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Role of the Virion Host Shutoff (*vhs*) of Herpes Simplex Virus Type 1 in Latency and Pathogenesis

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The herpes simplex virus type 1 (HSV-1) UL41 gene product, virion host shutoff (*vhs*), has homologs among five alphaherpesviruses (HSV-1, HSV-2, pseudorabies virus, varicella-zoster virus, and equine herpesvirus 1), suggesting a role for this protein in neurotropism. A mutant virus, termed UL41NHB, which carries a nonsense linker in the UL41 open reading frame at amino acid position 238 was generated. UL41NHB and a marker-rescued virus, UL41NHB-R, were characterized in vitro and tested for their ability to replicate in vitro and in vivo and to establish and reactivate from latency in a mouse eye model. As demonstrated by Western blotting (immunoblotting) and Northern (RNA) blotting procedures, UL41NHB encodes an appropriately truncated *vhs* protein and, as expected for a *vhs* null mutant, fails to induce the degradation of cellular glyceraldehyde-3-phosphate dehydrogenase mRNA. The growth of UL41NHB was not significantly altered in one-step growth curves in Vero or mouse $C_3H/10T1/2$ cells but was impaired in corneas, in trigeminal ganglia, and in brains of mice compared with the growth of KOS and UL41NHB-R. As a measure of establishment of latency, quantitative DNA PCR showed that the amount of viral DNA within trigeminal ganglia latently infected with UL41NHB was reduced by approximately 30-fold compared with that in KOS-infected ganglia and by 50-fold compared with that in UL41NHB-R-infected ganglia. Explant cocultivation studies revealed a low reactivation frequency for UL41NHB (1 of 28 ganglia, or 4%) compared with that for KOS (56 of 76, or 74%) or UL41NHB-R (13 of 20 or 65%). Taken together, these results demonstrate that *vhs* represents a determinant of viral pathogenesis.

Viral gene regulation during infection by herpes simplex virus type 1 (HSV-1) requires the coordinated regulation of all three temporal classes of viral genes: immediate-early (α), early (β), and late (γ) (20, 21). This is accomplished in part through the action of a 58-kDa phosphoprotein encoded by the viral UL41 gene which is known as the virion-associated host shutoff (*vhs*) protein (14, 24–26, 35, 36, 40, 42, 47, 52). Shutoff of macromolecular synthesis in HSV-infected cells occurs through two apparently independent mechanisms. Early shutoff occurs in the absence of viral gene expression and is due to the presence of approximately 200 copies of the *vhs* protein within the tegument of the infecting virus (14, 24, 40, 46). Shutoff can therefore occur immediately after viral infection, even though UL41 itself is expressed as a late gene (43). A secondary shutoff which requires viral gene expression occurs late during infection and appears to be dependent on the presence of the immediate-early protein ICP27 (12, 18, 33, 36).

Both host and viral RNAs are rapidly destabilized by an unknown mechanism in the presence of *vhs* (23, 37, 40, 52). All three temporal classes of viral messages and most cellular messages studied to date are destabilized by *vhs*, with the exception of rRNAs (38). An additional example of *vhs* resistance has been seen in cultured peripheral neurons, in which no significant shutoff of protein synthesis is observed following infection with HSV-1 up to 50 h postinfection (34). Viruses containing mutations in *vhs* have been isolated on the basis of their inability to cause degradation of host mRNA concomitant with an overaccumulation of immediate-early viral transcripts (24, 40, 52). Although *vhs* is not essential for virus growth, *vhs*-deleted mutants have slight but reproducible growth deficits compared with the wild type, that is they produce smaller plaques, have lower growth rates in mixed infections, and have two- to fivefold-reduced burst sizes (25, 40). In addition, it has been shown that the *vhs* protein can form a complex with the HSV transcriptional activator VP16, and it has been hypothesized that this interaction may modulate the activity of *vhs* during infection (49). Although a number of studies have addressed the role of *vhs* in lytic infections and in the cascade of viral gene expression, very little is known about the role of *vhs* in latency and pathogenesis. A recent study has found that a *vhs*-defective virus lacks intraperitoneal pathogenicity in newborn mice (2). In addition, recent sequence analysis (3) of the genomes of HSV-1, HSV-2, varicella-zoster virus, equine herpesvirus 1, and pseudorabies virus has revealed that each has a UL41 homolog. The neurotropic nature of these five viruses, coupled with the apparent absence of homologs in either the sequenced β- or γ-herpesviruses, suggests an important role for *vhs* in neurotropism.

In humans and in experimental animals the pathogenesis of HSV occurs in a series of discrete stages (for a review, see reference 16). Acute infection at peripheral sites involves viral replication and is followed by viral entry into neuronal termini. Virus is transported by intra-axonal flow to sensory ganglia, where further acute viral replication may occur and latency is established. Viral DNA remains episomal within neuronal nuclei, and the only readily demonstrable viral gene products found during latency are the latency-associated transcripts. The latent state may periodically break down in response to certain stimuli and lead to reactivation of virus and shedding of infectious virus at the periphery. The molecular mechanisms responsible for the altered regulation of viral gene expression

