The ICP0 Protein of Equine Herpesvirus 1 Is an Early Protein That Independently Transactivates Expression of All Classes of Viral Promoters

DAWN E. BOWLES, V. ROGER HOLDEN, YUHE ZHAO, AND DENNIS J. O’CALLAGHAN*

Department of Microbiology and Immunology, Louisiana State University Medical Center,
Shreveport, Louisiana 71130-3932

Received 22 November 1996/Accepted 25 March 1997

To assess the role of the equine herpesvirus type 1 (EHV-1) ICP0 protein (EICP0) in gene regulation, a variety of molecular studies on the EICP0 gene and gene products of both the attenuated cell culture-adapted Kentucky A (KyA) strain and the Ab4p strain were conducted. These investigations revealed that (i) the ICP0 open reading frame (ORF) of the KyA virus strain is 1,257 bp in size and would encode a protein of 419 amino acids, and in comparison to the ICP0 gene (ORF63) of the Ab4p strain of 1,596 bp (E. A. Telford, M. S. Watson, K. McBride, and A. J. Davison, Virology 189:304–316, 1992), it has an internal in-frame deletion of 339 bp; (ii) one early transcript of 1.4 kb predicted to encode the EICP0 protein and a late transcript of 1.8 kb are detected in Northern blot analyses using probes containing the ICP0 ORF; (iii) the KyA EICP0 protein (50 kDa) and the Ab4p EICP0 protein (80 kDa) are expressed as several species of early proteins that are first detected at 3 to 4 h postinfection by Western blot analyses of infected-cell polypeptides, using an antiserum generated to a TrpE fusion protein that harbors amino acids 46 to 153 of the EICP0 protein; and (iv) the EICP0 protein of both EHV-1 strains is a potent transactivator of EHV-1 genes. Transient expression assays using a simian virus 40 expression construct of the EICP0 protein of the KyA strain showed that the EICP0 protein independently transactivated chloramphenicol acetyltransferase reporter constructs under the control of the immediate-early promoter (3.9-fold), the early thymidine kinase promoter (95-fold), the late (g) promoter (12-fold), and the late (y1) promoter (21-fold). The finding that the EICP0 protein of the KyA virus can function as an activator of gene expression indicates that amino acids corresponding to residues 319 to 431 of the EICP0 protein are not essential for EICP0 transactivation of EHV-1 promoters.

As a causative agent of a number of equine ailments, including neurological disorders, respiratory tract infections, and abortions in pregnant mares, equine herpesvirus type 1 (EHV-1) represents an important worldwide pathogen of the horse (1, 14, 47, 48). In the laboratory, EHV-1 serves as an excellent model for the investigation of herpesvirus gene regulation during both productive and persistent infections (8, 33, 35, 58–61, 73). As with other alphaherpesviruses such as herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV-1), and pseudorabies virus (PRV), EHV-1 genes are temporally regulated and coordinately expressed in an immediate-early (IE), early (E), and late (L) fashion (6, 25, 26). The regulation of this cascade of gene expression is governed by the action of at least four characterized EHV-1 regulatory proteins: the sole IE protein (IEP) (27, 34, 35, 58–61), the EICP22 protein (formerly known as IR4 [31–33]), the EICP27 protein (formerly known as UL3 [72, 73]), and the α-TIF protein (16, 38, 50).

Once the EHV-1 IE gene IR1 has been activated by α-TIF, a 1,487-amino-acid (aa) phosphoprotein that exhibits significant homology to ICP4 of HSV-1, the open reading frame 62 (ORF62) protein of VZV, and other ICP4 homologs is synthesized (27). Like its HSV-1 counterpart, ICP4, the EHV-1 IEP is essential for productive infection, since inhibition of IEP production prevents expression of EHV-1 E and L genes (25, 26). Transient transfection assays revealed that the IEP is a bifunctional regulatory protein capable of negatively autoregulating its own promoter, independently activating expression of EHV-1 E promoters, and cooperating synergistically with EHV-1 early accessory regulatory proteins (EICP22 and EICP27) to activate expression of EHV-1 E and L (γ1) promoters (33, 58–60, 73). The IEP contains a potent transcriptional activation domain mapping within the amino-terminal 89 residues, a nuclear localization domain mapping to aa 963 to 970, and a DNA binding domain mapping to aa 495 to 597 (34, 35, 58, 61).

The other two identified EHV-1 regulatory proteins, EICP22 and EICP27, are first expressed at early times during infection and function as accessory regulatory proteins in transient transfection assays. The EICP22 protein cannot independently activate any EHV-1 promoter, acts synergistically with the EICP27 protein to activate the sole IE promoter, cooperates with the IEP to activate E promoters, and functions in conjunction with both the IEP and the EICP27 protein to activate expression of E and γ1 L promoters (33). The early EICP27 protein efficiently upregulates expression of the IE promoter but alone does not efficiently transactivate either E or L promoters (72, 73). However, EICP27 in conjunction with the IEP can increase expression of both E and L promoters (60, 73). Our ongoing studies indicate that the EICP22 protein, and possibly the EICP27 protein, functions to enhance the DNA binding capability of the IEP (34).

