Transcriptional Activation by the Human Cytomegalovirus Immediate-Early Proteins: Requirements for Simple Promoter Structures and Interactions with Multiple Components of the Transcription Complex

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We have utilized a number of well-defined, simple, synthetic promoters (upstream factor binding sites and TATA elements) to analyze the activation mechanisms of the human cytomegalovirus immediate-early (IE) proteins. We found that the 86-kDa IE protein (known as IEP86, IE2_{559aa}, or ppUL122a) can recognize and activate a variety of simple promoters, in agreement with the observation that it is a promiscuous activator. However, in the comparison of otherwise identical promoters IEP86 does have preferences for specific TATA elements (hsp70 > adenovirus E2 > simian virus 40 early) and specific upstream transcription factor binding sites (CAAT > SP1 ≈ Tef-1 > ATF; no activation with AP1 or OCT). In contrast, the 72-kDa IE protein (known as IEP72, IE1_{491aa}, or ppUL123) alone did not significantly activate the simple promoters under our experimental conditions. However, each promoter activated by IEP86 was synergistically affected by the addition of IEP72. In addition, the 55-kDa IE protein (IEP55, a splice variant form of IE2, IE2_{425aa}, or ppUL122b) repeatedly had a negative effect, downregulating the activation of promoters caused by IEP86 and the synergy of IEP86 and IEP72. We show that the ability of IEP86 to activate many simple promoters correlates not only with its previously described ability to interact with the TATA-binding protein (TBP) (B. A. Furnari, E. Poma, T. F. Kowalik, S.-M. Huong, and E.-S. Huang, *J. Virol.* 67:4981–4991, 1993; C. Hagemeyer, S. Walker, R. Caswell, T. Kouzarides, and J. Sinclair, *J. Virol.* 66:4452–4456, 1992; R. Jupp, S. Hoffman, R. M. Stenberg, J. A. Nelson, and P. Ghazal, *J. Virol.* 67:7539–7546, 1993) but also with its ability to interact with the transcription factors which bind to the upstream element of promoters it activated (e.g., SP1 and Tef-1 but not Oct-1). This ability to have multiple interactions with the promoter complex may be crucial for transcriptional activation, since the IE proteins cannot activate promoters having only a TATA element or only an upstream transcription factor binding site. In addition, we show that proteins which bind IEP86 also bind to IEP55. Thus, the negative effect on transcription noted with IEP55 may be the result of competition with IEP86 for interaction with the promoter complex. The synergy caused by IEP72 appears to be mediated by a more indirect mechanism. This is suggested by our observation that IEP72 could not bind to any of the proteins tested (TBP, Tef-1, or Oct-1) or to IEP86.

The human cytomegalovirus (HCMV) major immediate-early promoter (MIEP) controls the expression of a primary transcript that is alternately spliced and polyadenylated to form mRNAs encoding several different immediate-early (IE) gene products (Fig. 1). These include a set of proteins traditionally designated IE1 and IE2, which denote the two major coding areas within the total IE region (Fig. 1). However, due to the alternate splicing, these areas are capable of encoding several proteins (49). The major product of the IE1 region is a 72-kDa protein, hereafter termed IEP72. The IE2 region, or parts of it, are found in several alternately spliced transcripts (49). One of these transcripts produces an 86-kDa protein; another potentially encodes a 55-kDa protein (22, 35, 46, 48). Hereafter, these will be termed IEP86 and IEP55, respectively.

The role of these proteins as modulators of transcription from both cellular and viral promoters has been examined in many reports. However, the results of these studies are quite varied. This may largely be a consequence of the use of several

different cell types in these experiments, as well as a number of different test promoters. Hence, the variations in transcriptional effects may suggest cell type differences in the response to, or utilization of, the IE proteins. In a broad overview of the literature, it is often observed that IEP86 (or the IE2 product) is a promiscuous transcriptional activator (5, 10, 11, 15, 20, 21, 29, 32–34, 36, 37, 53, 55, 59, 60). However, a few experiments have shown some promoters and/or cell types in which IEP86 failed to activate whereas IEP72 (the IE1 product) caused activation (3, 6, 8, 12, 20, 23, 33, 47). In addition, instances in which both proteins activated the same promoters independently have been reported (5, 10, 15, 20, 21, 29, 32, 33, 55, 60). Still other reports have indicated that a combination of the IE1 and IE2 gene products is capable of activating both viral and cellular promoters (13, 24, 39, 44, 45, 54). There is somewhat more agreement when IEP86 and IEP72 are supplied together: activation is at least additive and most often synergistic (10, 12, 15, 29, 33, 34, 47, 53). Synergy is often reported in cases where the test promoter is activated by IEP86 and not IEP72, when tested individually.

Less is known about IEP55, a splice variant of IEP86 (Fig. 1). It is generally observed that IEP55 expression will decrease the level of activation seen with IEP86 and IEP72 (15, 29, 47).

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FIG. 1. The major IE region of HCMV. The major IE genomic region is depicted at the top, with the coding strategies for the IEP72, IEP55, and IEP86 proteins depicted below. IEP refers to the MIEP, and IE-1 and IE-2 are the traditional names for the major IE gene regions. The start site of transcription for the precursor RNA which undergoes alternate splicing and polyadenylation (PA) to yield the various IE mRNAs is depicted by the arrow. The various exons are numbered 1 through 7. Exonic regions containing known open reading frames are shaded. The promoter is not drawn to the scale of the open reading frames.