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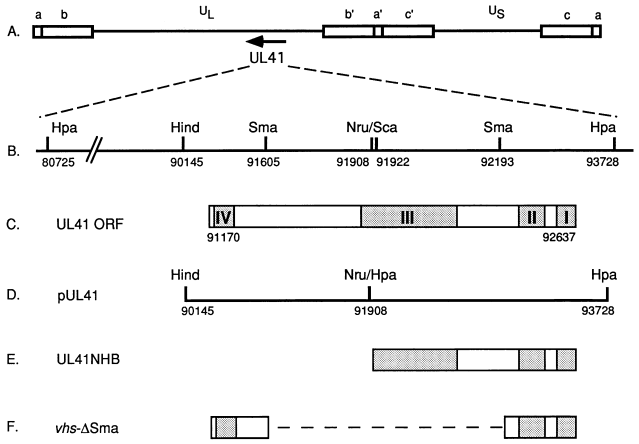

FIG. 1. Maps of *vhs* (UL41) ORF, plasmids, and viral mutants used in this study. (A) Prototypical arrangement of HSV-1 genome, showing unique long (U_L) and unique short (U_S) segments flanked by internal (a′, b′, and c′) and terminal (a, b, and c) repeats. The direction of transcription of UL41 is indicated by the arrow. (B) Expanded view of UL41 region showing selected restriction enzyme sites. Numbering is as described by McGeogh et al. (32). (C) Wild-type UL41 ORF showing conserved domains I to IV. The domains are those defined by Berthomme et al. (1) and are conserved among HSV-1, HSV-2, VZV, PRV, and EHV. (D) Plasmid pUL41 showing its limits and the position of the unique *Nru*I site which was used for the insertion of a nonsense linker (5) containing a unique *Hpa*I restriction site and stop codons in all three reading frames. (E) Limits of mutant *vhs* protein encoded by the virus UL41NHB. (F) Limits of the mutant *vhs* protein encoded by the virus *vhs*-ΔSma.

during the establishment of, maintenance of, and reactivation from latency remain poorly understood.

In this study, a virus containing a nonsense mutation in the UL41 gene was generated in order to elucidate a role for *vhs* in pathogenesis in a mouse model. A growth restriction for *vhs*-deficient viruses was shown in vivo but not in vitro, and *vhs*-deficient viruses also showed a significantly reduced ability to cause clinical disease, to establish latency, and to reactivate. These results therefore demonstrate a role for *vhs* in pathogenesis and latency.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were propagated as described previously (39). Murine $C_3H/10T1/2$ (ATCC CCL 226) cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (250 U of penicillin per ml and 250 μg of streptomycin per ml) in a 5% CO_2 humidified chamber at 37°C. Cell line 3-3 derived from Vero cells expresses a complementing level of ICP27, and the deletion mutant 5*dl*1.2 was grown on these cells as previously described (31). Growth and assay of the KOS strain of HSV-1 were carried out as described previously (39). The *vhs* mutant *vhs*-ΔSma was provided by Sullivan Read, University of Missouri at Kansas City (41). Viral mutants BGS41, UL41NHB, and *vhs*-ΔSma were propagated as described for the KOS strain. One-step growth curve experiments were performed at 37°C on confluent Vero or $C_3H/10T1/2$ cells (4).

Generation of viral mutants. The viral mutants used in this study were constructed from the parental strain KOS. Methods for constructing recombinants are described elsewhere (39). The KOS *Eco*RI A fragment was digested with *Hin*dIII and *Hpa*I, and the 3,595-bp fragment containing the entire UL41 open reading frame (ORF) as well as flanking sequence was cloned into pGEM5D3 (28) at the *Eco*RV and *Hin*dIII sites to generate plasmid pUL41 (Fig. 1). The β-galactosidase cassette from pHCMV-MP1-lacZ (provided by Paul Olivo, Washington University) was isolated as a 3,079-bp *Hin*dIII-*Sma*I fragment, ligated into the *Sca*I site within the UL41 ORF of pUL41, and designated as pGALSCA-11. pGALSCA-11 was cotransfected into Vero cells with infectious KOS DNA, and the progeny were screened by blue plaque selection (17) followed by Southern blot analysis for an altered *Sal*I digestion pattern. Blue plaque virus demonstrating the correct digestion pattern was plaque purified three times, and the high-titer stock prepared was designated BGS41. This virus was used for the subsequent selection of other viruses. pUL41HpaStop was constructed by the insertion of a nonsense linker (9) containing a *Hpa*I site and stop codons in all three ORFs into the *Nru*I site of pUL41. *Hin*dIII-linearized pUL41HpaStop was cotransfected into Vero cells with infectious BGS41 DNA, and the progeny were screened by white plaque selection followed by Southern blot analysis for an altered *Hpa*I digestion pattern. Virus demonstrating the expected *Hpa*I digestion pattern was plaque purified three times, and the high-titer stock prepared was designated UL41NHB. Marker rescue to produce UL41NHB-R was accomplished by cotransfection of infectious UL41NHB DNA with pUL41 and screening by Southern blotting as described for UL41NHB. Southern blot analysis of viral DNA was performed essentially as described previously (39, 44). pUL41 was labeled with ^{32}P by nick translation for use as a probe in the plaque purification of BGS41, UL41NHB, and UL41NHB-R.