A fifth potential EHV-1 regulatory protein, encoded by the U1.63 gene of the Ab4p strain of EHV-1, is the EHV-1 ICP0 protein (EICP0) (18, 20, 65). ICP0 protein homologs have been identified and characterized in HSV-1 (see review in
and PRV (9, 66). The ICP0 proteins of these herpesviruses contain a conserved cysteine-rich zinc finger (C4HC4) type or RING finger motif near the amino terminus, a motif found in numerous viral and cellular proteins (see reviews in references 24, 56). This RING finger is important for the transactivating functions of both the HSV-1 and VZV ICP0 proteins (7, 17, 43). The RING fingers of the EICP0, HSV-1 ICP0, and VZV ICP0 proteins bind zinc stably (18), and the transactivation ability of the BICP0 protein (BHV-1 homolog of the ICP0 protein) was found to be zinc dependent (23).

The 775-aa HSV-1 ICP0 nuclear phosphoprotein, the best studied of all ICP0 homologs, functions independently as a general activator of viral and cellular promoters and interacts synergistically with the HSV-1 ICP4 protein to activate expression of viral E and L promoters, as determined by transient transfection studies in a number of laboratories (10, 21). The HSV-1 ICP0 protein is not essential for viral replication in tissue culture, but the growth of many ICP0 mutant viruses appears to be dependent on the multiplicity of infection (MOI), cell type, and stage of the cell cycle (5, 51, 63, 71). The mechanism by which HSV-1 ICP0 functions to transactivate a variety of viral and cellular promoters is not known. This protein has been reported to bind to single-stranded and double-stranded DNA but is not able to form a stable complex with DNA in solution (19). Purified recombinant RING finger polypeptides derived from ICP0 proteins do not bind significantly to DNA or RNA probes in gel shift experiments (18). Interestingly, the activity of a cellular DNA-dependent protein kinase, one of many proteins involved in RNA polymerase II transcription, is affected by the HSV-1 ICP0 protein (37). Protein-protein interactions may be important for ICP0 function, as this protein is known to interact with a 135-KDa cellular protein (41, 42) and with the HSV-1 ICP4 viral regulatory protein (70).

The functions of the other ICP0 homologs are less well characterized, but current evidence indicates that these proteins are also involved in gene regulation. The VZV ICP0 equivalent, the ORF61 protein, independently transactivates VZV IE, E, and L promoters as well as heterologous viral promoters in transient transfection assays (45). In addition, a cell line expressing the ORF61 protein can complement an HSV-1 ICP0 deletion virus, suggesting that the ORF61 protein is the functional equivalent of the HSV-1 ICP0 protein (44). The PRV EP0 protein is an early nuclear protein that can activate IE, E, and L promoters in transient transfection assays (66). The BHV-1 ICP0 homolog, BICP0, is an IE, zinc-binding nuclear protein that functions in transient transfection assays as either a transactivator or a transrepressor of BHV-1 promoters, depending on the target promoter (23).

To begin to understand the role of the EICP0 protein during viral infection, we have performed a variety of molecular studies of all classes of EHV-1 promoters.

**MATERIALS AND METHODS**

**Viruses and cell culture.** NBL6 cells (ATCC CCL 57) were grown in Eagle’s minimum essential medium (EMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, and 5% fetal bovine serum (FBS). Rabbit kidney (RK) cells were grown as described previously (61). The KyA strain of EHV-1 was propagated in L-M suspension culture at a low MOI of 0.01 PFU per cell and assayed for infectivity by plaque titration (49). The Ab45 strain of EHV-1 (65) was propagated in NBL6 cells at a low MOI of 0.5 PFU per cell and assayed for infectivity by plaque titration (49).

