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Characterization of the Regulatory Functions of the Equine Herpesvirus 1 Immediate-Early Gene Product

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Use of the translation-inhibiting drug cycloheximide has indicated that the equine herpesvirus 1 (EHV-1) immediate-early (IE) gene, the sole EHV-1 IE gene, encodes a major viral regulatory protein since IE mRNA translation is a prerequisite for all further viral gene expression (W. L. Gray, R. P. Baumann, A. T. Robertson, G. B. Caughman, D. J. O'Callaghan, and J. Staczek, Virology 158:79–87, 1987). An EHV-1 IE gene expression vector (pSVIE) in combination with chimeric EHV-1 promoter–chloramphenicol acetyltransferase (CAT) reporter constructs was used in transient transfection assays to characterize the regulatory functions of the IE gene product. These experiments demonstrated that (i) the EHV-1 IE gene product is a bifunctional protein capable of both positive and negative modulation of gene expression; (ii) the IE gene product possesses an autoregulatory function which represses the IE promoter; (iii) IE autoregulation is dependent on IE promoter sequences mapping within positions −288 to +73 relative to the transcription initiation site (+1) of the IE gene; (iv) the IE gene product can independently activate the EHV-1 *tk* promoter (an early promoter) by as much as 60-fold; (v) two EHV-1 beta-gamma (leaky late) promoters, those of IR5 (gene 5 in the inverted repeat) and the glycoprotein D gene, demonstrate a requirement for both the IE gene product as well as a gene product encoded within the EHV-1 *Xba*I G fragment for significant activation; and (vi) the IE gene product is capable of activating heterologous viral promoters.

Equine herpesvirus 1 (EHV-1), a member of the family *Herpesviridae* and subfamily *Alphaherpesvirinae*, is the causative agent of a number of equine pathological states, including neurological disorders, respiratory infections, and epizootic abortion in pregnant mares (1, 39). The infectious EHV-1 virion is enveloped, possesses an icosahedral capsid, and harbors a linear, double-stranded DNA molecule of approximately 150 kbp. The genomic structure of EHV-1 (Fig. 1A) has been extensively examined (29, 52, 60, 63). The viral chromosome consists of a long region (L) covalently linked to a short region (S). The long region contains mostly nonrepetitive unique sequences (U_L), while the short region is composed of a segment of unique sequence complexity (U_S) flanked by inverted repeat sequences (IR_S and TR_S). The S region inverts with respect to the L region, thus giving rise to two isomeric forms of the viral genome.

During a productive lytic infection, the genes of EHV-1 are coordinately expressed and temporally regulated in an immediate-early (IE), early, and late fashion (6, 21) analogous to that of herpes simplex virus type 1 (HSV-1) (30). Gray et al. (21, 22) have reported a single IE transcript, 41 to 45 early transcripts, and 18 to 20 late transcripts. The 6-kb polyadenylated IE transcript maps to the inverted repeats of the S region; therefore, the IE gene exists in two copies per viral chromosome (map units 0.78 to 0.82 and 0.96 to 1.00). S1 nuclease and primer extension analyses have been used to map both the 3′ and 5′ termini of the EHV-1 IE mRNA and have revealed that splicing occurs within the untranslated leader sequence (25, 26). Interestingly, an early 4.4-kb transcript which is 3′ coterminal with the IE mRNA has been reported and is believed to encode a truncated form of the IE protein (27). The EHV-1 IE gene (including 5′- and 3′-flanking regions) has been sequenced (24). A major open reading frame (ORF) of 1,487 codons (4,461 bp) is present which, when translated, predicts a proline- and alanine-rich polypeptide of 155 kDa. Four antigenically cross-reactive EHV-1 IE polypeptides (ranging in size from 200 to 125 kDa) have been reported and characterized (5, 6, 48). The largest of the IE polypeptides (IE1) is the most abundant species and represents the major phosphorylated form of the protein (5). Two regions of approximately 50% homology (at the amino acid level) exist between the EHV-1 IE protein and ICP4 of HSV-1 and the ORF62 gene product of varicella-zoster virus (VZV) (24). It is postulated that the EHV-1 IE gene product is the functional equivalent of the ORF62 gene product and ICP4. Analyses of temperature-sensitive ICP4 mutants have indicated that ICP4 is an essential gene and that functional ICP4 molecules are required for progression of the lytic cycle into early and late phases (11, 59).

A detailed understanding of the regulatory events during productive EHV-1 infection is lacking. Early studies demonstrated a viral DNA replication-mediated change in viral RNA synthesis patterns (7, 8, 31). More recent work has indicated that, in addition to a late DNA replication-mediated event, expression of the EHV-1 IE gene is essential to the lytic program in that inhibition of IE mRNA translation by cycloheximide precludes the detectable expression of all other viral genes (21). This finding implicates the IE gene product as a key regulatory factor, and the IE gene (IR1) has, therefore, become the subject of detailed investigation.

In this study, we have used transient transfection assays to characterize the regulatory functions of the IE gene. An IE gene expression vector was constructed by placing the IE ORF under the control of the simian virus 40 (SV40) early promoter and enhancer elements. LM cells were transfected with the EHV-1 IE expression vector and chimeric reporter gene constructs containing various EHV-1 promoter ele-

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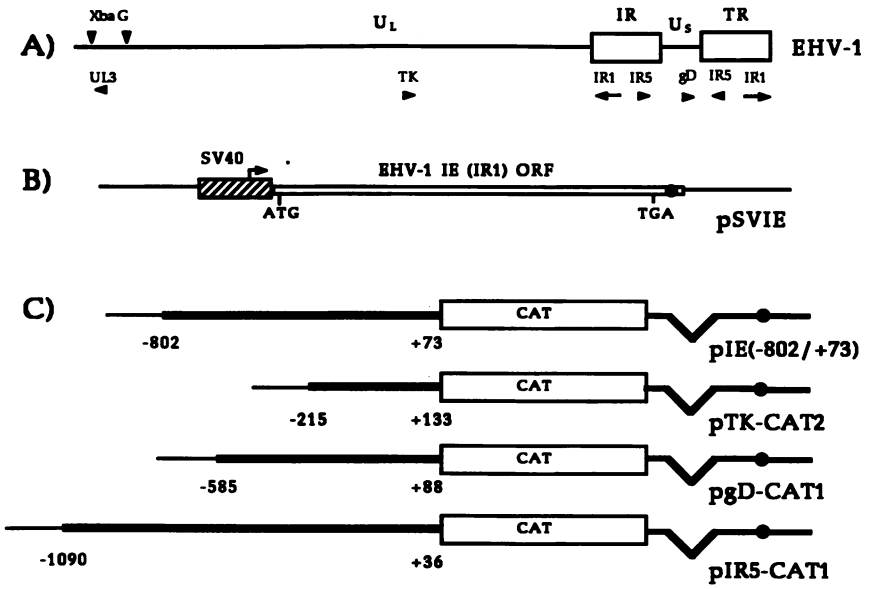

