Expression and Function of the Equine Herpesvirus 1 Virion-Associated Host Shutoff Homolog

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The ability of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) to repress host cell protein synthesis early in infection has been studied extensively and found to involve the activities of the UL41 gene product, the virion-associated host shutoff (vhs) protein. To date, UL41 homologs have been identified in the genomes of three other alphaherpesviruses: equine herpesvirus 1 (EHV-1), varicella-zoster virus, and pseudorabies virus, but very little is known about the putative products of these homologous genes. Our earlier observations that no rapid early host protein shutoff occurred in EHV-1-infected cells led us to test EHV-1 vhs activity more thoroughly and to examine the expression and function of the EHV-1 UL41 homolog, ORF19. In the present study, the effects of EHV-1 and HSV-1 infections on cellular protein synthesis and mRNA degradation were compared at various multiplicities of infection in several cell types under an actinomycin D block. No virion-associated inhibition of cellular protein synthesis or vhs-induced cellular mRNA degradation was detected in cells infected with any of three EHV-1 strains (Ab4, KyA, and KyD) at multiplicities of infection at which HSV-1 strain F exhibited maximal vhs activity. However, further analyses revealed that (i) the EHV-1 vhs homolog gene, ORF19, was transcribed and translated into a 58-kDa protein in infected cells; (ii) the ORF19 protein was packaged into viral particles in amounts detectable in Western blots (immunoblots) with monoclonal antibodies; (iii) in cotransfection *vhs* activity assays, transiently-expressed ORF19 protein had intrinsic vhs activity comparable to that of wild-type HSV-1 vhs; and (iv) this intrinsic vhs activity was ablated by in vitro site-directed mutations in which either the functionally inactive HSV-1 *vhs1* UL41 mutation (Thr at position 214 replaced by Ile [Thr-214→Ile]) was recreated within ORF19 or two conserved residues within the putative poly(A) binding region of the ORF19 sequence were altered (Tyr-190, 192→Phe). From these results we conclude that EHV-1's low vhs activity in infected cells is not a reflection of the ORF19 protein's intrinsic vhs activity but may be due instead to the amount of ORF19 protein associated with viral particles or to modulation of ORF19 protein's intrinsic activity by another viral component(s).

Equine herpesvirus 1 (EHV-1) is a ubiquitous pathogen of horses; infection results in respiratory disease and may lead to abortion and paresis (1). EHV-1 is a member of the *Alphaherpesvirinae* subfamily and shares many genetic and biologic properties with other subfamily members, including the human pathogens herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) and varicella-zoster virus (VZV). These properties include a genomic configuration typical of the alphaherpesviruses (35, 50), rapid growth in cultures derived from a variety of equine and nonequine cells, neurotropism, and the ability to establish latency in sensory ganglia and to reactivate from that tissue (41). In addition, EHV-1 establishes latent infections in other tissues, including lymph nodes, peripheral blood mononuclear cells, and the respiratory tract (41, 52).

The early suppression of host cell protein synthesis is a prominent feature of infections with many strains of HSV-1 and HSV-2 (11, 24). The shutoff is secondary to the destabilization and degradation of host mRNAs (2, 16, 32, 37, 39), and in general, HSV-1 strains inhibit host protein synthesis somewhat more slowly than do HSV-2 strains (12). This inhibition has been demonstrated with multiplicities of infection (MOI) as low as 4 PFU per cell (17) and is apparent in infected cultures within 3 h postinfection (11, 39). In contrast, we have observed that EHV-1 infections do not elicit a rapid reduction

in radiolabeled cellular protein in pulse-labeling experiments; in fact, there is no abrupt early shutoff of host protein synthesis but rather a gradual decline throughout which some cellular protein synthesis persists (5).

The phenomenon of HSV virion-associated host shutoff (vhs) has been studied extensively and shown to be mediated by a tegument protein encoded by the HSV-1 UL41 gene (8, 16). Several lines of evidence supported this conclusion: mRNA destabilization does not occur during infections with HSV-1 strains that harbor mutations in UL41 (10, 13, 32, 33, 42); mRNA degradation occurs in the absence of viral gene expression, suggesting that active vhs protein is a component of and released by the incoming virion (9, 11, 12, 39, 49); and UL41 expression is sufficient to induce degradation of cotransfected reporter gene products in transient expression assays, indicating that other viral proteins are not required for vhs activity (13, 29).

While vhs activity varies considerably among HSV strains and is not essential for infectivity, it appears to play an important role in regulating the turnover of viral as well as cellular mRNAs (16, 25, 26). A number of HSV-1 vhs mutants have been identified and appear viable, although engineered null mutations result in virus titers which are reduced approximately 10-fold compared with that of the wild type (32, 44). Read and Frenkel (32) induced mutations in the HSV-1 KOS strain using 5-bromodeoxyuridine and identified a series of mutant viruses with altered vhs function. One of these HSV-1 mutants, *vhs1*, exhibited a failure to suppress host protein synthesis during infection in the presence of actinomycin D

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(Act D). The inactivating mutation in HSV-1 KOS *vhs1* subsequently was mapped and sequenced (18); this analysis revealed a single point mutation resulting in a threonine to isoleucine substitution at amino acid position 214 of UL41 (Thr-214→Ile). The *vhs1* UL41 open reading frame (ORF) was cloned into an expression vector recently and used in transient cotransfection assays to compare the intrinsic vhs activity of the mutant protein to that of wild type HSV-1 vhs (13). The *vhs1* mutant was inactive in reducing reporter gene expression compared with that of wild-type *vhs*, supporting the notion that the vhs1 protein lacks intrinsic vhs function.

In their genomic sequence analysis of EHV-1 strain Ab4, Telford et al. identified an open reading frame (ORF19) that has significant homology to HSV-1 UL41 (50) and to the UL41 homologs identified in VZV and pseudorabies virus (3). However, nothing is known about the expression of these UL41 homologs or the activities of their putative products. On the basis of our previous observations that EHV-1 did not appear to have significant vhs activity, the present study was initiated to compare directly the vhs activities of several EHV-1 strains to that of HSV-1 strain F at various MOI. Subsequent experiments were performed to determine whether EHV-1's inability to effect early host shutoff could be attributed to a lack of virion-associated ORF19 protein, which in turn might reflect inefficient ORF19 expression or subsequent packaging into viral particles, or to a reduced level of intrinsic vhs activity.

