Journal of Virology, July 1997, p. 4904–4914 Vol. 71, No. 7
0022-538X/97/$04.00+0
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The ICP0 Protein of Equine Herpesvirus 1 Is an Early Protein That Independently Transactivates Expression of All Classes of Viral Promoters

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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 EICP0 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 (γ1) IR5 promoter (85-fold), and the late (γ2) glycoprotein K 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 Ab4p 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), expression of EHV-1 genes is 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 422 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 U_L63 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

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reference 21; 10), VZV (43, 44, 45, 46, 62, 63), BHV-1 (23, 67), and PRV (9, 66). The ICP0 proteins of these herpesviruses contain a conserved cysteine-rich zinc finger (C_3HC_4 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, 53, 64, 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 on the EICP0 gene and gene products. Here, we report the DNA sequence of the EHV-1 Kentucky A (KyA) strain EICP0 gene and show that there is an internal, in-frame deletion in this gene in comparison to the Ab4p strain EICP0 gene, demonstrate that the EHV-1 EICP0 gene is transcribed as an early message, and show that the EICP0 gene product exists as a series of proteins that are synthesized early during infection. Finally, transient transfection analyses indicate that the KyA EICP0 protein functions as a potent transcriptional activator 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 Ab4p 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, 73). The construction of the pGEMR4 plasmid used in in vitro transcription-translation experiments was described previously (31). The generation of the EHV-1 KyA strain clones Sma3 (formerly known as pCS-3), pS245, and Sal4 has been described previously (2). The Sma3 subclone (map units 0.765 to 0.777) is the third-largest *Sma*I subclone of the EHV-1 KyA strain *Eco*RI C fragment (map units 0.74 to 0.87). Sma3 was sequenced and found to contain 1,795 bp which included the KyA strain ICP0 ORF of 1,257 bp as well as 258 bp of upstream and 280 bp of downstream sequences. The pS245 clone is a truncated form of the Sma3 clone and contains bp 1 to 1231 of the Sma3 clone. The Sal4 clone (map units 0.755 to 0.795) contains bp 1231 to 1795 of the Sma3 clone in addition to 618 bp that were sequenced. Nucleotides (nt) 393 to 719 of the Sma3 subclone (corresponding to aa 46 to 153 of the KyA and Ab4p ICP0 proteins) were amplified by PCR using primers 5′CTGTCT*GAATTC*TTGCAAAGTGCCGGTCGAATCTGTGG3′ and 5′GGTACG*GGATCC*AGTGTATCCATAACAATGTTCCGC3′ which created *Eco*RI and *Bam*HI sites (italicized) on the 5′ and 3′ termini of the fragment, respectively. The PCR product was cleaved with restriction enzymes *Eco*RI and *Bam*HI, and the resulting fragment was cloned in frame adjacent to the TrpE coding region in the pATH22 vector (36) to create the ICP0Nterm construct. The ICP0Nterm plasmid was transformed and maintained in *Escherichia coli* TB1 by using standard methods (54). The pGEMICP0K plasmid that was used in in vitro transcription-translation reactions was generated by digestion of the Sma3 clone with *Pvu*II (nt 212) and *Eco*RV (nt 1702) and subsequent ligation of the 1,491-bp fragment into the *Sma*I site of the pGEM3z 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 Sma3 subclone with *Pvu*II (nt 212) and *Eco*RV (nt 1702) and subsequent ligation into the *Sma*I site of the pSVSPORT1 vector (Life Technologies, Gaithersburg, Md.). The ICP0 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 Sma3 clone and the 5′ portion of the Sal4 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 (10^8) were pretreated with phosphonoacetic acid (PAA) at a concentration of 100 or 200 μg per 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 2×10^6 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, 10^8 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 a cloned EHV-1 fragment (Sma3 or pS245) that had been nick translated with [α-^{32}P]dGTP and [α-^{32}P]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 antiserum. To generate an EICP0-specific antiserum, 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 program of the PC/GENE package (IntelliGenetics) to identify potential antigenic sites on the EICP0 protein. A potential antigenic region on the KyA and Ab4p EICP0 proteins was identified between aa 70 and 90. Nucleotides 393 to 719 of the Sma3 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 ICP0Nterm clone.

Induction and isolation of the TrpE-ICP0 fusion protein were 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 in the rear legs. For all booster injections, 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 (28). 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 (6×10^6) 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 (p.i.) in radioimmunoprecipitation 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]); 7.5×10^6 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 8.0], 150 mM NaCl, 0.5% Tween 20) containing 10% nonfat powdered milk and then incubated with the TrpE-ICP0 antiserum at a dilution of 1:7,500 in TBST or the EHV-1 α-TIF monoclonal antibody L3A (38; a gift from Gretchen Caughman) at a dilution of 1:1,000 in TBST for 30 min. The blots were washed three times for 10 min each in TBST and incubated with secondary antibody (anti-rabbit IgG [Fc]-alkaline phosphatase conjugate [Promega] or goat anti-mouse IgG [heavy plus light chain]-alkaline phosphatase conjugate [Life Technologies]) at a dilution of 1:10,000 or 1:5,000, respectively, for an additional 30 min. The membranes were washed in TBST for three 10-min washes, and the 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 MgCl) containing nitroblue tetrazolium (0.33 mg/ml; Life Technologies) and 5-bromo-4-chloro-3-indolylphosphate (0.165 mg/ml; Life Technologies).

