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Mutational Analysis of the Herpes Simplex Virus Virion Host Shutoff Protein: Evidence that vhs Functions in the Absence of Other Viral Proteins

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Herpes simplex virus (HSV) virions contain one or more factors that trigger rapid shutoff of host protein synthesis and accelerated decay of cellular and viral mRNAs in infected cells. HSV isolates bearing mutations at the virion host shutoff (*vhs*) locus (gene UL41) are defective for both processes, indicating that the vhs protein is required; however, it is not clear whether the role of vhs in shutoff is direct or indirect and if other virion components are also necessary. We therefore used a transient-cotransfection assay to determine if the vhs protein displays activity in the absence of other viral gene products. We found that a *vhs* expression vector strongly suppressed expression of a cotransfected *lacZ* reporter gene and that this effect was eliminated by the *vhs1* point mutation that abolishes virion-induced host shutoff during HSV infection. Further evidence for the biological relevance of the transfection assay came from the demonstration that five *vhs* in-frame linker insertion mutations yielded concordant results when assayed in cotransfected cells and following transfer into the viral genome: three mutations eliminated activity in both assays, while two had no effect. On the basis of these results, we conclude that the vhs protein can trigger host shutoff in the absence of other HSV proteins. The cotransfection assay was used to rapidly assess the activities of a panel of linker insertion mutants spanning the vhs polypeptide. All mutations that mapped to regions conserved among the vhs homologs of alphaherpesvirus inactivated function; in contrast, four of five mutations that mapped to regions that are absent from several vhs homologs had no effect. These results further support the biological relevance of the transfection assay and begin to delineate functional domains of the vhs polypeptide.

Herpes simplex virus (HSV) is a large enveloped DNA virus. The HSV type 1 (HSV-1) genome encodes over 70 polypeptides which are expressed in a highly regulated cascade during infection of mammalian cells. Inasmuch as expression of viral genes belonging to three distinct temporal classes (immediate early, early, and late) must be properly coordinated for a successful lytic infection to occur, it is not surprising that HSV-1 has evolved a number of regulatory proteins that modulate HSV-1 gene expression in conjunction with cellular factors (reviewed in reference 31). Several of these viral regulatory proteins are components of the infecting virion and are therefore strategically poised to influence gene expression immediately following infection.

One of the most striking effects of the HSV virion-associated regulators is rapid suppression of host protein synthesis (reviewed in reference 3). Translational shutoff occurs in the absence of de novo viral gene expression and is accompanied by disruption of host polyribosomes and accelerated turnover of cellular mRNAs (4, 7, 10, 23, 24). Read and Frenkel (28) isolated a series of viable HSV-1 mutations (termed *vhs*, for virion-induced host shutoff) that lead to defects in these processes (18, 42), and Kwong et al. (19) subsequently mapped one of these mutations (*vhs1*) to gene UL41. This assignment supported earlier studies indicating that a locus responsible for the difference in shutoff potency between HSV-1 and HSV-2 maps to a region that includes gene UL41 (8). More recently, the critical role of the UL41 gene product was confirmed by demonstrations that (i) targeted disruptions of the UL41 locus lead to a vhs-negative phenotype (6, 29, 37) and (ii) HSV-1 recombinants bearing the HSV-2 UL41 gene display the stronger HSV-2 shutoff phenotype (5). Therefore, we refer to the UL41 gene product as the vhs protein below. vhs is a ca. 58-kDa phosphoprotein that is packaged into the virion tegument (22, 35) and is therefore presumably delivered into the infected cell following fusion of the viral envelope with the host plasma membrane. vhs mutants are viable in tissue culture but display a 5- to 10-fold reduction in virus yield (28, 37) and altered patterns of viral protein synthesis during infection (28). Thus, vhs plays an important role in the HSV lytic cycle. Viral mRNAs are also destabilized in the presence of functional vhs (5, 9, 18, 25, 26), but some evidence suggests that the vhs activity of the infecting virion is downregulated by a newly made viral protein, thereby allowing accumulation of viral mRNAs after host mRNAs have been degraded (4–6, 9).

Little is known of the mechanism of virion-induced host shutoff or the relationship between vhs-dependent translational arrest and accelerated mRNA turnover. Perhaps the simplest hypothesis is that translation is inhibited because host mRNAs are rapidly degraded. According to this hypothesis, shutoff stems from vhs-dependent RNase activity. Consistent with this view, studies have shown that mRNAs need not be actively translated in order to be susceptible to vhs-dependent attack and that mRNAs that have runoff polysomes following treatment with agents that block polypeptide chain initiation are also destabilized (34, 42). Moreover, two groups have described in vitro systems for vhs-dependent accelerated mRNA

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turnover (17, 41). However, others have shown that virion-induced translational arrest precedes mRNA decay in some in vivo situations (24, 34), raising the possibility that translation arrest and mRNA decay both stem from a common upstream cause or that vhs-dependent shutoff involves multiple mechanisms.

Although the vhs protein is clearly required for virion-induced host shutoff, this observation places relatively few restrictions on possible modes of vhs action. Thus, the currently available data do not distinguish between hypotheses as diverse as the proposal that vhs acts directly (for example, by activating a cellular RNase) and the possibility that it mediates the virion packaging of another HSV protein that triggers shutoff. Moreover, recent data suggest that other viral proteins are involved in virion-induced shutoff. For example, a viral mutant bearing an inactivating lesion in the UL13 gene (which encodes a protein kinase) displays a *vhs* null phenotype, even though the mutant virions contain normal amounts of vhs protein (27). In addition, previous work from our laboratory has shown that vhs forms a complex with the virion transactivator VP16 (36), raising the possibility that VP16 modulates vhs function.

To clarify the role of the vhs protein in the shutoff process, we tested whether it displays biological activity in the absence of other HSV proteins. We found that vhs strongly inhibited expression of a reporter gene in transiently cotransfected mammalian cells and that this effect displayed the same mutational sensitivity spectrum as virion-induced host shutoff. On the basis of these data, we conclude that vhs is directly involved in virion-induced shutoff and that it is sufficient to induce at least some aspects of this process when expressed in isolation. The transfection assay was then used to scan a number of *vhs* mutations for their phenotype, leading to preliminary identification of several functionally important and two dispensable regions of the vhs polypeptide.

MATERIALS AND METHODS

Cells and virus. Monolayers of Vero cells (obtained from the American Type Culture Collection) were maintained in α minimal essential medium (αMEM; Gibco) supplemented with 5.0% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), and 1.0% penicillin-streptomycin (Gibco). Uninfected Vero cells were maintained at 37°C with 5.0% CO_2 in a humidified incubator. Virus-infected Vero cells were maintained at 37°C with 6.5% CO_2 in a humidified incubator.

Plasmids. Plasmid pRSVβ-gal, obtained from B. Rosenberger through J. P. Capone, contains the complete *Escherichia coli lacZ* open reading frame (ORF) linked to the Rous sarcoma virus long terminal repeat and simian virus 40 3′ mRNA processing signals.