MATERIALS AND METHODS

Viruses and cells. EHV-1 (Kentucky A and D strains) and EHV-1 strain Ab4 (a gift from J. Mumford) were propagated and assayed for infectivity in rabbit kidney (RK13) cells and equine dermis (ED) cells, respectively, by methods previously described (5). HSV-1 (strain F) was propagated and assayed for infectivity in Vero cells by the same methods. RK, Vero, and baby hamster kidney (BHK-21) cells were cultivated in Eagle's minimal essential medium (EMEM) supplemented with nonessential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml), and 5% fetal bovine serum (FBS). Primary hamster embryo (HE) cells were obtained from explants of hamster embryo tissue (LSH inbred strain) as described previously (34) and cultivated in EMEM–10% FBS. Extracellular virions were purified by two rounds of dextran-10 rate velocity centrifugation followed by potassium tartrate isopycnic separation (30), and the purity of each virion preparation was verified by its lack of reactivity in immunoblots probed with monoclonal antibodies (MAbs) against actin, tubulin, and the EHV-1 major DNA-binding protein as described previously (4, 22).

Quantitation of protein synthesis in infected cells. RK and BHK cells were mock infected or infected with EHV-1 strains KyA and KyD or HSV-1 strain F at 10 and 50 PFU per cell in the presence of 10 µg of Act D per ml and maintained in the drug throughout infection to prevent viral and cellular transcription. At 5 h postinfection (p.i.), nascent proteins were radiolabeled with 20 µCi of [^{35}S]methionine per ml for 1 h. Cells were solubilized in Laemmli's sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol [19]) and boiled, and samples representing equal numbers of cells were separated by SDS–9% polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gels were dried, and labeled proteins were detected by autoradiography. Quantitation of cellular protein synthesis was determined by liquid scintillation counting of trichloroacetic acid (TCA)-precipitable counts from equivalent aliquots of labeled cell lysates.

Quantitation of RNA synthesis in infected cells. The 700-bp β-tubulin probe (American Type Culture Collection, Rockville, Md.) and the 1.15-kb β-actin cDNA probe (a gift from E. Howard) were radiolabeled with [α-^{32}P]dCTP by the random primer labeling method (Oligolabeling Kit; Pharmacia, Piscataway, N.J.). The human 28S rRNA oligonucleotide probe (Clontech, Palo Alto, Calif.) was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega, Madison, Wis.).

ED or BHK cells were mock infected or infected with EHV-1 strain KyA or Ab4 or HSV-1 strain F (20 PFU per cell) in the presence of 10 µg of Act D per ml. Total RNA was extracted at 3 or 6 h p.i. with RNAzol B (Cinna/Biotecx, Friendswood, Tex.). Five micrograms of each sample was separated by electrophoresis in 1.5% agarose-glyoxal gels, and the RNA patterns were transferred to nylon membranes. Hybridization conditions and temperatures were 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS containing either 50% formamide (β-tubulin at 42°C or β-actin at 65°C) or 10% formamide (28S rRNA at 42°C). RNA/DNA hybrids were detected by autoradiography. Specific mRNA levels were quantitated by liquid scintillation counting of excised membrane regions corresponding to autoradiographic bands.

Analysis of ORF19 transcription. An ORF19 strand-specific oligonucleotide probe (spanning the ORF19 amino acid codons 238 to 246; synthesized by Midland Certified Reagents, Midland, Tex.) was end labeled as described above. Total RNA was extracted from mock- or EHV-1 KyA-infected (15 PFU per cell) RK cells (in the absence of Act D) at 6 h p.i. and subjected to Northern (RNA) blot analysis as described above. Hybridization conditions were 6× SSC–1% SDS–10% formamide at 57°C.

Preparation of ORF19 protein for immunization. *Sca*I-linearized pCMV19 template was PCR amplified with PCR primers P1 (CGGGATCCAAGAGAA TGGGACTGTTTGGGACT) and P2 (CTCTAGATGCATGATCGAGCGGCC GCC) to produce a 1.7-kb DNA fragment spanning ORF19. This PCR product was digested with *Bam*HI and *Hin*dIII and inserted into the polylinker of pET30(a) vector (Novagen, Madison, Wis.). The resulting pET-19 expression plasmid contained the entire ORF19 coding sequence fused in frame to six histidine codons at the N terminus (referred to hereafter as 6×His-ORF19). *Escherichia coli* BL21(DE)3 was transformed with pET19, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the expressed product was purified by Ni-nitrilotriacetic acid affinity chromatography according to the manufacturer's procedure (Novagen). The ORF19-containing fractions were identified by SDS-PAGE, pooled, dialyzed against volatile buffer (50 mM NH$_4$HCO$_3$, 0.1% SDS), and lyophilized. Lyophilized ORF19 protein was resuspended in phosphate-buffered saline, and the protein concentration was determined by the micro-bicinchoninic acid protein assay (Pierce Inc., Rockford, Ill.). Aliquots of all preparations were reelectrophoresed on analytical SDS-PAGE gels to verify molecular weight and purity prior to use as an immunizing antigen.

MAb preparation. MAbs specific for EHV-1 ORF19 protein were produced as described previously (4, 22) with 200 µg of purified ORF19 protein for primary immunizations followed by weekly 100-µg boosts. Resulting hybridomas were tested for production of EHV-1 ORF19-specific antibodies by enzyme-linked immunosorbent assay and immunoblot analyses. Selected hybridomas were cloned twice by limiting dilution and retested for reactivity by immunoblot analysis. MAbs were subtyped with a subisotyping kit (Hyclone, Logan, Utah).

Construction of reporter and expression plasmids. For the reporter plasmid, pCMV-βgal (Clontech) contains the human cytomegalovirus (HCMV) immediate-early (IE) gene promoter controlling the *E. coli lacZ* ORF. For HSV-1 vhs expression plasmids, pCMV-vhs and pCMV-vhs1 (gifts from J. R. Smiley [13]) contain the HCMV IE promoter linked to HSV-1 KOS vhs and mutant vhs1 (Thr-214→Ile) ORFs, respectively. For the EHV-1 ORF19 expression plasmid, an EHV-1 ORF19 expression vector bearing ORF19 driven by the HCMV IE promoter (pCMV-19) was constructed by insertion of a 1.76-kb *Hin*dIII fragment spanning ORF19 (derived from the EHV-1 strain KyA genomic clone p*Bam*-HIN; a gift from Dennis O'Callaghan) into the polylinker region of the pcDNA3/ Amp vector (Invitrogen). Restriction enzyme analysis identified those clones with the desired 5′ orientation of the HCMV IE and bacteriophage T7 promoters.