In vitro transcription-translation. In vitro transcription-translation reactions to express the ICP0 protein were performed by using the TNT-coupled transcription-translation reticulocyte lysate system as instructed by the manufacturer (Promega). Briefly, 1 μg of plasmid pGEMICP0K or pGEMR4 was incubated with 25 μl of rabbit reticulocyte lysate, 1 μl of SP6 polymerase, and [^{35}S]methionine (40 μCi), and reaction mixtures were incubated for 2 h at 30°C; 25 μl of the lysates was subjected to immunoprecipitation with rocking at 4°C overnight, using 15 μl of purified TrpE-ICP0 antiserum or 15 μl of TrpE-IR4 antiserum (31). Then 35 μl of protein A-Sepharose beads (Sigma Chemical Company, St. Louis, Mo.) was added, and the mixture was incubated overnight with rocking. The following day, the beads were pelleted by centrifugation and 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 μg of Sma3 plasmid DNA, 1 μg of Ab4p or 1 μg of KyA genomic DNA as the template, and 0.25 μg of each of the primers XN-1 (5′AGACTCCAGACCAGCAC3′), XN-2 (5′CGC GCAAACTCCCGCAG3′), and XN-3 (5′GGGCTGGTGGACGCTAT3′). The PCR amplification conditions consisted of an initial denaturation of 2 min at 95°C followed by 40 thermal cycles of 95°C for 2 min, 55°C for 2 min, and 75°C for 3 min. The amplified DNA was separated on a 1.0% agarose gel and stained with ethidium bromide.

Transfections and chloramphenicol acetyltransferase (CAT) assays. Transient transfections were performed as described previously (33, 58). Briefly, L-M cells were plated at a density of 4×10^6 per 60-mm-diameter tissue culture dish in EMEM supplemented with 5% FBS and allowed to grow overnight at 37°C in a 5% CO_2 incubator. The cells were washed three times in serum-free EMEM, and Lipofectin-mediated DNA transfection was performed with the Lipofectin reagent (Life Technologies) in 3 ml of serum-free EMEM. The DNA to be transfected was mixed with the Lipofectin reagent (5:1, wt/wt), incubated for 15 min to allow liposome-DNA complexes to form, and then added in a dropwise fashion to the appropriate tissue culture dish. After 5 h of incubation, the medium was removed, fresh EMEM medium containing 5% FBS was added, and the cells were incubated an additional 60 to 62 h. The amount of the pIE-CAT, pTK2-CAT, and pIR5-CAT reporter constructs was 1.4 pmol, and the amount of pgK-CAT reporter construct was 2.0 pmol. All effector constructs (pSVICP0K, pSVIE, and pSVUL3) were transfected in amounts of 0.3 pmol. Total DNA was adjusted to 8.5 μg by addition of pUC19 DNA.

CAT assays were performed as described previously (33, 58). At 60 to 62 h posttransfection, the cells were harvested by being washed three times in phosphate-buffered saline, scraped into TEN solution (40 mM Tris-HCl [pH 7.5], 10 mM EDTA, 150 mM NaCl), and collected by centrifugation. The cell pellet was resuspended into 200 μl of 250 mM Tris-HCl (pH 8.0), and the cells were lysed by a cycle of five freeze-thaws. Lysates were heated for 10 min at 60°C and centrifuged, and the supernatants were collected for CAT assays. The total protein concentration from each transfection was determined by using the BCA protein assay reagent (Pierce), and sample volumes were adjusted to 158 μl by addition of 250 mM Tris-HCl (pH 8.0). To each sample, 2 μl of [^{14}C]chloramphenicol (0.1 μCi, 50 to 60 mCi/mmol; New England Nuclear Corp., Boston, Mass.) and 20 μl of acetyl coenzyme A (3.5 mg/ml) were added. After incubation of the reaction mixtures for 60 min at 37°C, chloramphenicol and its acetylated products were extracted with ethyl acetate, and the ethyl acetate was evaporated. The samples were resuspended in 30 μl of ethyl acetate, 15 μl of each sample were spotted onto thin-layer chromatography sheets (Silica gel 1B; J. T. Baker), and ascending chromatography was performed in thin-layer chromatography tanks with chloroform-methanol (19:1, vol/vol). The radioactivity associated with each spot was determined by liquid scintillation counting.

Nucleotide sequence accession number. The GenBank accession number of the DNA sequence reported in this paper is U81154.

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 (U_L) 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 U_L 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 Sma3 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 Sma3 subclone, and the stop codon is located at nt 1512 to 1514 of the Sma3 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 319 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 Sma3 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 E promoters (32, 72). The 3′ downstream regions were identical except for two 1-bp deletions in the KyA sequence corresponding to nt 110115 and 109934 of the Ab4p genomic sequence (Fig. 2). Further sequencing of Sal4 (Fig. 1), a clone that contains the 3′ portion of the EICP0 gene and extends further into the U_L region, revealed that the EHV-1 KyA strain contains potential latency-associated transcript (LAT) promoter elements identical to those described for the

