Transactivation of a Human Cytomegalovirus Early Promoter by Gene Products from the Immediate-Early Gene IE2 and Augmentation by IE1: Mutational Analysis of the Viral Proteins

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Expression from a human cytomegalovirus early promoter (E1.7) has been shown to be activated *in trans* by the IE2 gene products (C.-P. Chang, C. L. Malone, and M. F. Stinski, *J. Virol.* 63:281–290, 1989). Using wild-type and mutant viral proteins, we have defined the protein regions required for transactivation of the E1.7 promoter in IE2 and for augmentation of transactivation in the IE1 protein. Two regions of the IE2 proteins were found to be essential for transactivation. One near the amino terminus is within 52 amino acids encoded by exon 3. The second comprises the carboxyl-terminal 85 amino acids encoded by exon 5. The IE2 protein encoded by an mRNA which lacks the intron within exon 5 and the IE2 protein encoded by exon 5 had no activity for transactivation of the E1.7 promoter. Although the IE1 gene product alone had no effect on this early viral promoter, maximal early promoter activity was detected when both IE1 and IE2 gene products were present. The IE1 protein positively regulated its enhancer-containing promoter-regulatory region. The IE1 protein alone increased the steady-state level of IE2 mRNA; therefore, IE1 and IE2 are synergistic for expression from the E1.7 promoter. Like the IE2 proteins, the IE1 protein requires for activity 52 amino acids encoded by exon 3. IE1 also requires amino acids encoded by exon 4. Since the IE1 and IE2 proteins have 85 amino acids in common at the amino-terminal end encoded by exons 2 and 3, the difference between these specific transactivators resides in their carboxyl-terminal amino acids encoded by exons 4 and 5, respectively.

Human cytomegalovirus (HCMV) is a ubiquitous virus that causes serious infections in neonates as well as in immunocompromised adults. The virus contains a large double-stranded DNA genome of approximately 150×10^6 daltons or 230 kilobase pairs (8, 11, 24, 37). Transcription of the viral genome occurs in three sequential phases designated immediate early (IE), early, and late (7, 26, 43). IE transcription does not require prior viral protein synthesis. Early transcription requires IE viral protein synthesis and occurs before viral DNA replication. Many HCMV structural genes are transcribed at early as well as at late times after infection (4, 10, 25, 43). The sequential and regulated pattern of HCMV protein synthesis is influenced at both the transcriptional and the posttranscriptional levels (10, 16).

The viral chromosome has an approximately 800-base-pair region upstream of the major IE gene (IE1) that can strongly bind host cell transcription factors (12–14). This region is referred to as the major IE enhancer-containing promoter-regulatory region (3, 41; M. F. Stinski, C. L. Malone, T. W. Hermiston, and B. Liu, in E. K. Wagner, ed., *Herpesvirus Transcription and Its Regulation*, in press). Downstream of the major IE promoter-regulatory region are the IE1 (0.739 to 0.755 map units) and IE2 (0.732 to 0.739 map units) transcription units (39). IE1 codes for a 1.9-kilobase (kb) mRNA consisting of four exons, with the translation initiation codon in exon 2. IE2 codes for a family of mRNAs ranging in size from 2.25 to 1.4 kb (35, 39). The exons in IE2 can be linked to upstream exons 1, 2, and 3 in IE1 by alternate splicing events (35). Therefore, the IE2 gene codes for a family of gene products, some of which share the amino-terminal 85 amino acids coded for by exons 2 and 3 of IE1. The IE2 mRNAs can also differ at their 5' ends and by splicing events within the IE2 transcription unit (32). The

IE2 gene can be transcribed as a single exon, as in the 2.25-kb mRNA, or as two separate exons with an intervening sequence spliced out, as in the 1.7-kb mRNA. Whether IE promoters exist within the IE2 transcription unit is presently not known.

The IE1 protein consists of 491 amino acids, corresponding to 64 kilodaltons (kDa) (1, 34). The viral protein is heavily phosphorylated and moderately acidic, with a region rich in glutamic and aspartic amino acid residues positioned near the carboxyl-terminal end. These properties plus its proline amino acid content may explain its reported molecular mass of 68 to 72 kDa (2, 15, 27, 36). Several IE2 proteins ranging in molecular mass from 82 to 28 kDa have been identified by using antibodies made to synthetic peptides based on the IE2 amino acid sequence (19, 32). The largest IE2 protein consists of 579 amino acids (32, 35). The predominant IE2 protein of 82 kDa and a minor protein of approximately 54 kDa are phosphorylated. However, a smaller IE2 protein of 28 kDa is not phosphorylated (19). Another IE2 protein of 52 kDa, which is low in abundance, is discussed in this report.

