Human cytomegalovirus late protein encoded by ie2: a trans-activator as well as a repressor of gene expression

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In order to study the function of human cytomegalovirus (HCMV) immediate early gene 2 (ie2) (UL122) gene products made at late times during infection, cDNA clones were isolated from an expression library made with 74 h post-infection mRNA. Based on screening of the library, 1% of transcripts in infected cells at this time were ie2 region-specific, and transcripts encoding γIE2579aa, a 40K late gene product, were more abundant than those encoding IE2338aa, an α gene product made throughout infection. As expected, the cDNA capable of directing the expression of γIE2579aa was derived from a contiguous genomic region within exon 5 of the ie1/ie2 region. The cDNA clones encoding γIE2579aa and IE2579aa were compared for their ability to trans-activate viral and cellular promoters and to repress expression from the ie1/ie2 promoter via the ie2 cis-repression signal. Unexpectedly, γIE2579aa trans-activated a variety of test promoters when cotransfected with the major α gene product, IE1491aa*. Promoters derived from the cellular β-actin gene, the simian virus 40 early region and the human immunodeficiency virus were all responsive to γIE2579aa plus IE1491aa, although several β promoters derived from the HCMV genome were unresponsive. Thus, this abundant late product from the ie2 region may play a role in trans-activation in addition to its role as a repressor of α gene expression.

Introduction

Human cytomegalovirus (HCMV) is a medically important betaherpesvirus which causes significant illness in immunocompromised individuals and the developing fetus (Alford & Britt, 1993; Ho, 1991). The process of, and functions involved in, viral gene expression have long been studied in efforts to unravel the complex biology of HCMV (Stinski, 1983). In infected cells, the temporal regulation of α, β, and γ genes (also called immediate early, delayed early, and late genes, respectively) occurs in a cascade fashion (Mocarski, 1993; Stinski et al., 1991). The principal α genes, ie1 and ie2, encode products that are believed to be important in the regulation of subsequent phases of viral gene expression; this view is based primarily on their activity in transient transfection assays (Cherrington & Mocarski, 1989; Depto & Stenberg, 1989; Everett & Dunlop, 1984; Hermiston et al., 1987; Klucher et al., 1989; Pizzorno et al., 1988; Staprans et al., 1988; Stenberg et al., 1989). Immediately after infection, ie1 and ie2 are expressed from a common transcription start site controlled by the ie1/ie2 promoter–enhancer (Fig. 1). Alternative splicing of three common exons to one of two downstream exons leads to the accumulation of two abundant transcripts, one encoding the 491 amino acid αie1 product and the other encoding the 579 amino acid ie2 product (Stenberg et al., 1984, 1989). These proteins share 85 amino acids at their amino termini but differ in their remaining sequence owing to differential splicing. A γ gene is embedded in ie2 and encodes a protein of Mr 40K representing the carboxy-terminal 338 amino acids of the 579 amino acid protein (Fig. 1). The 40K product of the γie2 gene is the most abundantly expressed protein from the ie2 region (Pizzorno et al., 1991; Puchtler & Stamminger, 1991; Stenberg et al., 1985, 1989).

IE1491aa, also called p72, IE68, IE72 or the 72K major immediate early protein, and designated ppUL123 by a systematic nomenclature (Landini & Spaete, 1993), is a 491 amino acid phosphoprotein (Gibson, 1983; Stinski, 1977) expressed from a spliced transcript (exons 1, 2, 3 and 4) (Stenberg et al., 1984). Although expression peaks between 5 and 8 h post-infection (p.i.), the protein continues to be made throughout infection (Stenberg et al., 1984, 1989). IE1491aa has been shown by transient assay to activate expression from the ie1/ie2 enhancer via NF-κB sites (Cherrington & Mocarski, 1989; Sambucetti et al., 1989), possibly explaining an autoregulatory process that occurs during infection (Malone et al., 1990). Although by itself IE1491aa does not trans-

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activate any other CMV promoters that have been tested, this protein trans-activates the human immunodeficiency virus long terminal repeat (HIV LTR) (Biegelke & Geballe, 1991; Hagemeier et al., 1992a; Walker et al., 1992), as well as a number of promoters from cellular genes (Hagemeier et al., 1992a, b; Hunninghake et al., 1992; Iwamoto et al., 1990; Wade et al., 1992). In most cases, trans-activation is strongly enhanced when ie2 gene products are also present.

IE2$_{578a}$, also called p86, 82K, IE80 or IE86 (Hermiston et al., 1987; Pizzorno et al., 1991; Stenberg et al., 1989), has been designated ppUL122a (Landini & Spaete, 1993). IE2$_{578a}$ is expressed from a spliced transcript (exons 1, 2, 3 and 5) (Pizzorno et al., 1991; Stenberg et al., 1985, 1989). Like IE1$_{491aa}$, expression of this protein peaks within a few hours after infection but continues through to late times (Stamminger et al., 1991; Stenberg et al., 1985, 1989). IE2$_{578aa}$ is the principal $\alpha$ protein expressed from the ie2 gene; however, additional minor proteins are also made from this region (Baracchini et al., 1992; Hermiston et al., 1987; Pizzorno et al., 1988, 1991; Stenberg et al., 1989; Siinski et al., 1983). Besides $\alpha$ gene products, this region encodes at least one abundant $\gamma$ protein (Pizzorno et al., 1991; Puchtl & Stamminger, 1991; Stenberg et al., 1985, 1989), $\gamma$IE2$_{338aa}$, a protein that is also called IE2-p40 or the 40K form of IE2 (Pizzorno et al., 1991; Stenberg et al., 1989) and also ppUL122c (Landini & Spaete, 1993). This abundant gene product has been observed in many studies (Hermiston et al., 1987; Pizzorno et al., 1988, 1991; Stenberg et al., 1985) and has been predicted to be expressed from a 1.5 kb $\gamma$ transcript. Recent studies using a collection of IE2-specific monoclonal antibodies have shown that the open reading frame encoding this $\gamma$ protein is collinear with the carboxy-terminal 338 amino acids of IE2$_{578a}$ (Plachter et al., 1993), although it has not yet been shown to be the product of the 1.5 kb mRNA. The basis for assigning function to $\gamma$IE2$_{338aa}$ has been studies on truncated versions of IE2$_{578aa}$ (Hermiston et al., 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990).