Procedures with animals. Outbred CD-1 female mice (weight, 21 to 25 g; Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were anesthetized with ketamine and xylazine, their corneas were bilaterally scarified, and the mice were inoculated with 2×10^6 PFU of virus in a volume of 5 μl as previously described (39, 54). Eye swab material and trigeminal ganglia were assayed for virus as described previously (27), or ganglia were homogenized by using 1-mm-diameter beads in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, Okla.). Intracerebral inoculations were performed essentially as described previously (4, 29). In brief, mice were inoculated intracerebrally with 10 μl of a virus inoculum containing the appropriate titer of virus. At the indicated times postinfection, three mice infected with one of the indicated viruses were sacrificed, and their brains were removed, weighed, and stored at −80°C. Individual brains were homogenized by shaking in 4 ml of medium with 1-mm-diameter glass beads, clarified at 5,000 × *g* for 5 min, and assayed for virus on Vero or 3-3 cells. Reactivation of virus from latency was studied as described by Leib et al. (27). Briefly, mice were sacrificed on day 28 postinfection, and their trigeminal ganglia were removed. The ganglia were cut into eight pieces and explanted onto Vero cell monolayers. After 5 days in culture, explants were frozen, thawed, homogenized with a pellet pestle or with 1-mm-diameter glass beads in a Mini-Beadbeater-8, sonicated, and assayed for infectious virus on fresh Vero cell monolayers.

Western blot (immunoblot) analysis. The virus stock (10^8 PFU) was pelleted, resuspended in 2× sample buffer (40 mM Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 10% glycerol, 2 mg of bromophenol blue per ml, 40 mM β-mercaptoethanol), boiled, and separated on a 12% acrylamide gel by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose (Schleicher & Schuell) by using standard protocols (19). The filters were probed for UL41 polypeptides with a polyclonal antiserum prepared against a *vhs*-protein A fusion (supplied by Jim Smiley, McMaster University) at a 1:1,000 dilution. *vhs*-primary antibody complexes were visualized with a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit; Amersham).

Northern (RNA) blot analysis and mRNA degradation assay. Total cytoplasmic RNA was prepared from monolayer cultures of infected or mock-infected Vero cells at various times postinfection essentially as described previously (41). Monolayer cultures of 5×10^5 to 5×10^6 cells were infected at a multiplicity of infection (MOI) of 20 with the indicated viruses. Mock-infected plates received Vero cell lysate only. At various times postinfection, cells were washed twice with ice-cold phosphate-buffered saline and scraped into 200 μl of resuspension buffer (10 mM Tris [pH 7.9], 0.15 M NaCl, 1.5 mM $MgCl_2$, 100 U of RNAsin per ml). Cells were disrupted via 10 passages through a 25-gauge needle, and the nuclei were pelleted by low-speed centrifugation. Individual supernatants were removed to fresh tubes, and an equal volume of urea buffer (10 mM Tris [pH 7.9], 7 M urea, 0.35 M NaCl, 10 mM EDTA, 1% SDS) was added. Samples were extracted twice with phenol-chloroform (24:1) and twice with chloroform alone. Cold ethanol (2.5 volumes) was added, and the samples were precipitated overnight at −80°C. The RNA was pelleted, washed with 75% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water, and formaldehyde-containing loading dye was added. Electrophoresis of RNA was done with a 1.2% agarose gel cast in 1× MOPS (morpholinepropanesulfonic acid)–1% formaldehyde (5). The RNAs were transferred to nitrocellulose membranes by capillary blotting. Filters were baked for 2 h under vacuum at 80°C. pRGAPDH13 (obtained from Bill Parks, Washington University) (15) was digested with *Pst*I, and a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment isolated and labeled with [α-^{32}P]dCTP by a random priming kit (Promega, Madison, Wis.). The oligonucleotide GGGCTGGGCCTCGATCAGAAGGACTTGG, specific for the 28S ribosomal subunit, was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Hybridization solutions and conditions have been previously described (5). Filters were first probed for GAPDH, stripped, and then reprobed for the 28S ribosomal subunit. Autoradiographic films were scanned by using a densitometer (Bio-Rad model GS-670), and each GAPDH value was first normalized to the 28S ribosomal subunit signal at each time point and then expressed relative to the GAPDH level expressed by mock-infected cells, which was set to 100% at each time point.

Quantitative DNA PCR. Ganglionic DNA was prepared from mice at 28 to 33 days postinfection exactly as described previously (22), with appropriate precautions to prevent contamination of the samples. The DNA from each individual trigeminal ganglion was resuspended in 100 μl of TE (10 mM Tris chloride, 1 mM EDTA [pH 7.5]), and the DNA concentration was estimated both by determining the UV A_{260} and by electrophoresis in parallel with known amounts of DNA standards. PCR was performed essentially as described previously

(22) with the following modifications. In brief, 100-ng samples of ganglionic DNA were mixed with 100 pmol of primers specific for the HSV VP16 gene (CGGTACCTGCGCGCCAGCGTC and CAGCGGGAGGTTAAGGTGCTC) or mixed in parallel with 100 pmol of primers specific for the single-copy mouse adipsin gene (AGTGTGCGGGGATGCAGT and ACGCGAGAGCCCCACG TA). As standards, 100-ng samples of mouse tail DNA spiked with known amounts of HSV DNA were mixed with the VP16 primers. Reaction mixtures (100 μl) containing these DNAs in 20 mM Tris-HCl (pH 8.4)–50 mM KCl–2 mM $MgCl_2$–200 μM each deoxynucleoside triphosphate–2.5 U of *Taq* DNA polymerase were assembled and subjected to 35 cycles of PCR amplification. A hot start at 95°C for 5 min was followed by denaturation at 94°C for 45 s, annealing for 30 s at 55°C, extension at 72°C for 30 s, and a final additional extension at 72°C for 7 min. The sizes of the expected PCR products were 377 bp for VP16 and 43 bp for adipsin. For quantitation of the VP16 product, a 10-μl aliquot of each amplification reaction mixture was loaded on an 8% nondenaturing acrylamide gel, visualized by ethidium bromide staining, transferred to a nylon filter (Hybond-N+; Amersham), and UV cross-linked (Stratalinker; Stratagene). For quantification of adipsin, 2-μl aliquots of each amplification reaction mixture were used, and all other procedures were performed as described for the VP16 product. Filters were both prehybridized and hybridized as described previously (22). Probes were either an oligonucleotide specific for and internal to the HSV VP16 PCR product (TATGGGCCGCGTACGCCG) or an internal oligonucleotide probe specific for the mouse adipsin PCR product (AGTCGAAGGTGT GGTTAC). Probes were radiolabeled with ^{32}P by using T4 polynucleotide kinase as described previously (44). Filters were washed as described previously (22) and exposed to preflashed Kodak XAR-5 film. Quantitation of the PCR products was done by phosphorimaging (Molecular Dynamics).