**Generation of plasmids.** The effector constructs pSVIE and pSVUL3 and the reporter constructs pIE-CAT, pTK2-CAT, pIR5-CAT, and pgK-CAT used in transient transfection assays were described previously (33, 58, 61, 71). The construction of the pGEMPR4 plasmid used in vitro transcription-translation experiments was described previously (31). The generation of the EHV-1 KyA strain clone Sm3 (formerly known as pCS-3) was described previously (2). The Sm3 subclone (map units 0.765 to 0.785) of the third-largest Sm1 subclone of the EHV-1 KyA strain EcoRI C fragment (map units 0.74 to 0.87) was sequenced and found to contain 1,795 bp which included the Sm3 strain ICP gene ORF of 1,257 bp as well as 258 bp of upstream and 280 bp of downstream sequences. The pcs245 clone is a truncated form of the Sm3 clone and contains bp 1 to 1231 of the Sm3 clone. The Sm4 clone (map units 0.755 to 0.795) contains bp 1231 to 1793 of the Sm3 clone in addition to 618 bp that were sequenced. Nucleotides (nt) 393 to 719 of the Sm3 subclone (corresponding to aa 46 to 153 of the KyA and Ab45 ICP0 proteins) were amplified by PCR using primers 5′-CGTCTGATTTCTTTGaGAAGTGCGGT GCcAACTGCTGG3′ and 5′-GGTACGGGGTACCTGATCACTATCAAACTAAGTTCCGCG3′ which created EcoRI and BamHI sites (italicized) on the 5′ and 3′ termini of the fragment, respectively. The PCR product of this reaction was digested with EcoRI and BamHI, and the resulting fragment was cloned in frame adjacent to the TrpE coding region in the pATH22 vector (36) to create the ICPIP0term construct. The ICPIP0term plasmid was transformed and maintained in E. coli strain TB1 by standard methods. The plasmid that was used in in vitro transcription-translation reactions was generated by digestion of the Sm3 clone with PstI (nt 212) and EcoRV (nt 1702) and subsequent ligation of the 1,491-bp fragment into the Sm1 site of the pGEMZx vector (Promega, Madison, Wis.). The orientation of the ORF with that of the SP6 promoter was confirmed by DNA sequence analysis. Thus, plasmid pGEMICP0K contains the entire EHV-1 KyA strain ICP0 ORF under the control of the SP6 bacterial promoter. The pSVICP0K expression construct was generated by digestion of the Sm3 subclone with PstI (nt 212) and EcoRV (nt 1702) and subsequent ligation into the Sm1 site of the pSVSPORT1 vector (Life Technologies, Gaithersburg, Md.). The ICPIP0 Orf was cloned in the forward orientation with respect to the simian virus 40 (SV40) promoter, and the orientation was confirmed by DNA sequence analysis. Thus, plasmid pSVICP0K contains the entire KyA ICP0 ORF under the control of the SV40 promoter and enhancer.

**DNA sequencing.** The Sm3 clone and the 5′ portion of the Sm4 clone (described above) were sequenced by the dideoxy-chain termination method (55) with Sequenase version 2.0 (U.S. Biochemical, Cleveland, Ohio). The DNA sequence was analyzed by PC/GENE software (IntelliGenetics; International Biotechnologies, Inc., New Haven, Conn.).

**Isolation of early and late viral mRNAs.** To isolate EHV-1 early mRNA, L-M cells (106) were pretreated with phosphonoacetic acid (PAA) at a concentration of 100 or 200 μg/ml for 1 h at 37°C and were infected with the EHV-1 KyA strain at an MOI of 15 to 20 PFU per cell in the presence of PAA. Following a 2-h attachment period, the cells were diluted to 106 cells per ml, and the infection was continued in the presence of PAA for an additional 4 h. For the isolation of EHV-1 late mRNA, 106 L-M cells were infected at an MOI of 15 to 20 PFU per cell in the absence of any metabolic inhibitors and harvested at 8 h postinfection.

**Isolation of poly(A)+ mRNA and Northern hybridization.** Poly(A)+ mRNA was isolated from infected and mock-infected cells by using a Fast Track mRNA isolation kit (Invitrogen, San Diego, Calif.) as recommended by the manufacturer and as described previously (12, 30). For Northern hybridization, 3 to 5 μg of poly(A)+ mRNA from each kinetic class was fractionated on a 1.2% formaldehyde gel by standard techniques (54). After transfer of RNA to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.), the filters were probed with cloned EHV-1 fragment Sm3 or pcs245 that had been nick translated with α-[32P]GTP and α-[32P]dCTP, using a nick translation kit from Life Technologies. Strand-specific riboprobes were generated by using the pGEMICP0K construct and the Riboprobe Gemini system as instructed by the manufacturer (Promega). Molecular weight standards used in Northern blot experiments were obtained from Life Technologies.

**Induction and isolation of the TrpE-ICP0 fusion protein and generation of ICP0-specific antisera.** To generate an EICP0-specific antisera, a bacterial TrpE (pATH) fusion protein was used as the immunizing antigen. The predicted amino acid sequence of the EICP0 ORF was analyzed by using the Antigen Prediction program of the PC/GENE package (IntelliGenetics) to identify potential antigenic sites on the EHV-1 protein. A potential antigenic region on the KyA and Ab45 ICP0 proteins was identified between aa 70 and 90. Nucleotides 393 to 719 of the Sm3a subclone, corresponding to aa 46 to 153 of the EICP0 protein and containing this potential antigenic region, were cloned in frame to the trpE gene in the vector pATH22 to generate the ICPIP0term clone.

**Induction and isolation of the TrpE-ICP0 fusion protein.** The induction and isolation of the TrpE-ICP0 fusion protein performed by the method of Koerner et al. (36) as described in detail elsewhere (29). The
TrpE-ICP0 fusion protein was purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie brilliant blue staining. A gel slice containing the TrpE-ICP0 fusion protein was excised, emulsified in Freund’s complete adjuvant, and used to immunize two New Zealand white rabbits intramuscularly. For all boosts in the posterior leg, the gel slice containing the TrpE-ICP0 antigen was emulsified in Freund’s incomplete adjuvant, and boosters were given at intervals of 2 to 3 weeks. The TrpE-ICP0 antiserum was collected and prepared as described by Harlow and Lane (26). In addition, the immunoglobulin G (IgG) fraction of the antiserum was purified by use of a protein G-Sepharose column (Pharmacia Biotech).