The structure of IEP55 and its effect provide the attractive possibility that IEP55 acts in a manner similar to that of other transcription-repressing viral proteins that are alternatively spliced gene products of the proteins that they inhibit (for example, the adenovirus E1a 12S RNA product [31]). However, it is not clear whether IEP55 is expressed with other IE proteins during infection. Although its 1.7-kb mRNA is detectable during the IE period, the corresponding protein has been seen only after cycloheximide treatment of the infected cells (22, 35, 46, 48). Hence, the significance of its effect in viral infection remains speculative.

Overall, the above data suggest a pattern of activation by the individual IEP72 and IEP86 proteins that varies with conditions and promoters. Layered on this is the complexity that the effects of the individual IEP72 and IEP86 proteins can be synergistic in combination and can be downregulated by IE55. The observations (i) that IEP86 prefers specific TATA elements (4, 5, 15, 20, 21, 55) and (ii) that IEP86 can interact with the TATA-binding protein (TBP), and possibly other proteins (14, 20, 26), suggest that direct interaction with multiple components of the transcription complex may be a means of transcriptional activation.

In the following report, we analyze promoter activation in CV-1 cells to address several questions which arise from the above observations. (i) Is the promoter structure required by the IE proteins simple or complex; or is it relatively specific or quite variable? (ii) Among the promoter structures activated, does the apparent synergy between IEP86 and IEP72 always occur? (iii) To what extent do the IE proteins interact with the cellular transcription apparatus? To approach these questions, we have utilized a number of well-defined, simple, synthetic promoters (upstream factor binding sites and TATA elements) to analyze the activation mechanisms of the IE proteins. We found that IEP86 can recognize and activate a variety of simple promoters, in agreement with the prediction that it is a

promiscuous activator. However, in the comparison of otherwise identical promoters, IEP86 does have preferences for both specific TATA elements and specific upstream transcription factor binding sites. In addition, it requires both *cis* sites to activate the promoters. In CV-1 cells, none of the promoters was significantly activated by IEP72; however, each promoter activated by IEP86 was synergistically affected by the addition of IEP72. Also, under our experimental conditions, IEP55 repeatedly had a negative effect, causing a decrease in the level of activation by IEP86 and the synergy of IEP86 and IEP72.

The ability of IEP86 to activate many simple promoters and its ability to interact with TBP (14, 20, 26) are similar to our previous observations of transcriptional activation by simian virus 40 (SV40) large T antigen (16, 18, 19, 40). Specifically, T antigen activates many simple promoters and it interacts with TBP. In addition, T antigen has been shown to interact with the factors which bind to upstream promoter elements (19). Hence, there are multiple interactions with the promoter complex which appear to affect transcription through stabilization of the transcription complex (19). We show here that IEP86, but not IEP72, is quite similar in action. IEP86, in addition to binding TBP, can also interact with transcription factors which bind the upstream elements of the simple promoters it activates.

MATERIALS AND METHODS

Plasmids. A number of different plasmids were used for the *in vivo* expression of viral *trans*-acting proteins. Plasmids pIE55, pIE72, and pIE86 (gifts of Richard Stenberg) are plasmids in which the HCMV MIEP drives expression of the cDNAs encoding the HCMV IE proteins, IEP55, IEP72, and IEP86, respectively (47). In addition to these plasmids, we also used plasmids which expressed each IE protein individually but utilized a different promoter: pRSV55, pRSV72, and pRSV86, which contain the Rous sarcoma virus (RSV) long terminal repeat (LTR), were gifts of R. Stenberg (15); and pON2205 (72K) and pON2201 (86K), the gifts of E. Mocarski, express the 72- and 86-kDa proteins from the SR α promoter. Plasmid pRL43a (a gift of Gary Hayward) contains the genomic HCMV major IE region and is capable of expressing HCMV regions IE1 and IE2 from the MIEP (Fig. 1). This plasmid can potentially produce all spliced and polyadenylated forms of the IE mRNAs; hence, it can potentially express IEP55, IEP72, and IEP86 (36). pCMVTag is a plasmid in which the SV40 large T antigen is driven by the cytomegalovirus (CMV) MIEP (19). Plasmid pMSVP16 (a gift of S. McKnight) expresses the herpes simplex virus type 1 tegument transcriptional activator VP16, under the control of the murine sarcoma virus LTR.

Several plasmids contained specific promoters with attached reporter genes and were used as reporters for the effects of IE proteins on promoter activity. Among the reporter plasmids listed below, some were based on the chloramphenicol acetyltransferase (CAT) reporter gene and others were based on the β -globin reporter gene.

