RB, which is missing the N-terminal 384 aa but retains the regions required for E1A and T-antigen binding (13), also binds tightly to IE2 86 (Fig. 1B). Neither CREB (Fig. 1C), Fos, nor luciferase binds to the IE2 proteins (data not shown). These results indicate that the interactions of TBP and RB with the IE2 proteins are specific in nature and not due to a general stickiness of the proteins.

Since the IE fusion proteins were isolated from bacterial lysates, it was possible that the presence of contaminating DNA was mediating the interaction of TBP with the IE2 proteins. To verify that the observed protein-protein interactions were DNA independent, we examined the stability of the complex in the presence of ethidium bromide (EtBr) as

FIG. 2. Regions involved in TBP and RB interactions. TBP or RB, labeled in vitro with [35 S]methionine, was added to the indicated GST-IE86 proteins immobilized on glutathione-agarose beads. Following incubation for 1 h at room temperature, an aliquot was removed and the remaining beads were washed in NETN buffer. The proteins in the input (IN) and bound (BD) fractions were resolved by SDS-PAGE. The wild-type IE2 86 (IE86) and the indicated deletion mutants are illustrated, with the numbers at the top representing the positions of important amino acid residues. The three regions involved in TBP and RB interactions are shown as differentially shaded lines. The gels were quantitated with a Molecular Dynamics PhosphorImager, and the numbers indicate the percent binding of each mutant relative to that for wild-type GST-IE86. For RB, only the area of the gel showing the full-length RB protein is shown.

previously described (16). Figure 1D shows that incubating the fusion protein, bound to glutathione-agarose beads and complexed with TBP, in the presence of increasing amounts of EtBr had no effect on the association of TBP and IE2 86. As a control, the beads-GST complexes also were incubated with EtBr. As expected, EtBr did not promote the interaction of TBP with the GST protein alone. These results indicate that the association of TBP and IE2 86 is DNA independent.

Three distinct regions on IE2 86 mediate TBP binding. To delineate the regions of IE2 86 involved in TBP interactions, a set of internal deletion, carboxy-terminal truncation, or internal deletion/carboxy-terminal truncation mutants was constructed. A representative sample, which most clearly illustrates the regions required for TBP binding, is shown in Fig. 2. When expressed as a GST-fusion protein, the carboxy-terminal region of IE2 86 (aa 365 to 579) is not required for TBP interactions, since a series of sequential deletions up to aa 364 did not significantly affect TBP binding. The three regions involved in TBP interactions span aa 85 to 364 and are indicated in Fig. 2 as differentially shaded boxes. The removal of all three regions (aa 85 to 135, 136 to 290, and 291 to 364), in mutants IE86TM85 and IE86ΔMN, is required to completely abrogate TBP binding. Mutants IE86TM138, IE86ΔMX, and IE86ΔHS/XN, which each contain a single

binding region, still retain the ability to bind TBP, thus indicating that each of the three regions can independently bind to TBP. However, the regions do differ in the strength of their binding to TBP. Two of the domains (aa 136 to 290 and aa 291 to 364) are strong binding regions, with the binding of the proteins containing only one domain comparable to that observed for the wild-type IE2 86. The interaction of TBP with the third region, between aa 85 and 135, appears to be weaker, since the binding of TBP to the IE86TM138 mutant is reduced fourfold relative to wild-type IE2 86. Since the mutants that contained an internal deletion of two binding sites (aa 86 to 290) and carboxy-terminal deletions up to aa 364 still bound TBP (mutants IE86ΔMX, IE86ΔMX401, and IE86ΔMX364), we were able to localize one of the strong binding domains to the region within aa 291 to 364.