FIG. 1. (A) Schematic representation of the EHV-1 genome. The relative positions of the IE (IR1), *TK*, gD, IR5, and UL3 (ICP27 homolog) genes are indicated by horizontal arrows and arrowheads. Also, the relative position of the *Xba*I G fragment (which contains UL3) is shown. (B) Representation of the EHV-1 IE gene expression vector pSVIE. A 4,772-bp DNA restriction fragment (open rectangle) containing the IE (IR1) ORF was placed under the control of the SV40 early promoter and enhancer. The approximately 340-bp SV40 fragment (striped rectangle) also contains the SV40 origin of replication and late promoter. *cis*-acting polyadenylation functions are provided by EHV-1 sequences. The polyadenylation signal (AATAAA) is indicated by a filled circle. (C) General structures of four EHV-1 promoter–reporter constructs used in this study. The various EHV-1 promoter fragments are represented on the left by filled rectangles. Numerals indicate distances from the transcription initiation site (+1; values for pTK-CAT2, pIR5-CAT1, and pgD-CAT1 are approximate). The region encoding CAT is indicated by an open rectangle. Splicing (V shape) and polyadenylation (filled circle) functions are indicated on the right and are of SV40 origin.

ments linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. CAT enzyme activity was assayed as a measure of promoter responsiveness to IE gene expression. We demonstrate that the IE gene product is an autoregulatory *trans*-acting protein capable of both positive and negative modulation of target gene expression. In addition, it was determined that the EHV-1 *Xba*I G fragment, which contains a homolog of the HSV-1 UL54 (ICP27) gene (64), is a necessary cofactor in the IE-mediated induction of two EHV-1 promoters.

MATERIALS AND METHODS

Cells and virus. The Kentucky A strain of EHV-1 was propagated at low multiplicity of infection in LM cell suspension cultures and assayed for infectivity as previously described (38, 45). Suspension cultures of LM cells (murine fibroblasts) were maintained in YELP suspension medium (yeast extract, lactalbumin hydrolysate, peptone) containing 0.12% methyl cellulose, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5% fetal bovine serum. RK-13 (rabbit kidney) cells and COS-1 cells (African green monkey kidney cells expressing SV40 large T antigen) were maintained in Eagle minimum essential medium (EMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, and 5% fetal bovine serum.

Western immunoblot analysis. Infected- and transfected-cell monolayers were harvested in 1× sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8), vortexed, heated to 100°C for 5 min, sonicated, and centrifuged briefly in an Eppendorf microfuge. Lysate aliquots were subjected to SDS-polyacrylamide gel electrophoresis (34) on 7.5% polyacrylamide gels. Separated polypeptides were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.). The filters were rinsed in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and treated for 30 min in TBST containing 0.4% gelatin. The blocked filters were then incubated for 30 min with an EHV-1 IE-specific monoclonal antibody (MAb B3.3) diluted 1:1,000 in TBST. After several rinses in TBST, the filters were incubated for an additional 30 min with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega, Madison, Wis.). Following several rinses in TBST, reactive protein bands were visualized by incubation in AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing nitroblue tetrazolium (0.33 mg/ml; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and 5-bromo-4-chloro-3-indolyl phosphate 0.165 mg/ml; Bethesda Research Laboratories).

Plasmids. Cloning was performed by using standard procedures (35). The EHV-1 IE–CAT reporter recombinant, pIE(−1814/+73), was constructed by inserting an approximately 1.9-kb *Hin*dIII fragment containing the intergenic region between the EHV-1 IR1 (IE) and IR4 genes (see Fig. 5) into the *Hin*dIII site of pCAT-Basic (Promega). Plasmid pCAT-Basic contains the CAT gene and SV40-derived splicing and polyadenylation functions in a pUC19 background. Plasmid pIE(−1488/+73) was constructed by ligating the 3.2-kb *Spe*I-*Bam*HI fragment of pIE(−1814/+73) into the *Xba*I-*Bam*HI sites of pUC12. Plasmid pIE(−802/+73) was constructed by cloning the 1.45-kb *Eco*RI-*Bam*HI fragment of pFG65 (which contains IE gene sequences from −802 to +630) into the *Eco*RI-*Bam*HI sites of pAT153 to generate

pSR10, followed by ligation of a 1.7-kb *Hin*dIII-*Bam*HI fragment containing the CAT gene and SV40 splicing and polyadenylation signals (excised from pCAT-Basic) to the 4.4-kb *Hin*dIII-*Bam*HI fragment of pSR10. Plasmid pIE(−359/+73) contains the 2.1-kb *Xma*I-*Bam*HI fragment of pIE(−1814/+73) in the *Xma*I-*Bam*HI sites of pUC12. A 2.0-kb *Nde*I-*Bam*HI fragment of pIE(−1814/+73) was ligated to the 2.4-kb *Nde*I-*Bam*HI fragment of pBR322 to generate pIE(−288/+73). pSV2cat (20) contains the CAT gene of the bacterial transposon Tn*9* under the control of the SV40 early promoter and enhancer. The 1.8-kb *Sph*I-*Bam*HI fragment of pSV2cat was ligated to the 4.2-kb *Sph*I-*Bam*HI fragment of pIE(−1814/+73) to generate pIE(−1814/−343)SV. The intact EHV-1 *tk* gene (49) was kindly provided by J. Millar Whalley, Macquarie University, Sydney, Australia. Plasmid pTK-CAT2 contains *tk* promoter sequences from approximately −215 to +133 linked to CAT-coding sequences and SV40 splicing and polyadenylation functions (derived from pCAT-Basic) in a partial pAT153 background. Plasmid pgD-CAT1 was constructed by ligating a 0.8-kb *Nar*I-*Hin*dIII fragment (containing the EHV-1 glycoprotein D (gD) promoter from approximately −585 to +88) to the 5.0-kb *Cla*I-*Hin*dIII fragment of pAT-CAT. Plasmid pAT-CAT contains the 1.7-kb *Hin*dIII-*Bam*HI fragment of pCAT-Basic ligated to the 3.3-kb *Hin*dIII-*Bam*HI fragment of pAT153. Plasmid pIR5-CAT1 contains IR5 promoter sequences from approximately −1090 to +36 (relative to the cap site) linked to CAT-coding and SV40 splicing and polyadenylation functions (derived from pCAT-Basic) in a pUC background. pSV2neo (56) contains the aminoglycoside phosphotransferase II gene of the bacterial transposon Tn*5* under the control of the SV40 early promoter and enhancer. pSV12 was generated by cloning the multiple cloning site of pUC12 into the *Hin*dIII-*Eco*RI sites of pSV2neo, thus deleting the aminoglycoside phosphotransferase II gene and SV40-derived RNA processing signals. Plasmid pSVIE contains an approximately 4.8-kbp *Hin*dIII-*Eco*RI fragment (encompassing the EHV-1 IE ORF and polyadenylation signal) ligated to *Eco*RI-*Hin*dIII-digested pSV12. Plasmid pCAT-Promoter (Promega) contains the CAT gene under the control of an enhancerless SV40 early promoter. pXba-G contains the EHV-1 *Xba*I G fragment cloned into the *Xba*I site of pACYC184.