A *vhs* expression vector bearing the *vhs* ORF driven from the human cytomegalovirus (HCMV) immediate-early promoter (pCMV vhs) was constructed as follows. First, a previously described *Eco*RI-*Hin*cII fragment of HSV-1 KOS PAA^{R5} (13) DNA extending from just upstream of the *vhs* initiation codon to ca. 180 bp downstream of the polyadenylation signal (36) was inserted at the *Hin*dIII site of pRC/CMV (Invitrogen; after filling in the *Eco*RI and *Hin*dIII sites with the Klenow fragment of DNA polymerase I). The resulting *vhs* expression cassette was then excised with *Kpn*I and *Sal*I and cloned between the *Kpn*I and *Sal*I sites on pUC18 to yield pCMV vhs. A matched expression vector bearing the inactivating *vhs1* point mutation (pCMV vhs1) was generated by exchanging a 588-bp *Sma*I fragment of pCMV vhs with the corresponding fragment from pV1H (see below). A control plasmid bearing only the HCMV immediate-early promoter (pCMV) was constructed by cloning the 0.9-kb *Kpn*I-*Sal*I promoter-bearing fragment from pRC/CMV between the *Kpn*I and *Sal*I sites of pUC18.

Two plasmids (pUC8 vhs Xba 2 and pTKSB-X) were constructed to facilitate transfer of *vhs* mutations into the *tk* locus of the intact viral genome. pTKSB-X was derived from pTKSB, which bears a mutant form of the 2.0-kb *Pvu*II *tk* fragment from HSV-1 KOS in which 200 bp of *tk* sequences were deleted and replaced with a *Bam*HI linker (44). pTKSB-X was generated by inserting an *Xba*I linker at the *Bam*HI site of pTKSB. Test fragments bearing wild-type and mutant forms of the *vhs* ORF (flanked by *Xba*I sites in pUC vhs Xba 2; see below) were cloned into the *Xba*I site of pTKSB-X and then transferred into the HSV genome (see below). pUC8 vhs Xba 2 was constructed in three steps. First, pUC8 was modified by destroying the unique *Aat*II site through insertion of a *Bgl*II linker (construct pUC8 BglII). Then a 2.3-kb *Pst*I-*Hin*cII fragment of HSV-1 strain 17 DNA bearing the UL41 ORF and 0.6 kb of 5′ and 0.3 kb of 3′ flanking sequences was subcloned between the *Pst*I and *Sma*I sites of pUC8 BglII (construct pUC8 vhs). Finally, *Xba*I linkers were added at the unique *Eco*RI and *Hin*dIII sites within the polylinker sequences of pUC8 vhs, yielding pUC8 vhs Xba 2.

Construction of in-frame deletions and premature chain termination mutations. The vhs deletion mutants pCMV ΔApa and pCMV ΔSma were constructed by excising the internal *Apa*I and *Sma*I fragments, respectively, from the *vhs* coding sequences present in pCMV vhs. pCMV BamAmb contains an amber codon at the unique *vhs Bam*HI site. It was constructed by replacing the *vhs Spl*I-*Eco*RV fragment of pCMV vhs with the corresponding fragment of construct pvhs Bam Amb (36).

Cloning of the mutant *vhs* gene from HSV-1 KOS *vhs1* DNA. The UL41 ORF from HSV-1 KOS *vhs1* (28), bearing the *vhs1* point mutation, was subcloned from viral DNA as follows. HSV-1 KOS *vhs1* nucleocapsid DNA (prepared as described in reference 45) was digested with *Bgl*II and then ligated into the *Bam*HI site of pUC18. A clone bearing the *Bgl*II G fragment (pV1B) was then identified by colony hybridization using a *vhs Sma*I fragment (^{32}P labeled by random priming) as a probe. The 3.6-kb *Hin*dIII-*Hpa*I fragment bearing the *vhs1* gene was then subcloned between the *Hin*dIII and *Sma*I sites of pUC18, yielding plasmid pV1H. Kwong et al. (19) mapped the mutation that causes the defect in host shutoff in the *vhs1* isolate to a 265-bp *Nru*I-*Xma*III subfragment of the UL41 ORF; subsequent sequence analysis identified a single-base change resulting in a threonine-to-leucine substitution at vhs residue 214 (17a). In this work, we transferred the *vhs1* mutation from pVIH to expression vectors by replacing a 588-bp *Sma*I fragment of wild-type DNA with the corresponding fragment of *vhs1* mutant DNA. We therefore sequenced the entire 588-bp *Sma*I fragment of *vhs1* DNA and confirmed the presence of thymine-to-adenine change that converts threonine 214 to isoleucine. Compared with the published sequence of HSV-1 strain 17 (21), several other base changes were identified; however, these changes did not alter the predicted amino acid sequence relative to that of strain 17. Details of the sequence are available upon request. The presence of the *vhs1* point mutation was confirmed in all constructs by dideoxy sequence analysis (33, 48).

Construction of in-frame linker insertion mutations. pCMV vhs was digested with limited amounts of *Eco*RV, *Msc*I, *Nae*I, *Pvu*II, *Sca*I, *Sma*I, or *Rsa*I under predetermined optimal conditions for the generation of unit-length molecules (32). Unit-length molecules were isolated from agarose gels and ligated with an unphosphorylated *Xho*I linker (5′ CCCCTCGAGGGG), resulting in the insertion of a unique *Xho*I site into the plasmid. Successful linker insertions were identified by restriction analysis, and the locations of all linker insertions were verified by dideoxy sequence analysis across the linker insertion sites (33, 47, 48).

Construction of vhs mutants bearing the influenza virus HA epitope 12CA5. As described in Results, several of the vhs in-frame linker insertion mutants retained biological activity. To determine if these mutants would retain activity following additional modification, two constructs (pN138 and pS344) were further modified by in-frame insertion of sequences encoding an epitope of influenza virus hemagglutinin (HA) that is recognized by monoclonal antibody 12CA5. pN138 and pS344 were digested with *Xho*I and ligated with the complementary oligonucleotides 5′ TCGATACCCATACGACGTCCCAGACTACG CTGG and 5′ TCGACCAGCGTAGTCTGGGACGTCGTATGGGTA encoding the 12CA5 epitope (16, 46), to generate the constructs pCMV vhs N138-HA and pCMV vhs S344-HA, respectively. This modification results in the insertion of an additional *Aat*II site into the plasmid.