A group of related simple promoter constructions based on the hsp70 gene promoter were the gift of R. Kingston and have been described elsewhere (52). Briefly, each contains human hsp70 gene promoter sequences, from position -84 relative to the transcription start site, adjacent to the CAT gene (Fig. 2). This promoter region contains the hsp70 TATA element and an upstream CCAAT binding site. From this simple promoter, an array of other simple promoters was constructed through systematic replacement of the CCAAT motif at position -65 with other transcription factor binding sites (ATF, SP1, API, OCT, or a nonsense sequence [None in Fig. 2]). In addition,

	USE	TATA	SalI
<u>SacI</u>			
gagctcggtGA	<u>TTGGCT</u>	cagaagggaaagcggtctccgtgacg	<u>ACTTATAAAAG</u> gctcgac- CAT GENE
CAAT	GATTGGCT	ACTTATAAAAG	HSP70
ATF	TGACGTCA	CCTTAAGAGTC	Ad E2a
SP1	GGGGCGGGGC	TTTTATTTATG	SV40 EARLY
AP1	TGAGTCAG	TAGGCAGTCCA	NO TATA (None)
OCTA	ATTTCAT	CCCTCANTCT	INITIATOR (INR)
NONE	TAGGCAGT		

FIG. 2. Simple synthetic promoters. The simple promoters used to analyze promoter preference for the IE proteins were derived from the hsp70 promoter, which contains a CAAT upstream element (USE) 29 nt upstream of a TATA box (underlined sequences). The five upstream elements substituted individually for the hsp70 CAAT element and the three TATA boxes, initiator element (INR), and nonsense element (None) substituted individually for the hsp70 TATA element are listed. All combinations of the upstream and downstream elements were utilized (see the text for details). Ad E2a, adenovirus E2a.

for each of these upstream sites, different TATA elements (adenovirus E2, SV40 early, or a nonsense sequence [No TATA in Fig. 2]) were inserted in place of the hsp70 TATA element. A set of previously described TATA-less simple promoters were also used (16); for each upstream binding site, the TATA element was replaced with the sequence of the initiator element from the murine terminal deoxynucleotidyl-transferase gene (43).

Plasmids pβ6xB20 and its mutant derivatives dpm 2 (Sph⁻), dpm 7 (Oct⁻), and dpm 8 (Oct⁻ Sph⁻) contain six tandem copies of the wild-type or mutant SV40 late promoter region between bases 200 to 219, upstream of the human β-globin TATA box and gene, as previously described (51). All of these plasmids express β-globin mRNA as the reporter gene product. Plasmid pSP6β350 (51) was used to generate the antisense β-globin RNA used to detect β-globin mRNA in RNase protection assays (described below).

Fusions of the HCMV IE proteins were made with the glutathione binding site of glutathione S-transferase (GST). These GST fusion plasmids are pGEX-3xIE55, pGEX-3xIE72, and pGEX-3xIE86, encoding the fusions with IEP55, IEP72, and IEP86, respectively. They were constructed by PCR subcloning, with the pIE series of plasmids (described above) as templates. The PCR primers introduced single 5' and 3' terminal restriction sites (*Bam*HI and *Eco*RI, respectively) to the coding region of each full-length CMV cDNA to allow in-frame insertion into the plasmid pGEX-3X (Pharmacia). The sequences of the primers used (with restriction sites underlined and a base changed from the IE protein sequences in bold) were as follows: 5' primer for pGEX3x-IE55, -IE72, and -IE86, **CGCGGATCCTGGAGTCCTCTGCC**; 3' primer for GEX3x-IE55 and -IE86, **CGCAATTCTGAGACTTGTTCCTC**; and 3' primer for GEX3x-IE72, **CGCAATTCTGTCTAGCCTTGCTTC**.

Several plasmids were utilized for the in vitro transcription and translation of various proteins. Plasmid pETHIID directs the synthesis of human TBP and was a gift from Arnold Berk (27). Plasmid pXJ40Tef1A directs the synthesis of human Tef-1 protein and was a gift from Pierre Chambon (57). pBS-Oct 1 directs the synthesis of human Oct-1 protein (50). pBS-Sp1-F1 directs the synthesis of the full-length Sp1 protein and was a gift of Robert Tjian.

Cell culture and transfection. The African green monkey kidney cell line CV-1 was propagated and maintained in Dulbecco's minimal essential medium supplemented with 5%

fetal bovine serum at 37°C in 5% CO₂. Low-passage cells were plated at approximately 6 × 10⁵ cells per 100-mm-diameter plate for RNA analyses or 2 × 10⁵ cells per 60-mm-diameter plate for CAT analyses and grown overnight. Monolayers at approximately 70% confluence were transfected with 12 μg of DNA for RNA analyses or 10 μg of DNA for CAT analyses (plasmid pXJ40 E/B [19, 57] was used as a filler to equalize, when necessary, the total DNA used per transfection). Transfections were done by the calcium phosphate precipitation procedure using the *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) modification (7). The calcium phosphate precipitate was left on the cells for 17 to 20 h, and the cells were then shocked with 15% glycerol (in BES-buffered saline) for 1 min before fresh medium was added. For RNA preparation, cells were harvested 24 h after glycerol shock, as described below. For CAT protein preparation, the cells were harvested 48 h after glycerol shock, as previously described (28). Extract protein concentrations were determined by the Bradford method (Bio-Rad). It should be noted that although the presented experiments used CV-1 cells, many of the experiments discussed below have also been done with the human T-cell line Jurkat. The results of these experiments are essentially identical to the data obtained with CV-1. CV-1 cells are utilized because of their adherent nature and ease of transfection.