Transfection procedure. LM cells, plated at a density of 4×10^6 cells per 60-mm tissue culture dish in EMEM, were allowed to attach overnight at 37°C in a humidified atmosphere containing 5% CO_2. The next day, LM cell monolayers were subjected to liposome-mediated DNA transfection (16) by the use of Lipofectin reagent (Bethesda Research Laboratories) essentially as described by the manufacturer. Briefly, the monolayers were rinsed twice with serum-free EMEM, and a final 3 ml of serum-free EMEM was added to each plate. Transfecting DNA was combined with Lipofectin reagent (5:1, wt/wt), incubated for 15 min, and added to the appropriate tissue culture dish in a dropwise fashion. The monolayers were incubated for 5 h at 37°C in a 5% CO_2 atmosphere, after which time the medium was removed, replaced with EMEM containing 5% fetal bovine serum, and incubated for an additional 60 to 62 h. All plasmids containing CAT-coding sequences were transfected in equimolar amounts (1.4 pmol). Similarly, effector plasmids (i.e., pSVIE and pXba-G) were transfected in equimolar amounts (0.3 pmol). The final amount of DNA applied per transfection was adjusted to 8 μg by the addition of pUC12. Where indicated in the text, cells which did not receive pSVIE instead received an equimolar amount of pSV2neo as a control for promoter competition (pSV2neo contains the same SV40-derived promoter and enhancer elements as does pSVIE).

CAT assays. Transfected cells were harvested and assayed for CAT activity essentially as described by Rosenthal (50). Briefly, cell monolayers were rinsed with phosphate-buffered saline and then scraped into 1 ml of TEN solution (40 mM Tris-HCl [pH 7.5], 10 mM EDTA, 150 mM NaCl). The cells were pelleted in a microcentrifuge, resuspended in 200 μl of 250 mM Tris-HCl (pH 8.0), and subjected to five freeze-thaw cycles. Cell lysates were heated to 60°C for 10 min and cleared by microcentrifugation. The total protein concentration of each lysate was determined by using the BCA protein assay reagent (Pierce, Rockford, Ill.). Equal amounts of protein were assayed for CAT activity. Each sample was adjusted to 158 μl (with 250 mM Tris-HCl, pH 8.0), mixed with 2 μl of [^{14}C]chloramphenicol (0.1 μCi, 50 to 60 mCi/mmol; New England Nuclear Corp., Boston, Mass.) and 20 μl of 3.5 mg/ml acetyl coenzyme A, and then incubated at 37°C for 60 min. Chloramphenicol and its acetylated products were extracted into 1 ml of ethyl acetate, which was then evaporated by centrifugation in vacuo. Dried samples were resuspended in ethyl acetate, and equal portions were spotted onto thin-layer chromatography (TLC) sheets (silica gel IB; J. T. Baker). The reaction products were chromatographed for 2 h in a TLC tank containing chloroform-methanol (19:1, vol/vol). Air-dried sheets were exposed (at −70°C) to Kodak X-Omat XAR-5 film. Chloramphenicol and its acetylated products were excised from the developed TLC sheets, and the radioactivity associated with each spot was quantitated by liquid scintillation counting. To control for variations in transfection efficiency, multiple individual transfections were performed and assayed for CAT activity. Individual lysates, from each set of transfection experiments, were reassayed to provide a visual representation of the results.

RESULTS

EHV-1 IE polypeptide expression in COS-1 cells transfected with the EHV-1 IE gene expression vector pSVIE. An EHV-1 IE gene expression vector (pSVIE) was constructed by placing the major IE ORF under the control of the SV40 early promoter and enhancer. To determine the ability of pSVIE to direct the synthesis of full-length IE polypeptides, COS-1 cells (African green monkey kidney cells which express SV40 large T antigen) were transfected with pSVIE or a negative control plasmid (pSV12), and transfected-cell polypeptides were examined by Western blot analysis using an EHV-1 IE-specific MAb (B3.3). Plasmid pSV12 is the pSVIE parental vector and contains the SV40 early and late promoters, enhancer element, and origin of replication in a partial pBR322 background. The MAb detected an approximately 190-kDa IE polypeptide band within the lysates of EHV-1-infected LTK$^-$ cells (Fig. 2, lane 3) and a similarly migrating IE MAb-reactive band in the lysates of pSVIE-recipient, but not pSV12-recipient, COS-1 cells (lanes 1 and 2). A lower-molecular-size cross-reactive band of approximately 95 kDa was detected in both pSVIE-recipient and non-pSVIE-recipient COS-1 cells (lanes 1 and 2). IE polypeptides could not be detected by immunoblot analysis in LM cells transfected with pSVIE. The ability to detect IE polypeptide expression from pSVIE in COS-1 cells may be due to replication of this SV40-origin-containing plasmid within these cells, thus resulting in a higher plasmid copy number per cell than in transfected LM cells in which pSVIE

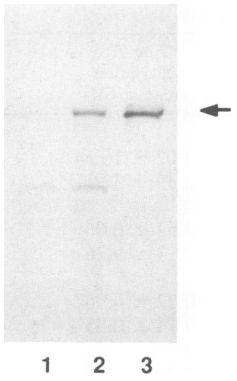

FIG. 2. Western blot analysis of COS-1 cells transfected with the EHV-1 IE gene expression vector pSVIE. To determine the ability of pSVIE to function as an expression vector, COS-1 cells were transfected with either 5 μg of the negative control plasmid pSV12 or 5 μg of pSVIE. Plasmid pSV12 is the pSVIE parental vector and contains the SV40 early and late promoters, enhancer element, and origin of replication in a partial pBR322 background. A positive control for IE polypeptide production was provided by LTK$^-$ cells infected with EHV-1 (multiplicity of infection of 20) and harvested 3 h postinfection. Lanes: 1, pSV12-transfected COS-1 cells; 2, pSVIE-transfected COS-1 cells; 3, EHV-1-infected LTK$^-$ cells. The arrow indicates the 190-kDa IE polypeptide band within the infected-cell lysate and the similarly migrating IE MAb-reactive band in pSVIE-recipient, but not pSV12-recipient, COS-1 cells.