One or more of the IE2 proteins can act *in trans* to enhance gene expression from either heterologous (6, 19, 29, 40) or homologous (4) inducible promoters. In contrast, the IE1 protein has little to no effect on heterologous inducible promoters (19, 29). Although the IE2 gene products can independently activate these inducible promoters, the presence of the IE1 gene product significantly augments the level of downstream expression (19, 40). Therefore, maximum stimulation of either heterologous or homologous inducible promoters occurs when both the IE1 and the IE2 genes are expressed (9, 19, 29, 31, 40). Two mechanisms have been proposed to explain these observations (19). (i) The IE1 and IE2 proteins act together at the E1.7 promoter to enhance downstream expression or (ii) the IE1 protein increases the

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level of IE2 protein expression at either the transcriptional or the posttranscriptional level and, consequently, increases the level of IE2 proteins. Using wild-type and mutant viral proteins, we have defined the IE2 protein regions required for transactivation of a homologous early promoter designated E1.7. We also define the IE1 protein regions required to positively regulate the major IE promoter and thereby increase the steady-state level of IE2 mRNA and ultimately the level of IE2 expression.

MATERIALS AND METHODS

Virus and tissue culture. Culturing of human foreskin fibroblast (HFF) cells and growth of HCMV (Towne strain) were done as previously described (36). COS-1 cells were cultured as described previously (33).

Plasmid constructions. Restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.), Klenow DNA polymerase and T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and T4 RNA ligase (New England BioLabs) were used according to manufacturer specifications. Plasmid DNAs were purified as described previously (42).

Plasmids pCC1 and pCC2 have been described previously (19, 33). Plasmid pCC1 (IE1) contains the IE promoter-regulatory region and the coding region of the IE1 gene followed by a polyadenylation signal, as found in the viral genome. Plasmid pCC2 (IE1) contains the same promoter-regulatory region and gene but in the opposite orientation relative to a simian virus 40 replication origin. In-frame deletions or carboxyl-terminal truncations were made (see Fig. 5 for details and for the number of amino acids deleted). Deletions *dIAva*, *dIBclI*, and *dIOxaNI* were constructed from pCC1. Plasmid pCC2 was the parent plasmid for deletions *dIE24* and *dIS12*. Plasmid pSVIE2 was previously designated pCSdlAcc (19). It lacks exon 4 of IE1 because of an *AccI*-*AccI* deletion, as described previously (4, 19). (See Fig. 2 for mutations introduced into pSVIE2 and the number of amino acids deleted. Amino acids beyond the mutation and coded for by a different open reading frame [ORF] are shown in parentheses.) Plasmid pCS (IE1 and IE2) was described previously (19). All of the above-mentioned plasmids have the simian virus 40 origin for DNA replication in T-antigen-containing COS-1 cells.

In-frame deletion plasmid pSVIE2dIPX was constructed by digestion with *PstI* and *XhoI*, creation of blunt ends with the Klenow polymerase, and ligation. Indicator plasmid pE1.7CAT contains a 2.2-kb *NaeI* fragment which includes the late promoter (P_L) and the early promoter (P_{E1}) of the E1.7 promoter (4) inserted into the *HindIII* site of pSVOCAT with *HindIII* linkers. A similar plasmid containing only P_{E1} of the E1.7 promoter was also used (see Fig. 1). No difference in transfection experiments was detected with plasmids containing P_L and P_{E1} or just P_{E1} alone.

Details of the construction of pCAT760 have been described previously (38). Sequences -760 to +7 of the IE promoter-regulatory region were inserted at the *HindIII* site of pSVOCAT and influenced the level of expression of the downstream *cat* gene as described previously (38).

Transfections. Transfections of HFF cells were performed on duplicate 100-mm plates. Transfections were done as described previously (4, 19), with the following exceptions: $2 \times N$ -2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid (HEPES)-buffered saline was pH 7.05, and 250 mM $CaCl_2$ was made fresh. The amounts of plasmid DNAs

added to transfection mixtures are noted in the figure legends. Equimolar DNA concentrations were determined by restriction endonuclease digestion, gel electrophoresis, and comparison of ethidium bromide-stained DNA fragments. Cells were harvested 48 h after transfection.

COS-1 cells were transfected as described previously (33). After 24 h, the cells were washed with saline and overlaid with 5 ml of low-methionine medium containing 2% dialyzed newborn bovine serum and 50 μ Ci of [35 S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml. Cells were harvested 48 h after transfection.