In transient assays, IE2$_{578aa}$ acts on promoters from both viral and cellular genes, and although this trans-activation is believed to be a key step in the viral regulatory cascade, transient assays suggest little specificity for target sites within CMV promoters (reviewed by Mocarski, 1993; Stenberg, 1994; Tevethia & Spector, 1989). The target for trans-activation varies with the promoter and the cell type used for assay (Biegelke & Geballe, 1991; Depto & Stenberg, 1992; Ghazal et al., 1991; Klucher et al., 1989; Klucher & Spector, 1990; Staprans et al., 1988; Walker et al., 1992). Furthermore, test promoters have been more responsive when IE2$_{578aa}$ is assayed in the presence of IE1$_{491aa}$, which has led to the suggestion that these two trans-activators cooperate during the viral regulatory cascade (Malone et al., 1990; Stenberg et al., 1990). This cooperation may result from either the combined action of IE1$_{491aa}$ and IE2$_{578aa}$ on a target promoter or the stimulation of IE2$_{578aa}$ expression by IE1$_{491aa}$ (Cherrington & Mocarski, 1989; Depto & Stenberg, 1989; Malone et al., 1990; Sambucci et al., 1989; Stenberg et al., 1990). It is possible that both mechanisms may contribute to trans-activation.

Transient assays have suggested that ie2 gene products function to repress iel and ie2 expression during viral replication (Pizzorno et al., 1988). Repression occurs through a cis repression signal (crs) located between -14 and +1 of the iel/ie2 transcriptional start site (Cherrington et al., 1991; Liu et al., 1991; Pizzorno & Hayward, 1990). Mutations that have been introduced into IE2$_{578aa}$ have shown that repression requires only the carboxy-terminal half of this large protein (Hermiston et al., 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990), which has led to the hypothesis that the abundant late gene product, IE2$_{338aa}$, functions exclusively as a repressor of $\alpha$ gene expression (Hermiston et al., 1990; Pizzorno & Hayward, 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990). Repression may involve direct binding of IE2$_{578aa}$ or IE2$_{338aa}$ to crs, thereby blocking RNA polymerase II-mediated transcription, an hypothesis that has been strengthened by reports demonstrating that recombinant IE2$_{578aa}$ (or derivatives that preserve the carboxy-terminal half of the protein) retain the ability to bind to the crs and to prevent efficient in vitro transcription from the iel/ie2 promoter (Jupp et al., 1993; Lang & Stamminger, 1993; Macias & Siinski, 1993). A lack of evidence that the carboxy-terminal half of IE2$_{578aa}$ can trans-activate has sustained the idea that IE2$_{338aa}$ functions solely as a repressor. Indeed, studies aimed at defining the activating domains of IE2$_{578aa}$ have concluded that the region representing IE2$_{338aa}$ is incapable of trans-activation in the absence of the amino-terminal 85 amino acids normally present in IE2$_{578aa}$ (Malone et al., 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990), even though this region carries all known domains for interaction with the cellular transcription machinery in addition to the DNA-binding domain (Chiou et al., 1993; Hagemeier et al., 1992a; Jupp et al., 1993; Yeung et al., 1993). Despite this, the only direct evidence that the carboxy-terminal half of IE2$_{578aa}$ may play a positive regulatory role comes from Gal4 fusion experiments performed in mammalian cells that revealed a trans-activation domain in this region (Pizzorno et al., 1991). One early report (Hermiston et al., 1987) suggested that the carboxy-terminal portion of IE2$_{578aa}$ may play an independent role for the region in trans-activation; however, more recent studies (Yeung et al., 1993) have
shown that this was probably due to a protein significantly longer than the natural \(\gamma IE2_{38}\).

We set out to isolate cDNA copies of \(\gamma\) transcripts encoded by the \(ie2\) gene to define more fully the activities of \(\gamma IE2_{38}\). We used a cDNA library made from polyadenylated RNA purified from cells at 74 h p.i. to identify expression clones of \(ie2\) region cDNAs and then studied the function of proteins expressed from these clones. We report here that \(\gamma IE2_{38}\) can indeed function as a trans-activator, particularly when introduced into cells along with \(IE1_{49}\). Thus, this abundant late gene product may be involved in both activation and repression during viral replication.