Western immunoblot analyses. L-M cells (6 × 10⁶) infected with EHV-1 KyA strain at 20 PFU per cell were harvested at 2, 3, 4, 6, 8, 10, 12, and 24 h postinfection. Lipid immunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.1% SDS, 0.5% deoxycholate, 1.0% Nonidet P-40) containing protease inhibitors (aprotinin [50 μg/ml], leupeptin [50 μg/ml], and phenylmethylsulfonyl fluoride [300 μg/ml]); 7.5 × 10⁶ RK cells infected with EHV-1 strain Ab4p at 20 PFU per cell were harvested at the same time points.

For PAA blocking experiments, L-M cells were infected with the KyA virus strain as described above except that the cells were pretreated with PAA (100 μg/ml) for 1 h prior to infection and infections were continued in the presence of PAA. Total infected-cell protein was determined by use of the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, Ill.). Equal amounts of protein within each experiment were analyzed by SDS-PAGE and Western blot analyses.

Separated proteins were transferred to nitrocellulose filters (Schleicher & Schuell) at 30 V overnight. Blots were blocked for 30 min in TBST (10 mM Tris-HCl [pH 9.5], 0.1 M NaCl, 5.0 mM MgCl₂) containing nitroblue tetrazolium proteins were visualized by incubating the membranes in AP buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5.0 mM MgCl₂) containing nitroblue tetrazolium. The following day, the beads were pelleted by centrifugation and washed three times. The samples were resuspended in 30 μl of purified TrpE-ICP0 antiserum or 15 μl of protein A-Sepharose beads (Sigma Chemical Company, St. Louis, Mo.) was added, and the mixture was incubated overnight with rocking. The mixtures were washed three times in radioimmunoprecipitation assay buffer, and the proteins were eluted in 1× Laemmli sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue, 62.5 mM Tris HCl [pH 6.8]). Samples were analyzed by SDS-PAGE, and the proteins were visualized by autoradiography.

PCR analysis. Genomic DNA of EHV-1 strains Ab4p and KyA was isolated as described previously (2). PCR analysis was performed with 1 μl of Smal plasmid DNA, 1 μg of Ab4p or 1 μg of KyA genomic DNA as the template, and 0.25 μg of each primer (5′ AAGCTTTTCGACGACTCACTATAGAG3′, 5′ AAGCTTTTCGACGACTCACTATAGAG3′, 5′GGGCTTGGTTGACCTAT3′). The PCR amplification conditions consisted of an initial denaturation of 2 min at 94°C followed by 40 thermal cycles of 95°C for 2 min, 55°C for 2 min, and 72°C for 3 min. The amplified DNA was separated on a 1.0% agarose gel and stained with ethidium bromide.

RESULTS

DNA sequence of the EHV-1 KyA strain ICP0 gene. The ICP0 (ORF63) gene of EHV-1 Ab4p strain was previously identified (65) and was designated the EHV-1 ICP0 homolog (EICP0) on the basis of limited amino acid homology with the VZV ORF61 and HSV-1 ICP0 proteins. The ORF63 gene is the last ORF in the unique long (U₈) region of the EHV-1 genome and is located before the inverted repeat sequences (Fig. 1). The Ab4p strain EICP0 gene is transcribed toward the U₈ terminus; the first base of the start codon is positioned at nt 111984, and the third base of the stop codon is positioned at nt 110386. This ORF consists of 1,596 bp and potentially encodes a protein of 532 aa with a predicted molecular weight of 58,627 (65). Here we report the sequence of the EICP0 gene of the tissue culture-adapted KyA strain and show that there is an internal in-frame deletion in the KyA strain EICP0 ORF. Based on the map location of the Ab4p EICP0 gene, the corresponding Smal subclone of the KyA strain (Fig. 1) was sequenced and found to harbor the entire KyA EICP0 ORF of 1,257 bp (Fig. 1 and 2) as well as 258 bp of 5′ sequences and 280 bp of 3′ sequences. The first in-frame ATG of the KyA EICP0 gene is located at nt 259 to 261 of the Smal3 subclone, and the stop codon is located at nt 1512 to 1514 of the Smal3 subclone (Fig. 2). Comparisons of the DNA sequences of the Ab4p and KyA EICP0 genes (Fig. 1C and 2) revealed several differences; the most significant is a 339-bp in-frame deletion in the KyA strain EICP0 ORF (deletion of Ab4p EICP0 ORF nt 1212 to 1551). This deletion of 339 bp suggests that the EICP0 ORF of the KyA strain is capable of encoding a protein of 419 aa with a predicted molecular weight of 46,828 that lacks aa 3 to 431 of the Ab4p EICP0 protein. In addition, three nucleotide differences in the KyA and Ab4p EICP0 ORFs were observed. Two of these resulted in a difference in the amino acid sequence such that valine 185 and serine 432 of the Ab4p EICP0 protein were changed to isoleucine 185 and proline 319, respectively, in the EICP0 protein of the KyA strain. A conservative nucleotide substitution was found at nt 925 of the Smal3 subclone. The 5′ upstream regions (Fig. 2) of both the KyA and Ab4p EICP0 genes were identical and included two potential TATA boxes, two octamer motifs, a CAAT box, and an SP1 binding site, which are cis elements detected in other EHV-1 promoters (32, 72). The 3′ downstream regions were identical except for two 1-bp deletions in the KyA sequence, which were transcribed to nt 110115 and 109934 of the Ab4p genomic sequence (Fig. 2). Further sequencing of SalI (Fig. 1), a clone that contains the 3′ portion of the EICP0 gene and extends further into the U₈ region, revealed that the EHV-1 KyA strain contains potential latency-associated transcript (LAT) promoter elements identical to those described for the...
Ab4p strain (Fig. 3) (3), including perfect conservation of two TATA boxes, two CAAT boxes, an SP1 binding site, and a latency promoter binding factor site.