Many transfection studies are internally standardized for transfection variations through the use of a second reporter gene on an independent promoter. In the case of transcriptional activation analyses using IEP86, T antigen, and other viral activators, this is a somewhat ineffectual undertaking since these activators exert some level of activation on most promoters (1). We have found that IEP86 and T antigen activated all of the normal promoters utilized for standardization purposes. Hence, these promoters are useless as internal controls. Therefore, we have used the approach of multiple repeats of experiments utilizing different cells and different DNA preparations in order to compensate for standard transfection variations. Side-by-side comparisons of data derived with internal standardization and data derived from multiple experiments are essentially identical, with comparable standard deviations (data not shown). In addition, many of the causes of transfection variability can be eliminated by careful preparation of cells and samples as described above. The transfection data presented herein are representative of at least three separate experiments in the case of CAT analyses, and the RNase protection data represent repeatable experiments. Additional discussion of our transfection methods can be found in reference 19.

RNA preparation and analysis. Total cellular RNA was prepared by the guanidinium thiocyanate method (58), and DNA was removed by treatment with RNase-free DNase (Boehringer Mannheim).

RNase protection was performed as previously described (2, 19). Plasmid pSP6β350 was used to generate a ³²P-labelled 350-nucleotide (nt) antisense β-globin RNA probe, which anneals to the second exon of the 700-nt β-globin RNA produced in transfection experiments using pβ6xB20 or its mutants. Properly initiated reporter RNA is represented by a 350-nt protected RNA after RNase protection analysis. Protected RNA was visualized by gel electrophoresis using a 5% polyacrylamide-urea gel. Some RNase protections with the β-globin probe yielded a second, shorter band, so that a doublet was produced. This is due to a nuclease-hypersensitive site within the hybrid, which is often recognized by the RNases during digestion, producing the smaller of the two bands (19).

CAT assays. CAT enzyme activity in transfected CV-1 cells

was determined by the method described by Gorman et al. (17). Equivalent amounts of extract protein were assayed, and the results were quantitated with a Phosphorimager (Molecular Dynamics).

In vitro transcription and translation. ^{35}S -labelled proteins for use in fusion protein binding assays were generated by the concerted in vitro transcription-translation system (Promega). Sixty microcuries of [^{35}S]methionine (Amersham; >1,000 Ci/mmol) were included in each reaction, and incorporation was measured by trichloroacetic acid precipitation.

Expression and purification of GST fusion proteins. The various pGEX-3x plasmids were transformed into *Escherichia coli* HB101, Y1090, or DH5 α . The specific strain used was determined by which strain yielded the largest amounts of intact fusion protein. The cells were induced, and fusion proteins were purified through binding to glutathione-agarose beads (Pharmacia) as previously described (19, 42).

Fusion protein binding assays. Fusion proteins bound to glutathione-agarose beads were washed with NETN+ (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*- α -tosyl-L-lysine chloromethyl ketone [TLCK]) three times to remove non-GST proteins. The bound proteins were subsequently incubated with 3% bovine serum albumin in 1 ml of NETN+ for 30 min at 4°C with rocking to block subsequent nonspecific protein binding to the beads. Under normal conditions, there is a much higher level of expression of the GST binding site alone than the fusion proteins. In order to compensate for this higher level of expression in binding assays, the volume of bacterial supernatant bound to beads was adjusted according to yields quantitated by silver staining of polyacrylamide gels. To each fusion protein bound to beads, 10^5 trichloroacetic acid-precipitable cpm of in vitro-produced protein was added in 1 ml of NETN+. This mixture was incubated at 4°C for 1 h with rocking and was then washed five times with 1 ml of NETN+ containing NaCl at each of various concentrations between 100 and 1,000 mM. The recovery and analysis of the bound ^{35}S -labelled proteins were performed by boiling in sodium dodecyl sulfate (SDS) sample buffer and electrophoresis on 10% polyacrylamide-SDS gels. Input protein lanes on the gels each contained 2×10^4 cpm of the proteins tested. Autoradiographic visualization of proteins was performed after treatment of gels with Amplify (Amersham). Quantitation was performed with a Phosphorimager (Molecular Dynamics).

RESULTS

trans activation of simple promoters by the HCMV IE proteins. In order to determine the simplicity and degree of variability of promoter structures utilized by the IE proteins, we asked whether the IE proteins could activate transcription from the series of simple promoters, diagrammed in Fig. 2, all operating the CAT reporter gene. These promoters are based on the hsp70 minimal promoter (16, 52). The hsp70 TATA element has been replaced with either the adenovirus E2a TATA, the SV40 early promoter TATA, the terminal deoxynucleotidyltransferase initiator element (INR) (5, 24, 49), or a nonsense sequence (No TATA). Each of these elements was paired with a variety of upstream stimulatory elements (USES) at which known transcription factors can bind. These include either a CAAT (from the original hsp70 promoter), ATF, SP1, AP1, or octamer site or a nonsense sequence (None). Adenovirus E1a and SV40 T antigen, both promiscuous viral activators, have been shown to activate transcription

FIG. 3. Preference for specific TATA elements by the HCMV IE proteins. Plasmid pRL43a was cotransfected into CV-1 cells with the five simple promoters shown in Fig. 2 containing an SP1 upstream element with the five different downstream elements: hsp70 TATA (HSP70), adenovirus E2a TATA (Ad E2a), SV40 early TATA (SV40 E), the terminal deoxynucleotidyltransferase initiator element (INR) or a nonsense sequence (None). Each promoter test plasmid contains the CAT reporter gene. The fold activation data are based on CAT activities measured in at least three separate experiments. The standard deviations of these data are indicated by the shaded area on top of each bar. Basal activity for each promoter was approximately 1% acetylation in a standard assay at 37°C.

from many of these simple promoters (16, 40, 52; also see further discussion below).