**Methods**

**Cells, virus and transient transfection assays.** Human foreskin fibroblast (HF) and COS-7 cells, and HCMV (Towne) were grown as previously described (Leach & Mocarski, 1989; Spaete & Mocarski, 1985a) in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% NuSerum (Collaborative Research) without antibiotics. HF cells were seeded to achieve 70% confluence into six- or 24-well cell culture dishes (Falcon) in medium supplemented with 100 U of penicillin/ml, 100 \(\mu\)g of streptomycin sulphate/ml, 0.66 mm-arginine, 1.48 mm-glutamine and 0.24 mm-asparagine, and transfected approximately 30 h later by the DEAE-dextran method as previously described (Spaete & Mocarski, 1985a; Stasiak & Mocarski, 1992). The effector:target ratio of DNA was 2:1, based on the amounts of DNA added and was maintained for each effector separately when multiple \(ie1\) and \(ie2\)-expression plasmids were used with a particular target construct. Briefly, for the experiments involving \(lacZ\) target constructs, 1 \(\mu\)g of each effector plasmid was added to 0.5 \(\mu\)g of target DNA, in 0.6 ml of DMEM (50 mm-Tris–HCl pH 7.5), and DEAE-dextran (Pharmacia) was added from a 20 mg/ml stock solution in HZO to a final concentration of 200 \(\mu\)g/ml. Each transfection mixture was distributed equally between four wells (approx. 10^5 cells each) of a 24-well cell culture dish and the cultures were incubated for 3-5 to 4 h at 37°C, washed once using medium containing 5% DMSO followed by the addition of 1 ml of medium. At 72 h post-transfection, \(\beta\)-galactosidase (\(\beta\)-gal) levels were measured by adding 4-methylumbelliferone-\(\beta\)-D-galactoside (MUG) to the medium at a concentration of 150 \(\mu\)g/ml and measuring the accumulated cleavage product after 4 h by a fluorometric assay as described previously (Geballe et al., 1986b). \(\beta\)-gal activity was expressed by calculating the ratio of MUG fluorescence after cotransfection of the effector and target plasmids to fluorescence levels after transfection of each target plasmid alone. In our initial experiments with \(ie1/ie2\) promoter target constructs, we determined that maximal expression occurred over 0-5 to 10 \(\mu\)g of effector DNA when the target DNA was held constant at 0.5 \(\mu\)g. Standard deviation was calculated from the mean of the four separate wells. All experiments were repeated at least five times with consistent results.

For the experiments involving chloramphenicol acetyltransferase (CAT) target constructs, DEAE-dextran transfections were performed as above except transfection mixtures (1 ml) containing 1 \(\mu\)g of a target plasmid and 2 \(\mu\)g of each effector plasmid were introduced into two wells (approx. 6.6 \(\times\) 10^5 cells) of a six-well cell culture dish. At 72 h post-transfection, cells were washed in PBS, scraped into PBS containing 0.5 mm-EDTA, pelleted, and resuspended in 100 \(\mu\)l of 250 mm-Tris–HCl pH 7.6. As described previously (Gorman et al., 1982), cells were lysed by three freeze/thaw cycles, cell debris was pelleted and 30 \(\mu\)l of each supernatant was assayed by incubation with acetyl coenzyme A and 0.125 \(\mu\)Ci of \(^{14}\)C-chlordirolabelled chloramphenicol (Amersham) for 1 to 3 h depending on the experiment. Acetylated products were fractionated by thin-layer chromatography, visualized by autoradiography, and excised from the plate for quantification using a scintillation counter. CAT activity was expressed as the percentage of input \(^{14}\)C-chlordirolabelled chloramphenicol converted to the 1- and 3'-monoacetylated forms as well as the increase in activation (the ratio of the CAT activity after cotransfection of the effector and target plasmid to the activity levels after transfection of a target plasmid alone). COS-7 cells were transfected by the same method except that 6 \(\times\) 10^5 cells were seeded into two wells of a six-well culture dish, and each well was transfected with 2 \(\mu\)g of DNA.

**Plasmid clones and cDNA library.** Whole cell RNA was prepared by the guanidinium isothiocyanate method (Sambrook et al., 1989) at 74 h p.i. from HF cells infected with HCMV (Towne). Polyadenylated mRNA was isolated using a oligo(dT)-cellulose column (Pharmacia). Polyadenylated RNA (3 \(\mu\)g) was reverse-transcribed using a cDNA synthesis kit (Pharmacia) according to the manufacturer's protocol. Following synthesis, the cDNA was passed over a Sephacryl S-400 column (Pharmacia) to eliminate excess linker DNA, ligated to BstXI adaptors (Invitrogen) with T4 DNA ligase, and cloned into the BstXI sites of the expression vector pME18S, a high copy 3-4 kb vector carrying the SRa promoter derived from pCD-SRa (Takebe et al., 1988).

To identify \(ie2\)-specific clones, the ligation mix was transformed into Escherichia coli MC161/WM1100 (Bio-Rad) and plated on LB agar plates (Sambrook et al., 1989) with 100 \(\mu\)g/ml ampicillin. Colonies were lysed and the DNA was bound to nitrocellulose using standard colony blot conditions (Sambrook et al., 1989). Putative positive colonies were identified by hybridization overnight at 42°C with 20% formamide, 1.0 M- NaCl, 1% SDS, 100 \(\mu\)g/ml yeast tRNA, 7 mm-EDTA and 0.1 mM-sodium phosphate buffer pH 8.0 using as a probe a 1.1 kb Clal–Stul fragment (Fig. 1) from pON303Acc (Cherrington et al., 1991) specific for \(ie2\) exon 5, radiolabelled with \(^{32}\)PdCTP and \(^{32}\)PdATP (Amersham), random synthetic hexanucleotide primers (Pharmacia) and Klenow polymerase (Sambrook et al., 1989).