Construction of recombinant HSV-1 KOS PAA^{R5} bearing a deletion in UL41. We constructed a derivative of HSV KOS PAA^{R5} lacking most of the *vhs* ORF as follows. The UL41 region of HSV-1 strain 17 DNA was subcloned as a *Hin*dIII-*Hpa*I fragment (using exactly the same cloning strategy as described previously for the generation of construct pVIH), generating pvhs17. The UL41 ORF in pvhs17 then was disrupted by a deletion/replacement in which 1.1 kb of UL41 coding sequences were replaced by a *lacZ* expression cassette. To this end, a 4.2-kb *Bam*HI fragment bearing the *E. coli lacZ* ORF driven by the HSV-1 ICP6 promoter (ICP6:*lacZ* cassette [11]) was cloned between *Apa*I and *Msc*I sites of pvhs17 (after flushing all ends with T4 DNA polymerase), generating pvhsβgal. The ICP6:*lacZ* cassette was inserted in the same orientation as the *vhs* ORF and replaced 1.1 kb of sequence encoding vhs amino acid residues 24 to 384. This deletion was then transferred into the UL41 locus of HSV-1 KOS PAA^{R5} by DNA-mediated marker rescue (38, 39). Viral recombinants expressing *lacZ* were selected as blue plaques in the presence of Bluo-Gal (Bethesda Research Laboratories) as described previously (37). The structure of the UL41 region of candidate recombinants was then examined by Southern blot analysis (2, 40). One isolate bearing the desired mutation, designated Pvhs(−), was chosen for further study.

Construction of viral recombinants bearing in-frame *vhs* linker insertion mutations. Several *vhs* linker insertion mutations were characterized following insertion of the modified *vhs* gene into the *tk* locus of the *vhs* null mutant virus, Pvhs(−). Viral recombinants were constructed in three steps. First, linker insertion mutations were transferred from the HCMV expression vector into a construct bearing the UL41 region of HSV-1 strain 17 flanked by *Xba*I sites (plasmid pUC8 vhs Xba 2, described above). In the case of constructs Sc243, S344-HA, and M384, this was accomplished by exchanging the 758-bp *Spl*I-*Eco*RV fragment of pUC8 vhs Xba 2 with the corresponding fragment from the HCMV expression clone. In the case of the constructs R27 and N138-HA, the 471-bp

*Apa*I fragment was exchanged. Second, the *vhs Xba*I fragments bearing the linker insertion mutations were subcloned into the *Xba*I site within the *tk* gene present on pTKSB-X (described above), in the *tk* antisense orientation. Third, the resulting *tk*-deficient deletion/insertion mutations were transferred into the *tk* locus of the *vhs* null mutant virus Pvhs(−) by DNA-mediated marker rescue followed by selection of *tk*-deficient progeny (38, 39). The structure of the *tk* locus of the resulting recombinant viruses was verified by Southern blot analysis (2, 40).

Cotransfection assay for vhs activity. Triplicate cultures of 1.5×10^5 Vero cells, in 5 ml of αMEM in 35-mm-diameter culture dishes, were transfected with 15 μg of pUC18 carrier, 15 μg of pRSVβ-gal reporter, and 100 to 500 ng of *vhs* expression vector, using a modification (15) of the calcium phosphate method (12). Cells were incubated with the DNA precipitate for 16 h, then washed thoroughly to remove the DNA precipitate, and incubated for an additional 24 h with fresh αMEM.

A colorimetric assay was used to quantitate the levels of β-galactosidase expression (30). Briefly, cells were washed once with phosphate-buffered saline and then once with TEN buffer (40 mM Tris [pH 7.5], 1 mM EDTA, 150 mM NaCl). The cells were lysed in 150 μl of ice-cold assay buffer (250 mM Tris [pH 8.0], 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and the lysate was immediately placed on ice. β-Galactosidase activity was determined by adding 40 μl of cell lysate to a microtiter well containing 275 μl of reaction mix (100 mM $NaPO_4$ [pH 7.2], 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, 10 mM *o*-nitrophenyl-β-D-galactopyranoside [Sigma]). The reaction mixture was incubated at 37°C for 20 to 60 min, and results were quantitated by measuring the A_{410} of each well in a Precision Microplate Reader (Molecular Devices).

To facilitate analysis of data from several independent experiments, the results were normalized to the levels of β-galactosidase activity in Vero cells transfected with 15 μg of pRSVβ-gal and 15 μg of pUC18 without competitor DNA. vhs mutants were considered inactive if the relative β-galactosidase activity was within 1 standard error of levels obtained with the pCMV vector alone.

Analysis of protein synthesis in infected cells. Relative protein synthetic rates were assessed by using a modification of a procedure described by Kwong and Frenkel (18). Cultures of Vero cells in 35-mm-diameter dishes were infected with 10 PFU per cell in 0.5 ml of αMEM and then labeled with [^{35}S]methionine for 1 h at the indicated times postinfection. Labeling was done by replacing the growth medium with 0.5 ml of labeling medium containing 25 μCi of [^{35}S]methionine. Labeling medium consisted of a 9:1 mixture of medium 199 lacking methionine and αMEM. Where indicated, actinomycin D (10 μg/ml) was added 20 min prior to infection and maintained continuously. Labeled cell extracts were prepared by lysing the cells in 200 μl of ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1% sodium deoxycolate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride), and the lysate was immediately transferred to a tube on ice. Labeled proteins were denatured by boiling in an equal volume of 2× sample buffer (125 mM Tris [pH 6.8], 600 mM 2-mercaptoethanol, 6% SDS, 20% glycerol, 0.005% bromophenol blue) and separated by electrophoresis through an SDS–12% polyacrylamide gel (20). The resolving gel was then infused with Enlightning (Dupont) for 30 min, dried under vacuum, and exposed to XAR5 autoradiographic film (Kodak).

Purification of HSV virions. Virions were purified from extracellular medium by sedimentation through a 5 to 15% Ficoll gradient, using a modification of the procedure described by Szilagyi and Cunningham (43). Briefly, five roller bottles of Vero cells at 75% confluency were infected with 0.025 PFU per cell and incubated at 37°C for 3 to 4 days. Medium from the infected cells was collected, and cellular debris was removed by centrifugation at $1,200 \times g$ for 30 min at 4°C. The supernatant was collected, and virions were pelleted by centrifugation in a V50.2 Ti rotor (Beckman) at $80,000 \times g$ for 2 h at 4°C. The pellets from each individual preparation were resuspended and combined into a total volume of 3.0 ml of STE buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 5 mM EDTA) and carefully layered onto a 30-ml preformed gradient of 5 to 15% Ficoll 400 (Pharmacia) prepared in STE buffer. The gradients were centrifuged in a SW28 rotor (Beckman) at $26,000 \times g$ for 2 h at 4°C. Two bands representing heavy and light HSV-1 particles centering at 3.0 cm into the gradient were carefully withdrawn by side puncture with a 20-gauge syringe needle. The virion fraction (5 to 7 ml) was diluted to 30 ml in STE buffer, and the virions were pelleted by centrifugation in a V50.2 Ti rotor (Beckman) at $80,000 \times g$ for 2 h at 4°C. The final pellet containing purified HSV-1 virions was carefully resuspended into 200 μl of STE buffer, and the protein content of each virion preparation was determined by using a Bradford protein assay (Bio-Rad) as described by the manufacturer.