In the binding experiments presented above, we noted that mutants IE86TM364, IE86ΔMX401, and IE86ΔMX364, as well as IE86ΔSX364 (Fig. 3), showed an increased level of binding to TBP compared with the full-length IE2 86. On the other hand, those mutants that terminate at aa 519 and 543 (IE86TM519 and IE86NheI) did not exhibit an increase in TBP binding (Fig. 4 and data not shown). This led us to speculate that the region between aa 365 and 519 may negatively influence the binding of TBP to GST-IE86. To test this

FIG. 3. Reverse binding assays reveal domains on the IE2 86 that negatively and positively influence complex formation with TBP and RB. Reverse binding assays were carried out as described for Fig. 2 except that GST-TBP was immobilized on glutathione-agarose beads prior to the addition of the indicated in vitro-translated, [³⁵S]methionine-labeled IE2 86. The results for the forward binding experiments are included for comparison. The gels were quantitated with a Molecular Dynamics PhosphorImager. For the binding of the IE2 86 to GST-TBP, the numbers represent binding relative to that observed for the IE86ΔSX mutant. For the binding of TBP to GST-IE, the numbers represent binding relative to that observed for wild-type GST-IE86.

hypothesis, we looked at the binding of the other IE2 gene product, IE2 55, to TBP. As discussed previously, the IE2 55 gene product differs from the IE2 86 gene product by a splicing event that removes the codons for aa 365 to 519. As shown in Fig. 2, IE2 55, when expressed as a GST-IE2 55 fusion protein, also binds increased levels of TBP compared with the full-length IE2 86. This fact, taken together with the results observed for the mutants that contain deletions in the carboxy half of IE2 86, suggests that the region between aa 365 and 519 may contain domains that negatively modulate the binding of TBP to the GST-IE2 proteins.

TBP binding regions of IE2 86 are also involved in RB interactions. HCMV infection stimulates DNA replication (34) and transcription of growth-related genes, such as that encoding dihydrofolate reductase (37). A recent study has suggested that this transcriptional stimulation may occur via the formation of an E2F-containing complex (37). The interaction of HCMV proteins with cellular regulatory proteins may therefore be one mechanism by which the virus stimulates cellular replication. Since one of the components of the E2F-containing complex is the RB gene product, a tumor suppressor protein that plays a role in cell cycle regulation (1), we were interested in determining whether IE2 86 interacts with RB.

Previous reports have shown that in vitro translation of the RB mRNA yields a nested set of N-terminal deletions which are the products of internal initiations within the RB coding

region (41). Rather than show the binding of the entire nested set of RB proteins in Fig. 2, we have chosen to show only the region of the gels that contain the full-length RB gene product. The results obtained for the binding of RB to the GST-IE fusion proteins are remarkably similar to those observed for TBP. Complete removal of the three regions, in mutants IE86TM85 and IE86ΔMN, is required to eliminate RB binding. The three regions can each bind RB independently, and the pattern of binding is identical to that for TBP: the two regions that were strong binding regions for TBP are also strong binding regions for RB. These results indicate that identical regions of IE2 86 are responsible for mediating the interactions with both TBP and RB. A similar increase in the level of binding to IE2 55 and the IE86TM364, IE86ΔMX401, IE86ΔMX364, and IE86ΔSX364 mutants, compared with the full-length IE2 86, was observed for RB as had been seen for TBP.

Interactions of IE2 86 with GST-TBP and GST-RB. The GST-IE fusion proteins used in the experiments described above were produced in bacteria and therefore would not have undergone the posttranslational modifications that could occur in eukaryotic cells. To determine if modification of the proteins might be important for the protein-protein interactions, we set up a series of reverse binding experiments. In these experiments, in vitro-translated, [³⁵S]methionine-labeled IE proteins were incubated with GST-TBP or GST-RB immobilized on glutathione-agarose beads. The vector expressing GST-RB

FIG. 4. One of the RB and TBP binding regions on IE2 86 is involved in transcriptional activation. U373MG cells were cotransfected with the indicated IE protein and the p729-CAT vector, which contains the HCMV UL112-113 promoter driving expression of the CAT gene. Cells were harvested after 48 h, and CAT activity was measured. The activity is given as percent activation by the mutant relative to the wild-type IE86 protein. The results of the in vitro TBP and RB binding assays, obtained as described for Fig. 2, are included as a reference and are presented as percent binding relative to wild-type IE2 86.

contains the coding region for the GST protein fused to the coding region for a truncated form of RB, lacking the amino terminal 384 codons. We have demonstrated that this truncated form of RB binds to GST-IE86 as efficiently as the full-length RB protein (Fig. 1B). Only the data for GST-TBP are shown, but the results obtained with GST-RB were identical.