Immunoprecipitation and denaturing gel electrophoresis. Cell pellets were solubilized in RIPA buffer (phosphate-buffered saline [pH 7.4], containing 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μ g of phenylmethylsulfonyl fluoride per ml, and 0.01% sodium azide) and cleared of debris. The cell lysate was diluted to 500 μ l with RIPA buffer containing 1 mg of ovalbumin per ml. For IE1 antigens, monoclonal antibodies E3 (Genetic Systems, Seattle, Wash.) and E13 (a gift from Y. Perol) were biotinylated as previously described (18) and added to 2×10^7 cpm of [35 S]methionine-labeled cell lysate. Antibody-antigen complexes were isolated by the addition of streptavidin-agarose (Bethesda Research Laboratories). Nonspecific proteins were removed by five washes with phosphate-buffered saline containing 0.1% Nonidet P-40. After a final wash with H_2O , proteins were eluted in 0.1 M glycine [pH 2.2]. After neutralization to pH 7.4, sample buffer was added and the proteins were fractionated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (36).

For IE2 antigens, three different polyclonal antisera were prepared and designated 1218, 1219, and F60B as described previously (19, 32). (See Fig. 2 for the locations of amino acids in the ORF of IE2 used to generate the synthetic peptides and ultimately the above-mentioned antisera.) Antibody 1218 was made with a synthetic peptide of amino acids 548 through 564; 1219 was made with amino acids 175 through 194; and F60B was made with amino acids 11 through 28. All amino acid numbers refer to the sequence of the 82-kDa IE2 protein. The peptides were synthesized with an Applied Biosystems peptide synthesizer. Antipeptide sera were prepared as described previously (19).

CAT assays. Chloramphenicol acetyltransferase (CAT) assays were performed on duplicate cultures as described by Gorman et al. (17). Several different plasmid DNA preparations were used for each experiment. CAT activities were determined in the linear portion of the enzyme reaction. Acetylated derivatives were separated from nonacetylated chloramphenicol by ascending chromatography with a chloroform-methanol (95:5) solvent. The plates were exposed to Kodak XAR-2 film. For quantitative comparisons, the radioactivity on the plates was quantitated with a thin-layer chromatography scanner or the plates were cut and the sections were counted in a scintillation counter.

RNase protection. For construction of the IE2 probe, plasmid pSP64 was digested with *SalI* and *BamHI* and ligated to the *BamHI*-*SalI* fragment of IE2 (see Fig. 2). This plasmid was digested with *SalI* and *MluI*, and the ends were filled in with the Klenow polymerase and circularized. The resulting clone, pSPSBdISM, was linearized with *AccI* and used as a template for SP6 RNA polymerase. Synthesis by SP6 RNA polymerase on linear pSPSBdISM DNA produced an antisense RNA probe of 306 nucleotides, in agreement with the predicted size. [32 P]RNA probe synthesis, hybridization, and RNase digestion conditions were as described

FIG. 1. Map locations of an HCMV early inducible promoter and the IE1 and IE2 genes. The map units for the prototype arrangement of the viral genome are shown. The components of the viral genome have been described previously (23). The early gene designated E1.7 has three promoters in tandem that determine three different transcription initiation sites and that are designated P_L , P_{E1} , and P_{E2} (4). The locations of E1.7 and the IE1 and IE2 viral genes relative to the *Xba*I restriction endonuclease map are shown.

by Krieg and Melton (22). Whole-cell RNA was harvested from five 100-mm plates 48 h after transfection as previously described (20). Fifty micrograms of RNA from each transfection was hybridized to the antisense RNA probe. Alternatively, cytoplasmic RNA was harvested from mock-infected cells or cycloheximide-blocked, infected cells (IE RNA) as described previously (39). Twenty micrograms of mock-infected cell RNA or 0.3 μ g of IE RNA was also hybridized to the IE2 [32 P]RNA probe. Hybridization was performed at 50°C overnight. The protected fragments were subjected to electrophoresis on 6% urea-polyacrylamide gels. The bands were detected by autoradiography, and the level of IE2 RNA was quantitated by scanning with a DU-8 spectrophotometer with the DU-8 gel-scanning Compuset module (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Transactivation of the E1.7 promoter by wild-type and mutant IE2 proteins. Figure 1 shows the HCMV (Towne) genome and the location of an early promoter (E1.7) in the large unique (U_L) component. Viral RNA from this promoter is present in infected cells at relatively high levels early after infection (4, 43). The locations of the IE1 and IE2 transcription units are also designated. We reported previously that expression from the E1.7 promoter was significantly enhanced by IE2 gene products alone (4).

