Site-Specific Binding of the Human Cytomegalovirus IE2 86-Kilodalton Protein to an Early Gene Promoter

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Received 14 February 1994/Accepted 28 May 1994

We have previously demonstrated that the human cytomegalovirus (HCMV) immediate-early region 2 86-kDa protein (the IE2 86 protein) is the major transactivator of the HCMV early promoter for the 2.2-kb class of RNAs (open reading frame UL 112-113). Here we show that specific stimulation of this promoter by IE2 86 in transient-expression assays requires sequences located between nucleotides (nt) –113 and –58 relative to the transcription start site; this is also the major regulatory region for this promoter during HCMV infection. To determine whether IE2 86 can bind to this promoter, a glutathione-S-transferase (GST)-IE2 86 fusion protein was incubated with the ³²P-labeled promoter and specific binding was assessed by retention of the protein-DNA complex on glutathione-agarose beads. DNase I footprint analysis was also used to map the sequences involved in the binding. Our results indicate that three regions, located between nt –286 and –257, nt –248 and –218, and nt –148 and –120, bind strongly to the IE2 86 protein and share sequence similarity with the previously identified *cis* repression signal located near the cap site of the major HCMV IE gene. In addition, there is a weaker binding region between nt –113 and –85, which shares some sequence homology with the *cis* repression signal element and the strong binding regions of the 2.2-kb RNA promoter but lacks one of the two CG dinucleotides present in all of the high-affinity binding sites. With a set of IE2 86 protein deletion mutants, we also show that the DNA-binding domain spans a large region in the carboxy-terminal half of the protein.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an important pathogen that is responsible for a variety of diseases in newborn and immunocompromised individuals (1, 32). As in other members of the herpesvirus family, the genome of HCMV is temporally expressed during the viral life cycle (10, 30, 54, 60, 61). The immediate-early (IE) gene products are synthesized immediately after viral infection and rely primarily on host factors for their expression. Early genes are transcribed prior to viral DNA replication, and their expression requires one or more viral IE gene products. Finally, late genes are transcribed at detectable levels only after viral DNA replication.

The major site of IE gene transcription is located in the long unique segment of the genome and includes two genetic units designated IE1 and IE2 (10, 19, 20, 52, 53, 55, 60, 62). At least three IE RNAs have been found to be transcribed from these regions. A 1.95-kb RNA, transcribed from the IE1 region, encodes a nuclear phosphoprotein of approximately 72 kDa (52). Two mRNAs of 2.25 and 1.7 kb arise from the IE2 region by differential splicing (53). These RNAs encode two proteins of approximately 86 and 55 kDa, respectively.

It has been demonstrated by transient-expression assays that HCMV early promoters, as well as heterologous viral and cellular promoters, can be activated by the IE1 and IE2 gene products (2–4, 7, 8, 11, 16, 19, 23, 28, 36, 39, 40, 49, 57, 59). The IE2 86-kDa protein (IE2 86 protein) plays a major role in activating HCMV early promoters as well as in repressing its own promoter (5, 18, 26, 37–39, 51). The ability of the IE2 86 protein to down-regulate specifically its own promoter, either

in transient-transfection assays or in vitro, is probably the result of a mechanism that includes direct DNA binding to a *cis* repression signal (CRS) near the cap site (6, 21, 25, 27, 65). The IE2 86 protein is also capable of interacting with several cellular proteins, including the TATA-binding protein (TBP) and the product of the retinoblastoma susceptibility gene (14, 17, 22, 47).

It has previously been shown that the IE2 86 protein is the major transactivator of the HCMV early promoter for the 2.2-kb class of RNAs (open reading frame UL 112-113), which encodes four nuclear phosphoproteins (24, 49, 50, 63, 64). In this study, we demonstrate that the IE2 86 protein is capable of binding to upstream regions of this promoter in a site-specific manner. We have mapped the DNA sequences that contact the IE2 86 protein as well as the regions of the IE2 86 protein that are required for binding DNA.

MATERIALS AND METHODS

Virus and cells. HCMV AD169 was obtained and propagated as previously described (56). Human U373-MG astrocytoma-glioblastoma cells were a gift from R. LaFemina (Merck, Sharpe and Dohme) and were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with a high glucose concentration and containing 5% fetal bovine serum and Mito Plus serum extender (Collaborative Research).

Molecular cloning. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., or Boehringer Mannheim Biochemicals and used as recommended by the manufacturers. Competent *Escherichia coli* DH5α organisms (Bethesda Research Laboratories) were transformed with recombinant plasmids as recommended by the supplier.