As before, a representative sample of the IE86 mutants is shown in Fig. 3, and for comparison we present the data for both the forward (TBP binding to GST-IE) and reverse (IE binding to GST-TBP) binding experiments. There were several important differences in the results obtained from the two types of experiments. First, in contrast to the results in the forward binding experiments, the full-length IE2 86 (IE86) bound poorly to the GST-TBP and GST-RB fusion proteins. However, following removal of the region between aa 136 and 290 (IE86ΔSX), which was previously shown to be a strong binding domain in the forward reactions, the IE2 protein bound efficiently to GST-TBP and GST-RB. These results suggest that this region acts negatively to influence the interaction of IE2 86 with GST-TBP and GST-RB fusion proteins in the reverse reactions but can act independently as a strong binding domain in the forward reactions. A second region on IE2 86 that negatively affected the binding in the reverse experiments was located at the carboxy terminus. Deletions of the carboxy-terminal region, together with the removal of aa 136 to 290 (IE86ΔSX544 and IE86ΔSX519), led to approximately a sixfold increase in relative binding compared with the removal of the negative region alone in the reverse binding experiments. In the forward reactions, the IE86ΔSX544 and IE86ΔSX519 proteins bind TBP and RB to levels similar to that observed for the full-length protein. Another difference

between the forward and reverse binding experiments involved the weaker binding domain between aa 86 and 135. Removal of this region did not significantly affect the binding to GST-TBP and GST-RB (data not shown), and this domain by itself (IE86ΔSN) was unable to mediate binding to either protein in the reverse binding experiments, although it could bind TBP and RB in the forward binding experiments.

Of the three binding regions identified in the forward reactions, only the strong binding region found between aa 291 and 364 is important for binding in both experiments. Deletion of this domain, in the context of the protein missing the negative region (IE86ΔMN and IE86ΔSN), reduced binding to background levels in the reverse binding experiments. Although this domain is important for binding in both types of reactions, analysis of carboxy-terminal deletion mutants revealed an additional requirement for the region between aa 401 and 519 for binding in the reverse experiments. As shown in Fig. 3, mutants that contained a deletion of aa 136 to 290 and terminated at aa 544 and 519 (IE86ΔSX544 and IE86ΔSX519) bound efficiently to TBP or RB in both experiments; however, the mutant that terminated at aa 364 (IE86ΔSX364) bound TBP or RB only in the forward reactions. Other mutants that contained a deletion of aa 85 to 290 and terminated at aa 364 or 401 (IE86ΔMX364 and IE86ΔMX401) also formed a complex with TBP when they were expressed as GST fusion proteins but did not bind to GST-TBP or GST-RB in the reverse experiments. Possible explanations for these divergent results are addressed in the discussion.

RB and TBP binding regions are involved in transcriptional regulation. Our laboratory and others have shown that IE2 86 can activate gene expression from a variety of cellular and viral promoters (14, 22, 27). To evaluate the role of the various

domains within IE2 86 in transactivation, transient transfection assays were carried out with the deletion mutants. The reporter plasmid used was an HCMV early promoter-CAT construct that contains the promoter for the family of RNAs encoded within the *EcoRI* R and d fragments (30). We have previously shown that this promoter is significantly stimulated by IE2 86 both in vivo and in vitro (14).

As shown in Fig. 4, the region between aa 291 and 364 (IE86ΔMX), which contains the strong TBP and RB binding region, is required for transactivation of the HCMV early promoter, and specific removal of this region (IE86ΔXN) reduced activation to background levels. The other two TBP and RB binding regions also appear to play some role in the activation of this promoter. Although removal of either the weak binding region (IE86ΔHS) or the other strong binding region (IE86ΔSX) had little to no effect on the ability of the protein to activate transcription, removal of both regions (IE86ΔMX) reduced the level of activation fourfold relative to wild-type IE2 86. In addition to the TBP and RB binding domains, other regions of the protein are functionally important. In this regard, removal of regions within the amino terminus (IE86ΔHM) and carboxy terminus (IE55 and IE86 NheI) also reduce activation to background levels.