RESULTS

Construction of nonsense mutant of UL41. In order to better define the effect of a mutation within one of the conserved domains of UL41 (3), a virus was constructed in which an ochre codon was inserted into conserved domain III (Fig. 1). A parent virus, BGS41, was constructed by the insertion of a cassette containing the human cytomegalovirus IE1 promoter driving β-galactosidase into the *Sca*I site of UL41. This blue-plaque parental virus was then cotransfected with pUL41Hpa Stop, a plasmid containing a nonsense linker (9) with stop codons in all three ORFs and a novel *Hpa*I site inserted into the *Nru*I site of UL41. Progeny were screened by white plaque selection followed by plaque purification and Southern blot analysis. The initial white plaque screening procedure greatly simplified plaque purification in that of five white progeny picked during the first round of plaque purification, two were plaque pure by the second round. A third round of plaque purification yielded UL41NHB. Isolation of marker-rescued virus was rapid in that 6 of 18 plaques picked from the transfection progeny contained the wild-type allele, consistent with a growth advantage for wild-type virus. Maps of the parental virus and mutant plasmid are shown in Fig. 1. The results of Southern blotting of DNA isolated from viral stocks are shown in Fig. 2. The introduction of the stop linker into the *Nru*I site resulted in the destruction of this site as well as the generation of a novel *Hpa*I site. An *Hpa*I digestion of wild-type viral DNA, when probed with nick-translated pUL41, is expected to yield a 13-kb fragment for wild-type KOS and marker-rescued UL41NHB-R viruses versus 11.2- and 1.8-kb fragments for recombinant UL41NHB. The sizes of the bands on this Southern blot coincided precisely with predicted values.

Detection of *vhs* polypeptides. Immunoblot analysis was performed to determine whether UL41NHB made a truncated *vhs* polypeptide with the expected size. A polyclonal antiserum raised against a *vhs*-protein A fusion (49) was used at a 1:1,000 dilution to detect *vhs* polypeptides in viral stocks. As shown in Fig. 3, this antiserum detected a protein with an apparent molecular mass of 58 kDa in KOS and UL41NHB-R viral preparations, in agreement with previous reports (41, 48). A protein with a mass of approximately 28 kDa was detected for UL41NHB (Fig. 3); the size of this protein is in close agreement with the predicted size of 26 kDa. No *vhs* polypeptides were detected with extracts from mock-infected cells or when

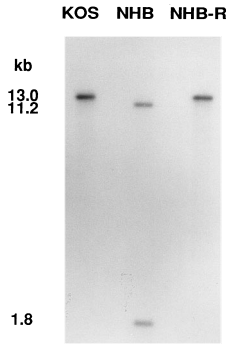

FIG. 2. Southern blot analysis of KOS, UL41NHB, and UL41NHB-R using pUL41 as probe. The positions of the fragments with the expected sizes resulting from an *Hpa*I digestion, 13-kb fragments for wild-type KOS and marker-rescued UL41NHB-R viruses and 11.2- and 1.8-kb fragments for the recombinant UL41NHB, are indicated on the left. ^{32}P-labeled pUL41 was used as a probe.

preimmune sera were used (data not shown). These results demonstrate that the insertion of the stop linker into the UL41 ORF in the context of the UL41NHB virus resulted in a truncation at the appropriate site in the UL41 protein.

Effect of UL41NHB mutation on accumulation of GAPDH. It has been previously shown that the failure to degrade cellular messages is the characteristic property of *vhs*$^-$ viruses (23–26, 37, 46, 52). UL41NHB was tested for its inability to degrade GAPDH mRNA in parallel with *vhs*-ΔSma, a *vhs* mutant previously shown to fail to degrade mRNA (41). GAPDH was chosen because it is a stable cellular message (15) and any effect seen would therefore not be due to inherent message instability. Vero cells were either mock infected or infected with an MOI of 20 with either KOS, UL41NHB, UL41NHB-R, or *vhs*-ΔSma, and cytoplasmic RNAs were harvested at 4, 8, and 12 h postinfection. The level of GAPDH mRNA was then analyzed by Northern blotting with a GAPDH probe. An oligonucleotide specific for the 28S ribosomal subunit was used as a loading control since 28S RNA is not affected by infection with HSV. As can be seen in Fig. 4, infection with KOS or UL41NHB-R leads to a decrease in the amount of GAPDH mRNA detectable over time. In contrast, UL41NHB and *vhs*-ΔSma are indistinguishable from each other and caused no significant degradation of GAPDH mRNA at any time point tested. These results show that UL41NHB is phenotypically a *vhs*$^-$ virus and that the lack of GAPDH degradation is due to the mutation in UL41.