Western blot (immunoblot) analysis. Proteins from infected cell extracts prepared by cell lysis in RIPA buffer or purified virions were separated by electrophoresis through an SDS–9% polyacrylamide gel (20) and transferred to a 0.45-μm-pore-size nitrocellulose filter (Schleicher & Schuell), using a standard protocol (14). The filters were incubated in blocking buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 1% Carnation instant skim milk powder) for 30 min at room temperature and then incubated for 1 h with a rabbit antiserum derived against a vhs-protein A fusion (36) diluted 1/500 in blocking buffer. Unbound antiserum was removed by washing the filter three times in TBS-T (20 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween 20) for 5 min per wash. Bound antiserum was reacted with donkey anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Amersham) diluted 1/5,000 in blocking buffer and incubated for 1 h at room temperature. The filters were washed with TBS-T as described previously, and bound conjugated antiserum was visualized by using the Renaissance chemiluminescence reagent (NEN) as described by the manufacturer.

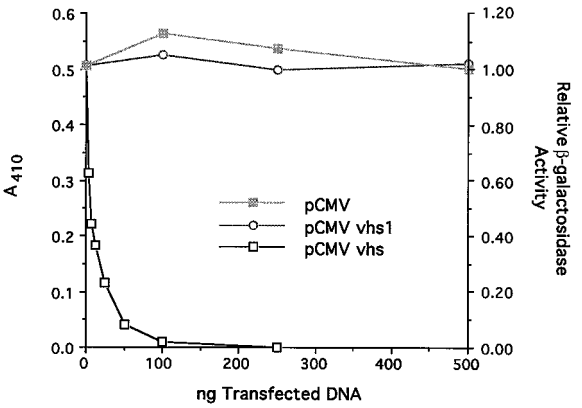

FIG. 1. Cotransfection assay for vhs activity. Triplicate cultures of Vero cells were cotransfected with 15 μg of the β-galactosidase expression vector pRSVβ-gal, 15 μg of pUC18 carrier DNA, and various amounts of pCMV vhs or pCMV vhs1. pCMV vector DNA was added to each sample to maintain a constant amount of HCMV promoter. Cell lysates were prepared at 2 days posttransfection, and the levels of β-galactosidase activity were determined as described in Materials and Methods. The level of β-galactosidase activity for each transfection was normalized to that obtained in Vero cells transfected with 15 μg of pRSVβ-gal and 15 μg of pUC18.

RESULTS

vhs inhibits reporter gene expression in cotransfected mammalian cells. Although substantial evidence indicates that the vhs polypeptide is required for virion-induced host shutoff, it is not known if it can trigger this process in the absence of other virion components. As one approach to answering this question, we examined whether vhs displays biological activity when expressed in isolation in a transient-transfection assay. If vhs is active on its own, then it seems reasonable to assume that it would terminate protein synthesis and globally destabilize mRNAs in the transfected cells. Although such an effect would be difficult to detect directly (because only a minority of cells are transfected), one should be able to indirectly monitor effects within the transfected subpopulation by including a constitutively expressed reporter gene in the transfection mixture. We therefore tested whether vhs inhibits expression of a cotransfected *lacZ* gene driven from the Rous sarcoma virus long terminal repeat. To this end, Vero cells were transfected with a constant amount of pRSVβ-gal reporter and increasing amounts of expression vectors encoding either wild-type or *vhs1* mutant forms of vhs driven from the HMCV immediate-early promoter (pCMV vhs and pCMV vhs1, respectively). The results (Fig. 1) indicated that the wild-type *vhs* expression vector dramatically suppressed expression of β-galactosidase, while the *vhs1* mutant vector had no effect. Similar results have been obtained by Read and colleagues using a chloramphenicol acetyltransferase expression plasmid as a reporter gene (27a).

These results raised the possibility that vhs can trigger host shutoff in the absence of other HSV proteins. However, given the indirect nature of the assay, we wished to more rigorously test the hypothesis that the negative effect on reporter gene expression in transfected cells is functionally related to virion-induced host shutoff during HSV infection. To this end, we first scored a variety of *vhs* mutations for their effects in transfected cells and then assayed a representative subset for their effects

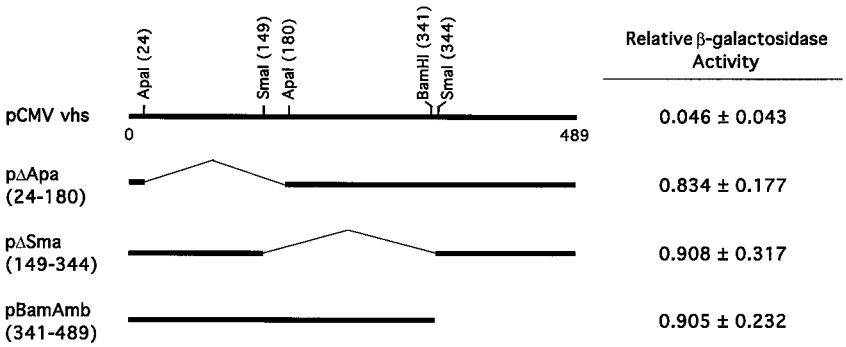

FIG. 2. Activities of vhs deletion mutants in the cotransfection assay. pCMV-based expression vectors encoding the indicated mutant forms of vhs (250 ng of each vector) were cotransfected in duplicate with 15 μg of the reporter plasmid, pRSVβ-gal, and 15 μg of pUC18 carrier DNA. The relative activity of each mutant was determined from at least three independent experiments as described in Materials and Methods.

on virion-induced shutoff following transfer into the intact HSV genome.

Analysis of vhs mutants in the transfection assay. We first examined three mutants that each lack about one-third of the vhs polypeptide and which in combination span nearly the entire *vhs* ORF (Fig. 2). These mutants (ΔApa, ΔSma, and BamAmb, lacking vhs residues 24 to 180, 149 to 344, and 344 to 490, respectively) had little or no effect on reporter gene expression, in that the levels of β-galactosidase expression obtained were within 1 standard error of those obtained in the absence of vhs (Fig. 2; Table 1). Presumably, these mutants encode inactive and/or unstable forms of vhs.

We then surveyed a set of 11 mutants bearing in-frame linker insertions across the *vhs* ORF (Table 1). These mutants were constructed by inserting a dodecameric *Xho*I linker at various restriction sites within the *vhs* ORF, resulting in insertion of either four or five amino acids (depending on the site of insertion). The mutants were designated by using a one- or two-letter abbreviation for the restriction site used for insertion, followed by the position of the last intact *vhs* codon preceding the insertion. For example, S344 designates an insertion at a *Sma*I site which interrupts the coding sequence immediately following codon 344. These mutants fell into two categories (Table 1): those that severely reduced vhs activity (R27, P192, Sc243, R243, P374, M384, and E461) and those which had little or no effect (N138, N166, N331, and S344). This analysis suggested a minimum of three mutation-sensitive regions within the *vhs* ORF, separated by two regions that tolerate insertions. The significance of these observations is discussed below.