To optimize expression from the E1.7 promoter, we performed a dose-response experiment with increasing amounts of pSVIE2 or enhancer control DNA (pInk760) (44) cotransfected with pE1.7CAT. Maximum expression downstream of the E1.7 promoter was detected with 4 to 5 μ g of pSVIE2 per 100-mm plate of cultured HFF cells (data not shown). To investigate transactivation by the IE2 gene products alone, we constructed a series of in-frame deletions

or carboxyl-terminal truncations in the IE2 ORF (Fig. 2). The plasmid for expression of the wild-type IE2 proteins was designated pSVIE2. Deletions in exons 2, 3, and 5 were designated according to either the number of amino acids removed from the ORF or the number of amino acids truncated at the carboxyl-terminal end (Fig. 2). In the latter, additional out-of-frame amino acids were acquired until a stop codon was encountered. Recombinant plasmid pSVIE2SJ was derived from a cDNA clone of an mRNA in which the intron in IE2 was spliced out. Therefore, this plasmid has the potential to code for the wild-type IE2 protein of 54 kDa but cannot code for the larger IE2 protein of 82 kDa. This IE2 protein is presumably coded for by a 1.70-kb mRNA (Fig. 2).

To substantiate that the wild-type and mutant IE2 recombinant plasmids actually coded for IE2 proteins on the basis of predictions of the size and maintenance of the correct ORF downstream of the mutations, we transfected COS-1 or HFF cells, pulse-labeled them with [35 S]methionine, solubilized them in RIPA buffer, and subjected them to immunoprecipitation as described in Materials and Methods. Polyclonal antisera were prepared to synthetic peptides based on three different locations in the IE2 ORF (Fig. 2) as discussed in Materials and Methods. All antisera (1218, 1219, and F60B) immunoprecipitated preferentially the large IE2 protein of approximately 82 kDa coded for by pSVIE2 (Fig. 3). The smaller IE2 proteins of 54, 52, and 28 kDa were also detected in transfected cells, but at a lower level relative to the 82-kDa protein. When epitopes were deleted in recombinant expression plasmids (Fig. 2), the predicted IE2 antigen was not detected (Fig. 3, dlHpa, lane 3; dlBstXI, lane 1; dlCla, lane 2; and dlXho, lane 1). In some cases, the mutated large IE2 protein migrated near the 54- to 52-kDa IE2 proteins, making the latter difficult to detect (Fig. 3, dlAva, dlCla, and dlXho). The synthesis of the wild-type or mutant

FIG. 2. Deletions in the IE2 ORF. The mRNA sizes (kilobases [kb]), the exons, and the molecular masses (kilodaltons [kDa]) of predicted and observed proteins are shown. Boldface lines represent the exons, and lightface lines represent the introns. Broken lines (top part of figure) indicate that linkages between the exons have not been established by exonuclease VII, cDNA, or primer extension analysis. RNA polyadenylation and cleavage sites and protein translation initiation and termination sites are shown. *, ●, and ▲, Locations of amino acid sequences used for the preparation of synthetic peptides to generate polyclonal antisera 1218, 1219, and F60B, respectively. The restriction endonuclease sites used to generate deletions or truncations in the recombinant plasmids are shown on a linear map downstream of the IE promoter-regulatory region (IE P/R). All plasmids are shown. The numbers of amino acids deleted by in-frame deletions or truncations are shown. The amino acid numbers in parentheses indicate additional amino acids present because of a frameshift until a stop codon was encountered. Dashes (bottom part of figure) indicate in-frame deletions. ×, Truncation. The activity of pE1.7CAT in the presence of IE2 gene products is given relative to the amount of CAT expression with enhancer control DNA (pInk760). Means were derived from three to seven separate experiments. S.D., Standard deviation.

IE2 proteins was confirmed by immunoprecipitation with at least two different antisera for each plasmid described. The predicted and observed sizes of the predominant wild-type and mutant IE2 proteins detected by immunoprecipitation are summarized in Table 1. All wild-type and mutant IE2 proteins were demonstrated to be synthesized on the basis of predicted sizes and to be relatively stable in COS-1 cells or in HFF cells (data not shown).

