Human Cytomegalovirus Immediate-Early Two Protein Region Involved in Negative Regulation of the Major Immediate-Early Promoter

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The immediate-early two (IE2) gene products of human cytomegalovirus negatively regulate gene expression from the major immediate-early promoter in permissive human fibroblasts. A mutational analysis of the IE2 proteins indicated that the carboxyl-terminal region is required for negative regulation. The IE2 proteins that lack amino acid residues 365 to 519, or the carboxyl-terminal amino acids failed to negatively regulate. Most of the amino-terminal portion of the IE2 protein was not required for negative regulation. A possible explanation of the negative effect on downstream expression by the IE2 proteins is discussed.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, has an approximately 230-kilobase-pair, double-stranded, linear DNA genome (9, 14, 22, 24). Like other herpesviruses, HCMV has a temporally regulated pattern of gene expression (8, 10, 27, 38, 41, 42). There are three broadly defined phases of gene expression which are based on the appearance of the viral RNAs in the cytoplasm, referred to as immediate early (IE), early, and late. The IE genes are defined by their transcription in the absence of prior viral protein synthesis (8, 26, 41). IE transcription is restricted to a few distinct regions of the HCMV genome (30, 41, 44). Once the IE genes are expressed, transcription occurs extensively throughout the viral genome (8, 27, 41).

Two IE transcription units have been designated IE1 and IE2 (38). Soon after infection, the IE1 mRNA and protein are expressed at approximately a fivefold higher level than those of IE2 (35, 38). Later, the steady-state level of the IE1 mRNA decreases, while the level of the IE1 protein, a 72-kilodalton (kDa) phosphoprotein, remains relatively constant (33, 36). IE2 codes for at least four proteins of 82, 54, 52, and 28 kDa (18, 25, 32). The higher-molecular-weight IE2 proteins are phosphorylated in human fibroblasts, but the 28-kDa protein is not phosphorylated (18). The largest IE2 protein consists of 579 amino acid residues.

The region upstream of the IE1 gene has a strong enhancer-containing promoter-regulatory region (3, 37, 40) which drives expression of the IE1 and IE2 genes (34, 35). There are a number of different repeat elements, some of which contain consensus binding sites for potential interaction with known eucaryotic transcription factors (3, 20, 37, 40; M. F. Stinski, C. L. Malone, T. W. Hermiston, and B. Liu, in E. K. Wagner, ed., *Herpesvirus Transcription and Its Regulation*, in press). Both repetitive and nonrepetitive sequences may be involved in the regulation of IE gene expression, as suggested by DNA footprinting and in vitro transcription experiments (15-17, 40).

The IE1 protein positively regulates the major IE promoter (MIEP) to enhance expression of IE2 (25). The IE2 gene products transactivate both heterologous (6, 18, 29, 39) and homologous (5) early promoters in both nonpermissive and permissive cells. In contrast, the IE2 gene products were reported to negatively regulate the MIEP in nonper-

missive Vero cells (29). In this study, we have defined the regions of the IE2 protein required for a negative effect on expression of the MIEP in permissive human fibroblasts.

The organization of the IE1 and IE2 genes located between 0.751 and 0.732 map units of the Towne strain of HCMV is shown in Fig. 1. The observed mRNA sizes and the predicted structures of the individual viral RNAs, polyadenylation signals, translation initiation start sites, and stop codons are designated and have been previously described (34, 35). The predicted molecular weights of the proteins, the apparent molecular weights of the proteins observed by immunoprecipitation using polyclonal or monoclonal antisera, and the sites recognized by three different polyclonal antisera are also shown. Upstream of the IE1 and IE2 genes is the promoter-regulatory region which controls expression of the IE1 and IE2 genes (32, 34, 35).