In activating these simple promoters, both E1a and T antigen show preferences for specific TATA elements (16, 40, 52). (hsp70 TATA > adenovirus E2a TATA, with little activation from the SV40 early TATA or the nonsense element). Figure 3 shows the results of experiments to test TATA preference using the CMV IE proteins. SP1 was used as the USE with each TATA element, the initiator element, and no TATA (None). SP1 was chosen as the test USE because previous studies using these promoter constructions showed that an SP1 site was a USE preferred by SV40 T antigen (16, 40). In addition, mutations of the SP1 sites in the human immunodeficiency virus LTR had significant effects on activation by the HCMV IE proteins (data not shown).

Plasmid pRL43a, which is potentially able to produce all of the IE proteins (see Materials and Methods), was cotransfected into CV-1 cells along with the simple promoter reporter plasmids. As shown in Fig. 3, the SP1/hsp70 and SP1/E2a promoters displayed 10- and 7-fold increases, respectively, in CAT activity over basal promoter levels (basal activities for these simple promoters are equivalent and given the arbitrary value of 1). However, the SP1/SV40E, SP1/initiator, and SP1/nonsense constructs displayed no significant activation over their basal activities. In the case of the initiator, the inability to activate is not due to a failure of the SP1/initiator promoter to function since SV40 T antigen can activate this promoter (16).

To determine the effect of varying the USE on transcriptional activation by the CMV IE proteins, pRL43a was cotransfected with the simple promoter reporter plasmids in which various USEs, or the nonsense sequence, were linked to the hsp70 TATA (Fig. 2). The hsp70 TATA element was used because of its apparent preference in the preceding experiment. As shown in Fig. 4 (IE region panel), expression of the IE region with CCAAT/hsp70, SP1/hsp70, and ATF/hsp70 caused 14-, 9-, and 4-fold increases, respectively, in CAT

FIG. 4. Preference for specific upstream elements by the HCMV IE proteins. Plasmids which produced the individual IE proteins (IEP86 and IEP72) or the genomic IE region were cotransfected into CV-1 cells with the six simple promoters consisting of the six individual USEs (CAAT, ATF, SP1, AP1, OCT, and nonsense [NONE]) with the hsp70 TATA element (Fig. 2). Each promoter test plasmid contains the CAT reporter gene. The fold activation data are based on CAT activities measured in at least three separate experiments. The standard deviations of these data are indicated by the open area on top of each bar. Basal activity for each promoter was approximately 1% acetylation in a standard assay at 37°C. The plasmids producing the IE proteins utilized the HCMV MIEP; however, similar results were obtained from experiments where the IE protein cDNAs were transcribed from the promoter of the RSV LTR or from the SR α promoter (not shown).

activity above each promoter's basal activity level. In contrast, AP1/hsp70, OCTA/hsp70, and None/hsp70 displayed no significant increases in the presence of the IE proteins. The non-sense sequence experiment indicated that a TATA element alone cannot be activated.

Activation of simple promoters by IE proteins individually and in combination. We next asked what effects individual IE proteins or specific combinations of IE proteins would have on the activity of the simple promoters. Expression plasmids pIE55kd, pIE72kd, and pIE86kd, which express the indicated individual IE proteins (as indicated in Materials and Methods) were cotransfected individually or in combinations with the simple promoter plasmids containing the hsp70 TATA element. Whenever the entire IE region (CCAAT/hsp70, SP1/hsp70, and ATF/hsp70) showed activation, similar activation was observed with IEP86 alone (11-, 5.8-, and 3-fold, respectively; Fig. 4, IEP86 panel). Expression of IEP72 alone caused no activation (IEP72 panel). However, the addition of both IEP86 and IEP72 (IEP72 and IEP86 panel) produced a synergistic effect equivalent to an approximate 3.7-fold increase over the activity of each promoter in the presence of IEP86 alone. Even the promoters which showed little or no activation by IEP86 alone, AP1/hsp70 and OCTA/hsp70, had increased activity in the presence of IEP86 and IEP72. The effect of IEP55 was also determined in cotransfection experiments. IEP55 alone had no effect on promoter activity; however, for the promoters activated by IEP86 and synergized by

IEP86 plus IEP72, the addition of IEP55 decreased activity by two- to sixfold, depending on the promoter (data not shown).

Overall, these results indicate that (i) a very simple promoter structure can be activated by the IEP86 protein, (ii) this activation is synergized by IEP72 and repressed by IEP55, (iii) both a TATA element and a USE are required for activation, and (iv) there is a specific preference for particular TATA elements and USEs; this preference is very similar to that seen with SV40 T antigen (16, 40; see also the section on analogies below).