DISCUSSION

Studies from a number of laboratories have demonstrated that the HCMV IE2 86 can activate a variety of viral and cellular promoters (14, 22, 27) and therefore appears to play an important role in the regulation of the HCMV life cycle. One mechanism by which an activator can function is to bind specifically to a target sequence on the promoter. Although examination of the many promoters activated by the IE2 protein has failed to reveal any common sequence element other than the TATA box, this protein does have the ability to bind to DNA, as evidenced by the fact that IE2 86 can bind to a region within its own promoter (4, 17, 21). In this context, binding of IE2 86 to a specific sequence located on its promoter between the TATA box and the start site of transcription leads to inhibition. Whether this sequence can function as an activation domain when present in other promoters remains to be determined.

A second potential mechanism for gene activation involves the interaction of an activator with other cellular factors. This has been observed for viral proteins such as adenovirus E1A and herpes simplex virus VP16, which lack specific DNA binding ability and activate viral gene expression via a complex series of interactions with cellular proteins that bind to specific DNA targets (20, 24, 25). Hagemeyer et al. have recently demonstrated that IE2 86 can form a complex with TBP (8), and Jupp et al. have shown that the IE2 86 can also interact with promoter-bound TBP (12). These results, coupled with the observed DNA-binding ability of IE2 86, suggest that this protein has the potential to affect gene expression by using both direct and indirect interactions with the target DNA.

As a tool for studying the mechanism by which IE2 86 regulates gene expression, the coding regions for the IE proteins were cloned into the pGEX-KG vector, and the proteins were then expressed in bacterial cells as GST-IE fusion proteins which were easily purified by chromatography on glutathione-agarose. In a recent report, we demonstrated that the GST-IE86 fusion protein is fully functional in *in vitro* transcription assays (14). We have now used this bacterially produced GST-IE86 fusion protein to study its interactions with full-length TBP and RB. To delineate the regions of IE2 86 that are required for interactions with TBP and RB, we

constructed a panel of carboxy-terminal and internal deletion mutants of IE2 86. Of interest is the fact that the binding of TBP and RB is mediated by similar regions on IE2 86. We have elucidated three regions (aa 85 to 135, 136 to 290, and 291 to 364) that can each independently bind to TBP and RB (Fig. 2). Jupp et al. recently demonstrated that the region between aa 47 and 153 is involved in interactions with promoter-bound TBP (12). Although this region overlaps partially with the binding regions elucidated in this study, they did not detect binding of TBP to regions downstream of aa 154. It is possible that deletion of the amino-terminal 154 aa causes a global disruption in the structure of the IE2 86 protein and thereby inactivates the other binding domains. Alternatively, promoter-bound TBP may have part of its binding site for IE2 86 masked, thereby preventing TBP from binding to the remaining binding domains on IE2 86. It is unlikely that the binding observed in our experiments is due to the GST portion of the fusion protein, since GST alone does not bind TBP or RB (Fig. 1A and B). In addition, the binding is not due to a general stickiness of IE2 86, since CREB (Fig. 1C), Fos, and luciferase (data not shown) do not bind, nor is the binding mediated by the presence of contaminating DNA (Fig. 1D). Taken together, these data suggest that the interactions of TBP and RB with IE2 86 are specific in nature. A recent study has shown that a region in the carboxy-terminal domain of TBP is similar to a region within the pocket domain of RB (7). This homology may provide the basis for the common binding pattern of TBP and RB to IE2 86. Further support for this idea is provided by the recent report by Hateboer et al. (9) that suggests that TBP and RB also bind to overlapping domains on c-Myc and E1A.

