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 γIE2339aa, a 40K late gene product, were more abundant than those encoding IE2579aa, a 579 amino acid gene product made throughout infection. As expected, the cDNA capable of directing the expression of γIE2339aa was derived from a contiguous genomic region within exon 5 of the ie1/ie2 region. The cDNA clones encoding γIE2339aa 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, γIE2339aa trans-activated a variety of test promoters when cotransfected with the major z 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 γIE2339aa 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 z 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 z, 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 z 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; Kluchet al., 1989; Pizzorno et al., 1988; Starans 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 ie1 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 & Stammerger, 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 immuno-deficiency virus long terminal repeat (HIV LTR) (Biegalkie & 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\(^{491a}\), 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\(^{578a}\) is the principal a 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; Stinski et al., 1983). Besides a gene products, this region encodes at least one abundant y protein (Pizzorno et al., 1991; Puchtler & Stamminger, 1991; Stenberg et al., 1985, 1989), yIE2\(^{338a}\), 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 y transcript. Recent studies using a collection of IE2-specific monoclonal antibodies have shown that the open reading frame encoding this y 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 yIE2\(^{338a}\) has been studies on truncated versions of IE2\(^{578a}\) (Hermiston et al., 1990; Pizzorno et al., 1988, 1991; Stenberg et al., 1990).