Construction of plasmids p358CAT, p259CAT, p148CAT,

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p93CAT, and p42CAT, which contain various lengths of the early promoter for the 2.2-kb class of RNAs fused to the chloramphenicol acetyltransferase (CAT) gene, has been previously described (49). Plasmid p195CAT was constructed by cleaving p358CAT with *Xba*I and *Xho*I followed by blunt ending with the Klenow fragment of DNA polymerase I. This removed sequences between nucleotides (nt) -323 and -161. The cleaved plasmid was then purified by gel electrophoresis and religated. The deletion was verified by restriction enzyme analysis. pDMCAT, which lacks sequences between nt -113 and -58 of the 2.2-kb RNA promoter, was constructed with the Muta-Gene M13 in vitro mutagenesis kit version 2 (Bio-Rad) as follows. p358CAT was cleaved with *Sst*I and *Xba*I, and the resulting fragment, containing the sequence from nt -323 to +35 of the 2.2-kb RNA promoter, was cloned into M13mp19 previously cleaved with *Sst*I and *Xba*I. The resulting vector was transformed into *E. coli* CJ236 (*dut ung*). Uracil-containing single-stranded DNA was isolated and annealed to an antisense oligonucleotide encompassing 15 bp surrounding each side of the desired deletion: 5' CGCCTAGCAACACC CGTGAACGACACCGGA 3'. Second-strand synthesis was performed by using T7 DNA polymerase followed by ligation with T4 DNA ligase. The resulting vector was transformed into the MV1190 wild-type strain, in which the parental strand is not replicated. The newly obtained vector was further cleaved with *Sst*I and *Xba*I, and the resulting fragment was cloned back into the original p358CAT digested with *Sst*I and *Xba*I.

Construction of pSGIE86 and all of the GST-IE2 86 protein mutants has been previously described (24, 47).

DNA sequence analysis. The presence of the desired mutations in pDMCAT was verified by the dideoxy-chain termination method of DNA sequencing (43) with the GemSeq K/RT Sequencing System (Promega Biotec, Inc.) in conjunction with the M13 universal primer. [α -³⁵S]dATP (1,000 Ci/mmol) was purchased from Amersham.

Transient-expression assays. Human U373-MG astrocytoma-glioblastoma cell monolayers were transfected with plasmid DNAs by the DEAE-dextran technique previously described by Staprans et al. (49). In all experiments, duplicate flasks were transfected. In some experiments, cells were infected with HCMV at a multiplicity of infection of 5 24 h posttransfection. Cells were harvested at approximately 48 h posttransfection, and the contents of each flask were split into two parts: one for the analysis of CAT activity, and the other for the isolation of nuclear DNA and assessment of transfection efficiency as previously described (49). All of the constructs were assayed in at least two independent experiments.

DNA labeling for binding assays. DNAs from all the plasmids used were cleaved with *Sst*I and *Hind*III to obtain small restriction fragments containing the sequences of interest. The fragments were separated by electrophoresis through 5% NuSieve (FMC BioProducts) agarose gels and purified with the Mermaid kit (Bio101). Approximately 100 ng of plasmid was labeled with the Klenow fragment of DNA polymerase I and [α -³²P]CTP (ICN Radiochemicals). Oligonucleotides were annealed and, when needed, labeled with T4 kinase.

DNA-binding assays. Expression and analysis of the glutathione-S-transferase (GST) fusion proteins were carried out as previously described (24). For each condition tested, a range of approximately 16 to 300 ng of the given GST-IE protein was incubated, with rocking, in 800 μ l of buffer A (phosphate-buffered saline plus 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, 1 mM benzamidine, and 1 mM dithiothreitol) plus 25 μ l of 50% (vol/vol) glutathione-agarose beads for 15 min at 4°C. No differences in binding results were observed within this protein concentration

range. The beads were centrifuged, the supernatant was removed, and the pellet was washed twice with buffer A. The pellet was washed once more with binding buffer consisting of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 300 mM KCl, 20% glycerol, 0.1% Nonidet P-40, 10 μ M ZnSO₄, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, 1 mM benzamidine, and 1 mM dithiothreitol. After these washes, the beads were incubated for 30 min at 22°C with approximately 50,000 cpm of the indicated ³²P-labeled DNA fragment and 20 μ g of dA-dT. When nuclear extract was added to the incubation mix, the beads were incubated first with the labeled DNA for 30 min at 4°C and then approximately 200 μ g of U373-MG nuclear extract was added. After incubation for 30 min at 22°C, the beads were centrifuged and the supernatant was set aside and counted in a scintillation counter. The beads were washed twice with binding buffer, and their radioactivity was counted. Specific binding was assessed by retention of the protein-DNA complex on the glutathione-agarose beads. Results are expressed as the percentage of bound counts per minute over total counts per minute. GST control reactions were run in all the experiments. All the constructs or assay conditions were tested in duplicate in at least two independent experiments.