Construction of ORF19 amino acid substitution mutants. Oligonucleotide-directed mutagenesis was performed on a 1.2-kb *Eco*RI ORF19 internal fragment isolated from pCMV-19. Each mutated fragment was introduced into pTZ18U, an intermediate phagemid vector for generating single stranded DNA templates (14, 15), to produce the missense mutant plasmids pCMV-19* (Thr-221→Ile) and pCMV-19** (Tyr-190, 192→Phe) with the Muta-Gene In Vitro Mutagenesis Kit (Bio-Rad) following the manufacturer's recommendations. The mutagenic primers Mp1 (CACGCGTTGACGAATGGGAATCCGAGGTGCC GAA) and Mp2 (ATGAGGATCAGATCTATATCGGTAGTGTAG) were designed complementary to sense strand DNA for construction of pCMV19* and pCMV19**, respectively. The presence of the desired oligonucleotide-directed mutations was verified by DNA sequence analysis by the dideoxy termination method (38). After successful mutagenesis, the 1.2-kb *Eco*RI fragment of ORF19 containing the desired mutations was reintroduced into the parental plasmid (pCMV-19) for transient expression assays.

Cotransfection assays for vhs activity. Transient cotransfection assays were modifications of the β-galactosidase expression assays described by Jones et al. (13). Triplicate cultures of 5.0×10^5 RK cells in 35-mm-diameter culture dishes were cotransfected with 1.0 µg of the reporter plasmid, pCMV-βgal, and 10 to 75 ng of either the HSV-1 wild-type or mutant *vhs* plasmids pCMV-vhs or pCMV-vhs1, the EHV-1 wild-type ORF19 plasmid pCMV-19, or mutant plasmids, pCMV-19* or pCMV-19**, or 100 ng of the parent vector pcDNA3 with the Lipofectin reagent (Gibco/BRL) in serum-free EMEM as previously described (21, 45). Parental plasmid pcDNA3 was added to each test plasmid transfection mixture such that the total amount of CMV promoter-bearing plasmid was standardized to 1.1 µg. After a 12-h incubation, media were replaced with EMEM containing 5% FBS, and cells were cultured an additional 48 h and harvested as previously described (21). Samples were normalized for protein concentration based on the detergent-compatible protein assay (Bio-Rad), and aliquots corresponding to 20 µg of total protein were assayed for β-galactosidase activity by the method of Rosenthal (36). The results from three identical, independent experiments were analyzed statistically by one-way analysis of variance with Duncan's multiple range test; P of ≤0.05 was considered significant.
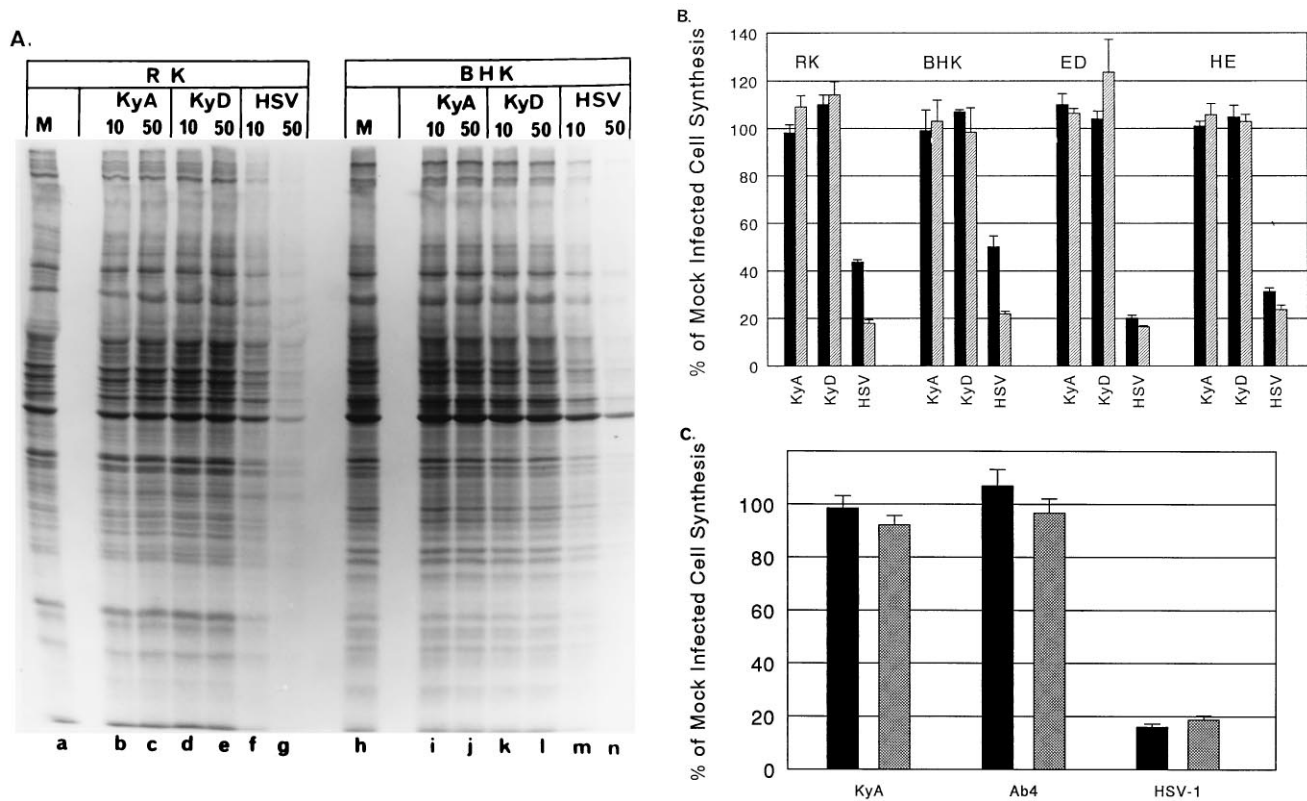
FIG. 1. Comparison of effects of EHV-1 and HSV-1 virion components on cellular protein synthesis. EHV-1 or HSV-1 infections were done in the presence of Act D and cellular protein synthesis was quantified by [^{35}S]methionine metabolic labeling (5 to 6 h p.i.), TCA precipitation, and liquid scintillation counting. (A) Autoradiograph of [^{35}S]methionine-labeled proteins extracted from RK and BHK cells infected with EHV-1 (strains KyA or KyD) or HSV-1 (strain F) at 10 and 50 PFU per cell. Lanes a and h are mock-infected RK and BHK cell lysates, respectively. (B) RK, BHK, ED, and HE cells were mock infected or infected with EHV-1 or HSV-1 at 10 (■) and 50 (▨) PFU per cell. The mean results (in counts per minute) from three independent samples were compared with those from mock-infected samples and expressed as the percentages of mock-infected cell synthesis; error bars represent 1 standard deviation. (C) ED cells were mock infected or infected with EHV-1 (strain KyA or Ab4) or HSV-1 (strain F) at 20 (■) and 100 (▨) PFU per cell, and the results are expressed as the percentages of mock-infected cell synthesis.