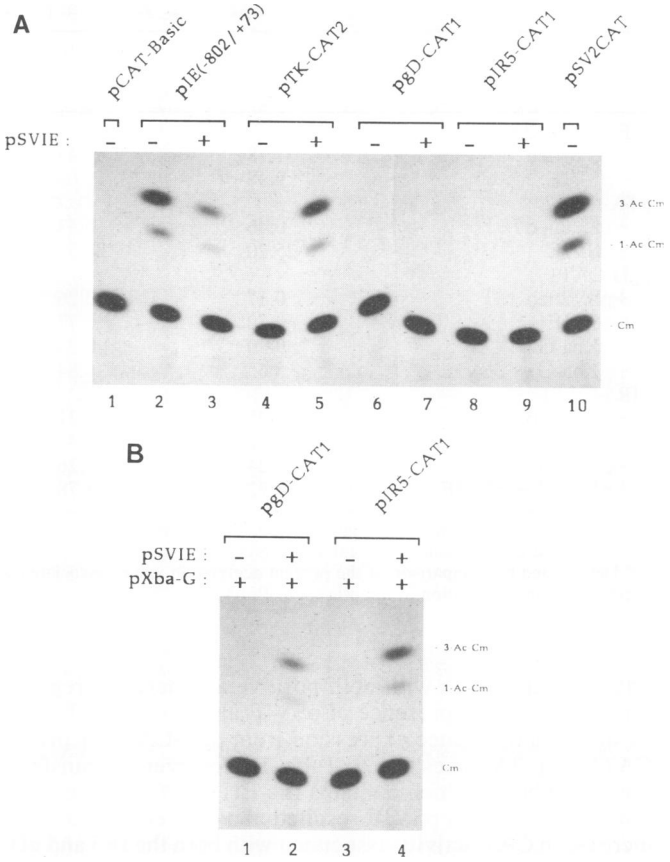

FIG. 3. (A) Effect of the EHV-1 IE gene product on various EHV-1 promoters. LM cells were cotransfected with the EHV-1 IE expression vector pSVIE and various EHV-1 promoter–reporter constructs representing different temporal classes of EHV-1 genes. The reporter constructs (indicated at the top) were applied in equimolar amounts (1.4 pmol). The cells received either pSVIE (0.3 pmol; lanes 3, 5, 7, and 9) or, to control for nonspecific effects due to promoter competition, an equimolar amount of pSV2neo (lanes 2, 4, 6, and 8). pCAT-Basic (lane 1) contains no eukaryotic promoter and was included as a negative control. pSV2cat (20) was included as a positive control. Transfected cells were harvested 60 to 62 h posttransfection and assayed for CAT activity. Samples 1 to 6 and 7 to 10 represent equal exposures of two separate autoradiographs. (B) Assay showing that two EHV-1 beta-gamma promoters require the presence of both pXba-G and pSVIE for full expression. LM cells were transfected with pgD-CAT1 or pIR5-CAT1 and either pXba-G or both pXba-G and pSVIE combined. The transfected cells were harvested and assayed for CAT activity 60 to 62 h posttransfection. Lanes 1 and 2 and lanes 3 and 4 were excised from the same autoradiograph. The effects of pSVIE alone on pgD-CAT1 and pIR5-CAT1 expression are shown in Fig. 3A. Cm, chloramphenicol; 1-Ac-Cm, 1-acetyl-chloramphenicol; 3-Ac-Cm, 3-acetyl-chloramphenicol.

is not replicated. Alternatively, the SV40 enhancer may not function as efficiently in murine LM cells as in simian COS cells.

EHV-1 promoters exhibit differential responses to IE gene expression. To test whether the EHV-1 IE gene product can act as a *trans*-acting factor and to ascertain its effect on promoters representative of different temporal classes of EHV-1 genes, LM cells were cotransfected with various EHV-1 promoter–CAT recombinants (Fig. 1C) and the EHV-1 IE expression vector pSVIE (Fig. 3A, lanes 3, 5, 7, and 9) or, to control for nonspecific effects due to promoter competition, an equimolar amount of pSV2neo (lanes 2, 4, 6, and 8). Plasmid pSV2neo (56) contains the same SV40-derived promoter and enhancer sequences as does pSVIE. Plasmids pIE(−802/+73), pTK-CAT2, pgD-CAT1, and pIR5-CAT1 contain promoter elements from the EHV-1 IE, thymidine kinase, gD, and IR5 genes, respectively. Northern blot analysis indicates that the EHV-1 *tk* gene encodes an approximately 1.3-kb early transcript (55). Robertson and Whalley (49) have sequenced the EHV-1 *tk* gene and have determined that a 3.15-kb *Bam*HI-*Pst*I subclone containing the *tk* ORF can restore the *tk*$^+$ phenotype to LMTK$^-$ cells. The EHV-1 gD gene, a beta-gamma (or leaky late) gene (18), encodes a glycoprotein showing significant homology to HSV-1 gD as well as the gD homologs of other herpesviruses (2, 17, 51). The EHV-1 IR5 gene, which encodes a 0.9-kb beta-gamma (or leaky late) transcript, exhibits homology to ORF64 of VZV and US10 of HSV-1 (28). Plasmid pCAT-Basic (Promega) contains the CAT gene and SV40-derived splicing and polyadenylation functions in a pUC19 background but lacks a eukaryotic promoter and was therefore used as a negative control. Plasmid pSV2cat (20) contains the CAT gene driven by the SV40 early promoter and enhancer and was used as a positive control.