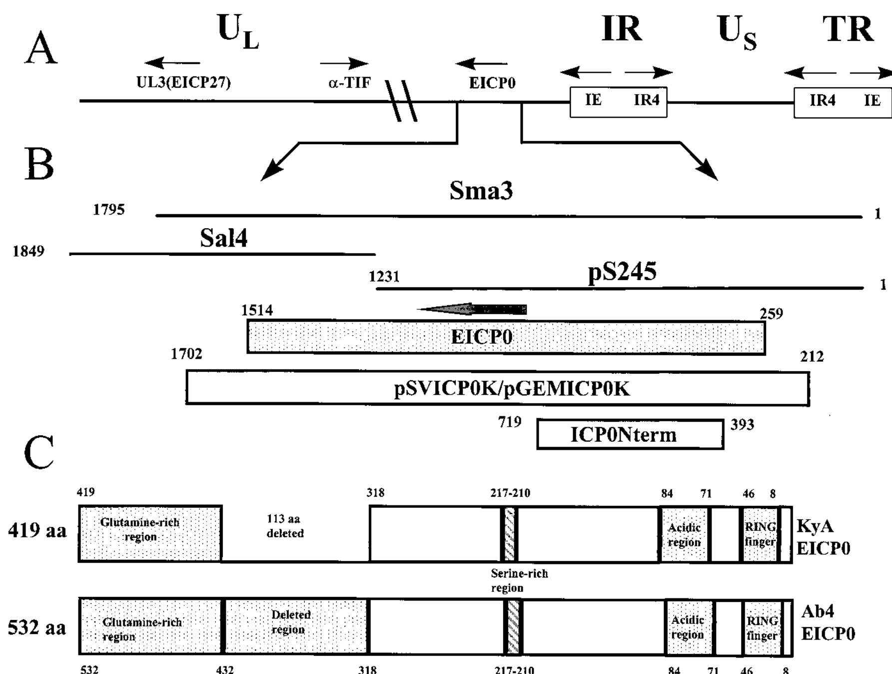

FIG. 1. (A) General organization of the EHV-1 genome depicting the unique long region (U_L), the inverted repeats (IR and TR), and unique short (U_S) segments. The relative locations of the UL3 (EICP27), α-TIF, EICP0 (U_L63), IE, and IR4 (EICP22) genes are indicated. (B) Expanded view of the Sma3 subclone (1,795 bp) of the EHV-1 KyA strain that contains the EICP0 ORF of 1,257 bp as well as 258 bp of upstream and 280 bp of downstream sequences. The pS245 clone contains bp 1 to 1231 of the Sma3 clone, and the Sal4 clone contains bp 1232 to 1795 of the Sma3 clone and an additional 618 bp. Plasmid pSVICP0K expresses the EICP0 protein under the control of the SV40 promoter; the pGEMICP0K construct contains the EICP0 ORF under the control of the SP6 promoter. Both plasmids contain bp 212 to 1702 of the Sma3 subclone. The ICP0Nterm plasmid was used in the generation of an EICP0-specific antiserum and contains bp 393 to 719 of the Sma3 subclone. The EICP0 ORF, shown by a large arrow, is located between bp 259 to 1514 of the Sma3 clone. (C) The predicted structural motifs of the Ab4p and KyA EICP0 proteins. The Ab4p EICP0 protein is 532 aa in size, and the KyA strain EICP0 protein is 419 aa in size. The RING finger motif of each protein is located between aa 8 and 46, a region enriched in acidic amino acids is located between aa 71 and 84, a serine-rich tract is located at aa 210 to 217, the region that is deleted in the KyA EICP0 protein corresponds to aa 319 to 431 of the Ab4p EICP0 protein, and a glutamine-rich region at aa 432 to 532 of the Ab4p EICP0 protein corresponds to aa 319 to 419 of the KyA EICP0 protein.