RESULTS

EHV-1 infection does not result in virion host shutoff activity. To substantiate and extend our earlier observations regarding the lack of early host shutoff during cytolytic EHV-1 infection, EHV-1's effect on cell protein synthesis was compared with that of HSV-1 in parallel infected cultures. RK and BHK cell cultures were infected with two EHV-1 strains, KyA and KyD, or HSV-1 (strain F) at an MOI of 10 or 50 PFU per cell in the presence of Act D. Proteins were pulse labeled with [^{35}S]methionine for 1 h at 5 to 6 h p.i. and solubilized immediately, and aliquots corresponding to equal cell numbers were subjected to SDS-PAGE (Fig. 1A). As expected, infection with HSV-1 caused an MOI-dependent decrease in cellular protein synthesis. However, neither EHV-1 strain effected a significant change in cellular protein synthesis compared with that of mock-infected cells. To eliminate the possibility that the lack of vhs activity in EHV-1-infected cells was cell type dependent, the experiment was repeated, and two additional cell types, primary HE and ED cells, were included. Protein synthesis was quantitated by TCA precipitation of cell lysate aliquots and liquid scintillation counting of associated [^{35}S]methionine. Again, HSV-1 infection caused a marked decrease in cellular protein synthesis in all cell lines (Fig. 1B). The magnitude of the decrease was dependent on the MOI; 50 PFU per cell induced approximately 80% inhibition. In contrast, [^{35}S]methionine incorporation into cellular proteins in EHV-1-infected

cells was essentially unchanged from that of mock-infected cells in each cell type tested.

Additional experiments in which cellular protein synthesis was monitored in Act D-blocked BHK cells infected with EHV-1 KyA at MOI of up to 1,000 PFU per cell or with HSV-1 at MOI of up to 200 PFU per cell revealed that whereas maximal inhibition (approximately 80%) was achieved in cells infected with HSV-1 at ≥50 PFU per cell, an EHV-1 dose of 200 PFU per cell resulted in approximately 22% inhibition of cell protein synthesis (data not shown). This level of inhibition did not change significantly with further increases in input virus, a finding which may reflect saturation of the cells with viral particles. Thus, it appeared that EHV-1 particles were capable of inducing minimal but detectable inhibition of host protein synthesis when supplied at high MOI. However, the significance of this observed inhibition is not yet clear; while it may be due to low-level vhs activity, it also could be the result of other unidentified effects of high multiplicities of EHV-1 infection.

Because of the possibility that the absence of vhs activity in EHV-1 strains KyA and KyD was simply the result of mutation or genetic rearrangement due to these strains' prolonged passage in culture, the activity of a low-passage, pathogenic EHV-1 isolate, strain Ab4, also was examined. The Ab4 virus stock has been propagated in equine cells only and was used at passage 9. As depicted in Fig. 1C, infection of ED cells with 20
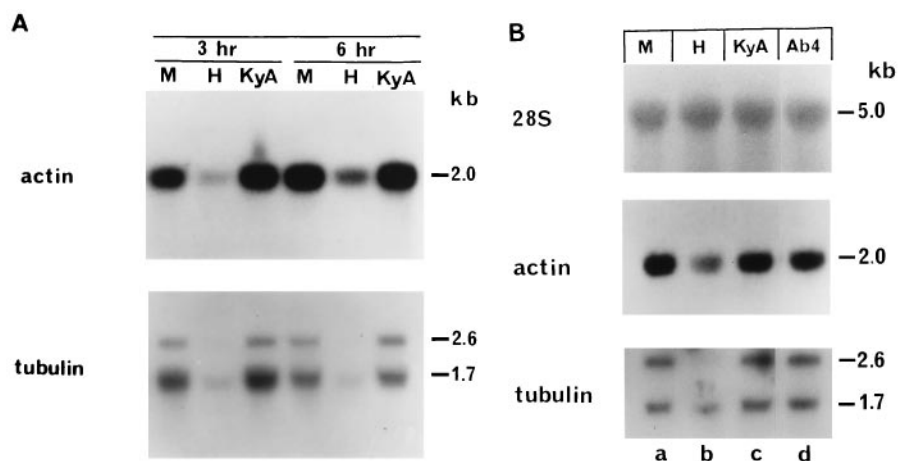
FIG. 2. Comparison of effects of EHV-1 and HSV-1 virion components on cellular RNA levels. (A) BHK cells were mock infected (lane M) or infected with EHV-1 (lane KyA) or HSV-1 strain F (lane H) (15 PFU per cell) as indicated in the presence of Act D. Total RNA was extracted at 3 and 6 h p.i., and Northern blot analysis was performed as described in Materials and Methods. The resulting autoradiographs are shown here with the specific mRNA bands identified by name and size. (See Table 1 for quantification of the effects in these specific bands.) (B) ED cells were mock infected (lane a) or infected with EHV-1 strain KyA or Ab4 (lanes c and d, respectively) or HSV-1 strain F (lane b) (20 PFU per cell) in the presence of Act D. Total RNA was extracted at 3 h p.i., and 5 μg of each sample was subjected to Northern analysis with ^{32}P-labeled probes complementary to cellular β-actin or β-tubulin mRNA or 28S rRNA probes. The RNA species identified by each of these probes are indicated by name and size.

or 100 PFU of EHV-1 Ab4 or KyA per cell resulted in no decrease in TCA-precipitable radiolabeled proteins compared with those in mock-infected cells, whereas similar doses of HSV-1 inhibited protein synthesis by more than 80%. These results suggest that the lack of vhs activity in EHV-1 strains KyA and KyD is not an artifact of prolonged culture conditions but rather is a characteristic EHV-1 feature.