The results presented in Fig. 3A and Table 1 indicate that the EHV-1 IE gene product is a *trans*-acting protein that can activate EHV-1 gene expression and can autoregulate its own promoter in a negative fashion. When pSVIE was cotransfected with the EHV-1 IE promoter–CAT recombinant pIE(−802/+73), an approximately fourfold decrease in CAT activity was observed (Fig. 3A, lanes 2 and 3; Table 1). Furthermore, pIE(−802/+73) responded to cotransfection with pSVIE in a dose-dependent fashion (Fig. 4). Cotransfection of pSVIE with pTK-CAT2 resulted in an approximately 60-fold induction of CAT activity (Table 1; Fig. 3A, lanes 4 and 5). Therefore, the EHV-1 IE gene product is a

TABLE 1. Effects of pSVIE on various EHV-1 promoters

Plasmids transfected	CAT activity[a]			Avg % acetylation (mean ± SD)	Fold change[b] (mean ± SD)
	1	2	3		
pIE(−802/+73)					
+pSV2neo	37.78	53.44	70.84	54.02 ± 16.54	
+pSVIE	9.48	11.08	16.91	12.49 ± 3.91	0.23 ± 0.07
pTK-CAT2					
+pSV2neo	0.46	0.53	0.58	0.52 ± 0.06	
+pSVIE	25.20	28.53	42.65	32.13 ± 9.26	61.78 ± 17.82
pgD-CAT1					
+pSV2neo	0.17	0.26	ND	0.22 ± 0.06	
+pSVIE	0.70	0.77	ND	0.74 ± 0.05	3.34 ± 0.23
+pXba-G	0.20	0.28	ND	0.24 ± 0.06	1.09 ± 0.25
+pXba-G + pSVIE	5.65	6.84	ND	6.24 ± 0.84	28.39 ± 3.83
pIR5-CAT1					
+pSV2neo	0.25	0.31	ND	0.28 ± 0.04	
+pSVIE	0.41	0.48	0.93	0.61 ± 0.28	2.16 ± 1.01
+pXba-G	0.24	0.26	0.32	0.27 ± 0.04	0.98 ± 0.15
+pXba-G + pSVIE	4.72	5.78	8.76	6.42 ± 2.10	22.93 ± 7.48

[a] Determined as percent of acetylation. Results of independent transfection experiments are shown. Since each set of transfections was performed in parallel, the results are presented in numerical order. ND, not done.

[b] Determined by comparison of the percent acetylation values associated with the target plasmid in the presence of the indicated effector plasmid versus the average percent acetylation in the presence of pSV2neo.

bifunctional protein with both positive and negative regulatory abilities. The presence of pSVIE alone was insufficient to significantly induce expression from pgD-CAT1 or pIR5-CAT1 (Fig. 3A, lanes 6 to 9; Table 1); however, cotransfection of pXba-G (which contains the EHV-1 *Xba*I G restriction fragment) with pSVIE resulted in a greater than 20-fold increase in CAT activity associated with both the IR5 and gD reporter constructs (Fig. 3B and Table 1). In contrast, pXba-G alone had no detectable effect on the IR5 or gD promoter. Cooperative induction of the IR5 and gD promoters by pXba-G and pSVIE cotransfection was also observed with *Xba*I-G in a pAT153 background (data not shown). The *Xba*I G fragment (Fig. 1A) is currently being sequenced in our laboratory and contains an ORF (UL3) which, when translated, predicts a polypeptide showing significant homology to the UL54 (ICP27) gene product of HSV-1 (64).

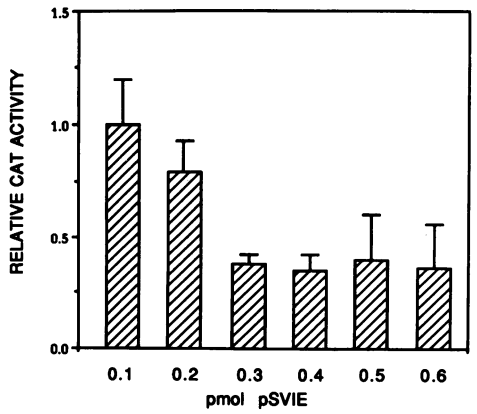

FIG. 4. Response of pIE(−802/+73) to increasing amounts of pSVIE. LM cells were transfected with 1.4 pmol of pIE(−802/+73) and the indicated amounts of pSVIE (no carrier DNA was added). Each transfection was performed in duplicate. The results are presented as fractions of the mean CAT activity observed for 1.4 pmol pIE(−802/+73) cotransfected with 0.1 pmol pSVIE (the activity of which was arbitrarily defined as 1).

Sequences within region −288 to +73 of the IE gene are required for IE autoregulation. To determine the effect of *cis*-acting elements on IE gene expression and autoregulation, LM cells were cotransfected with pSVIE (or pSV2neo) and various IE promoter-CAT recombinants containing sequential 5′ deletions within the IE promoter region (Fig. 5). The results (Fig. 6 and Table 2) show that the ability of the IE gene product to regulate its own promoter partitions with the smallest IE promoter construct tested [pIE(−288/+73); Fig. 6, lanes 9 and 10], indicating that sequences from −1814 to −288 do not play a significant role in IE autoregulation. To confirm that autoregulation is dependent on the remaining EHV-1 sequences (i.e., −288 to +73), nucleotides from −343 to +73 were deleted from the parental IE-CAT vector [pIE(−1814/+73)] and replaced with approximately 200 bp of SV40 sequence containing the SV40 early promoter minus the enhancer, thus generating pIE(−1814/−343)SV. Plasmid pIE(−1814/−343)SV was no longer down-regulated in the presence of pSVIE but was up-regulated by approximately fourfold (Fig. 6, lanes 11 and 12; Table 2). Taken together, these observations indicate that sequences within −288 to +73 of the IE cap site are required for IE gene product-mediated autoregulation. A comparison of the basal levels of promoter activity observed for pIE(−802/+73) and pIE (−359/+73) indicates that the promoter region between approximately −800 to approximately −360 (which contains a cluster of potential *cis*-acting elements; Fig. 5) makes a significant contribution to IE promoter function (Table 2). Sequences upstream of −802 had a mild inhibitory affect on basal levels of IE promoter activity [compare pIE(−1814/+73) and pIE(−1488/+73) with pIE(−802/+73)].