TABLE 1. Summary of activities of vhs mutants

Mutant plasmid[a]	Amino acids inserted[b]	Relative β-galactosidase activity[c]
pCMV	NA	1.196 ± 0.695
pvhs	NA	0.046 ± 0.043
pΔApa	NA	0.834 ± 0.177
pΔSma	NA	0.908 ± 0.317
pBamAmb	NA	0.905 ± 0.232
pR27	SPRGD	1.127 ± 0.212
pN138	PPRGG	0.041 ± 0.052
pN138-HA	PPRGGYPYNVPNYAG	0.086 ± 0.022
pN166	PSRG	0.026 ± 0.023
pP192	PLQG	0.966 ± 0.248
pvhs1	NA	1.238 ± 0.748
pSc243	SPRGD	0.771 ± 0.149
pR243	SPRGD	0.879 ± 0.252
pN331	PLQG	0.041 ± 0.034
pS344	PPRGG	0.143 ± 0.062
pS344-HA	PPRGGYPYNVPNYAG	0.107 ± 0.074
pP374	PLQG	1.223 ± 0.463
pM384	PRGA	1.025 ± 0.143
pE461	PLQG	0.887 ± 0.168

[a] Plasmid construction and nomenclature are as described in Results.

[b] Linker insertion mutagenesis resulted in the in-frame insertion of four to five amino acids, depending on the cleavage site and the frame of the insertion site. Two mutants, pN138 and pS344, were further modified through the insertion of a HA epitope tag, which resulted in the in-frame insertion of a total of 15 amino acids.

[c] Values were normalized to the levels of β-galactosidase activity obtained from Vero cells transfected with 15 μg of pRSVβ-gal reporter plasmid and 15 μg of pUC18 carrier plasmid. The results are represented as the mean β-galactosidase activity from at least three independent experiments of triplicate samples ± the standard error of the mean.

The existence of two regions within the *vhs* ORF that tolerate in-frame insertions suggested the possibility of generating biologically active versions of the vhs polypeptide bearing tags to facilitate detection and/or purification of the protein. To test the feasibility of this approach, we derived two second-generation constructs in which an influenza virus HA epitope was inserted into the unique *Xho*I site present in the linker of mutants N138 and S344, generating constructs N138-HA and S344-HA, respectively. As shown in Table 1, both HA-tagged derivatives retained vhs activity in the cotransfection assay.

Construction and characterization of HSV-1 KOS PAA^{R5} recombinants bearing selected *vhs* linker insertion mutations. To test the biological relevance of the foregoing results, we assessed the effects of a representative subset of the linker insertion mutations on virion-induced host shutoff following transfer into the intact HSV genome. This was accomplished by inserting the various modified *vhs* genes into the *tk* locus of a *vhs* null mutant virus, in the *tk* antisense orientation (Fig. 3; see Materials and Methods for details). A similar strategy has been used previously for expression of wild-type *vhs* from HSV recombinants (5). The *vhs* null HSV-1 PAA^{R5} recipient, Pvhs(−), was constructed by inserting an ICP6:*lacZ* cassette between *Apa*I and *Msc*I sites within the UL41 ORF. This insertion resulted in the deletion of *vhs* sequences encoding residues 24 to 384 of the 489-amino-acid vhs polypeptide (21) and dissociated sequences downstream of residue 384 from the UL41 promoter (Fig. 3A). As predicted (Fig. 3A), the mutation resulted in the replacement of the wild-type 2.2-kb PAA^{R5} vhs *Bst*EII fragment with a 5.4-kb fragment that comigrated with the corresponding band from the *vhs* disruption vector, pvhsβ-gal (Fig. 4A).