Nuclear extract preparation. Nuclear extracts were prepared from U373-MG cell monolayers by modifications of the methods used by Dignam et al. (12) and Shapiro et al. (44) as previously described (24).

Gel shift analysis. Following the binding of the DNA to the GST-IE constructs as described above, we eluted the DNA-protein complex from the beads by incubating the mixture for 10 min at 22°C in a solution of 50 mM Tris (pH 8), 20% glycerol, 10 mM glutathione, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM sodium metabisulfite, and 0.2 mM phenylmethylsulfonyl fluoride. The samples were then loaded on a 5% nondenaturing polyacrylamide gel. The gel was dried and exposed to autoradiography.

DNase I footprint analysis. Probes containing DNA sequences between nt -323 and -160 or between nt -160 and +35 relative to the 2.2-kb RNA start site were 5'-end labeled with T4 kinase on the noncoding strand and then subjected to a binding assay as described above. The DNA-protein complexes were eluted from the beads as described for the gel shift analysis and digested with various concentrations of DNase I for 1.5 min at 22°C. Reactions were stopped by addition of 2% sodium dodecyl sulfate, 200 mM EDTA, and 1 μ g of salmon sperm DNA. The samples were treated with phenol-chloroform, precipitated, resuspended in formamide loading buffer, and run on an 8% acrylamide-urea sequencing gel. As an internal sequence marker, G+A reactions were run in each gel (29).

Oligonucleotides. The CRS oligonucleotides used were 5' TAAGCAGAGCTCGTTTAGTGAACCGTCAGATCG CC 3' and 5' GGCGATCTGACGGTTCACTAAACGA GCTCTGCTTA 3'. For the cold-DNA competition assays, we used the protected DNA sequences revealed by the DNase I footprint analysis plus 3 to 5 flanking nucleotides on each side. The oligonucleotides used for competition of the high-affinity binding site encompassed between nt -148 and -120 were 5' TAGCGTTGCGATTTGCAGTCCGCTCCGGTGTCGTT C 3' and 5' GAACGACACCGGAGCGGACTGCAATCGC AACGCTA 3'. The oligonucleotides used for competition of the low-affinity binding site located between nt -113 and -85 were 5' TTCACCCAGTTACTTTAATAAACGTACTGTT TAACCA 3' and 5' TGGTTAAACAGTACGTTTATTAA AGTAACTGGGTGAA 3'. As nonspecific sequences for the

FIG. 1. Schematic representation of the early 2.2-kb RNA promoter deletion plasmids and their observed CAT activities following either HCMV superinfection or cotransfection with pSGIE86. Duplicate flasks of permissive U373-MG cells were cotransfected by the DEAE-dextran method with several deletion promoter constructs of the 2.2-kb RNA promoter and pSGIE86 (which encodes the IE2 86 protein) or were superinfected with HCMV. Cells were harvested 48 h posttransfection and assayed for CAT activity as described in Materials and Methods. CAT activity relative to p358CAT is shown. Nucleotide numbers relative to the transcription start site are indicated. ND, not determined.

binding assays, we used the coding region of the IE2 86 protein located between nt +140 and +165. The sequences of these oligonucleotides are 5' GATGGAGTCCTCTGCCAAGAGAAAGATG 3' and 5' GTCCATCTTTCTCTTGCGAGA GGAATCCA 3'. Nucleotide numbers are expressed in relation to the transcription start site at +1.

RESULTS

Analysis of 2.2-kb RNA promoter deletions. Previously we showed that the HCMV IE2 86 protein is the major transactivator of the HCMV early promoter for the 2.2-kb class of RNAs in U373-MG cells, which are fully permissive for HCMV infection (24). In those experiments, we used the reporter construct p729CAT, which contained the sequence from nt -694 to +35 relative to the transcription start site attached to the CAT gene. To define further the sequence requirements for transactivation, we analyzed the relative activation of a series of 5' deletion mutants of this promoter by the IE2 86 protein in transient-expression assays. For comparison, we also measured the activation of the deletion constructs by HCMV infection in U373-MG cells. As shown in Fig. 1, specific stimulation of the 2.2-kb early RNA promoter by the IE2 86 protein in transient-expression assays requires sequences located between nt -113 and -58 relative to the transcription start site. This domain is also a major regulatory region for this promoter during HCMV infection, although sequences -224 to -113 do contribute to full activation. The results obtained with the 5' promoter deletions and HCMV

superinfection in the U373-MG cells were very similar to those reported previously with human foreskin fibroblasts, which are also permissive for HCMV infection (49).