Effect of pSVIE on heterologous promoters. To determine the ability of the EHV-1 IE gene product to *trans* activate heterologous promoters, LM cells were cotransfected with pSVIE, or an equimolar amount of pSV2neo, and chimeric reporter plasmids containing promoter elements derived from either SV40 or HSV-1. Plasmids pPOH2 and pPOH3 (kindly provided by G. S. Hayward, Johns Hopkins University School of Medicine, Baltimore, Md.) are HSV-1 promoter–CAT recombinants containing the ICP4 promoter and

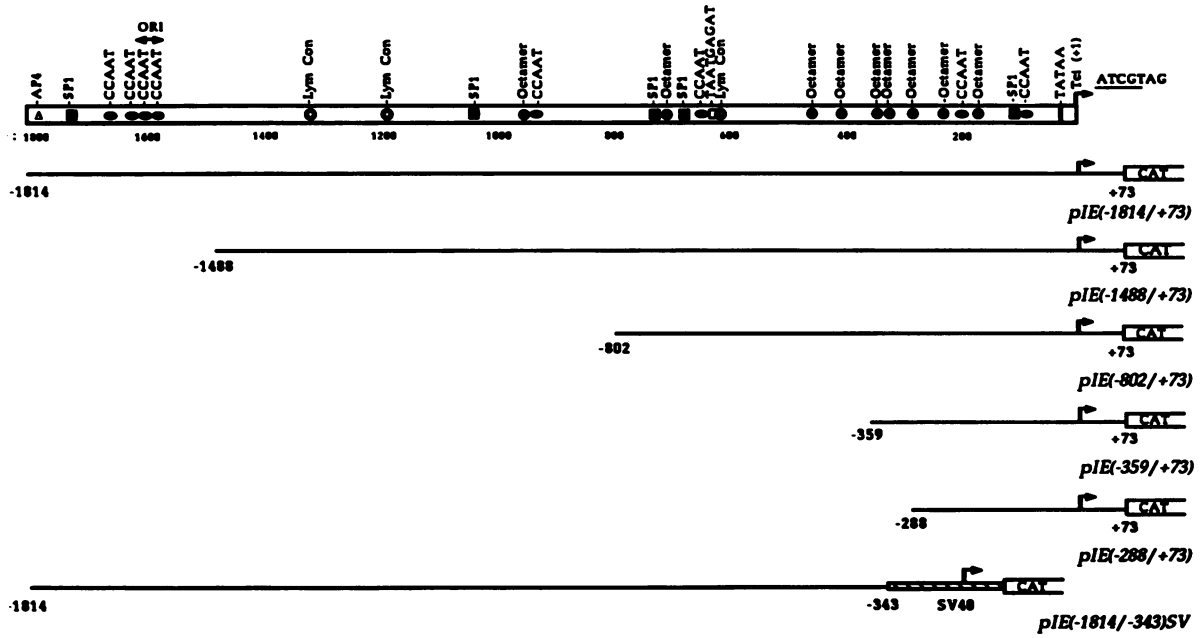

FIG. 5. Gene structure and constructs used. The open rectangle at the top represents the approximately 1,800-bp intergenic region between the IE gene (IR1) and the divergently transcribed IR4 gene (ICP22 homolog). The transcription initiation site of the IR4 gene has recently been mapped (28) and lies further to the left. A functional origin of replication (ORI) has been characterized (3) and maps approximately 1,600 bp from the IE transcription initiation site (Tci). Sequence data have indicated numerous potential *cis*-acting elements: octamer (ATGCAAAT), SP1 (GGGCGG), CCAAT (CTF binding), TAATGARAT, AP4 (CAGCTGTGG), and a consensus sequence occurring upstream of various lymphokine genes (Lym Con, GRGRTTYCAY [57]). ATCGTAG represents nucleotides −6 to +1. The underlined pentamer is conserved in close proximity to the cap site of the ICP4 gene as well as the ICP4 homologs of other alphaherpesviruses (62). Below are depicted deletion constructs used to examine the effects of the potential *cis*-acting elements on IE gene expression and autoregulation. The extent of the intergenic region contained within the constructs is indicated by the plasmid nomenclature. An approximately 415-bp fragment containing the EHV-1 TATA element and cap site, as well as the 73-bp untranslated leader, is deleted in pIE(−1814/−343)SV and is replaced by the SV40 early promoter (minus the enhancer element).

the *tk* promoter, respectively (40–42). In contrast to its effects on the EHV-1 IE promoter, the IE gene product did not negatively modulate the HSV-1 ICP4 promoter; instead, an approximately threefold increase in CAT activity was observed (Fig. 7 left, lanes 1 and 2; Table 3). A 17-fold increase in CAT activity was observed when the HSV-1 *tk*–CAT construct (pPOH3) was cotransfected with pSVIE (Fig. 7 left, lanes 3 and 4; Table 3). In addition, two SV40-CAT recombinants were tested for their responses to pSVIE. Plasmid pCAT-Promoter (Fig. 7 right, lanes 1 and 2) contains the CAT gene under the control of the enhancerless SV40 early promoter, while pSV2cat (Fig. 7 right, lanes 3 and 4) contains the CAT gene under the control of the SV40 early promoter and enhancer. The EHV-1 IE gene product activated the enhancerless SV40 promoter by 14-fold. These results (Table 3) demonstrate that the EHV-1 IE gene product is capable of *trans* activating a nonherpesvirus promoter in the presence or absence of enhancer sequences.

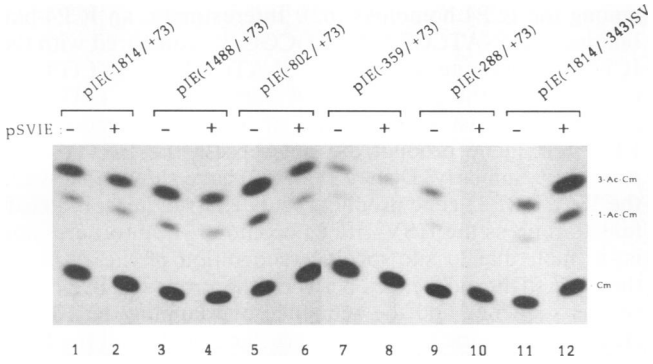

FIG. 6. Effects of *cis*-acting elements on IE gene autoregulation. LM cells were transfected with the indicated plasmids and either the EHV-1 IE expression vector pSVIE or an equimolar amount of pSV2neo. The extent of the IE promoter within each construct is indicated by the plasmid nomenclature and is diagrammed in Fig. 5. This composite figure shows samples excised from two separate autoradiographs which were exposed for equal amounts of time.