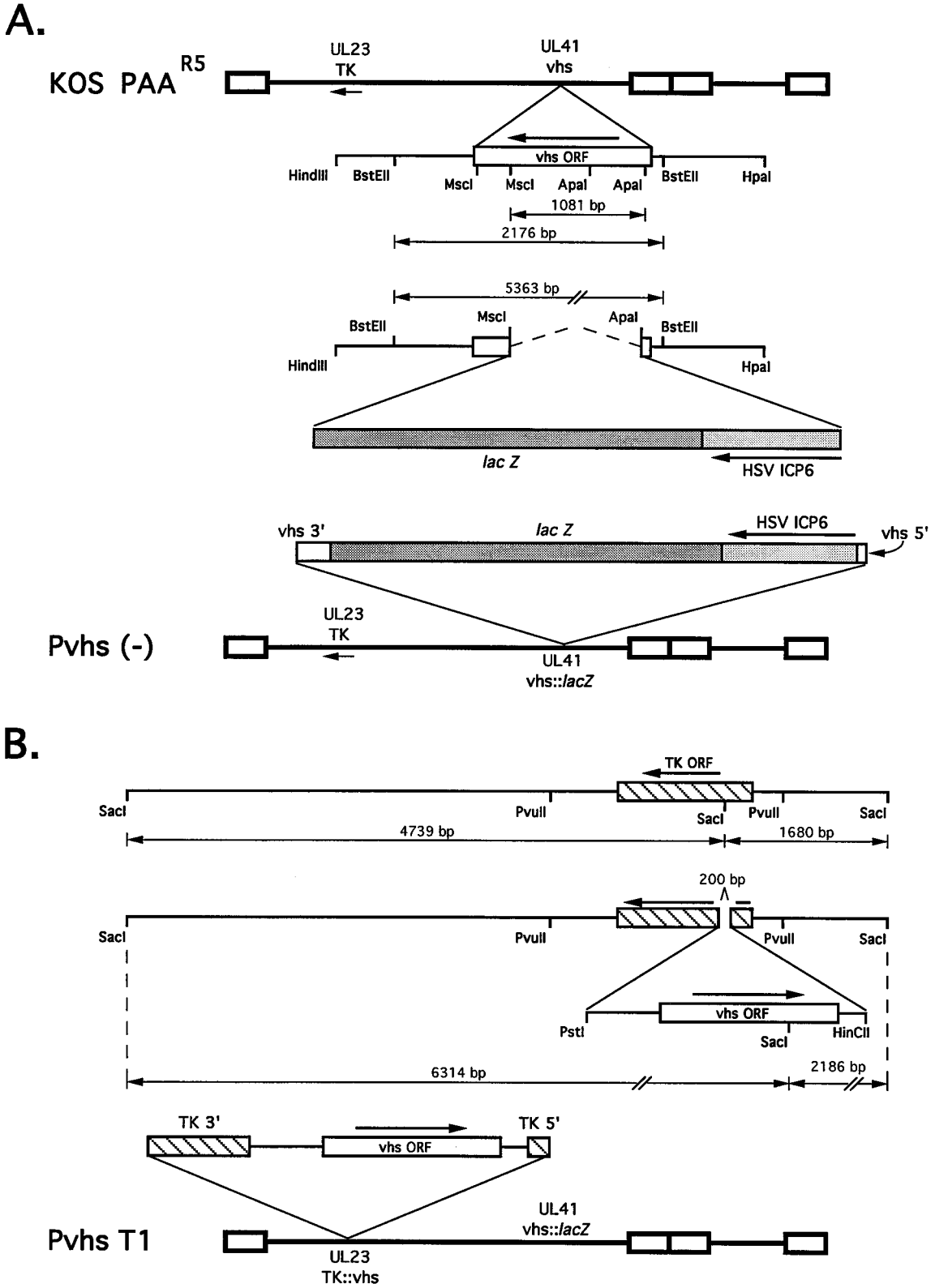

FIG. 3. Genomic structures of recombinant viruses expressing wild-type and mutant forms of vhs. The *vhs* ORF of HSV-1 KOS PAA^{R5} was disrupted by insertion of a *lacZ*:ICP6 cassette into the UL41 gene. (A) Structure of recombinant Pvhs(−). Below the schematic diagram of the PAA^{R5} genome is an expanded view of the 3.6-kb *Hin*dIII-*Hpa*I fragment bearing the *vhs* gene. A 1.1-kb *Msc*I-*Apa*I fragment containing *vhs* coding sequences was replaced with a 4.3-kb DNA fragment containing the *E. coli lacZ* ORF driven by the HSV-1 ICP6 promoter, generating recombinant Pvhs(−). (B) Construction of derivatives of Pvhs(−) bearing an additional copy of the *vhs* gene inserted at the *tk* locus. An expanded view of the *tk* region is shown. A 2.3-kb *Pst*I-*Hin*cII fragment bearing the *vhs* gene was inserted across the endpoints of a previously described 200-nucleotide deletion of *tk* coding sequences (ΔSB [44]), in the *tk* antisense orientation. Recombinant Pvhs T1 bears a wild-type version of the *vhs* gene at the *tk* locus.

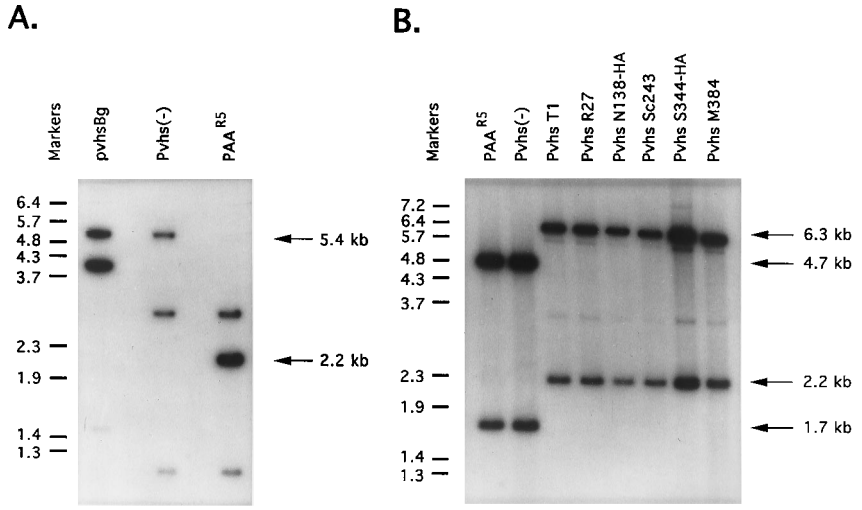

FIG. 4. Southern blot analysis of recombinant viruses. (A) Analysis of Pvhs(−). The indicated DNAs were cleaved with *Bst*EII, and the resulting fragments were resolved by electrophoresis through a 1% agarose gel, transferred to a GeneScreen membrane, and probed with the 3.6-kb *Hin*dIII-*Hpa*I *vhs* fragment (^{32}P labeled by random priming). The positions of the wild-type (2.2 kb) and mutant (5.4 kb) fragments are indicated. pvhsBg, plasmid used to derive Pvhs(−); Pvhs(−), viral recombinant bearing the *vhs* disruption. (B) Analysis of Pvhs(−) derivatives bearing an additional copy of the *vhs* gene inserted at the *tk* locus. The indicated DNAs were digested with *Sac*I, resolved by electrophoresis, and then probed with the 2.0-kb *Pvu*II fragment bearing the *tk* gene. The positions of the wild-type (1.7 and 4.7 kb) and *tk-vhs* fusion (2.2 and 6.3 kb) fragments are indicated by arrows. PAA^{R5}, parental HSV-1 KOS PAA^{R5} DNA; Pvhs(−), *vhs* null derivative of PAA^{R5}.

We derived six derivatives of Pvhs(−) which carry an additional copy of the *vhs* gene inserted into the *tk* gene. Pvhs T1 carries the wild-type *vhs* gene and served as a positive control. The remaining recombinants carry *vhs* genes bearing the R27, N138-HA, Sc243, S344-HA, and M384 mutations. As described above, N138-HA and S344-HA had little effect on vhs activity in transfected cells, while R27, Sc243, and M384 were functionally inert. Insertion of the various *vhs* genes into the *tk* locus resulted in replacement of the wild-type 1.7- and 4.7-kb *Sac*I *tk* fragments with novel *tk-vhs* fusion fragments of 2.2 and 6.3 kb (Fig. 4B), as predicted (Fig. 3B).

Accumulation and virion packaging of mutant vhs proteins. Before testing host shutoff phenotypes, we determined whether the various mutant forms of vhs accumulated in infected cells and were packaged into virions. To monitor accumulation in infected cells, samples prepared 14 h postinfection were separated on an SDS–9% polyacrylamide gel, transferred to nitrocellulose, and then analyzed by Western blotting with a polyclonal rabbit antiserum raised against a vhs-protein A fusion (Fig. 5A). In addition, monoclonal antibody 12CA5 (directed against the HA epitope) was used to confirm the expression of the two HA-tagged forms of vhs encoded by the viral recombinants Pvhs N138-HA and Pvhs S344-HA (Fig. 5B). The polyclonal serum reacted with two cellular proteins in mock-infected extracts. As predicted, it also detected a ca. 58-kDa protein in cells infected with the parental PAA^{R5} strain. Inasmuch as the 58-kDa band was eliminated by the Pvhs(−) mutation and restored by insertion of a wild-type *vhs* gene at the *tk* locus of derivative Pvhs T1, we conclude that it corresponds to the vhs polypeptide. Each of the viruses bearing insertions of a mutant *vhs* gene accumulated one or more vhs-related polypeptides (Fig. 5); moreover, the HA-tagged forms of vhs encoded by the N138-HA and S344-HA mutants reacted with monoclonal antibody 12CA5, while wild-type vhs encoded by Pvhs T1 did not (Fig. 5B; note that monoclonal antibody 12CA5 also reacted with a cellular protein present in mock-infected cells). In the case of Pvhs S344-HA (and possibly Pvhs N138-HA), the modified vhs protein comigrated with one of the cellular proteins that cross-reacted with the polyclonal antibody; however, the tagged versions of vhs encoded by both mutants were easily detected with 12CA5. We consistently observed that S344-HA gave rise to a stronger signal with 12CA5 than did mutant N138-HA. Two forms of vhs that differ in electrophoretic mobility were detected with mutants R27, N138-HA, S344-HA (probed with 12CA5), Pvhs M384, and possibly Pvhs Sc243. The existence of two electrophoretic forms of the wild-type vhs polypeptide that differ in the extent of phosphorylation has been recently reported (29); whether