Permissive HFF cells were cotransfected with plasmid pE1.7CAT and optimal concentrations of the wild-type or mutant IE2 plasmids (Fig. 2). Expression of the *cat* gene downstream of the E1.7 promoter was assayed as described in Materials and Methods. Reproducibility in different HFF cell isolates is shown by the results of three to seven separate experiments in Fig. 2. Enhancement of CAT expression by optimal concentrations of the wild-type IE2 proteins ranged from 42- to 109-fold. Considerable variability in transfection efficiencies of primary isolates of HFF cells led to a wide range in fold enhancement, reflected by the standard deviations. Deletions at the amino-terminal end (plasmids pSVIE2ΔHpa and pSVIE2ΔAva) greatly reduced transactivation of the E1.7 promoter by IE2 (Fig. 2). Plasmids pSVIE2ΔICla and pSVIE2ΔIPX had little effect on

transactivation of the E1.7 promoter. In contrast, deletions near the carboxyl-terminal end (plasmids pSVIE2ΔXho and pSVIE2ΔIBstXI) greatly reduced transactivation of the E1.7 promoter by IE2 (Fig. 2). Plasmid pSVIE2SJ failed to transactivate the E1.7 promoter (Fig. 2). Similar transactivation results for wild-type or mutant IE2 proteins were obtained with an indicator plasmid containing only the P_{E1} promoter (data not shown).

On the basis of these data, we propose that the large 82-kDa IE2 protein requires 52 amino acids encoded by exon 3 and 85 amino acids encoded by exon 5, which contains part of the intron. The smaller wild-type IE2 protein of 54 kDa encoded by an mRNA which lacks the intron had no independent activity for transactivation of the E1.7 promoter. The wild-type IE2 protein of 52 kDa encoded by exon 5 with the intron present also had no independent activity. Although a plasmid expressing only the 28-kDa IE2 protein was not tested, we assume that this IE2 protein would have no independent activity for transactivation of the E1.7 promoter since it would lack the amino-terminal domain.

A plasmid (pEnh10) that expressed only exon 5 of IE2 could transactivate the adenovirus E2 promoter (19). In contrast, no transactivation of the homologous E1.7 pro-

FIG. 3. Autoradiogram of immunoprecipitated wild-type and mutant IE2 proteins. COS-1 cells were transfected with 25 μ g of either wild-type or mutant plasmids and pulse-labeled with [35 S]methionine from 24 to 48 h after transfection. The plasmids used for wild-type or mutant IE2 gene expression are shown. See Fig. 2 for the construction of these plasmids. HFF cells were also infected with HCMV without cycloheximide treatment and pulse-labeled with [35 S]methionine from 1 to 6 h after infection. Cell lysis, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were done as described in Materials and Methods. IE2 antigens were immunoprecipitated with antiserum 1218 (lanes 1), 1219 (lanes 2), or F60B (lanes 3). The locations of the amino acid sequences used to generate synthetic peptides that were used to prepare the above-mentioned antisera are shown in Fig. 2. The 82- and 54- to 52-kDa IE2 antigens detected in HCMV-infected cells are shown. *, 52-kDa IE2 antigen expressed from pSVIE2dHpa. Mr, Protein molecular size standards (in kilodaltons [kDa]).

motor was detectable with this recombinant plasmid (data not shown).

Effect of IE1 on the major IE promoter-regulatory region. We previously demonstrated that transactivation of an inducible early promoter by IE2 gene products reached maximum levels when IE1 gene products were also expressed (19, 40). Augmentation may occur because of protein interactions between IE1 and IE2 at the E1.7 promoter. Alternatively, IE1 may increase the level of IE2 expression at the transcriptional or translational level and augmentation may be due to an increased level of the IE2 transactivator. Experiments were designed to test the latter interpretation with wild-type or mutant IE1 proteins. Figure 4 shows the locations of the mutations and the number of amino acids deleted.

The expression of wild-type and mutant IE1 proteins in transfected cells was evaluated in COS-1 or HFF cells by pulse-labeling with [35 S]methionine and immunoprecipitation with anti-IE1 monoclonal antibody E3 or E13 as described in Materials and Methods (Fig. 5 and Table 2). All wild-type and mutant IE1 proteins were demonstrated to be synthesized, based on predicted sizes. IE1 expression from plasmid pCC2 was always higher than that from plasmid

pCC1 because of the orientation of the HCMV enhancer-containing promoter-regulatory region relative to the simian virus 40 replication origin. Permissive HFF cells were cotransfected with pCAT760 plus enhancer control DNA (pLink760), plasmids expressing wild-type IE1 (pCC1 or pCC2), or plasmids containing the various mutations in the IE1 gene (Fig. 4). Figure 4 summarizes the results from three to seven separate experiments with four different HFF cell isolates. Wild-type IE1 protein expressed from either pCC1 or pCC2 always significantly enhanced expression downstream of the major IE promoter, but at a relatively low level. In contrast, deletions in the amino-terminal part (*dlAva*) or in the carboxyl-terminal part (*dlBclI*, *dlOxaNI*, *dlE24*, and *dlS12*) of IE1 failed to enhance expression of the downstream *cat* gene.