DISCUSSION

We have used short-term transfection assays utilizing an EHV-1 IE gene expression vector in combination with recombinant target promoter–CAT reporter constructs to demonstrate that the EHV-1 IE gene product encodes a regulatory protein capable of both *trans* activation and *trans* repression. The EHV-1 IE gene product, expressed from cloned copies of the IE ORF, was able to independently activate the EHV-1 *tk* promoter by as much as 60-fold and could activate the HSV-1 *tk* promoter by approximately 17-fold. These results indicate that the IE gene product can act as a strong *trans* activator of both EHV-1 and heterologous herpesvirus promoters. The IE gene product was also able to *trans* activate two EHV-1 beta-gamma promoters; however, in this case, there was a requirement for an

TABLE 2. Effects of IE promoter deletions on EHV-1 IE gene autoregulation

Plasmids transfected	CAT activity[a]			Avg % acetylation (mean ± SD)	Fold change[b] (mean ± SD)
	1	2	3		
pIE(−1814/+73)					
+pSV2neo	20.35	28.95	50.28	33.19 ± 15.41	
+pSVIE	19.77	19.82	20.46	20.02 ± 0.38	0.61 ± 0.01
pIE(−1488/+73)					
+pSV2neo	27.71	34.83	41.06	34.53 ± 6.68	
+pSVIE	20.96	22.33	25.78	23.02 ± 2.48	0.67 ± 0.07
pIE(−802/+73)[c]					
+pSV2neo	37.78	53.44	70.84	54.02 ± 16.54	
+pSVIE	9.48	11.08	16.91	12.49 ± 3.91	0.23 ± 0.07
pIE(−359/+73)					
+pSV2neo	5.05	5.73	7.63	6.14 ± 1.34	
+pSVIE	1.93	2.67	2.90	2.50 ± 0.50	0.41 ± 0.08
pIE(−288/+73)					
+pSV2neo	4.35	5.20	6.98	5.51 ± 1.34	
+pSVIE	0.28	0.29	0.34	0.30 ± 0.03	0.06 ± 0.01
pIE(−1814/−343)SV					
+pSV2neo	8.92	21.19	21.90	17.34 ± 7.30	
+pSVIE	65.49	82.72	86.72	78.31 ± 11.16	4.52 ± 0.65

[a] Determined as percent of acetylation. Results of independent transfection experiments are shown. Since each set of transfections was performed in parallel; the results are presented in numerical order.

[b] Determined by comparison of the percent acetylation values associated with the target plasmid in the presence of the indicated effector plasmid versus the average percent acetylation in the presence of pSV2neo.

[c] Reproduced from Table 1.

additional gene product (or products) of the EHV-1 *Xba*I G restriction fragment. It was also determined that the IE gene product can repress expression from its own promoter in *trans*. A series of 5′ IE promoter deletion constructs was used to map the *cis*-acting autoregulatory elements to a region within −288 to +73 of the IE transcription initiation site. The ability of the IE gene product to regulate its own promoter partitioned with pIE(−288/+73) (the smallest IE promoter element tested). However, when IE promoter sequences from −343 to +73 were replaced with the SV40 early promoter, IE autoregulation was completely abolished. Although it is possible that the IE gene product mediates autoregulation at the RNA level by interaction with the 73-bp untranslated leader region encoded by each of the IE promoter constructs used in this study, examination of the autoregulation of IE homologs in other herpesvirus systems suggests that IE autoregulation will, most likely, be mediated at the level of DNA binding. The ICP4 gene product of HSV-1 and the ORF62 gene product of VZV have been reported to down-regulate expression from their respective promoters (10, 42, 43, 47). Muller (37) has demonstrated that ICP4 can bind to its own cap site, and it has been proposed that this may be a common mechanism of autoregulation among the ICP4 homologs (62). Interestingly, an ICP4-like binding site 5′-ATCGTA(N)$_4$CGCGG-3′ [compared with the ICP4 binding site consensus 5′-ATCGTC(N)$_4$YCGRC-3′ (15)] overlaps the transcriptional start site of the EHV-1 IE gene. In contrast to its effects on its own promoter, the EHV-1 IE gene product did not repress the HSV-1 ICP4 promoter. Similarly, Disney et al. (10) have shown that while the VZV ORF62 gene product regulates its own promoter, it fails to repress the HSV-1 ICP4 promoter. If autorepression is, in fact, due to site-specific recognition of the cap site, then the slight differences between the proposed ICP4 consensus sequence and the sequence overlapping the EHV-1 IE cap site may be sufficient to preclude binding of the ICP4 consensus sequence within the ICP4 promoter by the EHV-1 IE gene product.

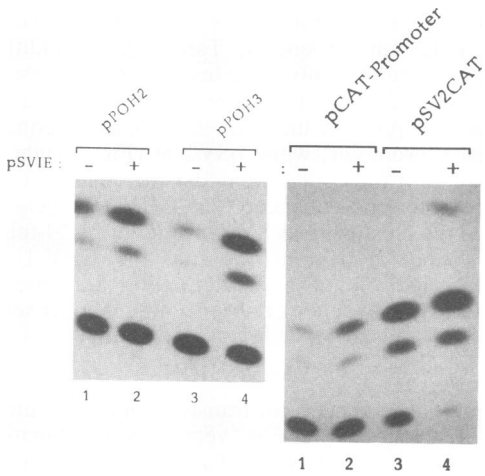

FIG. 7. Effects of the EHV-1 IE gene product on heterologous promoters. LM cells were cotransfected with pSVIE (lanes 2 and 4) or an equimolar amount of pSV2neo (lanes 1 and 3) and chimeric reporter plasmids containing promoter elements derived from either SV40 or HSV-1. The transfected cells were harvested and assayed for CAT activity as described in Materials and Methods. (Left) Plasmids pPOH2 and pPOH3 (40–42) are HSV-1 promoter–CAT recombinants containing the ICP4 promoter and the *tk* promoter, respectively. (Right) Plasmid pCAT-Promoter contains the CAT gene under the control of an enhancerless SV40 early promoter, while pSV2cat (20) contains the CAT gene under the control of the SV40 early promoter and enhancer.

The EHV-1 IE gene product, in the absence of other viral gene products, is capable of significantly activating the EHV-1 *tk* gene. The ability to activate certain promoters in the absence of ancillary viral gene products is a function common to both the ORF62 gene product and ICP4 which,

TABLE 3. Effects of pSVIE upon heterologous promoters

Plasmids transfected	CAT activity[a]			Avg % acetylation (mean ± SD)	Fold change[b] (mean ± SD)
	1	2	3		
pPOH2					
+pSV2neo	11.98	14.44	17.56	14.66 ± 2.80	
+pSVIE	26.31	37.14	59.70	41.05 ± 17.04	2.80 ± 1.16
pPOH3					
+pSV2neo	1.58	2.70	2.85	2.38 ± 0.69	
+pSVIE	30.18	44.91	46.68	40.59 ± 9.06	17.05 ± 3.81
pCat-Promoter					
+pSV2neo	1.60	4.12	ND[c]	2.86 ± 1.78	
+pSVIE	31.62	48.39	ND	40.00 ± 11.86	13.99 ± 4.15
pSV2cat					
+pSV2neo	77.20	96.32	ND	86.76 ± 13.52	—[d]
+pSVIE	97.22	98.06	ND	97.64 ± 0.60	—

[a] Determined as percent of acetylation. Results of independent transfection experiments are shown. Since each set of transfections was performed in parallel, the results are presented in numerical order.