In transient assays, IE2\(^{578a}\) 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 (Biegalkie & 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\(^{578a}\) is assayed in the presence of IE1\(^{491a}\), 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\(^{491a}\) and IE2\(^{578a}\) on a target promoter or the stimulation of IE2\(^{578a}\) expression by IE1\(^{491a}\) (Cherrington & Mocarski, 1989; Depto & Stenberg, 1989; Malone et al., 1990; Sambucetti 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\(^{578a}\) 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, yIE2\(^{338a}\), functions exclusively as a repressor of a 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\(^{578a}\) or yIE2\(^{338a}\) to crs, thereby blocking RNA polymerase II-mediated transcription, an hypothesis that has been strengthened by reports demonstrating that recombinant IE2\(^{578a}\) (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 & Stinski, 1993). A lack of evidence that the carboxy-terminal half of IE2\(^{578a}\) can trans-activate has sustained the idea that yIE2\(^{338a}\) functions solely as a repressor. Indeed, studies aimed at defining the activating domains of IE2\(^{578a}\) have concluded that the region representing yIE2\(^{338a}\) is incapable of trans-activation in the absence of the amino-terminal 85 amino acids normally present in IE2\(^{578a}\) (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 (Chiu 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\(^{578a}\) 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\(^{578a}\) 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 γIE2*38*. We report here that yIE2*38* can indeed function encoded by the ie2 gene to define more fully the activities of γIE2*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 yIE2*38* can indeed function as a trans-activator, particularly when introduced into cells along with IE1*51*. 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 μg 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 μg of each effector plasmid was added to 0.5 μg 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 H$_2$O to a final concentration of 200 μg/ml. Each transfection mixture was distributed equally between four wells (approx. 105 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, β-galactosidase (β-gal) levels were measured by adding 4-methylumbelliferyl-β-D-galactoside (MUG) to the medium at a concentration of 150 μg/ml and measuring the accumulated cleavage product after 4 h by a fluorometric assay as described previously (Geballe et al., 1986b). β-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 using 0.5 to 1.0 μg of effector DNA when the target DNA was held constant at 0.5 μg. 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 μg of a target plasmid and 2 μg of each effector plasmid were introduced into two wells (approx. 6.6 × 105 cells each) 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 μl 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 μl of each supernatant was assayed by incubation with acetyl coenzyme A and 0.125 μCi of 14C-radioisotalled 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 14C-radioisotalled 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 × 105 cells were seeded into two wells of a six-well culture dish, and each well was transfected with 2 μg 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 an oligo(dT)-cellulose column (Pharmacia). Polyadenylated RNA (3 μg) 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 SRα promoter derived from pcD-SRα (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 μg/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 μg/ml yeast tRNA, 7 mM-EDTA and 0.1 M-sodium phosphate buffer pH 8.0 using as a probe a 1:1 kb Clal–Stul fragment (Fig. 1) from pON303AAcc (Cherrington et al., 1991) specific for ie2 exon 5, radiolabelled with [α-32P]dCTP and [α-32P]ATP (Amersham), random synthetic hexanucleotide primers (Pharmacia) and Klenow polymerase (Sambrook et al., 1989). Candidate ie2 cDNA clones were grown in LB broth with 100 μg ampicillin/ml and plasmid DNA was purified from 5 ml overnight cultures by alkaline lysis (Sambrook et al., 1989). Southern blot hybridization using the same 1:1 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 digests 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 dideoxyribonucleotide chain termination sequencing method (Sequenase 2.0; US Biochemical) with 15 bp primers (5’ GTCGGGATATCCGG 3’ and 5’ AGGTATGATGTTCA 3’) homologous to vector DNA flanking the BstXI insertion sites of pME18S. The expression plasmids for IE2<sub>38</sub> (pON2206) and yIE2<sub>38</sub> (pON2203) 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 SRα promoter: pON2207 (IE2<sub>38</sub>; reverse orientation) and pON2204 (yIE2<sub>38</sub>; reverse orientation). An IE1<sub>51</sub> cDNA expression vector (pON2205) was constructed by inserting a 1549 bp EcoRI–Xbal fragment representing protein-coding sequences from exons, 2, 3 and 4 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, pON303AAcc, pON239, pON249, pON249rcs, pON284, pON283, pON2043, pON2044 and pON2046 (Cherrington et al., 1991; Cherrington & Mocarski, 1989; Geballe et al., 1986a; Spaete &
Fig. 1. Structure of HCMV (Towne) iel/iel 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 iel/iel 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–ClaI fragment) used to identify candidate cDNA clones is indicated by the bracket below the line. The iel/iel 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 (Spaete & Mocarski, 1985a), and the cDNA clones pON2205 (IE1491a cDNA), pON2206 (IE2579a cDNA) and pON2203 (IE2338a cDNA) SRα Promoter–enhancer.
carrying the E. coli lacZ gene, with the SV40 early polyadenylation signal and the following Kozak consensus start codons from the pSL300 polynucleotide added to the amino terminus of lacZ. GCAATGACATCATGACAGATC. pON826 was prepared by inserting a 1760 bp BamHI–XhoI fragment from plA2 (Larhammar et al., 1983) into BamHI–XhoI-digested pUC6S, and after deleting 242 bp between the two Psrl sites within the intron from the murine major histocompatibility complex (MHC) class II A2 gene to generate pON830, pON833 was prepared by inserting a 236 bp Sau3AI–YhoI fragment into SalI/Bgl-II-digested pMT11 (Spaete & Mocarski, 1985a). pON836 was prepared by inserting a 3.2 kbp EcoRI-Smal fragment from pON830 into Smal/EcoRI-digested pON833, which added an intron from the A2 gene to the lacZ construct. pON828 was prepared by inserting a 0.7 kbp Psrl fragment from pACT-CAT (Nudel et al., 1983) into Psrl-digested pUC120 (Vieira & Messing, 1991) and subsequently pON835 was made by moving a 0.6 bp RsaI–BamHI fragment into Smal/Bgl-II-digested pIC20H (Marsh et al., 1984). pON834 was constructed by inserting a 270 bp Sau3AI fragment from pSRα-CAT (Takebe et al., 1988) into BglII-digested pC20H and then moving a 0.6 kbp SalI fragment from pON835 into this plasmid to create pON837. pON838 was made by inserting a 3.6 kbp EcoRV-BglII fragment (after filling the 5’ overhang on the BglII site) into pON837, which had been digested by EcoRV and Smal. This plasmid placed the lacZ gene under control of the β-actin promoter (~600 to +28 relative to the transcription start site) fused to the R-U6 region of the HTLV-I LTR (+1 to +267 relative to the transcription start site), and a 230 bp fragment from the fourth intron of the murine MHC class II A2 gene. pON840 was constructed by removing a 0.5 kbp BamHI fragment from pON838, which generated a plasmid that had the β-actin promoter alone driving expression of lacZ and lacked any HTLV-I or MHC sequences. pON862 was made by inserting a 3.5 bp Cfr10–BamHI fragment from pON840 into Xmal/BamHI-digested pUC6S, thereby leaving a 138 β-actin promoter (~111 to +24 relative to the transcription start site) directing lacZ expression.