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 E 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

```
             SP1    CAAT BOX
                IE-BINDING SITE
Sma3 GGGGGTTTCCCGCCCACCAATCGCATTCAAACGGGTCCCCATGCTATACCCCTGAGATACAGATGTTCGA    70
Ab40 GGGGGTTTCCCGCCCACCAATCGCATTCAAACGGGTCCCCATGCTATACCCCTGAGATACAGATGTTCGA    70

   TATA BOX          OCTAMER                 OCTAMER
Sma3 TAAATTGGAACCGTATCCCGTGCAAATTTGGCATGCGTTCAAGATGCTAATTTTAAGCATATGGGTGGTG   140
Ab40 TAAATTGGAACCGTATCCCGTGCAAATTTGGCATGCGTTCAAGATGCTAATTTTAAGCATATGGGTGGTG   140

                                          TATA BOX
Sma3 TTGAATGGGCGTGCACAGCCACGTGAGTTTTGCAGCCAGATATATGAATGGCACCAAGCCTGGTTTTAAC   210
Ab40 TTGAATGGGCGTGCACAGCCACGTGAGTTTTGCAGCCAGATATATGAATGGCACCAAGCCTGGTTTTAAC   210

                                      IE-BINDING SITE
Sma3 AGCTGATACAGCCTTTTCATTTGGAAACTCTTCCAAATCGCTGCCACCATGGCAACTGTTGCAGAGCGAT   280
Ab40 AGCTGATACAGCCTTTTCATTTGGAAACTCTTCCAAATCGCTGCCACCATGGCAACTGTTGCAGAGCGAT   280
KyAP                                                 M  A  T  V  A  E  R      7
Ab4P                                                 M  A  T  V  A  E  R      7

Sma3 GCCCGATCTGCCTGGAGGACCCCAGCAACTACTCTATGGCGCTTCCATGCCTACATGCGTTCTGCTACGT   350
Ab40 GCCCGATCTGCCTGGAGGACCCCAGCAACTACTCTATGGCGCTTCCATGCCTACATGCGTTCTGCTACGT   350
KyAP C  P  I  C  L  E  D  P  S  N  Y  S  M  A  L  P  C  L  H  A  F  C  Y  V    31
Ab4P C  P  I  C  L  E  D  P  S  N  Y  S  M  A  L  P  C  L  H  A  F  C  Y  V    31
     *        *                                   *  *     *     *  *

Sma3 GTGTATTACGCGCTGGATACGACAAAACCCGACCTGTCCACTTTGCAAAGTGCCGGTCGAATCTGTGGTG   420
Ab40 GTGTATTACGCGCTGGATACGACAAAACCCGACCTGTCCACTTTGCAAAGTGCCGGTCGAATCTGTGGTG   420
KyAP   C  I  T  R  W  I  R  Q  N  P  T  C  P  L  C  K  V  P  V  E  S  V  V    54
Ab4P   C  I  T  R  W  I  R  Q  N  P  T  C  P  L  C  K  V  P  V  E  S  V  V    54
       *           *                    *  *  *  *

Sma3 CACACCATCGAGAGCGACAGCGAGTTTAAAGAAACAAAGGTGAGCGTGGACTTTGACTATGACAGCGAAG   490
Ab40 CACACCATCGAGAGCGACAGCGAGTTTAAAGAAACAAAGGTGAGCGTGGACTTTGACTATGACAGCGAAG   490
KyAP  H  T  I  E  S  D  S  E  F  K  E  T  K  V  S  V  D  F  D  Y  D  S  E      77
Ab4P  H  T  I  E  S  D  S  E  F  K  E  T  K  V  S  V  D  F  D  Y  D  S  E      77

Sma3 AAGACGAAGACAGTTTTGAGGGGCAATTTTTGGCCGTGGATTCTGGCGATGCTCCCGCCAATATCTCTGC   560
Ab40 AAGACGAAGACAGTTTTGAGGGGCAATTTTTGGCCGTGGATTCTGGCGATGCTCCCGCCAATATCTCTGC   560
KyAp E  D  E  D  S  F  E  G  Q  F  L  A  V  D  S  G  D  A  P  A  N  I  S  A   101
Ab4P E  D  E  D  S  F  E  G  Q  F  L  A  V  D  S  G  D  A  P  A  N  I  S  A   101

Sma3 GTGGAACGGACCCATGGCATTTGTGCCCCTCAACGCTAATGGGACTGCCGGGGCTCCCCGACTGCAGCCA   630
Ab40 GTGGAACGGACCCATGGCATTTGTGCCCCTCAACGCTAATGGGACTGCCGGGGCTCCCCGACTGCAGCCA   630
KyAP   W  N  G  P  M  A  F  V  P  L  N  A  N  G  T  A  G  A  P  R  L  Q  P   124
Ab4P   W  N  G  P  M  A  F  V  P  L  N  A  N  G  T  A  G  A  P  R  L  Q  P   124

Sma3 CTGGTGGACTGGCTGGTCGAAAGGCTCGACCAGCTATTCGAGACCCCAGAGCTAGCGTTGGTGATGCGGA   700
Ab40 CTGGTGGACTGGCTGGTCGAAAGGCTCGACCAGCTATTCGAGACCCCAGAGCTAGCGTTGGTGATGCGGA   700
KyAP  L  V  D  W  L  V  E  R  L  D  Q  L  F  E  T  P  E  L  A  L  V  M  R     147
Ab4P  L  V  D  W  L  V  E  R  L  D  Q  L  F  E  T  P  E  L  A  L  V  M  R     147

Sma3 ACATTGTTATGGATACACTGTGCGAGCATGGCTGTAACGAGGAAGAGCTCACTCGCCAGTTTTGGCCGAT   770
Ab40 ACATTGTTATGGATACACTGTGCGAGCATGGCTGTAACGAGGAAGAGCTCACTCGCCAGTTTTGGCCGAT   770
KyAP N  I  V  M  D  T  L  C  E  H  G  C  N  E  E  E  L  T  R  Q  F  W  P  M   171
Ab4P N  I  V  M  D  T  L  C  E  H  G  C  N  E  E  E  L  T  R  Q  F  W  P  M   171

Sma3 GTTTCACGAAGACACTGTGCCCTTTGTCACGGATCTCATCATCCAAGCAGAACTGTGCGTAGCGTCGAGA   840
Ab40 GTTTCACGAAGACACTGTGCCCTTTGTCACGGATCTCATCGTCCAAGCAGAACTGTGCGTAGCGTCGAGA   840
KyAP   F  H  E  D  T  V  P  F  V  T  D  L  I  I  Q  A  E  L  C  V  A  S  R   194
Ab4P   F  H  E  D  T  V  P  F  V  T  D  L  I  V  Q  A  E  L  C  V  A  S  R   194

Sma3 CCGATCTTGCCAATAGCCCGCGGAAGAGGGGTAGAATACATTGACTCCTCTTCATCGAGCTCTTCGTCTG   910
Ab40 CCGATCTTGCCAATAGCCCGCGGAAGAGGGGTAGAATACATTGACTCCTCTTCATCGAGCTCTTCGTCTG   910
KyAP  P  I  L  P  I  A  R  G  R  G  V  E  Y  I  D  S  S  S  S  S  S  S  S     217
Ab4P  P  I  L  P  I  A  R  G  R  G  V  E  Y  I  D  S  S  S  S  S  S  S  S     217

Sma3 AGGAAGAAACAGACAGCGACATAGAGGTTGACCCCAACAACCTAACCGACCCAGAAGATACGAGCGATGA   980
Ab40 AGGAAGAAACAGAGAGCGACATAGAGGTTGACCCCAACAACCTAACCGACCCAGAAGATACGAGCGATGA   980
KyAP E  E  E  T  D  S  D  I  E  V  D  P  N  N  L  T  D  P  E  D  T  S  D  E   241
Ab4P E  E  E  T  D  S  D  I  E  V  D  P  N  N  L  T  D  P  E  D  T  S  D  E   241

Sma3 AACCAGCACAGACAATAGTTCTGCTCAGGCACCCCGCCAAGAAGACTCCAGACCAGCACGTGCACGACCG  1050
Ab40 AACCAGCACAGACAATAGTTCTGCTCAGGCACCCCGCCAAGAAGACTCCAGACCAGCACGTGCACGACCG  1050
KyaP   T  S  T  D  N  S  S  A  Q  A  P  R  Q  E  D  S  R  P  A  R  A  R  P   264
Ab4P   T  S  T  D  N  S  S  A  Q  A  P  R  Q  E  D  S  R  P  A  R  A  R  P   264

Sma3 GGGCCACCCACCAGAGGGCGCAGACGGGGGAGAAGGCCGGCTGCCCCAGGACCAGCTAGTCGGCGCAGCG  1120
Ab40 GGGCCACCCACCAGAGGGCGCAGACGGGGGAGAAGGCCGGCTGCCCCAGGACCAGCTAGTCGGCGCAGCG  1120
KyAP  G  P  P  T  R  G  R  R  R  G  R  R  P  A  A  P  G  P  A  S  R  R  S     287
Ab4P  G  P  P  T  R  G  R  R  R  G  R  R  P  A  A  P  G  P  A  S  R  R  S     287