The IE2 86 protein is capable of binding to several discrete domains of the 2.2-kb RNA promoter. Since specific DNA binding is critical for the function of numerous transcription factors, we developed a highly sensitive assay to determine whether the GST-IE2 86 fusion protein could bind to the 2.2-kb RNA promoter. For these assays, a GST-IE2 86 fusion protein, which is a fully functional transactivator for this promoter in *in vitro* transcription reactions (24), was bound to glutathione-agarose beads and further incubated with ³²P-labeled DNA fragments containing different regions of the 2.2-kb RNA promoter. The binding reaction was optimized with respect to the amount of labeled DNA and GST-IE2 86 fusion protein added, time of incubation, temperature, and salt concentration as described in Materials and Methods. The concentration of GST-IE2 86 protein in the multiple experiments performed ranged from 16 to 300 ng (corresponding to a protein-to-DNA molar ratio which ranged from 5:1 to 90:1). In this range, the results of the binding assays were the same regardless of the concentration of protein. DNA binding to GST protein alone was measured in each experiment as a negative control. We also tested binding in the presence of the nonspecific DNA competitors poly(dA-dT) · poly(dA-dT) and poly(dI-dC) · poly(dI-dC). Binding was not affected by the presence of poly(dA-dT) · poly(dA-dT) but was completely abolished by the presence of poly(dI-dC) · poly(dI-dC) (data not shown).

As shown in Fig. 2, the GST-IE2 86 fusion protein is capable of binding with different affinities to various domains upstream of the start site for transcription of the early 2.2-kb RNA promoter. The data indicate that there are at least two relatively high-affinity binding regions, one between nt -323 and -160 and the second between nt -160 and -113. The GST-IE2 86 protein also is capable of binding with lower affinity to the biologically relevant domain within nt -113 to -58. When binding assays were performed in the presence of U373-MG nuclear extract, binding of this lower-affinity site to the GST-IE2 86 protein was enhanced twofold. Although this is a modest increase, it has been consistently observed in multiple independent assays. In contrast, binding of the GST-IE2 86 protein to constructs containing regions upstream of -113 appeared to be independent of the presence of U373-MG nuclear extract. None of the DNA constructs bound to the GST protein alone, and no significant binding to the GST-IE2 86 protein was detected for regions downstream of nt -58. As an additional control, we showed that the GST-IE2 86 protein does not bind to a segment of another HCMV early promoter (the 1.2-kb RNA promoter), which contains an AP-1-binding site (58). Therefore, it is unlikely that the GST-IE2 86 protein binds to DNA through the GST component or simply binds nonspecifically to DNA segments which interact with other DNA-binding proteins.

Gel shift analysis of IE2 86 protein binding to DNA. To confirm that specific protein-DNA complexes were being formed in the above assays, we eluted the bound material from the beads with glutathione and subjected it to electrophoresis through a 5% nondenaturing acrylamide gel. As shown in Fig. 3, the DNA fragments tested were the wild-type sequences of the 2.2-kb early RNA promoter, containing either 160, 113, or 58 bp upstream of the transcription start site, in both the presence and absence of U373-MG nuclear extract. The eluted DNA migrated much more slowly than the free probe, indicating that the DNA is forming a complex with the GST-IE2 86 fusion protein. More of the slowly migrating complex was

FIG. 2. Binding of the GST-IE2 86 protein (IE86) and GST protein (GEX) to the various deletion fragments of the 2.2-kb early RNA promoter in the presence (+NE) or absence of U373-MG nuclear extract. Binding assays were also performed on a fragment of the HCMV early 1.2-kb RNA promoter which contains an AP-1-binding site (58). Binding assays were performed as described in Materials and Methods. Results are expressed as a percentage of bound label over total label added. Nucleotide numbers relative to the transcription start site are indicated at the ends of each deletion. ND, not determined.

found in the lanes where the probe included nt -160 to +35 than in the lanes where the probe was the fragment from nt -113 to +35. However, the amount of complex formed with the probe from nt -113 to +35 was enhanced by nuclear extract. As expected, no slowly migrating complex was detected when a fragment containing sequences -58 to +35 was used.