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 Clal–Stal fragment, an exon 5-specific probe (Fig. 1) that detected γIE2338aa and IE2579aa-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 kbp mRNA predicted to encode γIE2338aa, three candidate clones appeared to be derived from the 2.25 kbp mRNA expected to encode IE2579aa, and five clones did not correspond to either and were not further characterized. The predominance of 1.5 kbp 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 kbp γ transcript predominated over the larger, spliced IE2579aa transcript by approximately three- to fivefold at 74 h p.i.

Two γIE2338aa and two IE2579aa 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 SRα promoter (Fig. 1). The structure of the IE2579aa cDNA was identical to that of the 2.25 kbp 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 kbp cDNA corresponded to the γ mRNA previously predicted from transcript analysis (Stenberg et al., 1989). The IE2579aa and γIE2338aa 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).

γIE2338aa-mediated repression of α gene expression
The exon 5 region has been shown to encode an important domain of IE2579aa 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 γIE2338aa 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 SRα promoter rather than the iel/ie2 promoter–enhancer was used to drive expression from cDNAs. In addition to lacking a shutoff signal, the SRα 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 IE1p8aa expression plasmid (pON308) and to be repressed by an IE2579aa 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) IE1p8aa 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 (γIE2_{338aa}) or pON2206 (IE2_{579aa}). As was the case for activation, repression occurred whether genomic (pON308) or cDNA (pON2205) IE1 constructs were cotransfected with the ie2-expressing plasmids (Fig. 2). In the absence of IE1_{491aa}, γIE2_{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, γIE2_{338aa} exhibited the same repression characteristics as IE2_{579aa}. Control expression plasmids containing cDNAs for IE2_{579aa} or γIE2_{338aa} in the reverse orientation (pON2207 and pON2204) 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 γ 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

Repression 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 γIE2_{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 γIE2_{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 γIE2_{338aa} construct (pON2204) was tested, nor when an IE1_{491aa} frameshift mutation was
cotransfected with \( \gamma IE2_{338aa} \) (data not shown). When used alone, \( \gamma IE2_{338aa} \) (pON2203) failed to trans-activate expression (Fig. 3), even over a range of DNA levels (0.1 to 5.0 \( \mu g \); data not shown) suggesting that trans-activation by \( \gamma IE2_{338aa} \) was dependent on the presence of IE1_{491aa}. We observed this activity and dependence on IE1_{491aa} over a range of input DNA levels (0.5 to 4.0 \( \mu g \) of pON2203 (data not shown). Therefore, when present with IE1_{491aa}, \( \gamma IE2_{338aa} \) demonstrated a potential to trans-activate this \( crs \)-deficient promoter–enhancer, a result that is contrary to previous predictions of \( \gamma IE2_{338aa} \) function. \( \gamma IE2_{338aa} \) was able to cooperate as well with the IE1_{491aa} genomic (pON308) or cDNA (pON2205) constructs (Fig. 3 and data not shown).

IE2_{579aa} was consistently more active than \( \gamma IE2_{338aa} \) in trans-activation assays, and was able to trans-activate alone (Fig. 3). The reverse orientation IE2_{579aa} construct (pON2207) when introduced alone or with IE1_{491aa} had no activity.