Sma3 CCAGGCTGAGAAGACGCCAGCCCAGAACCAACTCCCGCACCAACGGGGGGGACAACGGGGAAATTATAGA  1190
Ab40 CCAGGCTGAGAAGACGCCAGCCCAGAACCAACTCCCGCACCAACGGGGGGGACAACGGGGAAATTATAGA  1190
KyAP A  R  L  R  R  R  Q  P  R  T  N  S  R  T  N  G  G  D  N  G  E  I  I  D   311
Ab4P A  R  L  R  R  R  Q  P  R  T  N  S  R  T  N  G  G  D  N  G  E  I  I  D   311

Sma3 CTTAACTCTGGACAGCGATGGG                                                 1212
Ab40 CTTAACTCTGGACAGCGATGGGGACACCGAACCTGCCGATGTTAGCGGCAGCCTGAATACCACCGATCAG  1260
KyAP   L  T  L  D  S  D  G                                                  318
Ab4P   L  T  L  D  S  D  G  D  T  E  P  A  D  V  S  G  S  L  N  T  T  D  Q   334

Sma3
Ab40 CCAGTGCTTATCCCCGACGAGGAAGAGGCGGCGCCCGCATCCCCACATACAAGCTCAAACTCTGCAATAA  1330
KyAP
Ab4P  P  V  L  I  P  D  E  E  E  A  A  P  A  S  P  H  T  S  S  N  S  A  I     357

Sma3
Ab40 TCTGCCTGGTGTCTGAACTCACGCCTGAGTCTGAAGAACCCCCCCGGGACCAACCCGTTGCACCATCTGG  1400
KyAP
Ab4P I  C  L  V  S  E  L  T  P  E  S  E  E  P  P  R  D  Q  P  V  A  P  S  G   381

Sma3
Ab40 CAGTTCTGCGGGTGAGCGGCCCATGCGGCCTAGATGCAGCCTGCGGGAGTTTGCGCGCAGGTTTATGGCA  1470
KyAP
Ab4P   S  S  A  G  E  R  P  M  R  P  R  C  S  L  R  E  F  A  R  R  F  M  A   404

Sma3
Ab40 CTGGCACCGCGAGACAGCTCCACGAGCGAAGCCGCGGGCCCCAGTCGCCTGGGTGCTGGCCCCCGCGCAA  1540
KyAP
Ab40  L  A  P  R  D  S  S  T  S  E  A  A  G  P  S  R  L  G  A  G  P  R  A     427

Sma3            CCTGTTGCGGTTGTTTTAGTCGACCGAAGCTCTGAGGGAGCAGGGCTATTTGGTGGGCG  1271
Ab40 CAGAGCCCTTCTCTGTTGCGGTTGTTTTAGTCGACCGAAGCTCTGAGGGAGCAGGGCTATTTGGTGGGCG  1610
KyAP             P  V  A  V  V  L  V  D  R  S  S  E  G  A  G  L  F  G  G  R   338
Ab4P T  E  P  F  S  V  A  V  V  L  V  D  R  S  S  E  G  A  G  L  F  G  G  R   451

Sma3 TTTCGCACAACACGTGCGCAGACGAACTGAGGATGAATCCGCTAGGCGGCGCGGCAATGTGCTTCTGAGA  1341
Ab40 TTTCGCACAACACGTGCGCAGACGAACTGAGGATGAATCCGCTAGGCGGCGCGGCAATGTGCTTCTGAGA  1680
KyAP   F  A  Q  H  V  R  R  R  T  E  D  E  S  A  R  R  R  G  N  V  L  L  R   361
Ab4P   F  A  Q  H  V  R  R  R  T  E  D  E  S  A  R  R  R  G  N  V  L  L  R   474

Sma3 CCGAGACGGCAGAGTGTGCCCCCGGTGCCCTACCCAGACATAGCGTCCACCAGCCCATTGATTAGACAAG  1411
Ab40 CCGAGACGGCAGAGTGTGCCCCCGGTGCCCTACCCAGACATAGCGTCCACCAGCCCATTGATTAGACAAG  1750
KyAP  P  R  R  Q  S  V  P  P  V  P  Y  P  D  I  A  S  T  S  P  L  I  R  Q     384
Ab4P  P  R  R  Q  S  V  P  P  V  P  Y  P  D  I  A  S  T  S  P  L  I  R  Q     497

Sma3 GGGGTCAGCGTGTCCGTGACCTGCAACGGGCGTTTCAGACACAGCCCGCTGAACCAGAGGAGATGCGATG  1481
Ab40 GGGGTCAGCGTGTCCGTGACCTGCAACGGGCGTTTCAGACACAGCCCGCTGAACCAGAGGAGATGCGATG  1820
KyAP G  G  Q  R  V  R  D  L  Q  R  A  F  Q  T  Q  P  A  E  P  E  E  M  R  C   408
Ab4P G  G  Q  R  V  R  D  L  Q  R  A  F  Q  T  Q  P  A  E  P  E  E  M  R  C   521

Sma3 TCCCCACAACTGCCAAAGATACCGGAGAAACCAATAACTTTTTTACCACACCTGTAAAGTTTTGTACCCC  1551
Ab40 TCCCCACAACTGCCAAAGATACCGGAGAAACCAATAACTTTTTTACCACACCTGTAAAGTTTTGTACCCC  1890
KyAP   P  H  N  C  Q  R  Y  R  R  N  Q                                      419
Ab4P   P  H  N  C  Q  R  Y  R  R  N  Q                                      532

           PolyA+                                                GC RICH
Sma3 CATTTTTAAATAAAGCTGTTTTTTTAAAAAGTTTACTATCAGTCTGAGAGAGTGTTA&TAGTCGCCGCCC  1620
Ab40 CATTTTTAAATAAAGCTGTTTTTTTAAAAAGTTTACTATCAGTCTGAGAGAGTGTTAATAGTCGCCGCCC  1960

Sma3 TGGGTATATGGAACCTATTACAAAAACCTATGCAGGGGTGTGGGTGGGGGCAACCGCGAAAGGGGGAGCT  1690
Ab40 TGGGTATATGGAACCTATTACAAAAACCTATGCAGGGGTGTGGGTGGGGGCAACCGCGAAAGGGGGAGCT  2030

Sma3 TTAAAAATAGATATCTTTGGGTTTAATTGCTCTGCAGCATTATGACGATTACACAGTTCCGTTTGAGAAA  1760
Ab40 TTAAAAATAGATATCTTTGGGTTTAATTGCTCTGCAGCATTATGACGATTACACAGTTCCGTTTGAGAAA  2100

Sma3 GCAGATTTCTAAAGCTGCAAGATATAAG&CCCGGGG                                   1795
Ab40 GCAGATTTCTAAAGCTGCAAGATATAAGGCCCGGGG                                   2136
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FIG. 2. Comparisons of the Ab4p and KyA EICP0 genes and predicted translation products. Sma3 refers to the Sma3 clone that contains the DNA sequence of the KyA EICP0 gene. Within the Sma3 subclone, the first ATG is located at nt 259 to 261, and the stop codon is located at nt 1512 to 1514. Ab40 designates the DNA sequence of the Ab4p EICP0 gene (65). There is a 339-bp in-frame deletion in the KyA EICP0 gene corresponding to bp 1212 to 1551 of the Ab4p ORF. KyAP designates the predicted amino acid sequence of the KyA EICP0 protein of 419 aa. Ab4P designates the Ab4p EICP0 protein sequence of 532 aa. Potential *cis*-acting elements in the promoter region are labeled and include (i) two potential TATA boxes (located at nt −78 to −75 and −188 to −183 relative to the first ATG), (ii) two octamer motifs (−145 to −138 and −169 to −162), (iii) an SP1 binding site (−250 to −245), (iv) a potential CAAT box (−242 to −238), and (v) two potential binding sites for the IE protein (35) located at −12 to −7 immediately upstream of the first ATG and at −239 to −234 overlapping the CAAT box. Asterisks indicate the conservation of the RING finger motif in the EICP0 protein of both the KyA and Ab4p strains; the ampersand represents the site of single nucleotide deletions in the 3′ untranslated region of the KyA EICP0 gene sequence.

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 pGEMICP0K and pGEMR4 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. When