The results obtained in the reverse binding experiments (³⁵S-labeled IE proteins binding to GST-RB/TBP) are surprising in some respects. The full-length IE2 86 did not bind efficiently to either GST-RB or GST-TBP, and significant complex formation occurred only when the region between aa 136 and 290 was removed. However, this region is a strong binding domain in the forward reactions. The region between aa 136 and 290 is rich in serine and threonine residues and possesses casein kinase substrate motifs (42), thus suggesting that this region may be highly phosphorylated. We also have preliminary evidence that casein kinase II can phosphorylate the GST-IE2 86 protein *in vitro* and that this phosphorylation occurs mainly in the region between aa 136 and 290 (29). Other evidence that this region may be posttranslationally modified is that the difference in size observed for the *in vitro*-translated IE2 86 mutant that is missing aa 136 to 290 compared with the full-length IE2 86 is greater than that expected for a deletion of 154 aa (data not shown). One can speculate that phosphorylation of this region may affect the ability of this region alone or of the entire IE2 86 to interact with other proteins. For example, it has been shown that the ability of the Elf-1 transcription factor to bind to RB is determined by the phosphorylation state of the RB protein (38). The question of whether cycling of the phosphorylation state of IE2 86 is a mechanism by which the interactions with other proteins, and ultimately gene expression, are regulated needs to be addressed. Of the other two binding regions identified in the forward experiments, the strong binding domain between aa 291 and 364 is required for binding in the reverse experiments (compare IE86ΔMX and IE86ΔMN in Fig. 3), while the weak binding domain between aa 85 and 135 appears to be nonfunctional (compare IE86ΔSX, IE86ΔMX, and IE86ΔSN in Fig. 3).

Although it is clear that the region between aa 291 and 364 is required for binding in both types of reactions, a mutant that is missing the negative region (aa 136 to 290) and terminates at aa 364 (IE86ΔSX364) does not bind to GST-TBP, but it does

bind to TBP and RB in the forward experiments. The differential results obtained in the forward and reverse binding experiments may be explained, at least in part, by the presence of a dimerization domain in the region between aa 388 and 542 in IE2 86 (4, 5). The domain required for IE2 86 binding to TBP and RB lies between aa 291 and 364, but dimerization of the protein may be a requirement for this interaction to occur. Dimerization could be mediated either by the domain on IE2 86 or by the GST component of the fusion protein, which also can form dimers (15, 36). This would explain why the IE86 Δ SX364, IE86 Δ MX364, and IE86 Δ MX401 mutants, which lack the IE2 86 dimerization domain but have an intact binding region, bind to RB and TBP in the forward but not in the reverse experiments. With these mutants, the GST moiety on the GST-IE fusion proteins may be sufficient to bring two IE2 86 molecules together and allow binding to occur even if the dimerization domain within the IE2 86 protein is deleted. Further support for this hypothesis is provided by the observation that the IE86 Δ SX519 and IE86 Δ SX544 mutants, which retain the dimerization domain, bind RB and TBP in both types of reactions. Interestingly, certain mutants of IE2 86 (IE86TM364, IE86 Δ SX364, IE86 Δ MX364, and IE86 Δ MX401) display a higher level of binding to TBP when expressed as GST fusion proteins. The feature shared by these mutant proteins is that they are deleted for the dimerization domain on the IE2 86 protein and therefore would have only the dimerization domain provided by the GST protein. In comparison, the full-length IE2 86 protein has two dimerization domains present when it is expressed as a GST fusion protein, one at the amino terminus provided by the GST moiety and the second at the carboxy terminus of IE2. The presence of the two domains may somewhat hinder the ability of IE2 86 to bind to TBP and RB by locking together two GST-IE86 molecules. Removal of the dimerization domain in the IE86 Δ SX364, IE86 Δ MX364, and IE86 Δ MX401 mutants could free one end of the molecule and allow increased interactions of TBP to the GST-IE molecules. This idea is supported by the fact that IE2 55, which has the dimerization region removed by a splicing event, also binds increased amounts of TBP compared with the full-length IE2 86 (Fig. 2).

Further evidence to support our interpretation of the data obtained in the reverse experiments is found in the study of Caswell et al. (2). In their study, full-length IE2 86 bound weakly to a GST-TBP construct, while a mutant lacking the N-terminal 290 aa bound strongly. In addition, a truncated form of IE2 86 that covers aa 290 to 390 did not bind GST-TBP, while IE2 86 fragments that extended from aa 290 to aa 504 or 542 also bound TBP strongly. Their inability to detect binding with fragments spanning aa 1 to 290 and 290 to 390 may be due to the presence of the negative region and the absence of the dimerization domain, respectively.