DNase I footprinting. We conducted DNase I footprint analysis to identify the sequences protected by the IE2 86 protein. Probes containing DNA sequences within nt -323 to -160 or nt -160 to +35 relative to the 2.2-kb RNA start site were 5'-end labeled on the noncoding strand with T4 kinase and then subjected to a binding assay as described above. The DNA-protein complexes were eluted from the beads with glutathione and digested with various concentrations of DNase I as described in Materials and Methods. As shown in Fig. 4, the IE2 86 protein completely protects three regions located between nt -286 and -257, nt -248 and -218, and nt -148 and -120. The region between nt -113 and -85 is also protected by the IE2 86 protein but to a lesser extent. Similar results were obtained when the DNase I footprint analysis was performed on DNA-protein complexes formed in solution and not further purified on the beads (data not shown). The various degrees of protection observed were consistent with the results of the above binding assays with the promoter deletions and confirmed the specificity of the binding. Addition of U373-MG nuclear extract to the footprint assay did not alter the footprint obtained by the IE2 86 protein alone (data not shown).

FIG. 3. Gel shift analysis of the GST-IE2 86 fusion protein-DNA complex. Binding of the DNA to the GST-IE2 86 fusion protein was performed in the presence or absence of U373-MG nuclear extract (NE) as described in Materials and Methods, followed by elution of the DNA-protein complex from glutathione-agarose beads and electrophoresis through a 5% nondenaturing acrylamide gel. The DNA fragments tested were wild-type sequences of the early 2.2-kb RNA promoter containing sequences between nt -160, -113, or -58 and nt +35 (shown as -160, -113, and -58 at the top of each lane). Lane 1 contains only free probe (nt -160 to +35). Lanes 3, 5, and 7 had nuclear extract added to the binding assay.

Binding of the IE2 86 protein to the CRS element located near the cap site of the major IE gene has been previously reported (6, 21, 25, 27, 65). Inspection of the DNA sequences protected by the IE2 86 protein on the 2.2-kb RNA promoter revealed several striking similarities to the previously reported 14-nt CRS motif (37) (Fig. 5). The three high-affinity binding regions of the 2.2-kb RNA promoter and the CRS element all have invariant CG residues at both ends of the consensus 14-nt sequence. In contrast, the low-affinity binding region located between nt -113 and -85 lacks one of the conserved CG dinucleotides. The internal section of the consensus domain is A+T rich in the CRS element and all of the 2.2-kb RNA promoter-protected sequences, with an A or T at positions 4, 6, and 11. In addition, all of the protected sequences in both the CRS element and the 2.2-kb RNA promoter include an extended region of 4 to 5 nt upstream and 10 to 12 nt downstream of the critical 14-nt region on the noncoding strand.

Competition studies. To determine the specificity of binding of the IE2 86 protein to the 2.2-kb RNA promoter, we performed competition assays with an excess of unlabeled oligonucleotides representing the protected regions revealed by the DNase I footprint analysis. Since the three high-affinity binding regions bounded by nt -286 to -257, nt -248 to -218, and nt -148 to -120 have similar DNA sequences, we chose to study for specificity purposes the high-affinity region within nt -148 to -120 and the low-affinity binding region within nt -113 to -85. As shown in Fig. 6A, addition of a

FIG. 4. DNase I footprint analysis of the 2.2-kb RNA promoter bound to the GST-IE2 86 protein. Binding of the DNA to the GST-IE2 86 fusion protein, elution of the complex from glutathione-agarose beads, DNase I treatment, and electrophoresis through an 8% sequencing gel were performed as described in Materials and Methods. Lanes 1 to 3 show sequences protected within nt -160 to +35. Lanes 4-6 show sequences protected within nt -323 to -160. Lanes 1 and 4 show the G+A sequencing reactions, lanes 2 and 5 contain DNA alone, and lanes 3 and 6 contain DNA plus GST-IE2 86 protein. Sequences protected by the IE2 86 protein are underlined and labeled A through D.

500-fold excess of unlabeled high-affinity binding-site oligonucleotide (nt -151 to -115) completely blocks the binding of a labeled probe containing nt -160 to +35 to the GST-IE2 86 protein. However, no competition was seen with a 500-fold excess of unlabeled nonspecific DNA, thus confirming the specificity of the DNA-protein interaction. When we incubated the GST-IE2 86 protein with a labeled construct containing sequences -113 to +35 (which includes the low-affinity binding site) in the presence of a 500-fold excess of unlabeled oligonucleotide representing the low-affinity binding site (nt -118 to -81), we observed a fivefold drop in binding compared with that in assays in which no competing oligonucleotide was added (Fig. 6B). There is some competition of binding of the GST-IE2 86 protein to the low-affinity binding site by a 500-fold excess of nonspecific DNA, but the level of competition is significantly lower than that obtained with the specific oligonucleotide. Binding sites located between nt -148 and -120 and between nt -113 and -85 on the 2.2-kb RNA promoter also compete with each other for the binding of the IE2 86 protein. Addition of a 500-fold excess of the strong

binding-site oligonucleotide (nt -151 to -115) completely abolishes binding of the IE2 86 protein to the weak binding site of the 2.2-kb RNA promoter, and the low-affinity binding-site oligonucleotide (nt -118 to -81) can also compete with binding to the high-affinity binding site, albeit to a lesser extent (Fig. 6A and B).