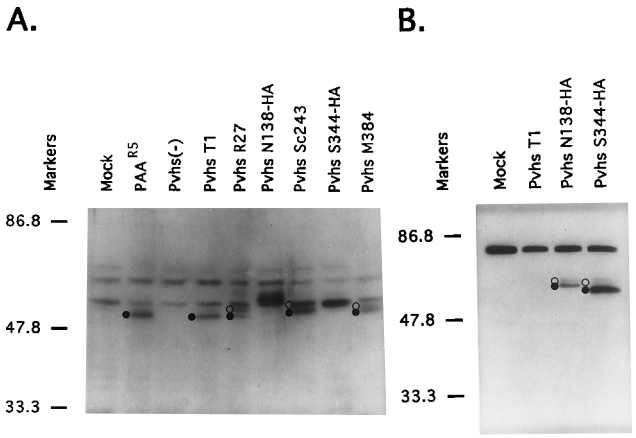

FIG. 5. Accumulation of wild-type and mutant vhs polypeptides in infected Vero cells. Vero cells were infected with 10 PFU per cell and harvested 14 h later. Cell extracts prepared in RIPA buffer were resolved on an SDS–9% polyacrylamide gel, transferred to nitrocellulose, and then probed with a rabbit antiserum raised against a vhs-protein A fusion protein (A) or HA epitope-specific monoclonal antibody 12CA5 (B). In some samples, two forms of the vhs polypeptide were detected: one of predicted mobility indicated by the closed circles, and one of slightly slower mobility indicated by the open circles. The constructs used were PAA^{R5} (parental HSV-1 KOS), Pvhs(−) (*vhs* deletion mutant of PAA^{R5}), Pvhs T1 [wild-type *vhs* expressed from the *tk* locus of Pvhs(−)], and various *vhs* linker insertion mutations expressed from the *tk* locus of Pvhs(−). Sizes are indicated in kilodaltons.

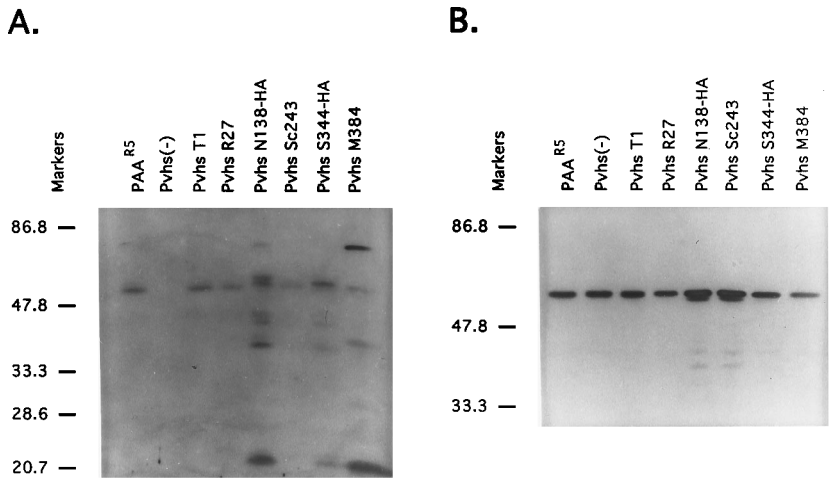

FIG. 6. Detection of wild-type and mutant vhs proteins in purified virions. Virions were purified from infected Vero cell supernatants by sedimentation through a 5 to 15% Ficoll gradient; 2.5 (A) or 1.0 (B) μg of protein from purified virions was separated on an SDS–9% polyacrylamide gel, transferred to nitrocellulose, and probed with a rabbit antiserum raised against a vhs-protein A fusion (A) or a monoclonal antibody specific for VP16 (LP1) (B). The constructs used were PAA^{R5} (parental HSV-1 KOS DNA), Pvhs(−) (*vhs* deletion mutant of PAA^{R5}), Pvhs T1 [wild-type *vhs* expressed from the *tk* locus of Pvhs(−)], and various *vhs* linker insertion mutations expressed from the *tk* locus of Pvhs(−). Sizes are indicated in kilodaltons.

these correspond to the two forms detected in the present work remains to be determined.

We next examined extracts prepared from purified virions to determine if the mutant forms of vhs were packaged into virus particles (Fig. 6A). The polyclonal serum detected the 58-kDa vhs protein in extracts prepared from PAA^{R5}; as predicted, the band was absent in Pvhs(−) and restored in recombinant Pvhs T1. vhs-related bands of ca. 58 kDa were observed in all recombinants bearing linker insertions. In the case of recombinants T1, R27, N138-HA, Sc243, and S344-HA, these ran as a doublet of closely spaced bands. Recombinants N138-HA and M384 also displayed a band of ca. 74 kDa, as well as several lower-molecular-mass species, and recombinant S344 displayed at least three low-molecular-mass bands that comigrated with those present in N138-HA. The origin of these additional signals remains unknown. As a control, the virion extracts were also examined with the VP16-specific monoclonal antibody LP1 (Fig. 6B). Each sample gave rise to the predicted VP16 signal at 65 kDa. This result confirmed the presence of virions in the Pvhs(−) sample and indicated that roughly comparable amounts of mutant virions were loaded.

Although precise quantitative comparisons are difficult, these results indicate that all of the mutant forms of vhs analyzed were incorporated into virions.

Host shutoff phenotypes of linker insertion mutants. We next determined the effects of the R27, N138-HA, Sc243, S344-HA, and M384 mutations on virion-induced host shutoff. Vero cells were infected with 10 PFU of the various mutants per cell in the presence of actinomycin D (to block viral gene expression), and the levels of host protein synthesis were determined at 5 h postinfection (Fig. 7). As expected, PAA^{R5} and Pvhs T1 dramatically inhibited host protein synthesis, while Pvhs(−) had no effect. The linker insertion mutants fell into two categories, as predicted by the cotransfection assay: N138-HA and S344-HA strongly inhibited cellular protein synthesis, while R27, Sc243, and M384 had no effect. Controls indicated that each mutant expressed the normal profile of viral polypeptides when Vero cells were infected in the absence of actinomycin D, confirming that infectious virus was present in each sample. Taken together, these data strongly corroborate the results obtained with the cotransfection assay.