Since enhancer regions compete for available cellular factors during transient gene expression (43) and since both the IE2 and indicator genes are driven by the MIEP, a plasmid (pLink760) containing only the MIEP in vector DNA was used as an enhancer control DNA. To demonstrate the effect on expression by either enhancer competition or IE2 gene products, various concentrations of plasmid pLink760 or pSVIE2 (Fig. 1) were used in cotransfection experiments with 2 µg of pCAT760, as previously described (25). Plasmid pCAT760 has the chloramphenicol acetyltransferase (CAT) gene inserted downstream of the MIEP, as previously described (37). Figure 2 shows the mean relative CAT activity for five separate experiments. The variability between experiments was relatively low. Expression of CAT from pCAT760 decreased in a dose-response fashion in the presence of pLink760, which demonstrates that enhancer competition does occur (Fig. 2, lanes 1, 3, and 5). However, expression of IE2 gene products from pSVIE2 significantly reduced the level of CAT expression to below that observed with pLink760 (Fig. 2, compare lanes 1, 3, and 5 with lanes 2, 4, and 6, respectively). Substitution of sonicated salmon sperm DNA (ssDNA) for the test DNA (pSVIE2) or the enhancer control DNA (pLink760) resulted in a higher level of CAT expression, which was due to a lack of enhancer competition (Fig. 2, lane 7).

Cotransfection experiments were also used to test the effect of IE2 on the steady-state level of IE1 mRNA. Human foreskin fibroblast cells were cotransfected with pCC2,

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FIG. 1. HCMV IE genes 1 and 2 and plasmid constructions. The MIEP upstream of IE1 and its cap site have been described previously (34). Structures of the IE1 and IE2 RNAs are shown. Thick lines define exons, and thin lines define introns. Exons are numbered 1 through 5. Transcripts whose 5' ends are undefined are shown (— = — = —). Start sites for translation initiation, stop codons, and polyadenylation signals are demarcated. The IE RNA sizes, predicted protein sizes by ORF, and observed protein sizes by immunoprecipitation with monospecific antisera are designated. The locations of amino acid sequences used for preparation of synthetic peptides to generate polyclonal antiserum as previously described (25) are indicated (*, ●, ▲). Truncations (X) or deletions (---) in IE2 are as previously described (25). The corresponding restriction endonuclease sites and amino acids deleted are listed with the corresponding plasmid name on the right. In some cases, a switch in the ORF added additional amino acids (+16, +49) until a stop codon was encountered. The locations of the IE1 and IE2 genes (in map units) are given at the bottom. The expected and observed molecular masses of the largest wild-type and mutant IE2 proteins are designated as reported previously (25).

which expressed IE1 and either enhancer control DNA (pLink760), or pSVIE2, as previously described (18, 25). For RNase protection experiments, a 279-base-pair *AccI*-to-*EcoRV* DNA fragment from pCC2 (IE1) was cloned into vector plasmid pGEM blue (Promega Biotec, Madison, Wis.). The resulting clone, pGEMIE1, was linearized with *HindIII* and used as a T7 RNA polymerase template to synthesize a 319-nucleotide antisense RNA probe. [³²P]RNA probe synthesis, hybridization, and RNase digestion conditions were as described by Krieg and Melton (23). Cytoplasmic RNA from each transfection, mock cytoplasmic RNA, or IE cytoplasmic RNA prepared as previously described (35) was hybridized to the IE1 [³²P]RNA probe. Hybridization was performed at 48°C overnight. The protected fragments were subjected to electrophoresis and detected by autoradiography. IE2 gene expression decreased the steady-state level of IE1 mRNA significantly (Fig. 3, compare lanes 2 and 3). IE1 mRNA was detected in HCMV-infected cells but not in mock-infected cells (Fig. 3, compare lanes 4 through 6). In agreement with the reduction of CAT expression from the MIEP by IE2 gene products (see Fig. 2), these data indicate that IE2 decreases the steady-state level of IE1 mRNA in the cytoplasm.