EHV-1's inability to effect virion-associated host shutoff was confirmed in experiments comparing the effects of EHV-1 and HSV-1 infections on the levels of two cellular mRNAs, β-actin and β-tubulin. BHK cells were infected with EHV-1 KyA or HSV-1 (20 PFU/cell) in the presence of Act D. Total RNA was extracted at 3 and 6 h p.i. and subjected to Northern analysis. Results indicated that the levels of both cellular mRNA species were markedly lower in HSV-1-infected cells than in mock-infected cells, whereas neither β-actin nor β-tubulin mRNA was reduced by EHV-1 infection (Fig. 2A). Quantitation of the radioactive probe hybridized to each cellular mRNA species revealed that, compared with RNA extracted from mock-infected cells, HSV-1 infection resulted in a 65% reduction in β-actin at 3 and 6 h p.i. and a 71 and 74% reduction in β-tubulin at 3 and 6 h p.i., respectively (Table 1). In contrast, levels of these cellular mRNAs in EHV-1-infected cells were not decreased; in fact, they were consistently higher than those in mock-infected cells (Table 1). Similar results were obtained in assays of ED cell cultures infected with EHV-1 Ab4 strain compared with EHV-1 KyA or HSV-1 and harvested at 3 h p.i. (Fig. 2B). As previously reported (26), HSV-1 virion components had no effect on 28S rRNA stability; equivalent band intensities in all lanes also verified equal sample loading (Fig. 2B). Together, these experiments demonstrated that neither protein nor RNA levels were reduced by EHV-1 virion components.

EHV-1 ORF19, the HSV-1 UL41 homolog, is transcribed in vivo. Other than the identification by Telford et al. (50) of an EHV-1 open reading frame (ORF19) with homology to HSV-1 UL41, nothing is known about the putative EHV-1 vhs gene. Therefore, Northern analysis of EHV-1-infected cells was performed to determine whether the lack of EHV-1 vhs activity could be attributed to a failure in expression of ORF19. RK

cells were either mock infected or infected with EHV-1 (KyA) in the absence of Act D. Total RNA was harvested at 6 h p.i. and subjected to Northern analysis with a ^{32}P-end-labeled ORF19 strand-specific oligonucleotide probe. As shown in Fig. 3, a single 2.1-kb RNA species was detected in the EHV-1-infected sample but not in the mock-infected-cell-derived RNA. ORF19 is 1,493 bp in length; thus, a 2.1-kb transcript is within the expected size range for its transcript. These results indicated that a transcript corresponding to ORF19 was expressed during EHV-1 infection.

ORF19 protein is present in EHV-1 virions. To determine whether the identified ORF19-specific transcript was translated and whether the resulting protein was packaged into virions, strategies to produce ORF19-specific antibodies were employed. An expression plasmid, pET-19, encoding a 6×His-ORF19 product was constructed as described in Materials and Methods. The 6×His-ORF19 protein was expressed, purified, and used as an immunogen to produce ORF19-specific MAbs.

TABLE 1. Quantitation of effects of EHV-1 and HSV-1 infections on levels of cellular mRNAs[a]

mRNA source and infection	Radioactivity at:			
	3 h p.i.		6 h p.i.	
	cpm	Ratio	cpm	Ratio
β-Actin				
Mock	417.2	1.00	754.2	1.00
HSV-1 (F)	144.8	0.35	260.2	0.35
EHV-1 (KyA)	799.6	1.92	985.6	1.31
β-Tubulin				
Mock	131.4	1.00	100.8	1.00
HSV-1 (F)	37.8	0.29	26.6	0.26
EHV-1 (KyA)	164.6	1.25	128.4	1.27

[a] Membrane regions corresponding to the hybridized bands in Fig. 2A were isolated and the cpm associated with each band was determined by liquid scintillation counting. The fold change in counts per minute is expressed as the ratio of counts per minute for infected cells to those for mock-infected cells.
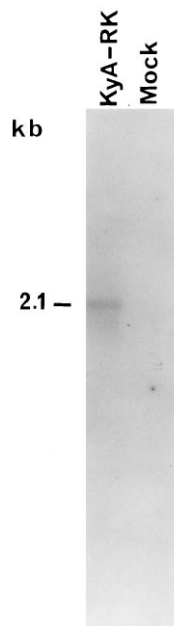
FIG. 3. ORF19 is expressed in EHV-1-infected RK cells. RK cells were mock infected or infected with EHV-1 KyA (15 PFU per cell) in the absence of Act D. RNA was extracted at 6 h p.i., and Northern blot analysis was performed with a strand-specific oligonucleotide probe complementary to the sequence spanning amino acid codons 238 to 246 of ORF19. The single 2.1-kb RNA species detected in the EHV-1-infected sample is indicated.

allele had no effect on β-galactosidase production, EHV-1 ORF19 and HSV-1 vhs exhibited equivalent shutoff activity.

The statistical analysis also revealed that, while there was no significant difference between the pcDNA3 control value and any of the pCMV-vhs1 doses, the values for pCMV-vhs1 at 10 and 75 ng were significantly different from each other. This trend toward differences between pCMV-vhs1 doses is consistent with results of assays in which larger amounts of pCMV-vhs1 resulted in increased inhibition of β-galactosidase production; a maximum of approximately 40% inhibition was achieved with 300 ng of transfected pCMV-vhs1 DNA (data not shown). This finding is in contrast to the results of Jones et al. (13) and Pak et al. (29), which revealed no inhibition by the *vhs1* allele at the doses used in their transient expression assays. Because of differences such as culture sizes, reporter plasmids, effector/reporter plasmid ratios, and transfection methods, the results of the three studies are not directly comparable. It is possible that our high-dose results are due to some effect unrelated to vhs activity, and further investigation is needed to determine the significance of this finding.