On the basis of these data, we concluded that the IE1 protein positively regulated expression from the major IE promoter. Regions of the IE1 protein encoded by exon 3 and most of exon 4 were required for this activity. It is notable that the IE1 and IE2 proteins both required for transactivation 52 amino acids encoded by exon 3.

Effect of the IE1 mutation on the augmentation of transactivation by IE2. The expression of heterologous (19, 20) or homologous (4) promoters was enhanced to the highest level when IE1 and IE2 were cotransfected with plasmid pCS (IE1 and IE2). In plasmid pCS, the transcription of both IE1 and IE2 is driven by a single IE promoter, as in the viral genome. Since the IE1 protein increases expression downstream of the major IE enhancer-containing promoter, could the augmentation by IE1 of the IE2-directed transactivation of the E1.7 promoter be explained simply by a higher expression of the IE2 protein? If so, a mutation in exon 4 (which encodes the IE1 protein) which leads to the synthesis of a mutant IE1 protein but a wild-type IE2 protein (pCSdIS12) should transactivate the E1.7 promoter to about the same extent as does

TABLE 1. Molecular masses of the predominant wild-type and mutant IE2 proteins expressed in transfected cells^a

Plasmid	Molecular mass (kDa) of the following protein:							
	IE2	<i>dlHpa</i>	<i>dlAva</i>	<i>dlCla</i>	<i>dlPX</i>	<i>dlXho</i>	<i>dlBstXI</i>	IE2SJ
Expected	82	52	74	68	77	51	72	54
Observed	82	52	68	66	73	60	78	54

^a The largest of the possible IE2 proteins was the highest in quantity. The smaller proteins (54, 52, or 28 kDa) were detectable only after longer incubation of the autoradiogram.

FIG. 4. Deletions in the IE1 ORF. Downstream of the major IE promoter-regulatory (P-R) region the exons for the IE1 gene are designated 1 through 4. The translation initiation and stop codons are shown. The relative positions of the exon junctions and kilobases (kb) of DNA are shown. The locations of in-frame deletions or truncations and the numbers of amino acids (a.a.) deleted are shown. Dashes indicate in-frame deletions. ×, ORF truncation. The exact locations of the mutations are indicated in base pairs. The mean percent conversion of chloramphenicol to acetylated derivatives was derived from three to seven separate experiments with four different HFF cell isolates. S.D., Standard deviation.

the IE2 protein alone. This theory was confirmed by the following experiment. HFF cells were cotransfected with 1 μ g of pE1.7CAT and 0.1 μ g of enhancer control DNA (plink760), pSVIE2, pCS (IE1 and IE2), or pCSdIS12 (mutant IE1 and wild-type IE2). Plasmid pCS activated the E1.7 promoter to a four-fold higher level (28% conversion) than did either pSVIE2 (8.0% conversion) or pCSdIS12 (9.4% conversion). An IE1 mutation which could not enhance expression from the major IE promoter also could not augment transactivation of the E1.7 promoter.

Effect of IE1 on IE2 steady-state mRNA. The synthesis of the 2.25-kb mRNA which codes for the large IE2 protein of 82 kDa was driven by the major IE promoter (Fig. 2). Cotransfection experiments were used to test the effect of IE1 on CAT expression from pCAT760 or on the steady-state level of IE2 mRNA expression from pSVIE2dIXho. Because the wild-type IE2 proteins can repress expression

from the major IE promoter region (29), we selected a plasmid with a mutation that truncated the ORF of the large IE2 protein (pSVIE2dIXho; Fig. 2) and eliminated repression. Plasmid pSVIE2dIXho codes for the same-sized mRNA as does the wild-type IE2 gene (data not shown). HFF cells were cotransfected with pSVIE2dIXho and either enhancer control DNA (plink760) or pCC2 (IE1). At the same time, the efficiency of the transfection was tested by cotransfecting pCAT760 with either enhancer control DNA (plink760) or pCC2 (IE1). Cell lysates were obtained for the CAT assay. Whole-cell RNA was harvested and used in an RNase protection experiment as described in Materials and Methods. Figure 6A demonstrates that wild-type IE1 gene expression enhanced the level of CAT expression from the major IE promoter at least fivefold. Figure 6B (lane 3 or 7) demonstrates that wild-type IE1 gene expression increased the steady-state level of IE2 mRNA approximately fivefold. The size of the protected RNA was identical to the size of the protected infected-cell IE RNA (Fig. 6B, lane 5). Figure 6B (lane 2 or 6) also demonstrates that without IE1 gene expression, a lower level of steady-state IE2 mRNA was detected. We concluded that one mechanism of IE1 augmentation of IE2 transactivation involved IE1 increasing the level of IE2 expression by positive regulation of the major IE promoter.