**Enhancer sequences not required for trans-activation by IE2_{338aa} or IE2_{579aa}**

Because we had found that \( \gamma IE2_{338aa} \) trans-activated only when present with IE1_{491aa}, we investigated whether the target included enhancer elements such as the IE1_{491aa}-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 \( \gamma IE2_{338aa} \) or IE2_{579aa} alone or in combination with IE1_{491aa}, we employed a series of lacZ target constructs carrying deletions through the ie1/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_{491aa}-responsive elements in the enhancer (Cherrington \\& Mocarski, 1989).

IE2_{579aa} (pON2206) alone or in combination with IE1_{491aa} trans-activated all ie1/ie2 promoter–enhancer targets tested (Fig. 4a and b). When introduced into HF cells with IE1_{491aa}, 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 ie1/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 ie1/ie2 promoter–enhancer were cotransfected into HF cells with effecter 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. \( \beta \)-gal activity was determined and expressed as described in the legend to Fig. 2.
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 IE2<sub>579a</sub> plus IE1<sub>491a</sub> (Fig. 4b). IE2<sub>579a</sub> alone produced a five- to 10-fold trans-activation (Fig. 4a). Consistent with observations on other target promoters (Biegalke & 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 IE2<sub>579a</sub> alone or in combination with IE1<sub>491a</sub> 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 IE1<sub>491a</sub> alone (Cherrington & Mocarski, 1989).

Likewise, γIE2<sub>338aa</sub> (pON2203) plus IE1<sub>491a</sub> 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 IE2<sub>579a</sub> trans-activation by γIE2<sub>338aa</sub> (pON2203) plus IE1<sub>491a</sub> was independent of enhancer elements such as the 18 bp repeat even though trans-activation by IE1<sub>491a</sub> 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 γIE2<sub>338aa</sub> alone (not shown). Trans-activation levels with γIE2<sub>338aa</sub> plus IE1<sub>491a</sub> were not as great as with IE2<sub>579a</sub> plus IE1<sub>491a</sub>, 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 IE1<sub>491a</sub> plus either γIE2<sub>338aa</sub> or IE2<sub>579a</sub> is distinct from the mechanism of trans-activation by IE1<sub>491a</sub> alone. The target for the activity of the ie2 gene product is promoter-proximal and distinct from the upstream enhancer element required by IE1<sub>491a</sub>. IE1<sub>491a</sub> autoregulation of the ie1/ie2 enhancer does not appear to contribute in any detectable manner to trans-activation seen when IE1<sub>491a</sub> cooperates with γIE2<sub>338aa</sub> or IE2<sub>579a</sub>.

Promoter responsiveness to trans-activation by IE2<sub>338aa</sub> and IE2<sub>579a</sub>

To investigate further the trans-activation potential of γIE2<sub>338aa</sub> and to compare its activity to that of IE2<sub>579a</sub>, additional promoters were evaluated for activation with or without IE1<sub>491a</sub>. 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 promoter 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–U<sub>5</sub> linked to lacZ, was not trans-activated to any significant degree by IE1<sub>491a</sub>, alone, but was strongly trans-activated by either γIE2<sub>338aa</sub> or IE2<sub>579a</sub> in the presence of IE1<sub>491a</sub> (Fig. 5a). γIE2<sub>338aa</sub> (pON2203) plus IE1<sub>491a</sub> (pON2205) trans-activated this promoter approximately 12-fold. IE2<sub>579a</sub> (pON2206) plus IE1<sub>491a</sub> exhibited nearly a 20-fold trans-activation. Lower levels of trans-activation were observed with IE2<sub>579a</sub> when introduced in the absence of IE1<sub>491a</sub>, but γIE2<sub>338aa</sub> 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 γIE2<sub>338aa</sub> functioned as a trans-activator only when present with IE1<sub>491a</sub>.