Another interesting result from the binding studies relates to the observation that the IE86 Δ SX519 and IE86 Δ SX544 mutants bind to GST-TBP six- to sevenfold more efficiently than the IE86 Δ SX and IE86 Δ MX mutants (Fig. 3). In addition to the casein kinase substrate motifs found between aa 136 and 290 (42), the carboxy-terminal region of IE2 86 may also contain consensus sites for phosphorylation by casein kinase. As has been suggested for the region between aa 136 and 290, binding may be inhibited due to phosphorylation events at the carboxy terminus of the molecule. However, the degree of inhibition differs for the two regions. The presence of aa 136 to 290 completely abrogates binding to GST-TBP, while the presence of the carboxy-terminal region reduces binding but does not abolish it completely. This may be because the potential phosphorylation sites at the carboxy terminus are

fewer in number than those found between aa 136 and 290. Another explanation would be the position of the phosphorylated regions within the folded protein. It has been previously reported that the extreme carboxy terminus of the IE2 protein (aa 544 to 579) is highly acidic and can function as an activator when expressed as a Gal4 fusion protein (26). It is possible to speculate that this region may interact with a region of the protein that is modified in the *in vitro* translation reaction but not in the bacterial cell. This potential interaction may also serve to inhibit, to some degree, the ability of the IE proteins to bind GST-TBP.

In this report, we have demonstrated that the ability of the IE2 86 protein to bind to TBP and RB correlates with its *in vivo* activation properties. The data show clearly that the region required for binding in the forward and reverse experiments *in vitro* (aa 291 to 364) is also essential for the transcriptional activation of an HCMV early promoter. However, other regions of IE2 86 are also necessary for full function, as indicated by the IE2 86 mutants that have retained the ability to bind TBP and RB but have lost the ability to transactivate. Our results are in agreement with those of others who have also identified regions of IE2 86 that are required for gene activation (22, 26, 31). Taken together, the data suggest that the region between aa 291 and 364 is necessary but not sufficient for the proper function of IE2 86.

Finally, the question arises of why IE2 86 contains three independent binding sites for TBP and RB. As discussed previously, one of the sites may act to regulate the binding to a second site via a phosphorylation-dephosphorylation pathway. Additionally, since IE2 86 is a multifunctional protein acting as an autoregulator and a transactivator, as well as potentially playing a role in cell cycle progression, it is likely that the protein interacts with a wide spectrum of cellular proteins other than TBP and RB. The presence of multiple sites may therefore allow IE2 86 to interact simultaneously with two or more proteins, fulfilling two functions at once, i.e., TBP for transactivation and RB for cell cycle progression. On the other hand, carrying out one function may require the interaction with more than one protein at a time, and thus the presence of multiple binding sites may allow IE2 86 to act as a bridge between two separate proteins. Hagemeyer et al. have suggested that since RB and TBP have sequence homology, they may also be functionally homologous (7). Perhaps then IE2 86 requires the simultaneous interaction with both TBP and RB for proper function. In a series of preliminary experiments, we have also found that IE2 86 can interact with both the A/B pocket (aa 384 to 776) and carboxy-terminal region (aa 768 to 928) of RB independently (28). In contrast, the adenovirus E1A protein or simian virus 40 T antigen binds only to the RB A/B pocket (13), and c-Abl binds only to the RB carboxy-terminal region (40).

The exact nature by which IE2 86 functions still remains to be elucidated. However, the data presented here, showing the interaction with important cellular regulatory proteins, provide further support for the hypothesis that the mechanism of action of IE2 86 is a complex one involving the interaction with a broad spectrum of cellular regulatory proteins and factors. The finding that both RB and IE2 86 interact with each other as well as with other cellular proteins via at least two separate domains also suggests that IE2 86 may regulate gene expression through the formation of multimeric protein complexes containing cellular regulatory factors and components of the basal transcription complex.

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