Replication kinetics. (i) In vitro. The replication kinetics of KOS, UL41NHB, and UL41NHB-R were examined in Vero

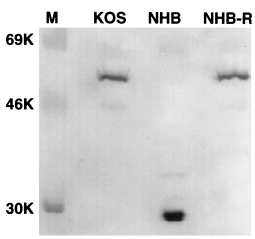

FIG. 3. Western blot analysis of *vhs* (UL41) protein from infected Vero cell lysates containing KOS, UL41NHB, or UL41NHB-R. The rabbit antiserum used was raised against a *vhs*-protein A fusion (49). The expected sizes of the *vhs* polypeptides are 58 kDa for KOS and UL41NHB-R and 26 kDa for UL41NHB. Molecular size markers (in thousands [K]) are indicated on the left. The filter was imaged and digitized on an Alpha Inotech Corporation IS-1000 digital imaging system.

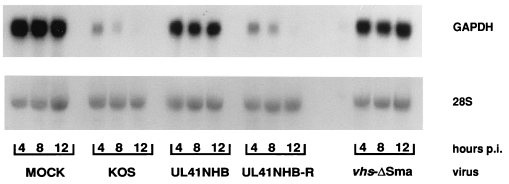

FIG. 4. RNA degradation assay by Northern blot analysis. Cytoplasmic RNA was extracted from KOS-, UL41NHB-, and UL41NHB-R-infected Vero cells at 4, 8, and 12 h postinfection (p.i.). (Top) Autoradiographic image of Northern blot probed for GAPDH; (bottom) autoradiographic image of blot shown above after it had been stripped and reprobed for 28S ribosomal subunit.

and mouse embryo cells (C_3H/10T1/2 cells). As shown in Fig. 5A, KOS, UL41NHB, and UL41NHB-R all replicated efficiently in Vero cells in a one-step growth curve. The yields and growth kinetics of UL41NHB were not significantly decreased from those of KOS and UL41NHB-R in these cells. These results are in agreement with the findings of Read et al. (41), who showed that *vhs*-ΔSma had only a marginal effect on virus growth in culture. Examination of the growth kinetics of the viruses in contact-inhibited C_3H/10T1/2 cells revealed similar results in that the growth of UL41NHB was comparable to that of KOS or marker-rescued viruses (Fig. 5B). These results indicate that the growth of UL41NHB was not severely restricted in Vero cells or in nondividing cells of murine origin.

(ii) In vivo. The growth of KOS, UL41NHB, and *vhs*-ΔSma was examined in the brains, corneas, and trigeminal ganglia of mice. Viral titers in the brains of mice were examined following intracerebral inoculation with 10^5 PFU (approximately 2×10^5 PFU/g [wet weight] of brain) of wild-type and mutant viruses. As shown in Fig. 6, virus replication in brain tissues was assayed for 5 consecutive days. These results show that the titers of *vhs* mutant viruses found in brains were 5- to 100-fold lower than the titers of KOS in brains at all time points. In an independent experiment, the growth of UL41NHB-R was shown to be indistinguishable from that of the wild type while UL41NHB remained significantly reduced (data not shown). One concern, however, was that at no point in any of these experiments did the viral titer rise above the concentration of the input inoculum. A possible explanation for this observation was that the virus recovered from the brains of mice inoculated with these viruses represented a residual inoculum which had not been cleared from the brain rather than de novo viral replication. To examine this possibility, mice were inoculated with 5×10^5 PFU of the ICP27-deleted virus 5*dl*1.2. Since ICP27 is an essential gene and this virus is unable to replicate outside of a complementing cell line, the decay of input virus

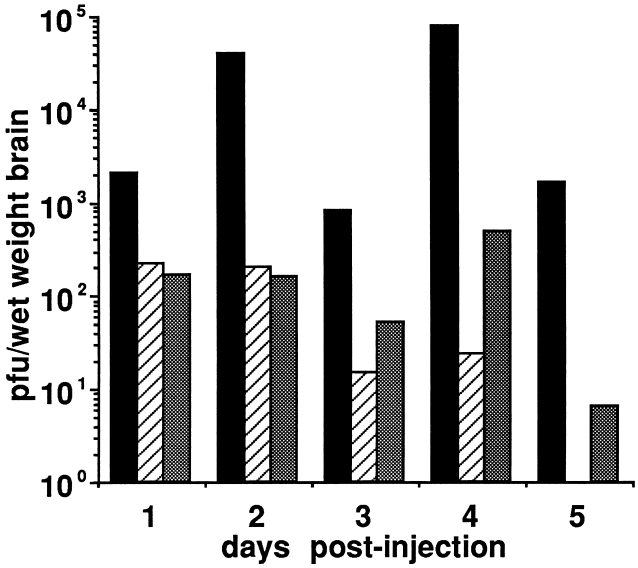

FIG. 6. Growth of KOS, UL41NHB, and *vhs*-ΔSma in mouse brain. Mice were inoculated intracerebrally with 10^5 PFU/g. Each bar represents the logarithmic mean number of PFU of virus obtained from three brains per time point, graphed as PFU per gram (wet weight) of brain versus days postinfection. ■, KOS; ▨, *vhs*-ΔSma; ▨, UL41NHB.

in the brain could be accurately assessed. At 3 days postinfection, no 5*dl*1.2 virus was detectable when brain homogenates were plated on the complementing 3-3 cell line, whereas control infections with KOS yielded 10^4 PFU of wild-type virus per g (wet weight). This result demonstrates that the titers detected in brains represent replicated virus and not the inoculum.