DISCUSSION

Transactivation of heterologous (6, 19, 29, 40) or homologous (4, 9) inducible promoters by HCMV IE gene products

TABLE 2. Molecular masses of the wild-type and mutant IE1 proteins expressed in transfected cells

Plasmid	Molecular mass (kDa) of the following protein:						
	pCC1	d1Ava	d1Bcl	d1OxaNI	pCC2	d1E24	d1S12
Expected	68	62	49	46	68	50	40
Observed	68	55	42	44	68	50	38 ^a

^a Expression of a mutant IE1 protein from pCC2dIS12 has been reported previously (31).

FIG. 5. Autoradiogram of immunoprecipitated wild-type or mutant IE1 proteins from transfected cells. COS-1 cells were transfected with 25 μ g of either wild-type (pCC1 or pCC2) or mutant plasmids and pulse-labeled with [³⁵S]methionine from 24 to 48 h after transfection. The mutant plasmid constructions are shown in Fig. 4. Cell lysis, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were done as described in Materials and Methods. Lanes: 1, mock transfected; 2, pCC1 (IE1); 3, pCC1d1Ava; 4, pCC1d1Bcl; 5, pCC1d1OxaNI; 6, pCC2 (IE1); 7, pCC2d1E24. IE1 WT and 68 designate the wild-type IE1 protein.

FIG. 6. Effect of IE1 on the steady-state level of IE2 RNA. Cells were cotransfected with either 2 μ g of pCAT760 or 2 μ g of pSVIE2dIXho (Fig. 2) plus either 5 μ g of enhancer control DNA (plink760) or 5 μ g of pCC2 (IE1) as described in the text. Cells were harvested 48 h after infection and assayed for either CAT activity or IE2 RNA as described in Materials and Methods. (A) CAT assay of transfected cell lysates. Lanes: 1, pCAT760 plus plink760; 2, pCAT760 plus pCC2 (IE1). The percent conversion of [14 C]chloramphenicol to its acetylated derivatives is shown. (B) RNase protection assay. Whole-cell RNA was isolated from transfected cells and hybridized to a 306-nucleotide 32 P-labeled antisense RNA probe. Mock-infected or cycloheximide-blocked, HCMV-infected-cell cytoplasmic RNA was also hybridized to the same probe. After RNase treatment, the protected RNA was fractionated in 6% urea-acrylamide gels as described in Materials and Methods. The structures of the IE2 mRNA, riboprobe, and protected fragment are shown below panel B. Lanes: 1, standard molecular size markers; 2, RNA from pSVIE2dIXho-plink760-transfected cells; 3, RNA from pSVIE2dIXho-pCC2 (IE1)-transfected cells; 4, RNA from mock-infected cells; 5, RNA from HCMV-infected cells; 6 and 7, longer exposures of lanes 2 and 3, respectively; 8, undigested 32 P-labeled RNA probe. AS, Acceptor site. Numbers at left are in nucleotides.

reaches maximal levels when both IE1 and IE2 genes are expressed. The IE2 gene products alone can enhance downstream expression of the HCMV E1.7 promoter (4). The HCMV IE2 transcription unit codes for several viral proteins ranging in molecular mass from 82 to 28 kDa (19, 32). We

were able to demonstrate the expression of wild-type and mutant IE2 proteins, and in each case, the largest IE2 protein was predominantly expressed in the transfected cells. A similar observation was made in HCMV-infected cells. The largest IE2 protein is a phosphorylated protein of 579 amino acids. Deletion analysis of recombinant plasmids demonstrated that amino acids 26 to 78 encoded by exon 3 and amino acids located downstream of amino acid 497 are required for transactivation. These amino acid regions are present in the 82-kDa protein. Since the wild-type IE2 protein of 54 kDa which contains the amino-terminal region failed to enhance expression from the E1.7 promoter, even when expressed at relatively high levels (Fig. 3), we propose that some or all of the amino acids specified by exon 5, which contains the intron, are required for transactivation by the large IE2 protein. The 52-kDa protein, which is expressed from an AUG encoded by exon 5, contains the carboxyl-terminal domain but lacks the amino-terminal domain. This IE2 protein also failed to transactivate the homologous E1.7 promoter. Using protein truncations only, Pizzorno et al. (29) found that the carboxyl-terminal end of IE2 was required for transactivation of heterologous promoters in nonpermissive Vero cells. Our data indicate that the amino acids encoded by exon 5 and a region within 52 amino acids of the amino terminus encoded by exon 3 are required for transactivation of a homologous promoter in permissive HFF cells. Our deletion plasmids code for more than a single protein; consequently, we cannot eliminate complementation by two different proteins, such as the 54- and 52-kDa IE2 proteins. However, the ORF that specifies the 82-kDa IE2 protein contains all the regions which are important for transactivation of the E1.7 promoter.