```
               5' END SMA 3 CLONE OR NEAR 3' END ICP0 GENE

                 SmaI
SAL4 KyA   GGCCCGGGGGAACCACTGCACCTGCAGAAATCTCCTTTTCCCAAACAGCG    50
Ab4  LAT   GGCCCGGGGGAACCACTGCACCTGCAGAAATCTCCTTTTCCCAAACAGCG    50

SAL4 KyA   CCTTGAAACACGATGCTGTAATAATCGCCTTAAAACCAAACCAAAGCCTT   100
Ab4  LAT   CCTTGAAACACGATGCTGTAATAATCGCCTTAAAACCAAACCAAAGCCTT   100

SAL4 KyA   GACCCCTTTAATGCAACCGAGTTAGAAGACCCCCAAGCGTTTACCTATTC   150
Ab4  LAT   GACCCCTTTAATGCAACCGAGTTAGAAGACCCCCAAGCGTTTACCTATTC   150

SAL4 KyA   CATGTACAGCCTACCCTAAAACATTTGCATGGGGGAGATCTTTTGCCAAT   200
Ab4  LAT   CATGTACAGCCTACCCTAAAACATTTGCATGGGGGAGATCTTTTGCCAAT   200

SAL4 KyA   CTTAAACCCACAGTGCATAGCATAATCCCATAGATATTTTTAGAATGGAA   250
Ab4  LAT   CTTAAACCCACAGTGCATAGCATAATCCCATAGATATTTTTAGAATGGAA   250

SAL4 KyA   AACTGTTTACTGATGCTATTGCAGCTGGGCTTTCCACATCGTATTAATAA   300
Ab4  LAT   AACTGTTTACTGATGCTATTGCAGCTGGGCTTTCCACATCGTATTAATAA   300

                                                TATA BOX
SAL4 KyA   GGCGCCCCCTAATGTCCAGCGCGTGCATACCTATATACCACAAAAGCCCA   350
Ab4  LAT   GGCGCCCCCTAATGTCCAGCGCGTGCATACCTATATACCACAAAAGCCCA   350

                           LPBF
SAL4 KyA   CCTAAACAAAACACCCCCAGCTGGCTGTTTATGTGAACATCACCACCAAT   400
Ab4  LAT   CCTAAACAAAACACCCCCAGCTGGCTGTTTATGTGAACATCACCACCAAT   400

                                                    CAAT BOX
SAL4 KyA   ACCAAGCACGGCGCCATGGCGGTATATTACCATGTGCCAATTGGGGGCTA   450
AB4  LAT   ACCAAGCACGGCGCCATGGCGGTATATTACCATGTGCCAATTGGGGGCTA   450

           CAAT    TATA
SAL4 KyA   ATTGGGGGTATATAATATGCATGCACTAAGAAATATGAAATGTTGCCCCA   500
Ab4  LAT   ATTGGGGGTATATAATATGCATGCACTAAGAAATATGAAATGTTGCCCCA   500

                SP1
SAL4 KyA   TTCATGCTACGCCCCCTCCCAAAGTCAACTGTAAATGCAACTTAGCTGAA   550
Ab4  LAT   TTCATGCTACGCCCCCTCCCAAAGTCAACTGTAAATGCAACTTAGCTGAA   550

SAL4 KyA   TTTTGGCTAGCGTCACAGTTGGCACCGATCCAAGCTTCTATTGATGTTTG   600
Ab4  LAT   TTTTGGCTAGCGTCACAGTTGGCACCGATCCAAGCTTCTATTGATGTTTG   600