Candidate \(ie2\) cDNA clones were grown in LB broth with 100 \(\mu\)g/ml ampicillin, and plasmid DNA was purified by 5 ml overnight cultures by alkaline lysis (Sambrook et al., 1989). Southern blot hybridization using the same 1 kb Clal–Stul probe, but under more stringent hybridization conditions (Spaete & Mocarski, 1985b), was used to identify candidate clones and to determine the approximate size of cDNA inserts. A series of restriction enzyme digestions was used to dissect predicted cDNA structure and orientation. Sequence analysis of the cDNA ends and of the predicted splice junctions was accomplished using the diodeoxynucleotide chain termination sequencing method (Sequenase 2.0; US Biochemical) with 15 bp primers (5' GCTGGGAATTCGGG 3' and 5' AGGATAATGATCGTACA 3') homologous to vector DNA flanking the BstXI insertion sites of pME18S. The expression plasmids for \(IE2_{38}\) (pON2206) and \(\gamma IE2_{38}\) (pON203) were found to contain cDNA inserts with the complete protein-coding sequence for each, extending from nucleotide +27 or +28, respectively, to the poly(A) tract. As a control, we also identified two clones with \(ie2\) cDNA in the reverse orientation relative to the SRa promoter: pON2207 (IE2_{38}; reverse orientation) and pON2204 (\(\gamma IE2_{38}\); reverse orientation). An \(IE2_{38}\) cDNA expression vector (pON2205) was constructed by inserting a 1549 bp EcoRI–Xbal fragment representing protein-coding sequences from exons, 2 and 3 of the HCMV (Towne) ie1 cDNA clone pie1 (Spaete, 1992) between the Xbal and EcoRI sites of pME18S.

Plasmids that have been previously described include pON308, pON303Acc, pON239, pON249, pON249crs, pON284, pON283, pON2043, pON2044 and pON2046 (Cherrington et al., 1991; Cherrington & Mocarski, 1989; Geballe et al., 1986a; Spaete &
Fig. 1. Structure of HCMV (Towne) ie1/ie2 locus and effector plasmids. The top line is a schematic representation of the viral genome showing unique (UL and US) regions as thin lines and repeated regions (a, b and c sequences) as thickened areas (Mocarski, 1993). A restriction map of the HCMV (Towne) XbaI E fragment with the ie1/ie2 locus is shown expanded below. The promoter–enhancer is indicated by the hatched box above this line and the exon 5-specific probe (a Stul–Clal fragment) used to identify candidate cDNA clones is indicated by the bracket below the line. The ie1/ie2 locus transcripts are indicated by thick arrows with splicing patterns of exons 1 to 5 (Stenberg, 1993; Stenberg et al., 1984, 1985, 1989) also called UL122 and UL123 (Chee et al., 1990) indicated. The protein-coding sequences are indicated by the shaded or open boxes. The bottom four lines show the effector constructs, IE1491a genomic clone pON308, and the cDNA clones pON2205 (IE491aa), pON2206 (IE2579aa) and pON2203 (IE2338aa). The SRα promoter (1762; Takebe et al., 1988) used to direct expression of the cDNA constructs is indicated by the striped box. The following constructs were used to prepare the rat β-actin promoter-containing indicator plasmids pON838, pON840 and pON862. Plasmid pON829 was prepared by inserting the SV40 polyadenylation signal (a 228 bp BamHI–BclI fragment from SV40 which had been converted into a PvuII–XbaI fragment) into PvuII/XbaI-digested pUC8s (Vieira & Messing, 1991). pON831 was prepared by inserting a 3115 bp BglII–DraI fragment from pMC1403 (Casadaban et al., 1980) into BglII/EcoRV-digested pSL300 (Brosius, 1989). pON832 was prepared by inserting a 3.3 kbp NcoI–DraI fragment from pON831 into NcoI/PstI-digested pON829 (after removing the 3’ overhang on the PstI site), which resulted in a construct.
Results and Discussion

Characterization of ie2 cDNAs isolated late in infection

A cDNA library made from polyadenylated RNA purified from cells at 74 h p.i. was screened by hybridization with a 1-1 kb ClaI–StuI fragment, an exon 5-specific probe (Fig. 1) that detected γIE2\text{2338aa}– and IE2\text{579aa}–specific clones. Hybridization identified 22 candidates from more than 1800 screened clones, suggesting that approximately 1% of mRNAs (cellular plus viral) present at this time were ie2-specific and confirming the abundant expression of this region at late times in infection (Pizzorno et al., 1991; Stenberg et al., 1989). The cDNA inserts from these clones were analysed by restriction enzyme digestion and Southern blot hybridization (data not shown). Based on this analysis, 14 candidate clones appeared to be nearly full-length copies of the 1-5 kb mRNA predicted to encode γIE2\text{2338aa}– three candidate clones appeared to be derived from the 2-25 kb mRNA expected to encode IE2\text{579aa}– and five clones did not correspond to either and were not further characterized. The predominance of 1-5 kb cDNA clones over any other ie2 species is consistent with previous reports of mRNA levels (Stenberg et al., 1989). Our analysis confirmed that the 1-5 kb γ transcript predominated over the larger, spliced IE2\text{579aa} transcript by approximately three- to fivefold at 74 h p.i.