The intron in exon 5 of IE2 can code for a single putative C_2H_2 zinc finger motif between amino acids 428 and 452. The carboxyl end of the large protein has a region of hydrophobic amino acids followed by a hydrophilic tail. The pSVIE2dIXho mutant retains the putative zinc finger motif, and the mutation only changes the last 79 amino acids of the large protein, yet this mutant IE2 protein failed to transactivate. Within the last 29 residues of the wild-type IE2 protein are seven negatively charged amino acids which may constitute an important component of the required carboxyl-terminal domain.

When the amino acids within the required amino-terminal region were displayed on an idealized alpha helix, hydrophobic residues were segregated to one side of the putative helix and hydrophilic amino acids were segregated to the opposite side. An amphipathic alpha helix has been postulated to be present within essential domains of transactivators in both procaryotes and eucaryotes (30).

The IE2 antigens are detected in the nuclei of infected cells (28). Exon 5 of the IE2 mRNA codes for two putative nuclear localization signals between amino acids 146 and 151 (Arg-Lys-Lys-Pro-Arg-Lys) and 322 and 327 (Arg-Lys-Lys-Lys-Ser-Lys) (32, 35). These nuclear localization signals would be present in both the large IE2 protein of 82 kDa and the intermediate IE2 proteins of 52 and 54 kDa. Since the 96-amino-acid deletion mutant pSVIE2dICla lacks the first nuclear localization signal and still enhanced expression from the E1.7 promoter, it is not required for nuclear localization.

Since the wild-type and mutant IE2 proteins were all synthesized at similar levels in transfected cells, the significant reduction in the level of transactivation is related to a deletion of essential amino acid domains, an alteration of secondary or tertiary structure, or a change in the spacing

between as-yet-undefined protein domains. Further investigation is required to determine how these IE2 amino acid domains interact in the transcription complex. For example, one domain could be involved in DNA binding and a second domain could be involved in protein-protein interactions.

One role for the IE1 gene product in the augmentation of transactivation by IE2 is to increase the steady-state level of the IE2 mRNA. Since the IE1 protein can enhance expression from the upstream major IE promoter-regulatory region (5), we tested the effect of wild-type and mutant IE1 gene products. Interestingly, the same amino-proximal region encoded by exon 3 and required for activity by the IE2 proteins was also required by the IE1 protein to enhance expression from the major IE promoter. Likewise, a second IE1 protein region was also required but was mapped to be broadly distributed through the amino acids encoded by exon 4. Since the IE1 protein has a great deal of secondary structure (33), any deletion in exon 4 might have perturbed this required secondary structure and possibly the tertiary structure. Although IE1 is also transported to the nucleus (28), putative nuclear localization signals have not been identified.

Since the IE1 and IE2 proteins have the same 85 amino acids at the amino-terminal end, the difference in the activity of these two HCMV IE proteins is determined by the splicing events that select either exon 4 for the IE1 protein or exon 5 for the IE2 proteins. The properties of the amino acids coded for by exon 4 are very different from those of the amino acids coded for by exon 5. Exon 4 codes for a higher content of hydrophobic amino acids towards the 5' end and for a very high content of hydrophilic amino acids towards the 3' end. The carboxyl-terminal side of the HCMV IE1 protein is rich in glutamic and aspartic acid residues (34). This property is also characteristic of the IE1 proteins specified by murine cytomegalovirus (21) and simian cytomegalovirus (T. Jeang and G. Hayward, personal communication). In contrast, exon 5 codes for IE2 proteins that are generally hydrophilic and have a great deal less secondary structure (32).

The effects of the two IE transactivators are quantitatively very different. The IE1 gene product has only a modulatory effect on expression from a very strong enhancer-containing promoter-regulatory region. Likewise, augmentation of transactivation is modulatory and only about fivefold. In contrast, the IE2 protein alone has a strong quantitative effect on the inducible homologous early promoter E1.7. Enhancement of downstream expression can be as much as 50- to 100-fold at optimal IE2 concentrations. Although the IE1 protein enhances expression from the major IE promoter (5), the IE2 protein suppresses expression (29). Therefore, the alternate splicing events produce proteins which have two different effects on expression from the major IE promoter.

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