EHV-1 ORF19 protein shares critical amino acids with HSV-1 vhs. Previous comparison of the sequences of five alphaherpesvirus UL41 homologs revealed four regions which are highly conserved (3); a portion of the third such region is depicted in Fig. 6. This sequence, which spans amino acids 178 to 223 in HSV-1 and 185 to 230 in EHV-1, contains a putative poly(A) binding sequence (LGYAYIN [46]) which has been proposed as a potential HSV vhs site for direct interaction with
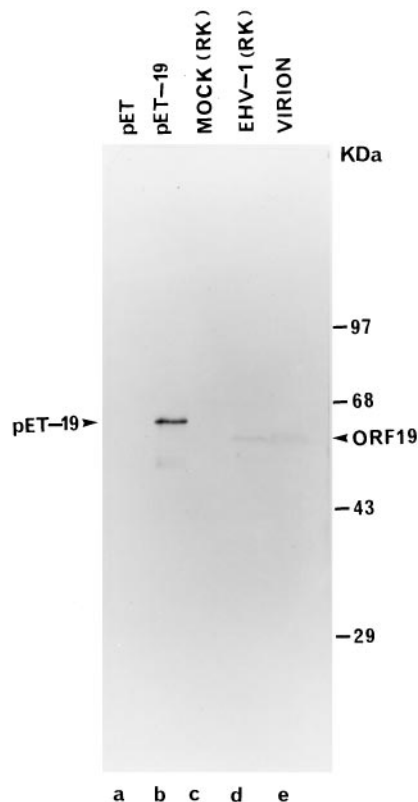
Immunoblot analysis revealed a weakly but reproducibly reactive protein with an apparent molecular mass of approximately 58 kDa present in EHV-1-infected RK cell lysates and in highly purified extracellular virions (Fig. 4). The virion preparations used here were characterized extensively previously and their purity was verified by their lack of reactivity in Western blots with MAbs against actin, tubulin, and the EHV-1 major DNA-binding protein (4). Thus, it appeared that detectable amounts of ORF19 protein are associated with EHV-1 particles.

EHV-1 ORF19 expression in transient cotransfection assays results in vhs activity. The ORF19 protein's intrinsic vhs activity was investigated by transient expression assays similar to those described recently by Jones et al. (13) and Pak et al. (29), in which cotransfection of HSV-1 vhs expression vectors with reporter gene constructs resulted in a reduction in reporter mRNA levels and in subsequent reporter enzyme activity. In the present study, RK cell cultures were cotransfected with increasing amounts of one of three expression vectors harboring the HCMV IE promoter-driven EHV-1 ORF19, wild-type HSV-1 *vhs*, or HSV-1 *vhs1* gene sequences (designated pCMV-19, pCMV-vhs, or pCMV-vhs1, respectively) or pcDNA3 as the vector control and a constant amount of pCMV-βgal reporter plasmid expressing the *E. coli lacZ* gene product β-galactosidase. These assays revealed that EHV-1 ORF19's ability to inhibit reporter gene expression was comparable to that of wild-type HSV-1 *vhs*, whereas the HSV-1 *vhs* mutant, *vhs1*, exhibited little or no vhs activity at comparable doses (Fig. 5).

Statistical analysis of these results indicated that there was no significant difference between the effects of pCMV-vhs and pCMV-19 at any dose, nor were there any significant differences among the doses of these two plasmids, whereas all doses of both plasmids were significantly different from all doses of pCMV-vhs1 and the pcDNA3 control (0 ng of effector plasmid). Thus, at doses at which expression of the HSV-1 *vhs1*

FIG. 4. ORF19 protein is present in EHV-1 virions. Aliquots of pET-transformed *E. coli* cell lysates, purified 6×His-ORF19 protein, mock- and EHV-1-infected cell lysates, and purified EHV-1 virions were separated by SDS–9% PAGE, and ORF19 protein was identified by immunoblot analysis with an ORF19-specific MAb. Purified 6×His-ORF19 (pET-19) and native ORF19 are indicated by arrowheads. The positions of molecular mass markers are indicated.
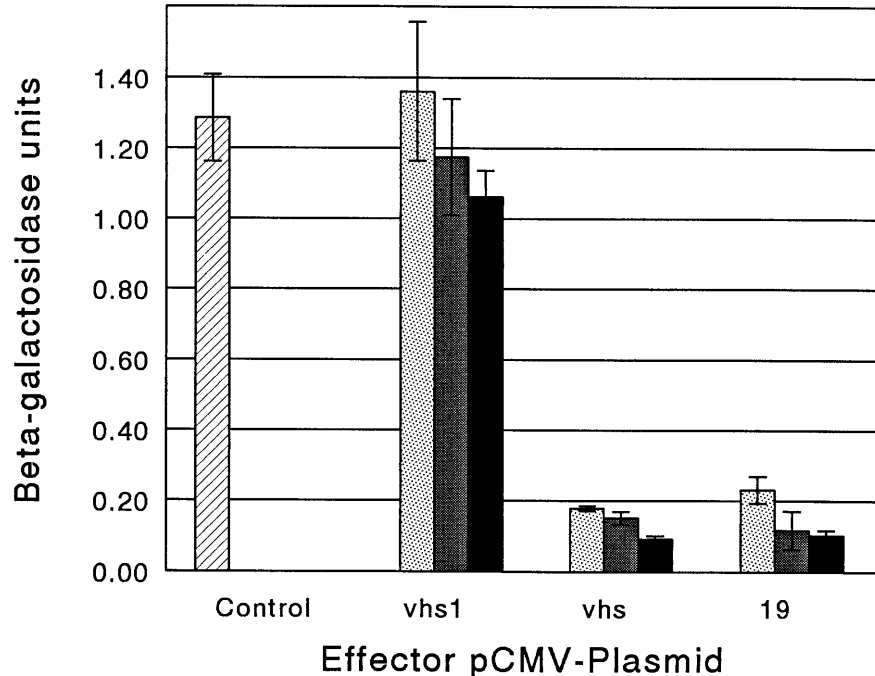
FIG. 5. EHV-1 ORF19 exhibits intrinsic vhs activity comparable to that of HSV-1 UL41 in transient expression assays. RK cells were cotransfected with 1.0 μg of the pCMV-βgal reporter plasmid and increasing quantities of either pCMV-19, pCMV-vhs, pCMV-vhs1, or pcDNA3 (control). Parental plasmid pcDNA3 was added to each transfection mixture such that the total amount of CMV promoter-bearing plasmid was standardized to 1.1 μg. The cultures were harvested at 48 h after transfection, and β-galactosidase assays were performed on cell extracts normalized to 20 μg of protein per sample as described in Materials and Methods. The β-galactosidase activity per sample was calculated, and the mean values for three independent experiments are depicted. Error bars represent 1 standard deviation. ▨, 0 ng; ▥, 10 ng; ▦, 50 ng; ■, 75 ng.