Acute viral replication in the mouse cornea was analyzed from 3 to 120 h postinfection following scarification and infection with 2×10^6 PFU per eye (Fig. 7). The replication of UL41NHB was reduced by approximately 100-fold compared with that of KOS or UL41NHB-R at all times tested. The replications of KOS and UL41NHB-R were indistinguishable, indicating that the growth defect is due to the mutation of *vhs*. The replication of *vhs*-ΔSma was reduced 1,000-fold relative to that of KOS at 48 h and was undetectable at 72 h postinfection, beyond which no further time points were tested. These results demonstrate a restriction to *vhs* mutant replication in the mouse cornea.

Viral replication in the trigeminal ganglion was determined

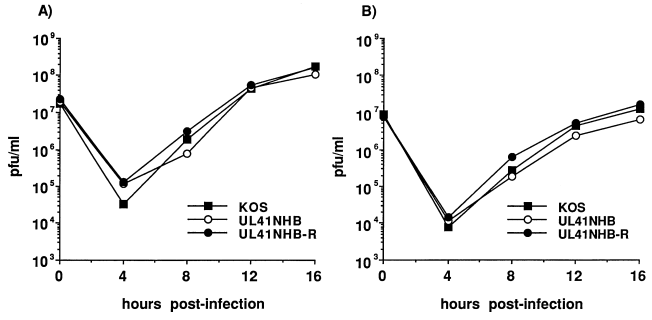

FIG. 5. One-step growth kinetics of KOS, UL41NHB, and UL41NHB-R in Vero (A) or contact-inhibited C_3H10T1/2 (B) cells. Cells were infected at an MOI of 5.

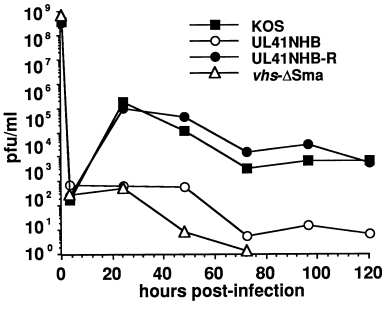

FIG. 7. Acute replication of KOS, UL41NHB, UL41NHB-R, and *vhs*-ΔSma in murine corneas following corneal inoculation with 2×10^6 PFU of virus per eye. Eye swabs were taken at 3, 24, 48, 72, 96, and 120 h postinfection. The values represent the logarithmic means for at least 10 eyes for KOS, 8 eyes for UL41NHB, 8 eyes for *vhs*-ΔSma, and 4 eyes for UL41NHB.

TABLE 1. Acute replication and establishment of and reactivation from latency of KOS, UL41NHB, UL41NHB-R, and *vhs*-ΔSma[a]

Virus	PFU/ml of[b]:		Mean no. of HSV-1 cells (range)[c]	No. of infected ganglia/no. tested (%)[d]
	Eye swab	Ganglion homogenate		
KOS	1.3×10^3 (30)	8.8×10^4 (26)	4 (3–5)	56/76 (74)
UL41NHB	3.7×10^1 (16)	0 (12)	0.2 (0.1–0.3)	1/28 (4)
UL41NHB-R	4.8×10^3 (4)	7.6×10^4 (8)	7.0 (3–12)	13/20 (65)
vhs-ΔSma	0 (8)	0 (8)	0.07 (0.05–0.1)	0/32 (0)

[a] These results represent data from five independent experiments for KOS, three independent experiments for *vhs*-ΔSma, and two independent experiments for UL41NHB.
[b] Samples were tested on day 3 postinfection. The number of samples assayed are given in parentheses.
[c] Mean number of HSV-1 genomes detectable in trigeminal ganglia at 28 to 33 days postinfection by quantitative PCR.
[d] Number of explanted latently infected trigeminal ganglia yielding virus per total number of ganglia assayed.

following corneal scarification and infection with 2×10^6 PFU per eye. While KOS was readily detectable (between 1×10^2 and 3×10^4 PFU/ml) on days 2 through 5 postinfection, infectious virus was never detected for *vhs*-ΔSma to 5 days postinfection. UL41NHB was similarly undetectable at all time points except for one ganglion at 4 days postinfection, which yielded 3.7×10^2 PFU/ml. To ensure that the infectious virus recovered from the ganglion was UL41NHB and not the result of contamination with KOS, a Southern blot analysis was performed on viral DNA isolated from the infected trigeminal ganglion (data not shown). The digestion pattern from this analysis was consistent with that of UL41NHB, demonstrating that UL41NHB was present in this trigeminal ganglion. To demonstrate that the lack of replication of UL41NHB was due to a mutation in UL41, the replication of UL41NHB-R in trigeminal ganglia at 3 days postinfection was examined and was indistinguishable from wild-type virus replication at that time (Table 1). Moreover, the extent of overt clinical signs (e.g., hair loss and swollen eyelids) during acute infection was indistinguishable between KOS and UL41NHB-R-infected mice, whereas UL41NHB- and *vhs*-ΔSma-infected mice appeared essentially uninfected.