To confirm that the observed 339-bp deletion in the KyA EICP0 gene was not a consequence of cloning, PCR analyses using primers flanking the deletion and internal to the deletion were performed with the Sma3 subclone, KyA genomic DNA, or Ab4p genomic DNA as the template (Fig. 4). In reactions performed with primers XN-1 and XN-3, which were designed to anneal to DNA sequences that flank the proposed deletion, a 695-bp amplified product was obtained with Ab4p genomic DNA as a template (Fig. 4, lane 6); in contrast, only a 374-bp product was observed with either the Sma3 subclone or KyA genomic DNA as a template (Fig. 4, lanes 2 and 4). In reactions performed with primers XN-1 and XN-2, where the XN-2 primer was designed to anneal to sequences deleted in the KyA EICP0 gene, an amplified product of 436 bp was detected only when Ab4p genomic DNA was used as the template (lane 7); no apparent amplified product was detected when KyA genomic DNA or the Sma3 subclone was used as the template for the PCRs (Fig. 4, lanes 3 and 5). These results are consistent with the observed 339-bp deletion in the sequenced Sma3 subclone. The results of both DNA sequencing and PCR analyses indicate that the KyA EICP0 gene should encode a protein of 419 aa that is 113 aa smaller than its Ab4p counterpart.

The EICP0 gene is expressed early during infection. To identify transcripts encoded by the EICP0 ORF, Northern blot analyses were performed on poly(A)+ mRNA isolated from KyA-infected cells under mock, early, and late conditions of infection (Fig. 5). The mRNA was probed with the nick-translated Sma3 clone, which contains the entire KyA EICP0 ORF, and the pS245 subclone, which contains a truncated portion of the EICP0 gene (Fig. 1). A single early transcript of approximately 1.4 kb was detected in the PAA-inhibited cells, using either the Sma3 or pS245 clone (Fig. 5A and B, lanes 2 to 4). This early transcript is of the expected size to encode the EICP0 protein, considering that it is initiated near the first TATA box and uses the polyadenylation signal located at nt 1560 of the Sma3 clone. Furthermore, the promoter regions of both the KyA and Ab4p EICP0 genes contain several potential cis-acting elements reminiscent of EHV-1 promoters (Fig. 2). An additional transcript of 1.8 kb was detected at late times, using either Sma3 or pS245 as a probe (Fig. 5A and B, lanes 5). To ensure that this was indeed a late transcript, Northern blot analyses were performed on mRNA isolated under conditions in which the PAA concentration was increased to 200 μg/ml. As can be seen in Fig. 5, the 1.8-kb transcript is detected only under late conditions of infection. Northern blot analysis of poly(A)+ mRNA isolated from Ab4p-infected cells indicated the presence of an early 1.6- to 1.7-kb transcript and a 2.0-kb late transcript (data not shown). The size differences between the KyA and Ab4p EICP0-specific transcripts suggest that these early and late transcripts are colinear rather than in an antisense orientation. To determine the orientations of the 1.4- and 1.8-kb transcripts, strand-specific riboprobes were generated by using the pGEMICP0K construct, which contains the EICP0 ORF under the control of the bacterial SP6 promoter. The two transcripts are colinear, since both the 1.4-kb early...
and 1.8-kb late transcripts were detected with the riboprobe generated from the T7 promoter (Fig. 5C) and were not detected with a riboprobe generated from the SP6 promoter (data not shown).

The EHV-1 EICP0 protein is expressed early during infection.