Two γIE2\text{2338aa}– and two IE2\text{579aa}– cDNA clones were characterized by DNA sequence analysis to verify that they were close to full-length and that the structure met that predicted by transcript analysis (Stenberg et al., 1984, 1985, 1989). Each set of clones had one representative with a cDNA insert in the forward and one in the reverse orientation relative to the SrZ promoter (Fig. 1). The structure of the IE2\text{579aa}– cDNA was identical to that of the 2-25 kb transcript characterized in cells infected for 8 h and held in the presence of cycloheximide to allow γ gene expression (Stenberg et al., 1985, 1989). Thus, this transcript remains unchanged during infection. The structure of the 1-5 kb cDNA corresponded to the γ mRNA previously predicted from transcript analysis (Stenberg et al., 1989). The IE2\text{579aa}– and γIE2\text{2338aa}– cDNA constructs were evaluated for expression in transfected COS-7 cells and polypeptides of M, 80K and 40K, respectively, were detected by immunoprecipitation with a monoclonal antibody (data not shown).

γIE2\text{2338aa}–mediated repression of α gene expression

The exon 5 region has been shown to encode an important domain of IE2\text{579aa}– responsible for binding to crs (Jupp et al., 1993; Lang & Stamminger, 1993; Macias & Stinski, 1993), interaction with transcription machinery (Hagemeier et al., 1992a; Jupp et al., 1993; Yeung et al., 1993) and dimerization (Chiou et al., 1993). We investigated whether γIE2\text{2338aa}– had the expected ability to repress gene expression via the crs sequence. To avoid shutoff of the expression plasmids used to encode ie2 gene products, the SrZ promoter rather than the iel/ie2 promoter–enhancer was used to drive expression from cDNAs. In addition to lacking a shutoff signal, the SrZ promoter–enhancer was significantly less responsive to IE2-mediated trans-activation than a crs-deficient iel/ie2 promoter–enhancer (see below). As targets, we chose two previously described lacZ reporter constructs with the crs, pON249crs and pON239 (Cherrington et al., 1991), because they had been shown to be trans-activated by an IE1\text{414aa} expression plasmid (pON308) and to be repressed by an IE2\text{579aa} expression plasmid (pON303ΔAcc; Cherrington et al., 1991; Cherrington & Mocarski, 1989).

Fig. 2 shows that the pON249crs target was trans-activated approximately four- to fivefold by either a genomic (pON308) or cDNA (pON2205) IE1\text{414aa} expression plasmid. Because both constructs functioned similarly, neither the genomic configuration, which included introns, nor the presence of the iel/ie2...
promoter-enhancer in pON308, which was autoregulated by IE1\_491aa, influenced the activity on the target construct. IE1-mediated trans-activation of pON249crs was repressed by either pON2203 (\(\gamma IE2\_338aa\)) or pON2206 (\(IE2\_579aa\)). As was the case for activation, repression occurred whether genomic (pON308) or cDNA (pON2205) IE1\_491aa constructs were cotransfected with the ie2-expressing plasmids (Fig. 2). In the absence of IE1\_491aa, \(\gamma IE2\_338aa\) reduced the expression of pON249crs by approximately 50 % (data not shown), consistent with previous reports in which derivatives of \(IE2\_579aa\) were used (Pizzorno et al., 1988, 1990; Hermiston et al., 1990; Stenberg et al., 1990). Thus, \(\gamma IE2\_338aa\) exhibited the same repression characteristics as \(IE2\_579aa\). Control expression plasmids containing cDNAs for \(IE2\_579aa\) or \(\gamma IE2\_338aa\) in the reverse orientation (pON2207 and pON2208) failed to repress. Target plasmid pON239 (Cherrington et al., 1991; Spaete & Mocarski, 1985a), carrying the natural ie1/ie2 promoter-enhancer and first exon/intron with crs in its natural context, was also tested and gave results similar to those obtained with pON249crs (data not shown). We conclude that the 338 amino acid \(\gamma\) gene product retains the full repression capability of the larger ie2 gene product. Our results are consistent with a number of previous analyses using ie2 exon 5 derivatives.

**Fig. 2. Repression of the ie1/ie2 promoter-enhancer by IE2\_579aa and \(\gamma IE2\_338aa\).** Target plasmid pON249crs (Cherrington et al., 1991) (0.5 \(\mu\)g) was cotransfected into HF cells alone (the first two bars) or with 1.0 \(\mu\)g of various effector plasmids expressing ie1 or ie2. Two separate experiments are shown; solid bars depict data obtained using IE1\_491aa genomic construct pON308 (Spaete & Mocarski, 1985a), and the hatched bars depict data using IE1\_491aa cDNA construct, pON2205. ie2 plasmids included pON2206 (IE2\_579aa), pON2203 (\(\gamma IE2\_338aa\)), pON2207 (IE2\_579aa, reverse orientation) or pON2204 (\(\gamma IE2\_338aa\), reverse orientation). \(\beta\)-gal activity was measured 72 h post-transfection from quadruplicate wells, with levels expressed as the mean fold increase in activation determined by dividing the activity of target plasmid cotransfected along with effector plasmid(s) by the activity of target plasmid alone. Standard deviation of the quadruplicate samples is indicated above the bars.

**Fig. 3. Trans-activation of the ie1/ie2 promoter-enhancer.** Target plasmid (0.5 \(\mu\)g) pON249 (Geballe et al., 1986a) carries a promoter-enhancer fragment (−1138 to −14 bp relative to the transcription start site) lacking the crs, and was cotransfected into HF cells alone or with 1.0 \(\mu\)g each of the ie1- and/or ie2-expressing plasmids indicated. \(\beta\)-gal activity was determined and expressed as described in the legend to Fig. 2.