To identify the IE2 proteins or protein regions required for negative regulation of the MIEP, various in-frame deletions or truncations of the IE2 open reading frame (ORF) were made (25) (Fig. 1). All mutants synthesized IE2 viral proteins

of the predicted size and antigenicity, as determined by immunoprecipitation and denaturing polyacrylamide gel electrophoresis (25). The wild-type and mutant IE2 proteins were made at the same levels and were also presumed to be transported to the nucleus, since the putative nuclear localization signals were not deleted, as previously discussed (25). The expected and observed molecular masses (in kilodaltons) of the largest wild-type and mutant IE2 proteins are summarized in Fig. 1. Wild-type IE2 or mutants of IE2 were cotransfected with pCAT760. All cotransfection experiments were standardized to the level of expression of pCAT760 that was cotransfected with the enhancer control DNA (pLink760), which was set at 100%. The wild-type IE2 gene products significantly decreased CAT expression from the MIEP, five- to sevenfold (Fig. 4, lanes 2). The wild-type 54-kDa IE2 protein which was encoded by a 1.7-kilobase mRNA, which lacks the coding intervening sequence of exon 5 (Fig. 1), did not negatively regulate the MIEP independently (Fig. 4A, lane 3). Mutant IE2 proteins coded by plasmids pSVIE2 dIAva, dICla, and dIPX significantly decreased expression from the MIEP (Fig. 4B, lanes 3 through 5). Therefore, deletions in part of exon 3 or in the 5' end of exon 5 did not inactivate the IE2 activity. In contrast, disruption of the IE2 ORF towards the carboxyl termini in plasmids pSVIE2 dIXho or pSVIE2 dIBstXI eliminated negative regulation by IE2 (Fig. 4B, lanes 6 and 7). Even though all but 16 amino acids coded by the intron of exon 5

FIG. 2. Negative regulation by IE2 of expression from the MIEP. Indicator plasmid pCAT760 was cotransfected into permissive HFF cells with the plasmid pLink760, which is a control containing the MIEP only; pSVIE2, which expresses the IE2 gene products; or sssDNA, which is carrier DNA. The bar graph shows averages of five separate experiments. The mean percent relative CAT activity, which represents the percent conversion of the unacetylated form of [14 C]chloramphenicol to the 3'-acetylated form, is shown. The amount of CAT expression from pCAT760 (2 μ g) in the presence of pLink760 (2 μ g) is defined as 100%. Lanes: 1, 2 μ g of pLink760; 2, 2 μ g of pSVIE2; 3, 5 μ g of pLink760; 4, 5 μ g of pSVIE2; 5, 10 μ g of pLink760; 6, 10 μ g of pSVIE2; 7, 10 μ g of sssDNA.

were present in the IE2 proteins from pSVIE2 dIBstXI, this was also not sufficient for negative regulation. Interestingly, the IE2 proteins truncated at the carboxyl terminus repeatedly activated CAT gene expression above the control. The IE1 and IE2 proteins have 85 amino acids in common that are coded by exons 2 and 3 (32, 35). One of the protein regions of IE1 required for positive regulation of the MIEP is the amino-terminal region (25). Therefore, in the absence of the IE2 carboxyl-terminal region, the remaining protein regions have a positive effect on the MIEP.

These data indicated that the carboxyl-terminal end of the IE2 protein, which lies between amino acid residues 365 and 519 and is specified by the coding intervening sequence of exon 5, and possibly the terminal 60 amino acid residues are required for negative regulation of the MIEP. These protein regions would be present in the IE2 82- and 52-kDa proteins (Fig. 1). The ORF that specifies the 28-kDa protein requires further investigation. The IE2 52-kDa protein encoded by exon 5 that contains the intron or the 28-kDa protein encoded by part of exon 5 is present at relatively low levels in transfected cells (25). Whether these IE2 proteins can negatively regulate expression from the IE promoter-regulatory region requires further investigation.

When the intron was absent, as in the 1.7-kilobase mRNA, the wild-type 54-kDa IE2 protein lacks amino acid residues 365 to 519. A putative C_2H_2 zinc finger motif is located between amino acids 428 and 452. These motifs have been implicated in mediating transcriptional control, and they are common to many of the hormone receptors (2, 11, 13). However, the DNA sequence that codes for this motif lies upstream of the *Bst*XI site. Truncation at the *Bst*XI site eliminates only 16 amino acids encoded by the intron of exon 5 and the remainder of the carboxyl-terminal end encoded by