To demonstrate that the antiserum generated against the TrpE-ICP0 fusion protein was specific for a protein originating from the EICP0 ORF, the pGEMICP0 and pGEMR constructs, which contain the EICP0 and the EICP22 (IR4) ORFs, respectively, were in vitro transcribed and translated together in a single reaction. This reaction yielded a diffuse protein band that migrated between the 43- and 68-kDa molecular weight markers and was assumed to be the EICP0 protein (Fig. 6, lane 2). The reaction mixture also contained a smaller protein that was presumed to be the EICP22 (IR4) protein (Fig. 6, lane 2), as confirmed by immunoprecipitation of the reaction mixture with EICP22 antiserum (31) (Fig. 6, lane 3). Incubation of the reaction mixture with the TrpE-EICP0 antiserum immunoprecipitated the diffuse protein band migrating between the 43- and 68-kDa markers (Fig. 6, lane 4). The shift in apparent molecular mass between the bands in lanes 2 and 4 may be due to the differences in the buffer composition.
the pGEMICP0K construct alone was used to prime the in vitro transcription-translation reaction, only the diffuse EICP0 protein was detected (Fig. 6, lane 5). When this reaction was subjected to the immunoprecipitation procedure with no antiserum (negative control), no proteins were visualized (Fig. 6, lane 6). Additional in vitro transcription-translation reactions were performed with only the pGEMICP0K construct, and incubation of the reaction mixtures with the EICP0 antiserum led to the immunoprecipitation of the same diffuse protein band (data not shown). These findings confirm the specificity of the EICP0 antiserum for the in vitro-translated product originating from the EICP0 ORF.

To determine the time at which the KyA and Ab4p EICP0 proteins are first expressed during infection and to determine the relative sizes of the two proteins, Western blot analyses using the EICP0 protein-specific antiserum were performed on infected cell extracts. As shown in Fig. 7, both the KyA and Ab4p EICP0 proteins are made early in infection and migrate as a series of proteins at approximately 50 and 80 kDa, respectively. A predominant 68-kDa band is detected nonspecifically by the EICP0 antiserum and is present in both mock-infected and infected cells. The KyA EICP0 protein can first be detected at 3 h p.i. (Fig. 7, lane 3), and the Ab4p EICP0 protein can be detected at 4 h p.i. (Fig. 7, lane 13). In agreement with predictions from the DNA sequence and PCR analyses, the EICP0 protein of EHV-1 KyA migrates as a series of proteins smaller than the Ab4p EICP0 protein (50 kDa versus 80 kDa). To confirm that the EHV-1 EICP0 protein is a member of the early kinetic class, proteins isolated from KyA-infected L-M cells treated prior to and during infection with PAA (to inhibit viral DNA synthesis) were analyzed for expression of the EICP0 protein is a potent activator of EHV-1 promoters.
EICP0 protein. As shown in Fig. 8A, the EHV-1 α-TIF protein, which is a late protein, was not detected in cells infected in the presence of PAA (lane 4) but was detected at 6 and 10 h p.i. in cells that were not treated with PAA (Fig. 8A, lanes 5 and 6). In contrast, in the blot probed with the EICP0 antiserum, the EICP0 proteins were detected as early as 4 h p.i. in the presence or absence of PAA (Fig. 8B, lanes 2 and 3). Taken together, these results show that the EICP0 protein of EHV-1 is an early gene product and therefore is encoded by the sole EICP0 early transcript of 1.4 kb.

The EHV-1 EICP0 protein is a potent transactivator. Since the EHV-1 EICP0 protein is homologous to the ICP0 proteins of HSV-1, VZV, BHV-1, and PRV, and each of these viral proteins can function as a potent transactivator, it was of interest to examine the regulatory function of the EHV-1 EICP0 protein. L-M cells were cotransfected with a plasmid expressing the EICP0 ORF under the control of the SV40 promoter (pSVICP0K) in conjunction with plasmids containing EHV-1 IE, E, and L promoters linked to the CAT gene. Results of these assays revealed that the KyA EICP0 protein activated expression of the IE promoter approximately 3.9-fold over basal levels (Fig. 9A). As expected from previous work (73), the EHV-1 IC27 protein (pSVUL3) also activated expression of the IE promoter but could not completely overcome the negative autoregulatory effect of the IE protein on the IE promoter (pSVIE-pSVUL3). To address whether the EICP0 protein was able to activate expression from an EHV-1 E promoter, the pTK2-CAT reporter construct containing the EHV-1 thymidine kinase (TK) promoter was used as a representative E promoter. The KyA EICP0 protein was able to independently activate expression of the TK promoter 95-fold over basal levels (Fig. 9B); this level of activation was greater than that obtained for the IE-expressing construct alone (25-fold) and even was greater than that observed (52-fold) by the combination of the constructs expressing the IE and UL3 (EICP27) proteins. Thus, the EHV-1 EICP0 protein is a very potent activator of this representative E promoter.