A very important consideration regarding the above data comes from the suggestion that one function of IEP72 is the specific activation of the CMV MIEP (3, 8, 41, 47). Some of the plasmids used in the experiments described herein expressed the IE proteins from this promoter. Hence, it was possible that the synergy noted with IEP72 and IEP86 was simply due to increased production of IEP86 due to activation of the CMV IE promoter. For this reason, we have repeated the experiments presented with vectors which express the IE proteins from different promoters (the RSV LTR and the SR α promoter; see Materials and Methods). The overall trends in activation were identical regardless of the promoter expressing the IE proteins (data not shown). This is in agreement with previous results (15), in which IE protein expression from the promoter of the RSV LTR resulted in synergistic activation of the human immunodeficiency virus type 1 LTR.

It should be noted that although the experiments presented were done with CV-1 cells, all of the experiments whose results are shown in Fig. 4 and some of the experiments discussed below have also been done in the human T-cell line Jurkat. The results of these experiments are essentially identical to the data obtained for CV-1 cells.

Analogies between activation by CMV IE proteins and SV40 T antigen. The spectrum of promoters activated by IEP86 and SV40 T antigen are quite similar. The preference for TATA elements is the same, whereas USE preferences, although similar, show characteristics unique to each protein (CMV: CAAT > SP1 > ATF and shows no activation with AP1 and OCT; T antigen: SP1 > ATF > AP1 and shows no activation with CAAT and OCT [16, 40]). SV40 T antigen also prefers to utilize promoters containing Tef-1 sites (19). For example, the USE in the SV40 late promoter (Fig. 5A) needed for T antigen-mediated transcriptional activation is a region containing two Tef-1 elements overlapped by an octamer element, known as the Oct/Tef element (OTE; Fig. 5B). Within the OTE, it is the Tef-1 sites which are required for activation by T antigen (19). In addition, a simple promoter containing the β -globin TATA element and six upstream OTE motifs (Fig. 5C, OTE-TATA promoter) is substantially activated by T antigen (19). This activation was shown to be due to the ability of T antigen to make multiple contacts with the promoter complex through interactions with both the Tef-1 protein and factors of the basal transcription complex, most prominently TBP (19). The CMV 86-kDa protein has previously been shown to interact with TBP (14, 20, 26). This finding, coupled with the requirement for an upstream element to activate simple promoters, suggests that IEP86, like SV40 T antigen, may also be able to interact with multiple components of the transcription complex. To approach this question, we first determined that the IE proteins activated the SV40 late promoter driving the CAT gene in CV-1 cells: IEP86 alone activated (18- $\pm$ 3-fold), IEP72 alone produced a modest activation (3- $\pm$ 1-fold), and together IEP72 and IEP86 synergized in activation (68- $\pm$ 7-fold).

Knowing that the late promoter was activated, we next utilized the simple OTE-TATA promoter (Fig. 5C) to deter-

FIG. 5. SV40 late promoter and OTE. (A) Promoter region of SV40. The elements of the overlapping early and late promoters as well as the origin of replication (ORI) are indicated. The elements defined for early promoter activity, the 21-bp repeat region (21s) and the 72-bp repeat enhancers (72s), are indicated. The T-antigen-responsive late promoter elements, the OTE and the initiator region (IR) are shown. Late transcripts start at multiple sites in the IR, as depicted by the arrows, with the thickness of the arrow representing the relative frequency of use. SV40 nucleotide (nucs) numbering is used. (B) Sequence of the OTE. The 19-nt OTE between SV40 nt 199 and 218 within the second 72-bp repeat is shown. The positions of the overlapping octamer (Oct) and Tef-1 protein binding sites are shown above the sequence. Below the wild-type sequence are the sequences of three mutant derivatives of the OTE in which mutations (underlined sequences) destroy the octamer binding site (Oct⁻ or -O), the Tef-1 binding sites (Tef⁻ or -T), and both the Tef-1 and octamer binding sites (Tef⁻ Oct⁻ or -T, -O). (C) Simple OTE-TATA promoter. The simple promoter contains six tandem copies of either the wild-type OTE sequence or the several mutant OTE sequences linked 36 nt upstream of the human β -globin TATA box and reporter gene.

mine whether the IE proteins were activated through the Tef-1 sites similarly to T antigen. Figure 6 shows quantitative nucleic acid protection analysis of the β -globin RNA produced in CV-1 cells from the OTE-TATA promoter or its mutants (Fig. 5B and C). The wild-type promoter alone (Fig. 6, lane WT Basal) has a very low basal activity. However, cotransfection of pRL43a caused a marked increase in β -globin RNA (Fig. 6, lane WT + IE). To differentiate between utilization of the Tef-1 sites and the OCT site within the OTE, mutant simple promoters (51) which were constructed identically except for specific point mutations which disrupt the function of either the OCT site (Oct⁻, -O), the Tef sites (Tef⁻, -T), or both (Oct⁻ Tef⁻, -T,-O) (Fig. 5B [51]) were used. Figure 6 shows that mutation of the Tef-1 sites eliminated β -globin RNA production in the presence of the IE proteins (lane -T+IE). However, mutation of the OCT site did not affect β -globin