SAL4 KyA   GCATATCCTGTCCCGTCGAC                                 620
Ab4  LAT   GCATATCCTGTCCCGTCGAC                                 620
```

FIG. 3. Sequence of the Sal4 clone and alignment of the LAT promoter elements of the Ab4p and KyA strains. The EHV-1 KyA Sal4 genomic clone was sequenced (see Materials and Methods) and was found to contain LAT promoter elements identical to those of Ab4p (3). These elements include an SP1 site, two potential TATA boxes, two potential CAAT boxes, and a latency promoter binding factor site (LPBF).

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

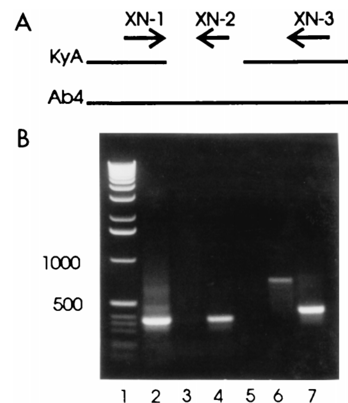

FIG. 4. Confirmation by PCR analyses of the deletion within the EICP0 gene of the EHV-1 KyA genome. (A) Schematic diagram of the relative positions of the XN-1, XN-2, and XN-3 primers used for PCR analysis. The XN-1 and XN-3 primers annealed to DNA sequences that flank the deletion in the KyA EICP0 gene. The XN-2 primer annealed to sequences that are contained only within the Ab4p EICP0 gene. (B) PCR products resolved on a 1.0% agarose gel stained with ethidium bromide. Lane 1, 1-kb DNA ladder as markers (Life Technologies); lane 2, Sma3 subclone of the KyA virus amplified with XN-1 and XN-3 primers; lane 3, Sma3 subclone of the KyA virus amplified with XN-1 and XN-2 primers; lane 4, KyA genomic DNA amplified with XN-1 and XN-3 primers; lane 5, KyA genomic DNA amplified with XN-1 and XN-2 primers; lane 6, Ab4p genomic DNA amplified with XN-1 and XN-3 primers; lane 7, Ab4p genomic DNA amplified with XN-1 and XN-2 primers. Sizes are indicated in base pairs.

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

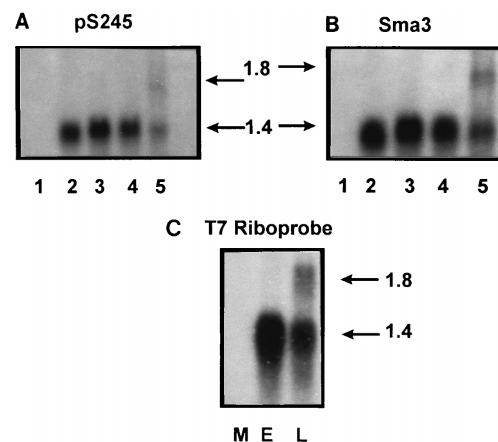

FIG. 5. Northern blot analyses of poly(A)$^+$-selected mRNA isolated from L-M cells infected with EHV-1 KyA strain under early and late conditions. Clones Sma3 (A) and pS245 (B) were ^{32}P labeled by nick translation and hybridized to mRNA isolated from mock-infected L-M cells (lane 1), cells harvested at 4 h p.i. following PAA treatment (lanes 2 to 4), or cells harvested at 8 h p.i. that were not treated with metabolic inhibitors (lane 5). Lanes 2 and 3 contain RNA isolated from cells infected in the presence of 100 μg of PAA per ml, and lane 4 contains RNA isolated from cells infected in the presence of 200 μg of PAA per ml. A T7 riboprobe generated from the pGEMICP0K construct was used as a probe and hybridized to mRNA isolated from mock-infected cells (C, lane M), cells harvested at 4 h p.i. following PAA treatment (lane E), or cells harvested at 8 h p.i. that were not treated with metabolic inhibitors (lane L). Sizes are indicated in kilobases.