Since the EHV-1 EICP0 protein is expressed early in infection and efficiently activates expression of the EHV-1 IE and TK promoters, it was of interest to determine if the EICP0 protein could also activate the expression of representative L promoters. Previous studies established that expression of EHV-1 L promoters requires the presence of both the IE and EICP27 proteins or the combination of the IE, EICP27, and EICP22 proteins (33, 58, 60, 73). None of these EHV-1 regulatory proteins alone can significantly activate expression of any L promoter tested to date. To assess the ability of the EICP0 protein to activate expression of L promoters, the IR5-CAT reporter construct was used as a representative y1 L promoter. As shown in Fig. 9C, the EICP0 protein was able to activate expression of this promoter approximately 85-fold. A plasmid (pSVICP0KREV) in which the EICP0 ORF was cloned in the reverse orientation relative to the SV40 promoter was used as a control, and it failed to activate expression of this promoter above basal levels. A second EHV-1 L promoter, the

FIG. 6. SDS-PAGE analysis of in vitro-transcribed and -translated and immunoprecipitated proteins synthesized from the pGEMICP0K and pGEMR4 constructs, which express the EHV-1 IECP0 and IR4 (EICP22) proteins, respectively. Both constructs were in vitro transcribed and translated in a single reaction (lanes 2 to 4), or the pGEMICP0K plasmid was in vitro transcribed and translated alone (lanes 5 and 6). Protein size markers in kilodaltons are shown, and arrows indicate the locations of the EICP0 and IR4 proteins. Lane 1, a negative control reaction performed with no plasmid; lane 2, in vitro-transcribed and -translated IR4 (EICP22) and EICP0 proteins; lane 3, in vitro-transcribed and -translated IR4 (EICP22) and EICP0 proteins immunoprecipitated with IR4 antiserum; lane 4, in vitro-transcribed and -translated IR4 (EICP22) and EICP0 proteins immunoprecipitated with EICP0 antiserum; lane 5, in vitro-transcribed and -translated EICP0 protein, no immunoprecipitation; lane 6, immunoprecipitation of the in vitro-transcribed and -translated EICP0 protein with no antiserum as a negative control.

![Figure 6](http://jvi.asm.org/)

FIG. 7. Western blot analysis of EICP0 protein synthesis in EHV-1 KyA-infected L-M cells (A) and Ab4p-infected RK cells (B). Mock-infected (lanes M; 12 h shown as example) and infected cells were harvested at 2, 3, 4, 6, 8, 10, 12, and 24 h p.i. (lanes 2 to 9 for KyA infection; lanes 11 to 18 for Ab4p infections), blotted to nitrocellulose, and probed with the EICP0 antiserum. Protein concentrations were determined by the Pierce BCA protein assay, and equal amounts of protein were analyzed in all lanes. Sizes are indicated in kilodaltons.

![Figure 7](http://jvi.asm.org/)
glycoprotein K (gK) promoter, shown to be a true late or γ2 promoter since expression of the gK transcript is dependent on prior DNA replication (72), was also tested. As shown in Fig. 9D, the EICP0 protein also activated expression of the pgK-CAT reporter construct, and levels of activation were as high as 21-fold. To date, EICP0 is the only EHV-1 protein shown to be able to independently activate expression of the γ1 and γ2 classes of L promoters. Thus, the KyA EICP0 protein can independently activate expression of all classes of EHV-1 promoters and is a potent activator of E and L genes. In light of its ability to activate expression of L genes, EICP0 appears to play an important, possibly essential, role in EHV-1 replication. The ability of the Ab4p EICP0 protein to activate E and L promoters was also examined (data not shown). The results indicate that the EICP0 proteins of both EHV-1 strains activate expression from these promoters equally well, indicating

FIG. 8. Western blot analyses of the α-TIF protein (A) or the EICP0 protein (B) in L-M cells infected with the EHV-1 KyA strain. Infections were performed in the presence of PAA (lanes 2 and 4) or in the absence of PAA (lanes 3, 5, and 6). Mock-infected cell extracts (lanes M) served as a negative control. Infected cells were harvested at the indicated time points, blotted to nitrocellulose, and probed with either the α-TIF L3A monoclonal antibody (A) or the EICP0 antiserum (B). Protein concentrations were determined by the Pierce BCA protein assay, and equal amounts of protein were analyzed in all lanes. Sizes are indicated in kilodaltons.

FIG. 9. Assays to detect transactivation of representative EHV-1 IE, E, γ1L, and γ2L promoters linked to the CAT reporter gene by effector constructs expressing the IE, UL3 (EICP27), or EICP0 protein of EHV-1 KyA. L-M cells were transfected with each promoter-CAT reporter construct and 0.3 pmol of either pSVIE, pSVUL3, pSVICP0K, or pSVICP0KREV. Transfected cells were harvested at 62 h posttransfection, and CAT activity was measured. Each transfection was performed in triplicate. Error bars show the standard deviations. (A) Transactivation of the EHV-1 IE reporter construct (1.4 pmol); (B) transactivation of the EHV-1 E pTK2-CAT reporter construct (1.4 pmol); (C) transactivation of the EHV-1 γ1L IR5 promoter (pIR5-CAT; 1.4 pmol); (D) transactivation of the EHV-1 γ2L gK promoter (pgK-CAT; 2.0 pmol).
that aa 319 to 431 of the Ab4p EICP0 protein are not essential for this transactivation activity.