[b] Determined by comparison of the percent acetylation values associated with the target plasmid in the presence of the indicated effector plasmid versus the average percent acetylation in the presence of pSV2neo.

[c] ND, not done.

[d] —, Value falls outside the linear range of the assay.

by transient transfection experiments, have been shown to activate their homologous *tk* promoters in the absence of other viral gene products (13, 19, 32, 41). Another characteristic common to the EHV-1 IE gene product, ICP4, and the ORF62 gene product is the ability to cooperate with other viral gene products in the activation of certain viral promoters. Two types of cooperation have been noted. The first may be described as synergistic cooperation in which each of a set of *trans*-acting factors is individually capable of activating a target promoter but in combination yield a far greater level of activation. Cooperation of this type has been observed between the HSV-1 ICP0 and ICP4 gene products (12, 13, 45a). A second type of cooperation is exemplified by two or more viral gene products which individually may have little or no effect on a specific target promoter but together can achieve significant activation of the promoter. In transient transfection assays, for example, ICP4, ICP0, and ICP27 have little effect on the HSV-1 major capsid protein (VP5) promoter when the effector genes are transfected individually or in pairs; however, the VP5 promoter is significantly activated when the three *trans* activators are present simultaneously (13, 54). A similar situation was observed with the EHV-1 IE expression vector, the EHV-1 *Xba*I G fragment, and the EHV-1 gD and IR5 promoters. Significant activation of the gD and IR5 promoters occurred only when both the IE expression vector and the *Xba*I G fragment were present in the transfection experiment. Sequence analysis of the EHV-1 *Xba*I G fragment has revealed an ORF (UL3) which exhibits significant homology to the UL54 (ICP27) gene of HSV-1 (64). ICP27 is an essential HSV-1 gene product capable of both positive and negative modulation of gene expression, and it plays a particularly important role in the activation of late genes (46, 53, 54, 58). Ongoing experiments have shown that the EHV-1 UL3 gene product alone can function with the IE protein to *trans* activate EHV-1 late promoters, specifically those of the gD and IR5 genes. Interestingly, the EHV-1 UL2 gene product, a homolog of HSV-1 UL55, may further enhance *trans* activation of these late promoters. The interactions among the proteins encoded by the IE (IR1), UL2, and UL3 genes are now being investigated.

The EHV-1 IE gene product differs from ICP4 in its effect on the SV40 promoter. O'Hare and Hayward (41) have used transient cotransfection assays to examine the effect of HSV IE gene products on the SV40 early promoter and enhancer. Specifically, a plasmid bearing the HSV-2 ICP0, ICP4, and ICP27 genes failed to significantly enhance expression of the CAT gene contained within pSV2cat. Similarly, a plasmid bearing the HSV-2 ICP0, ICP4, and ICP47 genes failed to significantly activate an enhancerless SV40-CAT construct (pA10CAT). Sekulovich et al. (54) have assayed the effects of a construct bearing the HSV-1 ICP4 gene cotransfected with pSV2cat. It was observed that ICP4 alone had no effect on CAT activity associated with pSV2cat. This finding is in contrast to the effect of the EHV-1 IE gene product on the SV40 regulatory region. An enhancerless SV40 early promoter–CAT recombinant (pCAT-Promoter) was induced by approximately 14-fold in the presence of pSVIE. Similarly, pSV2cat (which includes the SV40 enhancer) was up-regulated by the EHV-1 IE gene product. The pseudorabies virus IE gene product has also been reported to activate an enhancerless SV40 early promoter in transient transfection assays (23). The different effects of ICP4 and the EHV-1 IE gene product on the SV40 promoter may indicate a more promiscuous *trans*-activating ability for the EHV-1 IE gene product. It is unlikely that the stimulatory effects observed with the SV40 promoter in combination with the EHV-1 IE effector plasmid are due to recombination between effector and reporter promoter elements, since the basal activities of the SV40 reporters were determined in the presence of pSV2neo (in a molar amount equal to that of pSVIE). Everett and Dunlop (14) have shown that infection with EHV-1 can activate transfected reporter plasmids bearing the HSV-1 gD or rabbit β-globin promoter; however, the specific EHV-1 *trans* activators involved were not identified. We have shown that the EHV-1 IE gene product can significantly activate the heterologous HSV-1 *tk* promoter. Similarly, the VZV ORF62 gene product has also been shown to activate the HSV-1 *tk* promoter (4, 33).

The EHV-1 IE gene product has structural as well as functional similarities to both ICP4 and the VZV ORF62 gene product. In a sequence comparison of the HSV-1 ICP4 and VZV ORF62 regulatory proteins, McGeoch et al. (36) divided the ICP4 gene product into five regions based on the

degree of homology. Regions 2 and 4 share approximately 50% homology each between ICP4 and the ORF62 proteins, while regions 1, 3, and 5 share very little homology (except for a conserved serine-rich stretch within region 1). The EHV-1 IE gene has been sequenced in its entirety (24), and an examination of the predicted protein sequence of the EHV-1 IE gene has indicated that regions 2 and 4 are conserved within the EHV-1 IE protein. In fact, EHV-1 regions 2 and 4 are more similar to their HSV-1 and VZV counterparts than the respective HSV-1 and VZV domains are to each other (24). It has been postulated that the evolutionarily conserved regions of the ICP4 and ORF62 proteins may represent functional domains. Systematic mutational analyses of ICP4 (9, 43, 44) have confirmed the involvement of region 2 (amino acids 315 to 484) and, to a lesser extent, region 4 (amino acids 797 to 1224) in both positive and negative regulatory functions. Moreover, Wu and Wilcox (61) have demonstrated that a TrpE-ICP4 fusion polypeptide containing ICP4 amino acids 262 to 490 (which encompass region 2) can specifically bind probes which bear an ICP4 binding site. It is reasonable to assume that the evolutionarily conserved regions of the EHV-1 IE protein will play similar functional roles.

In this report, we have demonstrated that the EHV-1 IE gene encodes a regulatory factor with functional capabilities common to at least two other alphaherpesvirus gene products (ICP4 of HSV-1 and the ORF62 gene product of VZV). These common functional capabilities include negative autoregulation, the ability to activate certain promoters in the absence of ancillary viral gene products, and the ability to cooperate with ancillary viral gene products to activate a subset of viral promoters. Overall, we believe that this information will contribute to a greater understanding of the ICP4 family of alphaherpesvirus regulatory proteins and provide greater insight into the developmental program of EHV-1 productive infection and the mechanisms of altered viral gene expression in EHV-1 persistently infected cells.

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