mRNA (53); it also includes residue 214, the single amino acid substituted in the HSV-1 *vhs1* mutant. Because it appeared that transiently expressed EHV-1 ORF19 protein was comparable to HSV-1 vhs in intrinsic vhs activity, it was of interest to determine whether creation of the *vhs1* mutation at the corresponding position in ORF19 would likewise ablate its intrinsic vhs activity. The corresponding amino acid substitution (EHV-1 Thr-221→Ile, compared with HSV-1 Thr-214→Ile) was introduced into ORF19 by in vitro site-directed mutagenesis, and the resulting construct was designated pCMV-19*. In addition, a second ORF19 mutant construct (designated pCMV-19**) was produced by substitution of two conserved tyrosine codons within the putative poly(A) binding sequence (amino acid positions 190 and 192) with phenylalanines. Transient expression assays were performed to compare the effects of 50 ng of pCMV-19, pCMV-vhs, pCMV-vhs1, or pCMV-19*, or pCMV-19** on expression of pCMV-βgal, and the results are

given in Table 2. Statistical analysis of the data indicated that cotransfections with pCMV19* or pCMV19** resulted in β-galactosidase expression which was not significantly different from that obtained in control pcDNA3 or pCMV-vhs1-transfected cultures. Thus, creation of the *vhs1* mutation at the corresponding ORF19 residue resulted in *vhs1*-like activity. These results indicated that ORF19 protein's intrinsic activity can be abrogated by alteration of an amino acid known to be critical in HSV-1 vhs, as well as by substitution of residues in a suspected RNA interaction domain.

DISCUSSION

Systematic attempts to detect vhs activity in three EHV-1 strains revealed that vhs activity was severely impaired in EHV-1 infections compared with HSV-1 (strain F) infections. Two of the EHV-1 strains, KyA and KyD, have been passaged

FIG. 6. Comparison of EHV-1 ORF19 amino acid substitution mutants and HSV-1 *vhs1* shutoff activity in transient expression assays suggests shared critical amino acids. Comparison of the highly conserved central region of ORF19 with its counterpart proteins of HSV-1, HSV-2, VZV, and pseudorabies virus (PRV) with the Genetics Computer Group software program. Identical amino acids in all five viruses at a given position are shaded. *, ORF19 amino acid substitutions generated by site-directed mutagenesis as described in Materials and Methods. See Table 2 for quantitation of β-galactosidase activity.

TABLE 2. Effect of cotransfected plasmids on pCMV-βgal
transient expression[a]

Effector plasmid	β-Galactosidase activity (U) ± SD
pcDNA3 (control)	1.285 ± 0.074
pCMV-vhs	0.151 ± 0.018
pCMV-19	0.117 ± 0.055
pCMV-vhs1	1.175 ± 0.165
pCMV-19*	1.101 ± 0.135
pCMV-19**	1.113 ± 0.097

[a] RK cells were cotransfected with 50 ng of the effector plasmid indicated and 1.0 µg of the pCMV-βgal reporter plasmid as described in the legend to Fig. 6.

extensively in a variety of cell cultures; thus, it could be argued that results from assays using these viruses may not reflect EHV-1 vhs activity in general. However, identical results were obtained with EHV-1 Ab4, a pathogenic strain isolated from a clinical case of paresis and used by Telford et al. as the initial virus stock for EHV-1 genomic sequencing (50). The Ab4 stock used in the present study had been propagated only in cell cultures of equine origin and was used at passage 9. Therefore, we conclude that recent pathogenic EHV-1 isolates as well as established laboratory strains fail to exhibit significant vhs activity in infected cell cultures. The two most straightforward explanations for this finding are that the ORF19 protein either is not present in virions or is present as a viral component but is intrinsically inactive. However, further analyses revealed that (i) the EHV-1 vhs homolog gene, ORF19, is transcribed and translated in infected cells; (ii) the ORF19 protein is packaged into viral particles in detectable amounts; (iii) in cotransfection vhs activity assays, transiently-expressed ORF19 protein was comparable to wild-type HSV-1 vhs in reducing reporter gene expression; and (iv) ORF19 protein's vhs activity was ablated by in vitro site-directed mutations that introduced amino acid substitutions within a highly conserved region. Thus, from these findings it appeared that neither simplistic explanation is entirely adequate, and the underlying mechanisms are likely more complex.

The presence of the ORF19 protein in EHV-1 virions was established by determining that the ORF19 protein was detectable in Western blots of highly purified viral particles. While it is possible that the lack of vhs activity during EHV-1 infection is due to a reduced concentration of the ORF19 protein per viral particle compared with that of HSV-1 vhs, no direct conclusions can be drawn from the present study concerning the relative concentration of the virion-associated ORF19 protein compared with that of HSV-1 vhs. However, in this regard, it should be noted that wild-type HSV-1 vhs also is a relatively minor viral component; it has been estimated that there are approximately 200 vhs molecules per viral particle (46, 47), compared with 1,000 to 2,000 copies of the abundant tegument protein, VP16, the α gene transinducing factor (αTIF [23]). Further studies to compare the relative amounts of these proteins as well as the transcriptional efficiencies of UL41 and ORF19 are underway.