**DISCUSSION**

In this paper, we report the DNA sequence of the KyA strain EHV-1 EICP0 gene of 1,257 bp and note that in comparison to the Ab4p strain EICP0 gene of 1,596 bp, the KyA EICP0 gene has an internal in-frame deletion of 339 bp. The KyA strain of EHV-1 is a murine fibroblast (L-M) cell-adapted strain that has been passaged in tissue culture since 1962 (52) and lacks at least six ORFs in addition to the 339-bp deletion in the EICP0 ORF. ORF1 and ORF2 (65), which encode proteins of unknown function, are absent in the KyA genome due to a 1,283-bp deletion near the U1 portion of the genome (68, 69). ORF7, the VZV ORF15 equivalent, has a 1,207-bp deletion located 5' to the c ORF (40). Three ORFs in the unique short segment (ORF72, ORF74, and ORF75) which encode glycoprotein I, glycoprotein E, and a 10-kDa ORF of unknown function, respectively, are absent as a result of a 3,859-bp deletion (22). Our recent studies have shown that the KyA strain of EHV-1 is avirulent in both the equine (39) and the BALB/c mouse (13) and induces protective immunity upon challenge with pathogenic EHV-1 strains. These results suggest that one or more of the deletions within the KyA genome are responsible for the attenuation of this vaccine candidate strain. Although a segment of 113 aa is absent in the KyA EICP0 protein, the EICP0 protein of the KyA strain is a potent transactivator of all classes of EHV-1 promoters and has activities similar to those of the EICP0 protein of the Ab4p strain. Whether this deletion has some influence on the virulence of EHV-1 remains to be determined; however, it is interesting that both an HSV-1 ICP0 deletion mutant virus (d1403) and an HSV-1 mutant virus (d701) lacking certain cis elements of the ICP0 promoter (subsequently expressing less ICP0 protein) exhibited a reduction in virulence in the mouse model (11, 15). In addition, a RING finger-containing protein of equine influenza virus is an important determinant of virulence for this poxvirus pathogen of mice (57).

Both transcriptional and translational analyses revealed that EICP0 is an E gene. Northern blot analysis of mRNA isolated from lytically infected cells treated with metabolic inhibitors indicates that the KyA EICP0 gene belongs to the early kinetic class of viral genes. One early transcript of 1.4 kb originating from the EICP0 promoter was detected under early conditions in which PAA was used to prevent viral DNA synthesis. Further experiments revealed that the EICP0 protein was made in the presence of PAA, indicating that the 1.4-kb transcript encodes the EICP0 protein. The 1.8-kb transcript detected under late conditions of infection could be 5' or 3' coterminal with the EICP0 1.4-kb transcript, since a strand-specific riboprobe detects both transcripts.

Western blot analyses using an EICP0 antiserum and KyA- and Ab4p-infected cell extracts indicate that the KyA EICP0 protein is approximately 50 kDa in size and is smaller than the Ab4p EICP0 protein of approximately 80 kDa. This size difference in the two proteins supports findings from both the DNA sequence and PCR analyses, indicating that the KyA EICP0 ORF should encode a protein smaller than the Ab4p EICP0 ORF product. Both proteins are first expressed at early times during infection, and the KyA EICP0 protein is expressed in the presence of PAA. These observations support the assignment of the 1.4-kb early transcript as the messenger that encodes the EICP0 protein. The KyA and Ab4p EICP0 proteins exist as multiple protein species that may result due to differences in the amount of posttranslational modifications such as phosphorylation. Indeed, recent evidence (4) indicates that the KyA EICP0 protein is phosphorylated, and a serine-rich region located at aa 210 to 217 of both the KyA and Ab4p EICP0 proteins may represent a major site for phosphorylation. An analysis of potential sites for posttranslational modifications using the Prosite program (PC/GENE software) indicates that four potential phosphorylation sites and two potential N-myristoylation sites are absent in the KyA EICP0 protein due to the 113-aa deletion. Such differences in posttranslational modification may account for some of the size differences observed between the KyA and Ab4p EICP0 proteins.

In conclusion, the EICP0 protein of EHV-1 is a very potent activator of EHV-1 promoters and likely is a promiscuous transactivator since it can activate expression of the heterologous ICP6 promoter of HSV-1 (20). Since the EICP0 protein is the only regulatory protein capable of activating y2 gene expression, it may play an essential role in EHV-1 gene programming. Recently, we have generated several EHV-1 EICP0-expressing cell lines to complement an EICP0 null mutant virus and thereby ascertain whether the EICP0 protein is essential for EHV-1 replication in cell culture and, if not, whether the deletion of this gene in pathogenic EHV-1 strains results in a reduction or loss of virulence.

**ACKNOWLEDGMENTS**

We thank Suzanne Zavecz for excellent technical assistance. We also thank Gretchen Caughman for the kind gift of the o-TIF monoclonal antibody L3A.

This investigation was supported by research grants from the National Institutes of Health (AI-22001) and the U.S. Department of Agriculture (92-37204-8040).

**REFERENCES**


2. Baumann, R. P., J. Staczek, and D. J. O’Callaghan. 1987. Equine herpesvi-
rus type 1 defective interfering (DI) particle DNA structure: the central region of the inverted repeat was deleted from DI DNA. Virology 159:137–
146.


4090.


220.


3282.