(Hermiston et al., 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990). Furthermore, consistent with previous results using genomic constructs that expressed IE2\_579aa (Cherrington et al., 1991), we found that repression by ie2 gene products was dominant over ie1-mediated trans-activation. Thus, our results are consistent with previous reports suggesting that both \(\gamma IE2\_338aa\) and IE2\_579aa repress via the crs and support the long-held impression that an important function of \(\gamma IE2\_338aa\) is to sustain repression of the ie1/ie2 promoter-enhancer at late times of infection. Recent reports have verified that IE2\_579aa and the carboxy-terminal half of IE2\_579aa can bind to the crs (Chiu et al., 1993; Lang & Stamminger, 1993; Macias & Stinski, 1993). We predict that the authentic \(\gamma IE2\_338aa\) would most likely bind to crs under the reported conditions as well.

**Trans-activation by \(\gamma IE2\_338aa\)**

Repression of the ie1/ie2 promoter-enhancer has been linked to a 15 bp sequence between −14 and +1 of the ie1/ie2 transcription start site (Cherrington et al., 1991; Liu et al., 1991; Pizzorno & Hayward, 1990). To verify that the repression we had observed from \(\gamma IE2\_338aa\) was dependent on crs, we tested the target plasmid pON249 (Geballe et al., 1986a), which lacked the crs and should escape repression by ie2 gene products (Cherrington et al., 1991). This construct is known to be trans-activated by IE1\_491aa (Cherrington & Mocarski, 1989), but we found that \(\gamma IE2\_338aa\) plus IE1\_491aa trans-activated expression three- to fourfold above levels achieved by IE1\_491aa alone (Fig. 3). This increase was not seen when the negative control \(\gamma IE2\_338aa\) construct (pON2204) was tested, nor when an IE1\_491aa frameshift mutation was
cotransfected with γIE2<sub>238aa</sub> (data not shown). When used alone, γIE2<sub>238aa</sub> (pON2203) failed to trans-activate expression (Fig. 3), even over a range of DNA levels (0.1 to 5.0 µg; data not shown) suggesting that trans-activation by γIE2<sub>238aa</sub> was dependent on the presence of IE1<sub>491aa</sub>. We observed this activity and dependence on IE1<sub>491aa</sub> over a range of input DNA levels (0.5 to 4.0 µg) of pON2203 (data not shown). Therefore, when present with IE1<sub>491aa</sub> γIE2<sub>238aa</sub> demonstrated a potential to trans-activate this crs-deficient promoter-enhancer, a result that is contrary to previous predictions of γIE2<sub>238aa</sub> function. γIE2<sub>238aa</sub> was able to cooperate as well with the IE1<sub>491aa</sub> genomic (pON308) or cDNA (pON2205) constructs (Fig. 3 and data not shown).

IE2<sub>579aa</sub> was consistently more active than γIE2<sub>238aa</sub> in trans-activation assays, and was able to trans-activate alone (Fig. 3). The reverse orientation IE2<sub>579aa</sub> construct (pON2207) when introduced alone or with IE1<sub>491aa</sub> had no activity.

Enhancer sequences not required for trans-activation by IE2<sub>238aa</sub> or IE2<sub>579aa</sub>

Because we had found that γIE2<sub>238aa</sub> trans-activated only when present with IE1<sub>491aa</sub>, we investigated whether the target included enhancer elements such as the IE1<sub>491aa</sub>-responsive 18 bp repeat (Cherrington & Mocarski, 1989) or, alternatively, elements closer to the promoter. To investigate the role of the upstream enhancer elements in trans-activation by γIE2<sub>238aa</sub> or IE2<sub>579aa</sub> alone or in combination with IE1<sub>491aa</sub>, we employed a series of lacZ target constructs carrying deletions through the iel/ie2 enhancer (Cherrington & Mocarski, 1989). The series of constructs was derived from pON249, and therefore lacked crs, and had been previously employed to identify IE1<sub>491aa</sub>-responsive elements in the enhancer (Cherrington & Mocarski, 1989).

IE2<sub>579aa</sub> (pON2206) alone or in combination with IE1<sub>491aa</sub> trans-activated all iel/ie2 promoter-enhancer targets tested (Fig. 4a and b). When introduced into HF cells with IE1<sub>491aa</sub>, trans-activation levels ranged from approximately 15- to 40-fold on the various promoter constructs progressively deleting upstream regions of the enhancer. Responsiveness of the smallest target tested, a

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Fig. 4. Trans-activation of the iel/ie2 enhancer–promoter does not require the enhancer region. A series of target constructs lacking the crs (Cherrington et al., 1991; Cherrington & Mocarski, 1989) and reflecting a progressive removal of enhancer sequences from the CMV iel/ie2 promoter–enhancer were cotransfected into HF cells with effector plasmids pON2206 (a), pON2206 plus pON308 (b), pON2203 plus pON308 (c) or pON308 alone (d). The upstream endpoint of each deletion mutant is indicated in parentheses below the graph along with the number (#) of 16, 18 and 19 bp repeat elements carried by each. β-gal activity was determined and expressed as described in the legend to Fig. 2.
2344