Transient expression assays of vhs activity revealed that coexpression of EHV-1 ORF19 reduced β-galactosidase production to the same degree as did HSV-1 vhs coexpression, whereas identical doses of HSV-1 vhs1 had no effect on reporter gene expression. Furthermore, creation of the vhs1 mutation at the corresponding residue of ORF19 resulted in a construct with vhs1-like transient expression activity. Likewise, the substitution of two conserved tyrosine codons in the putative poly(A) binding region with phenlyalanines resulted in a

mutant ORF19 protein which was inactive in cotransfection assays. Because the cotransfection assay is an indirect assessment of vhs activity, it is possible that the effect on reporter gene expression induced by wild-type ORF19 resulted from some non-vhs activity. However, similar assays developed and validated by Jones et al. (13) and Pak et al. (29) have been used in the characterization of a number of HSV-1 UL41 mutants; in all cases there was a direct correlation between the phenotypes of the vhs mutants in the transient expression assay and their ability to induce virion-associated host shutoff after their transfer into the viral genome. The results of our studies indicate that the wild-type EHV-1 vhs homolog has intrinsic activity in cotransfection assays similar to that of HSV-1 vhs and that activity can be similarly destroyed by changes in critical amino acids. However, unlike HSV-1 UL41, ORF19's cotransfection assay phenotype does not correlate with EHV-1's virion-associated host shutoff activity.

One potential difference between HSV-1 vhs and ORF19 may lie in their ability to complex with other proteins. HSV-1 αTIF has been reported to interact directly with HSV-1 vhs (43); furthermore, a discrete 21-amino-acid αTIF binding domain has been identified recently in HSV-1 vhs (40). Interestingly, comparison of the aligned HSV-1 and EHV-1 protein sequences indicates that only 5 of these 21 amino acids are shared by the two proteins, suggesting that ORF19 may lack the primary sequence required for association with EHV-1 αTIF. If, as Schmelter et al. (40) hypothesized, αTIF plays a role in ensuring that vhs is efficiently packaged into the viral tegument, the lack of a corresponding interaction between EHV-1 αTIF and the ORF19 protein could contribute to low-efficiency packaging of the ORF19 protein into EHV-1 particles.

An alternative explanation for our findings is that a sufficient amount of intrinsically active ORF19 protein is present in EHV-1 particles but that activity is masked during infection due to other viral or infection-induced cellular factors. In this regard, Schmelter et al. (40) postulated that a second role for the HSV-1 vhs-αTIF interaction might be to regulate vhs function. This hypothesis was supported by the observation that infection of nonpermissive cells with the αTIF null mutant 8MA strain resulted in decreased early and late protein synthesis due to rapid degradation of viral mRNAs, while an 8MA derivative with a deletion of vhs exhibited normal protein levels (40, 51). These results suggest that the unrestrained mRNA degradation in the 8MA αTIF null mutant is due to the loss of αTIF-mediated regulation of vhs activity. This interpretation was further supported in subsequent studies by Lam et al. (20) which demonstrated that derivatives of the 8MA viral mutant harboring a truncated form of αTIF that was transactivationally incompetent but retained vhs binding activity could restore the normal pattern of viral protein synthesis at intermediate and late times p.i. Because EHV-1 ORF19 shares little homology with the reported αTIF minimal binding domain found in vhs, it may be less likely that an ORF19-αTIF complex is important in the regulation of ORF19's vhs activity; however, there are as yet no experimental data to exclude such a possibility. Also, it is possible that other regulatory proteins modulate ORF19 protein function during infection. Studies are underway to determine whether, like HSV-1's vhs and αTIF, EHV-1 ORF19 and EHV-1 αTIF form complexes and, if so, what effect this interaction may have during cytolytic infection. In addition, other proteins which may complex with ORF19 and mediate vhs activity are being examined.

At least one other HSV-1 structural component, the protein product of UL13, has been shown to be capable of modulating vhs activity. The UL13 gene product is a 57-kDa tegument

phosphoprotein (7, 28). Because the predicted UL13 protein sequence contains motifs with homology to known protein kinases, its potential role in phosphorylation of viral and host proteins has been examined with several UL13 mutant HSV-1 viruses (6, 27, 31). Overton et al. (27) observed that during UL13 mutant viral infection, overall host protein synthesis appeared comparable to that of mock-infected samples, a finding which suggested a loss of vhs activity in the UL13 mutants. Initially this effect was predicted to be due to defective phosphorylation of the UL41 gene product; however, no demonstrable changes in vhs protein phosphorylation levels were detected in lysates of UL13-negative virions, and there appeared to be no significant difference in the amounts of vhs protein in wild-type and mutant virions. Thus, the HSV-1 UL13 mutants have defective vhs function that is unrelated to the amount of UL41 gene product present in the virion (27). As discussed previously, transient cotransfection assays reported by Jones et al. (13) and Pak et al. (29) demonstrated that UL41 is sufficient to produce mRNA shutoff in transient cotransfection assays; thus, UL13 is not required for vhs-mediated shutoff activity. In their attempts to delineate an indirect role for UL13 in vhs function, Pak et al. hypothesized that UL13 may be required for viral uncoating (29). Alternatively, UL13 may phosphorylate and inactivate a virion-associated UL41 inhibitor and thereby prevent it from acting on vhs. Therefore, UL13 mutants would retain the active vhs inhibitor and thus be defective in vhs activity. Further examination of EHV-1's UL13 homolog gene product is needed to determine whether it plays a role in EHV-1's low vhs activity during cytolytic infection.

It has been assumed that the primary function of vhs and its homologs is to facilitate viral infection by inducing rapid shutoff of host protein synthesis and regulating viral mRNA turnover. However, since host shutoff activity appears minimal in cytolytic EHV-1 infection, it is possible that the ORF19 gene product (and, by analogy, the other vhs homologs) has other important functions that provide selective pressure for its genomic maintenance and ensure its continued expression in an intrinsically active form. In this regard, Strelow and Leib reported that infections with an HSV-1 strain containing a UL41 nonsense mutation resulted in a significant reduction in the establishment of latency and reactivation in the corneas and ganglia of experimental animals (47). Further studies with more narrowly defined UL41-mutant HSV-1 viruses suggested that vhs protein domains III and IV are required for both host shutoff activity and virulence (48). The mechanism(s) responsible for this effect and its significance for the other alphaherpesviruses with vhs homologs are unknown, but this finding may reflect additional roles for vhs in latency and pathogenesis that previously have been unrecognized. Clearly, there are many questions yet to be addressed concerning the vhs homologs' functions, interactions with other viral and cellular components, and mechanisms of action. Further characterization of the ORF19 protein's expression, functional domains, and role in various aspects of EHV-1 infection will enhance our ability to answer these questions.

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