FIG. 6. RNase protection analysis of β -globin mRNA produced from wild-type and mutant OTE promoters in the presence of IE proteins or herpes simplex virus VP16. Plasmid pRL43a or pMSVP16 was cotransfected, separately, in CV-1 cells along with the wild-type or mutant OTE promoter plasmid. Total cellular RNA was analyzed by RNase protection assay (see Materials and Methods), the 350-nt protected fragment is indicated by the arrow, and the source of the doublet is explained in Results and Materials and Methods. Lane 1: wild-type (WT) OTE promoter basal activity (pXJ40 filler plasmid used). Lanes 2 to 4: pRL43a (+IE) cotransfected with either the WT, Tef⁻ Oct⁻ (-T, -O), Tef⁻ (-T), or Oct⁻ (-O) OTE simple promoter reporter plasmids, respectively. Lanes 5 to 7: pMSVP16 cotransfected with either the WT, Oct⁻ (-O), or Tef⁻ (-T) OTE simple promoter reporter plasmids, respectively.

RNA production in the presence of the IE proteins (lane -O+IE). Mutation of each site, expectedly, resulted in no RNA production (lane -T, -O+IE). We used the herpes simplex virus transcriptional activator VP16 to ensure that the mutation of the Tef-1 sites did not affect the OCT element function and to show that the OCT mutations eliminated OCT function. Figure 6 shows that the Tef-minus promoter is activated by VP16 (lane -T+VP16), whereas the OCT-minus mutant promoter is not (lane -O+VP16). The doublet seen in some lanes has previously been shown to be a nuclease artifact and not the utilization of a new start site (19) (see Materials and Methods). These data show that the IE proteins can utilize a Tef-1 site but not an OCT site as a USE. This result is in agreement with the simple promoter data obtained by using the OCT/hsp70 TATA promoter (Fig. 4).

In the gel in Fig. 7, the OTE-TATA promoter reporter plasmid was cotransfected with the plasmids which produce the individual IE proteins. As we have seen in previous experiments, IEP86 stimulated β -globin RNA synthesis, IEP72 had no effect, and the combination of IEP72 and IEP86 had a synergistic effect on promoter activity. These data provide one more example of a simple promoter structure which is activated by the IE proteins and large T antigen.

IEP86 interacts not only with components of the basal transcription complex but also with the upstream bound factor. The simplicity and similarity of the promoters activated by IEP86 and SV40 large T antigen suggested that they may utilize a common mechanism for transcriptional activation. It has previously been shown that T antigen interacts with both components of the basal transcription complex and with the factors bound upstream (19). Each of these interactions appears to be necessary for activation to occur. Therefore, we prepared GST fusions of the IE proteins to determine whether similar binding occurred with the IE proteins. Using *in vitro* transcription and translation, we prepared [³⁵S]Met-labelled

FIG. 7. RNase protection analysis of β -globin mRNA produced from the wild-type OTE promoter in the presence of the individual IE proteins. The wild-type OTE promoter plasmid was cotransfected into CV-1 cells with the pXJ40 filler plasmid (Basal) or with pIE72 (72kDa), pIE86 (86kDa), or pIE72 and pIE86 together (72+86kDa). RNase protection assays were performed as described in the Fig. 6 legend and Materials and Methods.

TBP, Tef-1, SP1, and Oct-1 to test for binding to GST fusions of IEP86 and IEP55. TBP has previously been shown to interact with IEP86 (14, 20, 26) and is a positive control for binding; Tef-1 and SP1 might be expected to bind IEP86, since promoters containing their binding sites were well activated (Fig. 4 and 5); and Oct-1 may be expected not to bind IEP86, since promoters containing its binding site were not activated (Fig. 4 and 5).

Figure 8A shows that the glutathione binding site alone (Gst) does not bind any of the proteins. In agreement with previous data, the Gst-IE86 fusion binds to TBP; we extend this observation by showing that Gst-IE55 also binds to TBP. In addition, the data show that both Gst-IE55 and Gst-IE86 bind to Tef-1 but not to Oct-1, in agreement with the above predictions based on the promoters affected by the IE proteins (previous data from this laboratory indicated that the Oct-1 protein made in these experiments can interact with appropriate proteins [19]). In further agreement with the predictions, Fig. 8B shows that Gst-IE86 binds to SP1 in a manner similar to transcription factor YY1, previously shown to interact with SP1 (42).

In Fig. 9, the binding of IEP86 and IEP55 with TBP and Tef-1 was tested by washing the complexes in increasing salt concentrations. Binding with TBP is relatively stable up to 300 mM NaCl; however, a moderate amount of binding continues after a washing in 500 mM NaCl (especially in the case of IEP55). This indicates that the interaction is primarily ionic but may also have some nonionic nature. The binding of both proteins to Tef-1 is relatively unaffected by increasing the NaCl concentration, suggesting that the interaction is primarily nonionic.