D. E. Jenkins, C. L. Martens and E. S. Mocarski

62 bp promoter fragment (−14 to −75 relative to the transcription start site), was not reduced more than twofold from maximal levels. This target construct (pON2046), which has only a TATA box plus Sp1 and C/EBP binding sites and lacks enhancer activity (Lang et al., 1992), yielded a 20-fold trans-activation with IE2579a plus IE1491a (Fig. 4b). IE2579a alone produced a five-to 10-fold trans-activation (Fig. 4a). Consistent with observations on other target promoters (Biégalke & Geballe, 1991; Depto & Stenberg, 1992; Ghazal et al., 1991; Klucher et al., 1989; Klucher & Spector, 1990; Staprans et al., 1988; Walker et al., 1992), the trans-activation by IE2579a alone or in combination with IE1491a occurred through a relatively small target region on the promoter and was independent of the 18 bp repeats that have been shown to be responsive to IE1491a alone (Cherrington & Mocarski, 1989).

Likewise, γIE2338a (pON2203) plus IE1491a trans-activated all target plasmids including the smallest 62 bp promoter fragment (Fig. 4c). This construct (−14 to −103) was more highly trans-activated than pON2046, and constructs pON2043 (−14 to −131) and pON283 (−14 to −291) were trans-activated as much as 15-fold. As was the case for IE2579a, trans-activation by γIE2338a (pON2203) plus IE1491a was independent of enhancer elements such as the 18 bp repeat even though trans-activation by IE1491a alone was strictly dependent upon the presence of 18 bp repeats (Fig. 4d; Cherrington & Mocarski, 1989). We did not observe trans-activation of any construct by IE2579a alone (not shown). Trans-activation levels with γIE2338a plus IE1491a were not as great as with IE2579a plus IE1491a, although both sets of trans-activators gave a similar pattern of responsiveness on the deletion series. Taken together, these results show that the mechanism of activation by IE1491a plus either γIE2338a or IE2579a is distinct from the mechanism of trans-activation by IE1491a alone. The target for the activity of the ie2 gene product is promoter-proximal and distinct from the upstream enhancer element required by IE1491a. IE1491a autoregulation of the ie1/ie2 enhancer does not appear to contribute in any detectable manner to trans-activation seen when IE1491a cooperates with γIE2338a or IE2579a.

**Promoter responsiveness to trans-activation by IE2338a and IE2579a**

To investigate further the trans-activation potential of γIE2338a and to compare its activity to that of IE2579a, additional promoters were evaluated for activation with or without IE1491a. The cellular and viral promoters we used in this survey included those which have been reported to be trans-activated by HCMV infection and by ie1 and ie2 gene products.

The β-actin promoter has been shown to be a strong construct in a wide range of mammalian cells (Gunning et al., 1987). Our preliminary analysis suggested that a construct based on this promoter was responsive to ie1 plus ie2 gene products in transient assays. When tested, the target plasmid pON838, carrying the β-actin promoter/HTLV-I R–U5 linked to lacZ, was not trans-activated to any significant degree by IE1491a alone, but was strongly trans-activated by either γIE2338a or IE2579a in the presence of IE1491a (Fig. 5a). γIE2338a (pON2203) plus IE1491a (pON2205) trans-activated this promoter approximately 12-fold. IE2579a (pON2206) plus IE1491a exhibited nearly a 20-fold trans-activation. Lower levels of trans-activation were observed with IE2579a when introduced in the absence of IE1491a, but γIE2338a alone failed to trans-activate (Fig. 5). The responsiveness of this target promoter paralleled the results with the HCMV enhancer target plasmid and showed that γIE2338a functioned as a trans-activator only when present with IE1491a.

To assess the contribution of the HTLV-I sequences carried by this test construct and to assay a target that contained the β-actin promoter without the added complexity of the HTLV-I R–U5 sequence and the MHC class II intron, a plasmid (pON840) that had these elements deleted was prepared. This promoter construct showed a similar pattern of responsiveness although levels were approximately half those observed with pON838 (Fig. 5b). Finally, deletion of upstream sequences in the β-actin promoter, leaving only a TATA box, serum response element and CAAT box (pON862), did not alter the pattern of responsiveness to the trans-activators even though overall levels were further reduced (Fig. 5c). Thus, these results confirmed and extended those obtained using the 62 bp ie1/ie2 promoter construct, pON2046. Although they were ineffective or weak by themselves, the combination of γIE2338a and IE1491a produced a significant level of trans-activation even on minimal promoter targets. These results suggested that these trans-activators worked in concert rather than independently on the target promoter. These results also suggested that γIE2338a could play a role in the stimulation of cellular gene expression at late times in the viral replication cycle.

Additional target promoters from a variety of additional sources were analysed and some, but not all, were found to be responsive to γIE2338a. Again this activity was generally dependent on the presence of IE1491a. We assayed a number of CAT reporter gene constructs, including the HIV LTR (pHIV-CAT), the SV40 early promoter–enhancer (pSV2-CAT), the SV40–HTLV-I chimeric SRα promoter (pSRα-CAT), the ie2 promoter (pIE2PCAT), and the adenovirus E2 promoter (pEC). We found that three of these promoters,
pHIV-CAT, pSV2-CAT, and pSRα-CAT, responded to γIE2338aa, but to different degrees and with different requirements for IE1491aa. pHIV-CAT was trans-activated by γIE2338aa, IE2579aa, and IE1491aa much like the crs-minus HCMV enhancer–promoter (pON249). As shown in Table 1, IE1491aa independently trans-activated pHIV-CAT threefold above that of the target alone, but an 11-fold increase in trans-activation occurred when pHIV-CAT was cotransfected with γIE2338aa (pON2203) plus IE1491aa (pON308). Moreover, IE2579aa (pON206) plus IE1491aa (pON308) produced an even stronger response, a 57-fold increase in trans-activation above the basal level. IE2579aa alone yielded a much lower level of trans-activation, whereas γIE2338aa (pON2203) did not show any trans-activation on its own. These data may have implications for the effect of CMV infection on other viruses residing in the same host cell. The results with the HIV LTR suggested a potential role for both early and late ie2 gene products in activating HIV gene expression.