On the basis of the homology of the CRS element to the binding sites on the 2.2-kb RNA promoter, we were interested in determining whether the CRS element could compete for the binding of the sequences on the 2.2-kb RNA promoter and vice versa. As shown in Fig. 6A and B, addition of a 500-fold excess of oligonucleotides containing the CRS element completely abolished the binding of the IE2 86 protein to both high- and low-affinity binding sites on the 2.2-kb RNA promoter. In addition, as shown in Fig. 6C, addition of a 500-fold excess of oligonucleotides containing the strong binding site of the 2.2-kb RNA promoter (nt -151 to -115) also competed with the binding of the IE2 86 protein to the CRS element. Oligonucleotides containing the weak binding site of the 2.2-kb RNA promoter (nt -118 to -81) were also capable of

CRS	-18	agctCGTTT <u>AGTGAACCG</u> tcagatcgcc	+10
A	-286	ggttgCGGAGATAAGTCCGtgattagtcgg	-257
B	-248	ggaggCGGAAGGAAATCGgataacggcacg	-218
C	-148	cgttgCGATTTCAGTCCGctccggtgtc	-120
D	-113	ccagtTACTTTAATAAACGtactgtttaa	-85
CONSENSUS		<div style="display: flex; align-items: center; justify-content: center;"> <div style="text-align: center; margin-right: 10px;"> 1 4 7 10 14 CGGTTAGNAAACCG TAATT GGTT A G </div> <div style="text-align: center;"> <u>CGGTTAGNAAACCG</u> TAATT GGTT A G </div> </div>	

FIG. 5. Sequence comparison of the CRS element with the IE2 86 protein-binding sites located within the 2.2-kb RNA promoter. The first line represents the previously published sequence of the CRS element (25). Lines A through D represent the various DNA sequences within the 2.2-kb RNA promoter protected by the IE2 86 protein. The last line represents a consensus sequence corresponding to the high-affinity binding sites of the 2.2-kb RNA promoter and the CRS element. Capital letters represent the DNA domains that have similarity to the CRS element. Underlined sequences represent nucleotides in the CRS sequence that are conserved in the 2.2-kb RNA promoter sequences.

competing for the binding of the IE2 86 protein to the CRS element but only to a low level. These results suggest that the CRS element and both the strong and weak binding sites on the 2.2-kb RNA promoter compete for binding to the same region of the IE2 86 protein, with the binding of the CRS element being somewhat favored over that of the strong binding site on the 2.2-kb RNA promoter.

Characterization of the domains of the IE2 86 protein required for DNA binding. To determine the protein domains necessary for binding to the strong and weak binding sites on the 2.2-kb RNA promoter, GST-IE2 86 fusion protein constructs containing both carboxy-terminal and in-frame internal deletions were tested for complex formation with both sites. As can be seen in Fig. 7, mutants IE86dHM and IE86dHS, which lack amino acid residues 25 to 85 and 86 to 134, respectively, show the same DNA-binding levels as that of the intact IE2 86 protein. Mutants IE86dSX and IE86dMX, which lack residues 136 to 290 and 86 to 290, respectively, also bind DNA as well as the wild-type protein does. Therefore, removal of several regions near the N terminus of the protein does not significantly lower its DNA-binding properties. In fact, mutant IE86dMX reproducibly binds better to the low-affinity DNA region than does the wild-type protein. It is possible that deletion of amino acids 86 to 290 eliminates a domain in the IE2 86 protein that interferes with binding to the weak site. The DNA-binding domain appears to map to a broad region in the carboxy-terminal half of the protein, since mutants IE86dXN, IE2 55, and IE86/NheI, which lack amino acid residues 290 to 368, 365 to 519, and 544 to 579, respectively, all fail to bind DNA. In addition, the IE1 72 protein fails to bind to this promoter. These results support those of the DNA-binding competition assays described above, which also suggest that the same regions of the IE2 86 protein are required for binding to both the high- and low-affinity sites within the 2.2-kb RNA promoter.

FIG. 6. Competition analysis between the 2.2-kb RNA promoter-binding sites and the CRS element. Binding assays were performed as described in Materials and Methods. For a detailed description of the oligonucleotides used for competition, see Materials and Methods. As nonspecific DNA, we used the coding region of the IE2 86 protein encompassed by nt +140 to +165. nt -151 to -115 represent the high-affinity binding site, and nt -118 to -81 represent the low-affinity binding site. A 500-fold molar excess of the above oligonucleotides was used to compete for binding of the IE2 86 protein to sequences located within nt -160 to +35 (A), sequences bordered by nt -113 to +35 (B), and the CRS element (C). Results are expressed as percentage of bound label over total label added.