Many of the binding experiments discussed above have been repeated with the status of the proteins reversed, e.g., a GST fusion with TBP or Tef-1 and an in vitro-transcribed and -translated IEP86. All of these data (not shown) agreed with the results presented above. We have also tested a Gst-IE72

FIG. 8. Binding of IEP86 and IEP55 to cellular transcription factors. (A) GST fusion proteins of the IEP55 (Gst-IE55) and IEP86 (Gst-IE86) or the GST binding site alone (Gst) were assayed for binding to in vitro-transcribed and -translated (IVT/T) forms of the cellular transcription factors: Oct-1 (Oct), Tef-1 (Tef), and TBP. See Materials and Methods for details. The electrophoretic migration positions of the 35 S-labelled IVT/T proteins are indicated in the input lanes, which contain one-fifth of the amount of the IVT/T proteins used in each binding reaction. (B) Gst-IE86, Gst-YY1, or the GST binding site alone was assayed for binding to IVT/T Sp1. The position of the electrophoretic migration of Sp1 is indicated in the input lane, as described for panel A.

fusion and found that it binds none of the above proteins, nor does it interact directly with IEP86 (data not shown).

DISCUSSION

We have utilized a well-characterized transfection system to examine the transcriptional activation functions of the CMV IE proteins. Our data show that the promoter structure required for IE protein activation is very simple and can be quite variable, consisting of a TATA element and one of several upstream binding sites for transcription factors. Both of these elements, the TATA box and the USE, are essential for activation, which suggested the possibility of multiple interactions with components of the transcription complex, a situation we have previously studied in relationship to SV40 T antigen (16, 18, 19).

Further studies using the simple promoters with individual IE proteins showed that promoter activation was caused by IEP86 alone and not by IEP72 alone. However, IEP72 does affect promoter activity in concert with IEP86. Under these conditions, we have seen a synergy of effect resulting in a 3.5- to 5-fold increase in promoter activity over that provided by IEP86 alone. As mentioned in the introduction, these results

FIG. 9. Salt washes of Gst-IE55 or Gst-IE86 in complex with Tef-1 or TBP. 35 S-labelled TBP or Tef-1 prepared by *in vitro* transcription and translation were bound to Gst-IE55 or Gst-IE86 in NETN+ (100 mM NaCl). Complexes were then washed five times with NETN+ containing the indicated concentration of NaCl. Protein remaining in the complexes was electrophoretically analyzed.

agree with several previous reports but contrast with others, possibly because of differences in the cell types used in the various experiments.

The synergy caused by IEP72 is seen with all promoters activated by IEP86 alone. In fact, this effect of IEP72 appears to be tailored for IEP86. In a comparison of simple promoters activated by both IEP86 and SV40 T antigen, the synergy of activation by IEP72 occurred only with IEP86 and has never been observed with SV40 T antigen (data not shown).

The above data suggest intimate interactions between IEP86 and the factors bound to the elements of the simple promoters which it activates. We provide evidence that this is indeed possible. We verify the interaction of IEP86 with TBP (14, 20, 26). However, we also show that IEP86 interacts with the transcription factors which bind to USEs of promoters it activates (e.g., Tef-1 and SP1) and does not interact with transcription factors which interact with USEs of promoters it is unable to activate (e.g., Oct-1). Overall, these data suggest that transcriptional activation by IEP86 is mediated through multiple interactions with the promoter complex, i.e., interactions with the basal transcription complex (e.g., TBP) and interactions with the upstream bound factors (e.g., Tef-1 and SP1). The requirement for both of these interactions is indicated by the fact that IEP86 activates neither a promoter containing only an hsp70 TATA element nor one which contains only an upstream factor binding site (Fig. 3 and 4).

Interactions between IEP86 and factors in the transcription complex indicate that HCMV has evolved a transcriptional activation mechanism which is shared by other DNA viruses. The early, IE, or transactivator proteins of the papovaviruses, adenoviruses, and other herpesviruses have been shown to act, at least in part, through protein-protein interactions with factors involved in transcription. However, evidence also suggests that IEP86 binds to specific DNA sequences within the HCMV MIEP. This interaction appears to negatively regulate the MIEP and provide an autoregulatory function (25, 30, 56). This dual functioning of IEP86, seen by comparing DNA binding and protein-protein interactions, is similar to the known transcriptional effects of SV40 large T antigen. It has been well documented that T antigen autoregulates its own synthesis by binding to specific sequences within the SV40 early promoter (38), whereas it activates transcription of many promoters through interactions with factors of the transcription complex (16, 18, 19, 40).

In contrast to the effect of IEP86, its splice variant IEP55 reduced the level of activation caused by IEP86 or IEP86 and IEP72 together. This is intriguing, since we have shown that IEP55 interacts with TBP and Tef-1. Hence, IEP55 appears to maintain all of the binding domains of IEP86. This would suggest that the unique central region of IEP86 (Fig. 1) may provide the ability to form an activation domain or a domain for yet other critical protein-protein interactions. This region of IEP86 has been shown to be important for dimerization and DNA binding to the MIEP (9, 14, 25, 56). We speculate that the negative effect of IEP55 may be due to competition with IEP86 for binding to cellular proteins in the promoter complex.

Overall, our data provide a model for both positive and negative transcriptional control mechanisms mediated by IEP86 and IEP55, respectively. This model can account for the promiscuous activation of promoters by IEP86. However, under our experimental conditions, the mechanism of synergy mediated by IEP72 remains unknown. The lack of evidence for direct interactions between IEP72 and other transcription factors, or with IEP86, suggests a more indirect means of action.

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