The SV40 early promoter target, pSV2-CAT, was trans-activated by γIE2338aa (pON2203) plus IE1491aa (pON308) but not by γIE2338aa alone (Table 1). This target was not trans-activated by IE2579aa either alone or with IE1491aa, a result that was consistent with previous reports (Pizzorno et al., 1988). The SRα promoter was unique in that it was trans-activated by γIE2338aa alone, and that the combination of IE1491aa plus γIE2338aa resulted in even higher levels of expression (Table 1). Thus, this promoter, like the SV40 early promoter–enhancer, showed a much stronger response to γIE2338aa than to IE2579aa. It should be noted that the addition of the HTLV-I R–U5 region in either the SV40 (SRα-CAT) or the β-actin (pON838) promoter constructs resulted in a twofold increase in maximal levels to which these promoters responded without altering the overall pattern of the response.

We found that IE2579aa alone or with IE1491aa failed to trans-activate the adenovirus early promoter construct, pEC (Imperiale et al., 1985), although we tried a range of conditions and plasmid preparations (data not shown). Other workers have indicated that an ie2 genomic construct carrying exon 5 and potentially encoding a protein of 410 amino acids trans-activated the adenovirus 5 E2 promoter independently of IE1491aa (Hermiston et al., 1987). It is possible that the additional 72 N-terminal amino acids of the larger IE2 protein may be required for
trans-activation of the adenovirus E2 promoter, just as it appears to be important for trans-activation of the HIV LTR (Yeung et al., 1993).

To investigate whether γIE2338a regulated its own expression, we tested the target plasmid pIE2PCAT (Puchtler & Stamminger, 1991), which carried the promoter driving expression of the 1-5 kb mRNA. This promoter had been shown to be highly active at late times of infection but unresponsive to genomic ie1 or ie2 constructs when tested in transient assays (Puchtler & Stamminger, 1991). We also found that this construct was trans-activated in HCMV-infected cells but failed to respond to IE2579a or γIE2338a with or without IE1491a (Table 1). Thus, we found no evidence that γIE2338a autoregulated its expression. Furthermore, we tested pEC (Imperiale et al., 1985), which is similar to pIE2CAT, a construct that had been reported to respond to a truncated form of IE2579a (Hermiston et al., 1987). We did not detect any trans-activation by γIE2338a (data not shown).

Finally, three HCMV promoters known to be responsive to ie1 plus ie2 or to ie1, ie2 and trs1 gene products (Klucher et al., 1989; Klucher & Spector, 1990; Stasiak & Mocarski, 1992; Stenberg et al., 1990) were found to be unresponsive to γIE2338a plus IE1491a (data not shown). These included two early HCMV promoters, those of DNA polymerase and of the β2.7 genes, as well as the late HCMV promoter of the ICP36 (UL44) gene. All of these promoters were activated when IE2579a plus IE1491a were used under conditions (which included TRS1 for the UL44 promoter) established in previous reports. Taken together, the results from this series of experiments suggested that γIE2338a trans-activation is more restricted than that of IE2579a. It would appear from our small survey that the role of γIE2338a in the viral life cycle may not have to do with control of β or γ gene expression but rather it may play a role in activation of cellular genes during the later stages of infection.

This study demonstrates that γIE2338a can activate or repress gene expression and, when considering its abundance late during infection, suggests that it may play a key role in regulating gene expression during viral replication. When tested on a crs-deficient ie1/ie2 promoter or on a variety of other target promoters, we found that γIE2338a manifested the properties of a trans-activator of gene expression. Trans-activation by γIE2338a usually depended on the presence of IE1491a, but this combination did not trans-activate any of the HCMV β promoters tested. These results are in sharp contrast to those obtained with IE2579a which has been found to trans-activate by itself, to cooperate with IE1491a and to activate CMV β promoters. Under most conditions, γIE2338a exhibited lower levels of trans-activation, and more promoter specificity than did IE2579a. Considering the very high levels of expression of this protein late in infection, it is tempting to speculate that IE2338a will have an activity with some physiological relevance.

We thank M. Kirichenko at Stanford for cell culture work, J. Luh at DNAX for construction of the cDNA library, J. Vieira for constructing pON838, pON840 and pON862, G. Duke and P. Stasiak for isolation of CMV RNA, P. Stasiak for construction and analysis of pON2319, R. Spaete for a plasmid clone containing the IE1491a cDNA, M. Shepherd and B. Hoh for construction and analysis of pON2118, and G. Hayward and L. Pereira for antibodies. We are grateful to J. B. Petersen and L. C. Sambuceti for helpful advice. This work was supported by research grants from the ACS (MV230) and USPHS (AI20211). D. E. Jenkins was supported by USPHS predoctoral training grant (T32 AI07328).

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(Received 14 February 1994; Accepted 7 April 1994)