FIG. 7. Schematic representation of deletion mutants of the GST-IE2 86 protein and their relative binding activities to the 2.2-kb RNA promoter. The amino acid residues at the border of each deletion are indicated. Binding assays were performed as described in Materials and Methods. The DNA fragments tested were wild-type sequences of the early 2.2-kb RNA promoter containing sequences between nt -160 or -113 and nt +35 (shown as -160 or -113 at the top of each column). Equal amounts of protein were assayed for each construct.

DISCUSSION

The HCMV IE2 86 protein is capable of regulating the activity of multiple viral and cellular promoters, including its own (5, 6, 18, 23–28, 37–39, 49). Although formal genetic proof is lacking, it is believed that this protein probably plays a key role in the transcriptional regulation of HCMV infection. However, how this protein functions, whether by DNA-protein or protein-protein interactions or both, remains to be elucidated.

It has been proposed that IE2 86 mediates negative regulation of its own promoter via a short nucleotide sequence, the CRS, located between the TATA box and the transcriptional start site of this gene (5, 26, 37). In support of this hypothesis, several investigators (6, 21, 25, 27, 65) have demonstrated that the IE2 86 protein interacts directly with the CRS element and that this binding correlates with the repression properties of the protein. In this paper, we have addressed the question whether the IE2 86 protein also binds to an early HCMV promoter which is strongly activated by it. To determine whether the IE2 86 protein could bind to the 2.2-kb RNA promoter, we developed a highly sensitive assay which allowed us to detect both weak and strong DNA-protein interactions. In these assays a GST-IE2 86 fusion protein, which we have shown to be a fully functional transactivator for this HCMV early promoter in *in vitro* transcription reactions (24), was bound to glutathione-agarose beads and incubated with the labeled promoter. Specific binding was then assessed by retention of the protein-DNA complex on the glutathione-agarose beads and by DNase I footprint analysis.

Our studies show that the GST-IE2 86 fusion protein is capable of binding with different affinities to several distinct domains upstream of the transcription start site of the early 2.2-kb RNA promoter. There are high-affinity binding regions in the domains bounded by nt -286 to -257, nt -248 to -218,

and nt -148 to -120 and a lower-affinity binding region bounded by nt -113 to -85. Interestingly, this last domain appears to be the most biologically relevant on the basis of transfection and superinfection experiments. It is possible that although this site is not bound as strongly as the other upstream sites by the IE2 86 protein, its precise position and characteristic affinity allow it to play a critical role in transactivation. In addition, it is possible that in the context of a whole-HCMV infection, different IE2 86 binding sites play distinct roles at different times.

The DNase I protection data argue strongly that the interactions between the GST-IE2 86 protein and the 2.2-kb RNA promoter are specific. Although the data presented in Fig. 4 were obtained following DNase I digestion of DNA-protein complexes bound and eluted from glutathione-agarose beads, we have obtained similar results when the DNase I footprint analysis was conducted by incubating the labeled DNA with purified GST-IE2 86 protein in solution (data not shown). No binding to GST protein alone was observed under any circumstances. Therefore, it can be ruled out that the observed binding to the 2.2-kb RNA promoter is due to the GST protein. In addition, the IE1 72 protein, which does not have a major role in the transactivation of this promoter in U373-MG cells (24), fails to bind to the 2.2-kb RNA promoter. As an additional control, we showed that the GST-IE2 86 protein does not bind to a segment of another HCMV early promoter (the 1.2-kb RNA promoter) which contains an AP-1-binding site.

The previous failure to detect binding of the IE2 86 protein to promoters activated by it could be due to several factors. We have found that the IE2 86 protein-DNA interaction is sensitive to poly(dI-dC), which is commonly used as a nonspecific inhibitor in DNA-binding assays. This has also been reported regarding the binding of the IE2 86 protein to the CRS

element (6, 25). In addition, failure to detect DNA binding could have been due to the use of in vitro translated IE2 86 protein as reported by Chiou et al. (6), particularly if post-translational modifications of the protein in these reactions can interfere with its DNA-binding properties. Finally, we have found the presence of 300 mM KCl in the binding-assay mixture to be critical for stable DNA-protein binding activity. This is not an unprecedented finding, since it has been reported that Sp1 binds its target sequence only when high concentrations of KCl are present (42).