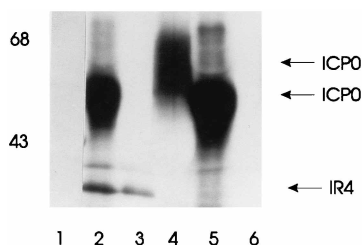

FIG. 6. SDS-PAGE analysis of in vitro-transcribed and -translated and immunoprecipitated proteins synthesized from the pGEMICP0K and pGEMR4 constructs, which express the EHV-1 EICP0 and IR4 (EICP22) proteins, respectively. Both constructs were in vitro transcribed and translated in a single reaction (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.

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 ICP27 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 obtained (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 γ1 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

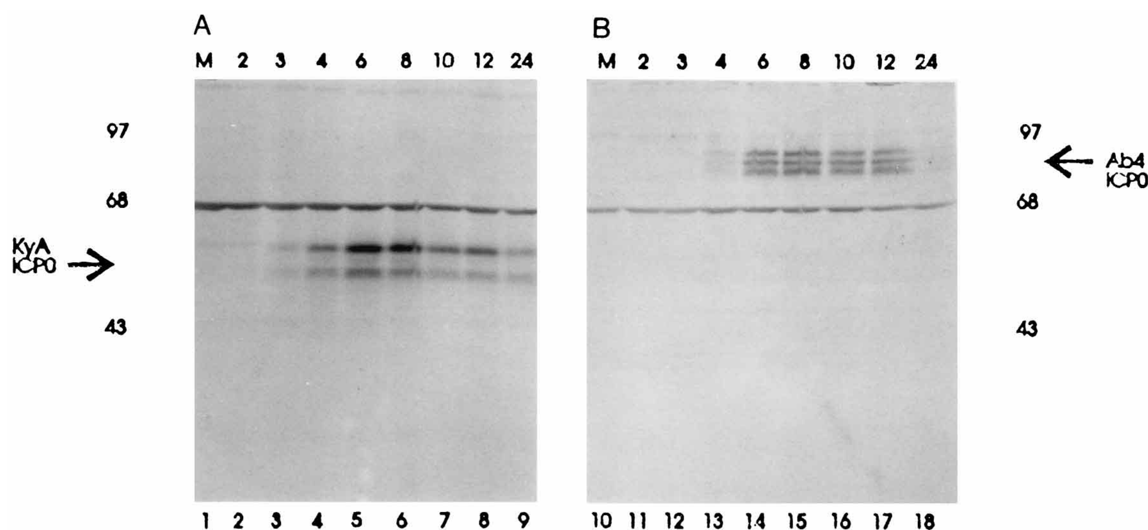

FIG. 7. Western blot analysis of EICP0 protein synthesis in EHV-1 KyA-infected L-M cells (A) and Ab4p-infected RK cells (B). Mock-infected (lanes M; 12 h shown as example) and infected cells were harvested at 2, 3, 4, 6, 8, 10, 12, and 24 h p.i. (lanes 2 to 9 for KyA infection; lanes 11 to 18 for Ab4p 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.

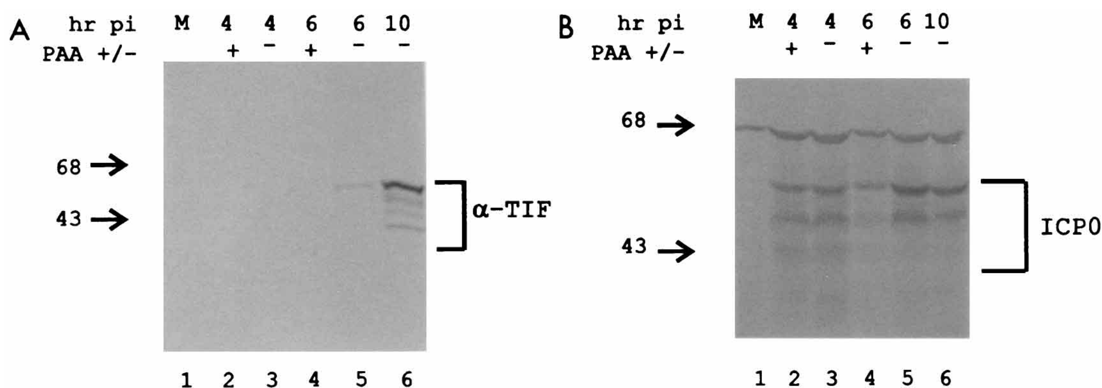

FIG. 8. Western blot analyses of the α-TIF protein (A) or the EICP0 protein (B) in L-M cells infected with the EHV-1 KyA strain. Infections were performed in the presence of PAA (lanes 2 and 4) or in the absence of PAA (lanes 3, 5, and 6). Mock-infected cell extracts (lanes M) served as a negative control. 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.

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

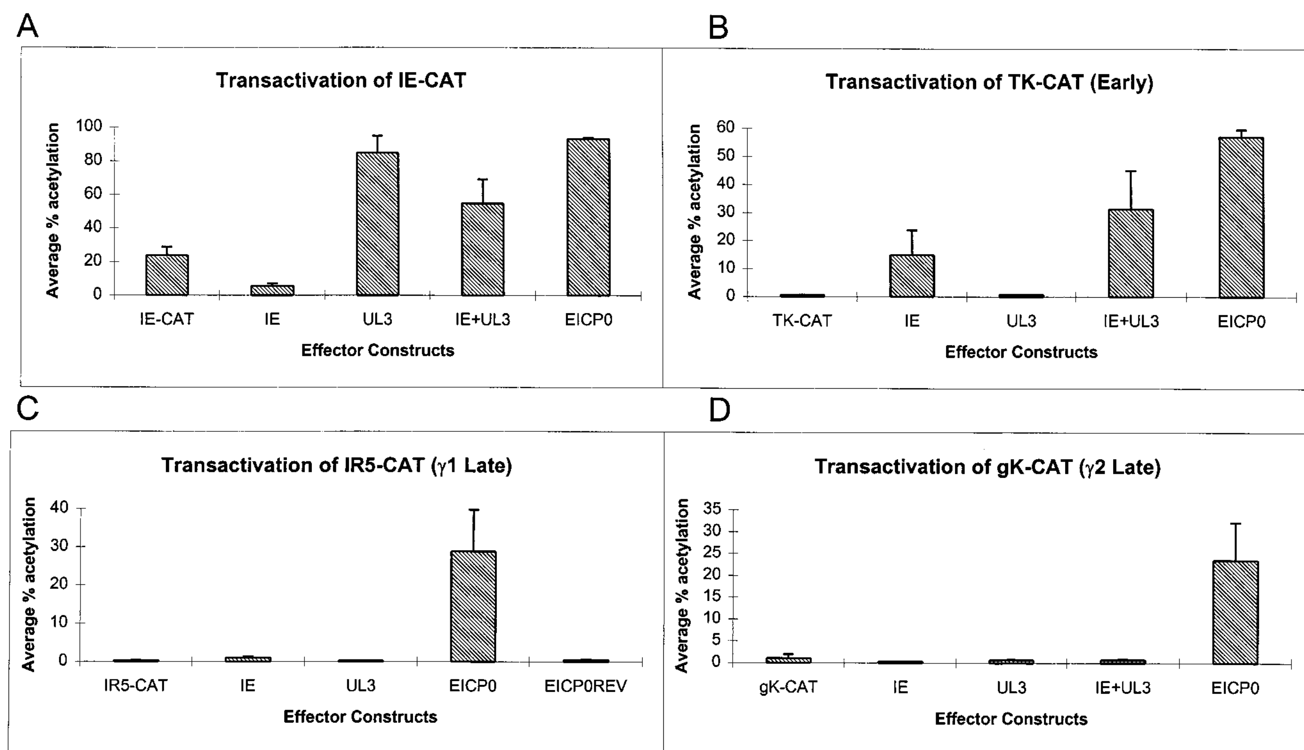

FIG. 9. Assays to detect transactivation of representative EHV-1 IE, E, γ1 L, and γ2 L promoters linked to the CAT reporter gene by effector constructs expressing the IE, UL3 (EICP27), or EICP0 protein of EHV-1 KyA. L-M cells were transfected with each promoter-CAT reporter construct and 0.3 pmol of 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 γ1 L IR5 promoter (pIR5-CAT; 1.4 pmol); (D) transactivation of the EHV-1 γ2 L 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 U_L portion of the genome (68, 69). ORF17, the VZV ORF15 equivalent, has a 1,207-bp deletion located 5′ to the gC 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 (*dl*1403) and an HSV-1 mutant virus (ΔTfi) 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 ectromelia 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 protein 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 γ2 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 α-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).

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