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–U<sub>5</sub> 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 γIE2<sub>338aa</sub> and IE1<sub>491a</sub> 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 γIE2<sub>338aa</sub> 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 γIE2<sub>338aa</sub>. Again this activity was generally dependent on the presence of IE1<sub>491a</sub>. 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. IE2338aa 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 γIE2\textsuperscript{338aa} 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 IE2\textsuperscript{579aa} or γIE2\textsuperscript{338aa} with or without IE1\textsuperscript{491aa} (Table 1). Thus, we found no evidence that γIE2\textsuperscript{338aa} autoregulated its expression. Furthermore, we tested pEC (Imperiale et al., 1985), which is similar to pE2-CAT, a construct that had been reported to respond to a truncated form of IE2\textsuperscript{579aa} (Hermiston et al., 1987). We did not detect any trans-activation by γIE2\textsuperscript{338aa} (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 γIE2\textsuperscript{338aa} plus IE1\textsuperscript{491aa} (data not shown). These included two early HCMV promoters, those of DNA polymerase and of the β\textsubscript{2}y genes, as well as the late HCMV promoter of the ICP36 (UL44) gene. All of these promoters were activated when IE2\textsuperscript{579aa} plus IE1\textsuperscript{491aa} 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 γIE2\textsuperscript{338aa} trans-activation is more restricted than that of IE2\textsuperscript{579aa}. It would appear from our small survey that the role of γIE2\textsuperscript{338aa} 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 γIE2\textsuperscript{338aa} 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 γIE2\textsuperscript{338aa} manifested the properties of a trans-activator of gene expression. Trans-activation by γIE2\textsuperscript{338aa} usually depended on the presence of IE1\textsuperscript{491aa}, but this combination did not trans-activate any of the HCMV β promoters tested. These results are in sharp contrast to those obtained with IE2\textsuperscript{579aa} which has been found to trans-activate by itself, to cooperate with IE1\textsuperscript{491aa} and to activate CMV β promoters. Under most conditions, γIE2\textsuperscript{338aa} exhibited lower levels of trans-activation, and more promoter specificity than did IE2\textsuperscript{579aa}. Considering the very high levels of expression of this protein late in infection, it is tempting to speculate that IE2\textsuperscript{338aa} will have an activity with some physiological relevance.

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## Table 1. Trans-activation of CAT constructs

<table>
<thead>
<tr>
<th>CAT construct</th>
<th>pHIV-CAT</th>
<th>pSRα-CAT</th>
<th>pSV2-CAT</th>
<th>pIE2P-CAT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector plasmid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Target alone</td>
<td>1† (0.05)</td>
<td>1 (0.90)</td>
<td>1 (0.35)</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>308</td>
<td>3 (0.12)</td>
<td>2 (1.46)</td>
<td>1 (0.18)</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>308 + 2206</td>
<td>57 (2.68)</td>
<td>5 (4.90)</td>
<td>1 (0.40)</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>308 + 2203</td>
<td>11 (0.11)</td>
<td>10 (8.75)</td>
<td>3 (1.10)</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>2206</td>
<td>6 (0.30)</td>
<td>2 (1.50)</td>
<td>1 (0.30)</td>
<td>2 (0.10)</td>
</tr>
<tr>
<td>2203</td>
<td>1 (0.04)</td>
<td>5 (4.05)</td>
<td>1 (0.40)</td>
<td>1 (0.06)</td>
</tr>
<tr>
<td>No DNA</td>
<td>1 (0.05)</td>
<td>0.3 (0.30)</td>
<td>0.8 (0.25)</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>HCMV 72 h p.i.†</td>
<td>ND§</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Values shown for this construct are from single samples not duplicates except for the HCMV 72 h p.i. results.
† Results are given as the fold increase in activity over that of the construct alone. The figure in parenthesis is the percentage conversion of radiolabelled chloramphenicol.
‡ Infection of cells with HCMV (Towne) at an m.o.i. of 5.
§ ND, Not determined.

References


Baracchini, E., Glezer, E., Fish, K., Stenberg, R. M., Nelson, J. A.


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