It is interesting that ICP4, which is encoded by herpes simplex virus type 1, has many properties in common with the HCMV IE2 86 protein. Both proteins are capable of binding DNA directly with high and low affinity depending on the sequence in question. ICP4 binds with high affinity to sequences corresponding to the consensus ATCGTCNNN NYCGRC and with lower affinity to a variety of sequences that are not represented by a single consensus (13, 31, 35). Both proteins autorepress by binding to a high-affinity site located near the cap site of their respective promoters (9, 34, 41). IE2 86 and ICP4 are also capable of activating multiple promoters which share little sequence homology among themselves (9, 15, 33). In addition, both proteins are capable of binding TBP and TFIIB (46). Finally, mutational analysis of both proteins has revealed a strong correlation between loss of DNA-binding activity and transactivation function (45). Nevertheless, the precise role that DNA binding plays in the stimulation of viral gene expression in the context of the viral genome for both ICP4 and IE2 86 remains to be determined.

Both the high- and low-affinity protein-binding regions of the 2.2-kb RNA promoter share some sequence homology with the CRS element (37) (Fig. 5). The three high-affinity binding regions of the 2.2-kb RNA promoter and the CRS element all have invariant CG dinucleotides at both ends of the consensus 14-nt sequence. In contrast, the low-affinity binding region within nt -113 and -85 lacks one of the conserved CG dinucleotides. The internal sequence of the consensus domain is A+T rich in both the CRS element and all of the 2.2-kb RNA promoter protected sequences. In addition, the protected sequence in the noncoding strand of both the CRS element and the 2.2-kb RNA promoter includes an extended region of 4 to 5 nt upstream and 10 to 12 nt downstream of the critical 14-nt region. On the basis of the observation that all the high-affinity binding sites of the 2.2-kb RNA promoter and the CRS element contain invariant CG dinucleotides at both ends of the 14-nt sequence while the low-affinity binding site of the 2.2-kb RNA promoter lacks one of them, it is likely that both CG domains surrounding an A+T-rich sequence are needed for strong binding to the IE2 86 protein. Moreover, the inhibition of DNA binding of the IE2 86 protein by the presence of poly(dI-dC) · poly(dI-dC), as reported by us and others (6, 25), further supports the importance of these CG dinucleotides. We also observe competition for binding of the IE2 86 protein to the low-affinity binding region by both the CRS element and the high-affinity binding site located between nt -151 and -115 of the 2.2-kb RNA promoter. These results suggest that the CRS element and both the strong and weak binding sites on the 2.2-kb RNA promoter compete for binding to the same region on the IE2 86 protein, with the binding of the CRS element being somewhat favored over that of the strong binding site on the 2.2-kb RNA promoter.

Analysis of the domains on the IE2 86 protein required for binding to the 2.2-kb RNA promoter indicates that when amino acids 290 to 368, 365 to 519, or 544 to 579 are deleted, the DNA-binding capability of the IE2 86 protein is lost. These regions also correspond to the domain required for binding of

the IE2 86 protein to the CRS (6). Recently, Sommer et al. analyzed the functional properties of the IE2 86 mutants described in this article (47). Their results showed that all of the IE2 86 protein mutants, which in our experiments do not bind DNA, also fail to activate the 2.2-kb RNA promoter. This suggests that the domain containing the DNA-binding activity of the IE2 86 protein is also very important for transactivation.

On the basis of the specific protection patterns revealed by DNase I footprint analysis, the competition binding studies, the inability of the GST-IE2 86 protein to bind to an unrelated DNA, the absence of binding of the GST protein alone to DNA, and the mutational analysis of the IE2 86 protein, we conclude that the IE2 86 protein is capable of binding to the upstream sequences of the 2.2-kb RNA promoter in a specific fashion. However, there is evidence that in addition to its DNA-binding ability, the IE2 86 protein can form a complex with itself and several other proteins including TBP and retinoblastoma protein (6, 14, 17, 22, 47). Preliminary experiments conducted in our laboratory suggest that the IE2 86 protein is also capable of interacting with TFIIB (48). It is therefore possible that the IE2 86 protein is serving as an anchoring and/or stabilizing agent for the TBP-DNA interaction (21) and the TBP-TFIIB-DNA interaction, as recently suggested by Smith et al. (46) for ICP4. Analysis of these kinds of interactions is in progress.

ACKNOWLEDGMENTS

We thank Chuck Clark for excellent technical assistance, Maarten Stuiver and Marc Van Dijk for their help with the footprint analysis, and Lyn Alkan for the preparation of the manuscript. We also thank Isaac Engel, Erik Wade, Valerie Koval, Tom Moreno, Françoise Jault, and Franziska Ruchti for stimulating discussions and critical reviews of the manuscript.

This investigation was supported by NIH grant CA 34729 and NSF grant MCB-9105990.

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