FIG. 3. Effect of IE2 on the steady-state level of IE1 RNA. Cells were cotransfected with 5 μ g of pCC2 (IE1) plus either 5 μ g of enhancer control DNA pLink760 or 5 μ g of pSVIE2, as described in the text. Cells were harvested and assayed for IE1 RNA as described in the text. Cytoplasmic RNA from transfected, mock-infected, or HCMV-infected cells in the presence of cycloheximide (200 μ g/ml) was hybridized to a 319-nucleotide 32 P-labeled antisense RNA probe. After RNase treatment, the protected RNA was fractionated in 6% urea-acrylamide gels. The structure of the IE1 mRNA, riboprobe, and protected fragment are diagrammed at the bottom of the figure. Lanes: 1, standard molecular size markers; 2, RNA (125 μ g) from cells transfected with pCC2 (IE1) plus pLink760; 3, RNA (125 μ g) from cells transfected with pCC2 (IE1) plus pSVIE2; 4, RNA (90 μ g) from mock-infected cells; 5, RNA (15 μ g) from HCMV-infected cells; 6, RNA (55 μ g) from HCMV-infected cells; 7, undigested 32 P-labeled RNA probe.

the 3' end of exon 5. Truncation at this site also eliminated negative regulation, which implies that amino acids encoded by the 3' end of exon 5 may also be required for negative regulation by IE2. A frame shift adds an additional 49 amino acids, which could also explain a lack of negative regulation by this mutant IE2 protein. The carboxyl-terminal end of the wild-type IE2 protein has an acidic domain (32).

On the basis of mutants analyzed in these experiments, the amino-proximal end of the IE2 protein appears not to be required for negative regulation. A deletion of 52 amino acids in the IE2 82-kDa protein (pSVIE2 dIAva) did not affect negative regulation by IE2. In addition, deletions of 25 (pSVIE2 dIPx) or 96 (pSVIE2 dICla) amino acids did not effect negative regulation. Therefore, only the carboxyl-terminal region of the IE2 protein is required to negatively regulate expression from the MIEP. The properties of the minimal regions required for negative regulation of the MIEP will require further investigation.

The MIEP of HCMV is located directly upstream of the IE1 gene and is required for the expression of the IE1 and IE2 genes (34, 35). The IE2 gene codes for one or more *trans*-acting regulatory proteins. These proteins enhance expression from heterologous (6, 18, 29, 39) and homologous (5) inducible promoters. Both the amino- and carboxyl-terminal regions of IE2 are required for transactivation of a representative HCMV early promoter, and both of these regions are in the largest IE2 protein of 579 amino acid residues (25).

The initial event in the formation of a transcription initiation complex is the recognition of the TATA box by TFIID

FIG. 4. Protein regions of IE2 required for negative regulation. The recombinant plasmids and their structures are diagrammed in Fig. 1. Indicator plasmid pCAT760 (2 μ g) was cotransfected with either 5 μ g of pLink760 or 5 μ g of wild-type or mutant IE2 plasmids into permissive HFF cells. Mean percent relative CAT activity is explained in the legend to Fig. 2. (A) Values are averages of three separate experiments. Lanes: 1, pLink760; 2, pSVIE2; 3, pSVIE2 SJ. (B) Values are averages of five separate experiments. Lanes: 1, pLink760; 2, pSVIE2; 3, pSVIE2 dIAva; 4, pSVIE2 dICla; 5, pSVIE2 dIPX; 6, pSVIE2 dIXho; 7, pSVIE2 dIBstXI.

(4, 7, 12, 28). The binding of transcription factors such as ATF, AP1, SP1, etc., is postulated to stabilize this interaction. The transactivating proteins encoded by most viruses appear to act at the level of transcription initiation by increasing the binding activity of promoter-specific factors on inducible promoters (1, 19, 21, 31, 45). Therefore, it seems plausible that the same viral proteins might also interact in the cell in a negative manner. The IE2 proteins could disrupt the formation of transcription initiation complexes on the MIEP either by binding to the viral DNA or by complexing with transactivator proteins already present in the cell. An overexpression of the IE2 protein could sequester transcription factors necessary for downstream transcription. The relevance of this potential regulation of the MIEP by IE2 to the HCMV infection process requires further investigation. It is hypothesized that the level of IE2 expression may influence the amount of IE1 and IE2 in the infected cell. This phenomenon could be related to the low rate of HCMV replication and to persistent infection.

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