Quantitative DNA PCR analysis of latently infected trigeminal ganglia. The ability of the viruses in this study to establish latency in murine trigeminal ganglia was examined by quantitative PCR analysis. Attempts to use the viral thymidine kinase (*tk*) gene as a target with primers previously described (22, 53) were unsuccessful because of high background signals apparent in the PCRs of DNA from uninfected control tissues, thereby precluding accurate quantitation at low DNA concentrations as seen with the *vhs* mutants. It is believed that these signals arose either from exogenous contamination with *tk* DNA being frequently cloned elsewhere in the laboratory or from priming and amplification of endogenous cellular *tk* homologous sequences. PCR primers which targeted the HSV-1 VP16 ORF were therefore selected, with the mouse single-copy adipsin gene as an internal standard (22). In order to establish a linear range for the assay, a standard curve was constructed by spiking 100-ng samples of mouse tail DNA with a known amount of genomic HSV-1 DNA (Fig. 8D). As can be seen, the signals as measured by PhosphorImager (Molecular Dynamics) analysis were linear over a wide range, from 0.01 to 10 copies of viral DNA per cell equivalent. After the linearity of the assay was demonstrated, trigeminal ganglia latently infected with the viruses in this study were harvested and examined for their HSV DNA contents. As shown in Fig. 8 and in Table 1, the amount of KOS DNA in latently infected ganglia ranged from four to five copies of viral DNA per cell equivalent with an average of five copies, on the basis of the assumption of 10^6 cells per trigeminal ganglion (22). Ganglia latently infected with UL41NHB-R showed a comparable amount of viral DNA (range of 3 to 12 copies; mean of 7 copies). These values are comparable to those obtained by others (22, 45). In contrast, UL41NHB-infected ganglia contained about 30-fold fewer genomes than KOS and 40-fold fewer than UL41NHB-R. *vhs*-ΔSma-infected ganglia contained about 80-fold fewer genomes than KOS. Specific bands for these mutants were clear on the original autoradiographs and phosphorimages but not on the photographs shown. These data show that *vhs* mutants can establish latency but with significantly reduced efficiency.

Explant cocultivation reactivation studies. As shown in Table 1, the reactivation frequency for KOS (56 of 76 ganglia, or 74%) was similar to previously published results (39) and comparable to that for UL41NHB-R marker-rescued virus (13 of 20, or 65%). In contrast, UL41NHB was reactivated in only 1 of 28 ganglia (4%) while *vhs*-ΔSma was not reactivated in any

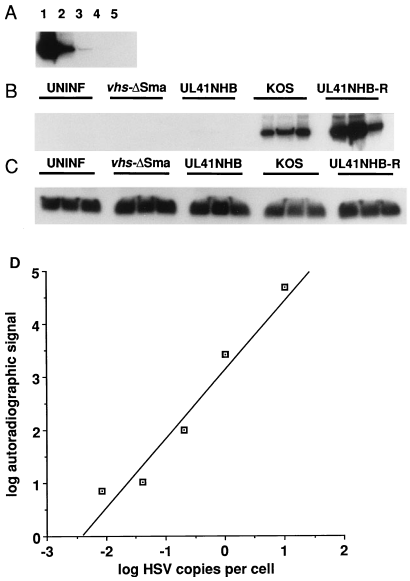

FIG. 8. Quantitative PCR analysis of HSV DNA extracted from latently infected trigeminal ganglia. The HSV-1 VP16 gene was used as a target for amplification with the single-copy mouse adipsin gene as an internal loading control. PCR was performed for 35 cycles, and all probes were internal to the primers as described in Materials and Methods. (A) Quantitative PCR analysis of uninfected mouse tail DNA spiked with HSV DNA. Lanes: 1, 10 copies; 2, 1 copy; 3, 0.2 copy; 4, 0.04 copy; 5, 0.008 copy of viral DNA per mouse cell equivalent. (B) PCR analysis of DNA from uninfected trigeminal ganglia or ganglia latently infected with *vhs*-ΔSma, UL41NHB, KOS, or UL41NHB-R. (C) Quantitative PCR analysis of mouse adipsin for each sample. (D) Standard curve showing signals for a range of copy numbers (0.008 to 10) of HSV-1 genomic DNA per mouse cell equivalent. Autoradiographs were scanned and analyzed by using a Molecular Dynamics PhosphorImager.

of 32 ganglia. These results indicate that the *vhs* mutants are significantly reduced in their ability to reactivate from latency. The ability of each of these viruses to reactivate was consistent with the amount of its DNA found in latently infected ganglia, suggesting that the defect in reactivation occurs at the level of establishment.

DISCUSSION

This study demonstrates an important role for the HSV-1 *vhs* function in pathogenesis. Although a role for *vhs* has been previously demonstrated during HSV-1 infection in cell culture, this is the first study of the role of *vhs* in latency. Consistent with a role in neuropathogenesis, the sequence analysis by Berthomme et al. (3) indicated that the UL41 gene has homologs among five neurotropic herpesviruses but not in the sequenced lymphotropic herpesviruses. Of the five neurotropic herpesviruses in which UL41 homologs have been sequenced (HSV-1, HSV-2, varicella-zoster virus, pseudorabies virus, and equine herpesvirus 1), HSV-1 (35, 40, 42), HSV-2 (11), and pseudorabies virus (51) have been examined and shown to cause rapid degradation of host RNA and shutoff of host macromolecular synthesis. There is, therefore, conservation of both sequence and function, suggesting that *vhs* is vital to some aspect of neurotropism.

The nonsense mutation introduced into UL41 in the virus UL41NHB rendered HSV-1 incapable of degrading host RNA at a high MOI. This nonsense mutation truncated the protein as predicted within conserved domain III (3). Clearly, mutation within this conserved domain does have a negative effect on *vhs* function, and, consistent with this, a number of published *vhs*-defective viruses contain mutations in this conserved domain (25, 41).

***vhs* is important for viral pathogenesis.** A recent report has shown that cultured sensory neurons are not susceptible to *vhs*-induced reduction in protein synthesis and that nerve growth factor differentiation of PC12 cells leads to decreased susceptibility to HSV-1 *vhs* (34). In that study it was proposed that the inability of HSV-1 to cause shutoff in neurons promotes the establishment of a latent state due to delayed or attenuated gene expression in these cells. These data might predict that mutation of *vhs* would have little or no effect on pathogenesis, especially since previous studies have shown that *vhs* is dispensable for replication and that viruses with weak shutoff can be neurovirulent (13, 30). In this study, however, it has been shown that mutation of UL41 results in a virus which exhibits reduced growth in corneas, trigeminal ganglia, and brains, minimal ability to cause clinical disease (data not shown), and reduced ability to establish latency and to reactivate.

The data of this study therefore apparently conflict with previous studies. For a number of reasons, however, the observations of this and previous studies are not mutually exclusive. First, the infection of cultured sensory neurons is clearly very different from the in vivo situation in which neurons are surrounded by a large number of nonneuronal cells. It is likely that the majority of viral replication in both peripheral nervous system and central nervous system (CNS) during acute infection occurs in nonneuronal cells and such cells are not present in any significant amount in neuronal cultures. Second, as pointed out by Nichol et al. (34), there may be a transport defect in cultured neurons of the UL41 gene product to the cell body, a defect which, because of anatomical differences, may not occur in vivo. Third, the proved neurovirulence of viruses which have weak shutoff phenotypes (13, 30) does not necessarily mean that *vhs* is not a major determinant of neurovirulence, since viruses with only partial activities of other gene products, such as thymidine kinase (7), latency-associated transcripts (39), and ICP0 (8), retain their abilities to be virulent and to reactivate. Low levels of gene activity can therefore be both necessary and sufficient for virulence, and the use of *vhs* null mutants in this study allowed a more accurate assessment of the role of *vhs* in disease.

What is the nature of the decreased pathogenicity of *vhs*$^-$ viruses? The observations that *vhs* plays a significant role in pathogenesis in the mouse ocular model and the mouse brain are consistent with a previous demonstration of a lack of intraperitoneal pathogenicity in newborn mice by *vhs*-defective viruses (2). What is the basis for this reduced pathogenicity in these disparate systems? One possible explanation is that the mutant UL41 gene products expressed by the viruses in this study fail to interact with the viral transactivator protein VP16. VP16 plays a crucial role in the transactivation of viral immediate-early genes and has been shown to interact with *vhs* (48). Mutational analysis has shown that amino acid residues 238 to 344 were necessary and sufficient for this interaction and that *vhs*-ΔSma fails to bind VP16, as assayed by immunoprecipitation (48). Although the transactivation domain of VP16 is not required for reactivation, VP16 as a whole is an essential structural component of the virus (56). If, as a result of a UL41 mutation, the virus packaged VP16 very inefficiently, this might result in inefficient viral replication, particularly at low MOIs, such as, for example, during reactivation. Studies of *vhs* and VP16 levels within purified mutant virions are in progress to investigate this issue, especially with respect to UL41NHB, in which the mutant *vhs* protein is predicted not to interact with VP16 (48). In most aspects of this pathogenesis study (i.e., corneal and ganglionic viral replication and establishment of and reactivation from latency), UL41NHB appears slightly more pathogenic than *vhs*-ΔSma, and it is therefore of interest to assess the relative levels of VP16 in these two viruses. It is noteworthy that many of the in vivo properties of the *vhs* mutants in this study are comparable to those of *in*1814, an HSV-1 mutant which lacks functional VP16 (1, 50, 55). The *in*1814 mutant grew poorly in eyes and ganglia and established latency very inefficiently, in that viral DNA was almost undetectable 28 days postinfection in latently infected ganglia (55). This study has shown that the properties for replication and the establishment of latency in *vhs* mutants are similar to those of *in*1814, but *in*1814 reactivated with an efficiency almost equivalent to that of the wild type. In contrast, *vhs* mutants reactivated with very low efficiency. This is consistent with the idea that *vhs* may play an additional role in promoting both the establishment of and reactivation from latency, independent of VP16 interactions, although the precise mechanism is unclear.

The ability to grow in the CNS is one distinguishing characteristic of neurovirulence (6, 10). That the levels of mutant virus seen in Fig. 6 represented actual (albeit reduced) replication and not a failure to clear the input inoculum was addressed by the intracerebral inoculation of the replication-incompetent ICP27 mutant 5*dl*1.2. No detectable virus was found 3 days postinfection, indicating that the input inoculum was indeed cleared from the brain and that the virus observed for the *vhs*$^-$ viruses therefore represented authentic, although decreased, amounts of replicated virus. In comparing acute replication in the periphery to acute replication in the CNS, it can be seen that, although both *vhs*$^-$ viruses are impaired in their levels of replication in these tissues, the deficit seen in the peripheral nervous system is greater than that seen in the CNS. The reasons for this difference are unknown although it is likely that the direct injection of virus into the CNS by intracerebral inoculation promotes a more robust infection in the

brain than that seen in the trigeminal ganglion, where the virus enters via the cornea and posterior ciliary nerves. Alternatively, the neurons of the CNS may be more susceptible to viral infection, because of either the lack of nonspecific immune factors or the presence of certain transcription factors presumed lacking in the peripheral nervous system.

Taken together, the data show that the *vhs* function is important for HSV pathogenesis in a mouse model system although the exact mechanism by which the UL41 gene product can affect the establishment of and reactivation from latency remains to be determined. These results are consistent with the conservation of the UL41 gene product in neurotropic herpesviruses, and further studies of the functional domains of the UL41 gene and its homologs should help to clarify its apparent requirement for pathogenicity in vivo.

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