DISCUSSION

Given the current state of knowledge, two of the most basic questions that one can ask about vhs are the following: Is it directly involved in virion-induced host shutoff mechanisms? If so, is it sufficient to trigger these processes? As one approach to answering these questions, we used a cotransfection assay to

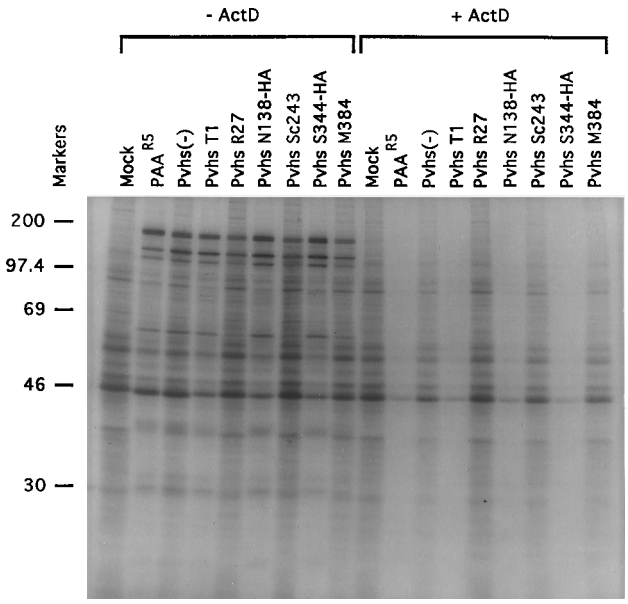

FIG. 7. Host shutoff activity of *vhs* linker insertion mutants. Vero cells were infected with 10 PFU per cell in the absence or presence of 10 μg of actinomycin D (ActD) per ml, as indicated. Ongoing protein synthesis rates were monitored at 5 h postinfection by incubating the cells with [^{35}S]methionine, in the presence of excess cold methionine, for 1 h. Cell lysates were prepared in RIPA buffer, separated on an SDS–12% polyacrylamide gel, and processed for autoradiography. Mock, mock-infected cells. The constructs used were PAA^{R5} (parental HSV-1 KOS DNA), Pvhs(−) (*vhs* deletion mutant of PAA^{R5}), Pvhs T1, [wild-type *vhs* expressed from the *tk* locus of Pvhs(−)], and various *vhs* linker insertion mutations expressed from the *tk* locus of Pvhs(−). Sizes are indicated in kilodaltons.

determine if vhs displays activity in the absence of other HSV gene products. We reasoned that if vhs is sufficient to trigger shutoff, then it should strongly inhibit expression of an otherwise constitutively active β-galactosidase reporter gene in cotransfected mammalian cells (through a combination of direct and indirect effects; see below). Our results confirmed this prediction. We then sought to determine if the negative effect in transfected cells reflects virion-induced host shutoff activity during HSV infection. A direct demonstration that this is so is not yet feasible, for at least three reasons. First, the primary biochemical function of vhs is currently unknown. Second, the time frame of the transfection assay greatly exceeds that required for the onset of host shutoff after virus infection (40 h versus ca. 30 min), making it extremely difficult to distinguish direct effects of vhs action from indirect effects of (for example) prolonged translational arrest. Third, only a minority of the cells in the transfected population express vhs (unpublished data). We therefore adopted a genetic approach to test the biological relevance of the transfection assay. To this end, we surveyed 15 vhs mutants in the transfection assay, to identify mutations that display varied phenotypes. We then assayed a representative subset of these mutations for their effects on virion-induced host shutoff following transfer into the viral genome. The results revealed a complete concordance between the two assay system: four mutants were inactive in both situations (vhs1, R27, Sc243, and M384), and two retained wild-type activity (N138-HA and S344-HA). Inasmuch as all six mutant polypeptides are incorporated into virions (see reference 29 for data on the vhs1 mutant), the results provide a strong indication that the activity detected in the transfection assay is required for virion-induced host shutoff. We therefore conclude that vhs plays a direct role in host shutoff and that it is sufficient to trigger at least some aspects of this process in the absence of other HSV gene products. This conclusion is in agreement with recent data indicating that vhs is the only viral protein required to induce translational arrest and accelerated mRNA turnover in an in vitro system (2a). Further studies will focus on determining the primary biochemical function of vhs and identifying possible cellular targets.

Although our data argue that vhs is sufficient to induce shutoff when expressed in isolation, the situation within infected cells appears to be quite different. Recent evidence indicates that a viral mutant lacking the UL13 gene product (a virion-associated serine-threonine protein kinase) displays a vhs-deficient phenotype, even though the mutant virions contain normal levels of wild-type vhs protein (27). The role of UL13 in virion-induced host shutoff function is not yet clear; however, our results suggest that it is likely indirect, perhaps serving to regulate interactions between vhs and other viral proteins. For example, vhs binds to the tegument protein VP16 (36), and recent data suggest that this interaction suppresses vhs function (20a). Therefore, an interesting possibility is that UL13 acts to disrupt the vhs-VP16 complex during the earliest stages of infection.

One advantage of the transfection assay described in this report is that it allows one to test the biological activity of mutant forms of vhs which cannot be packaged into virions. For example, Read et al. (29) have shown that a vhs derivative lacking sequences encoded by the internal *Sma*I fragment (mutant ΔSma) accumulates in infected cells but is not incorporated into virus particles. Therefore, although the mutant virions are defective for virion-induced host shutoff, this phenotype provides little information about the inherent activity of the mutant polypeptide. Our results demonstrate that the ΔSma deletion eliminates vhs function in transfected cells, arguing that this mutation inactivates the polypeptide as well as preventing its incorporation into virions.

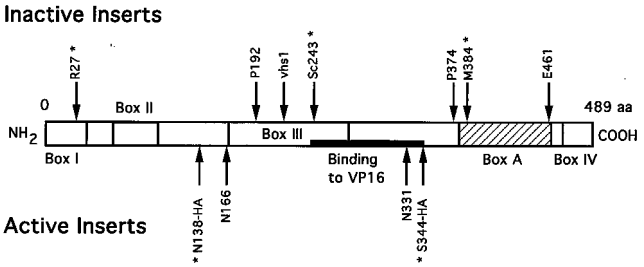

FIG. 8. Correlation between the mutational sensitivity spectrum of vhs and regions conserved among the UL41 homologs of alphaherpesviruses. A linear representation of the *vhs* coding region is shown. The shaded regions (boxes I to IV) and the hatched region (box A) represent regions in which ≥25 and 11% of the amino acid (aa) residues are invariant between five different alphaherpesviruses (1). The solid bar indicates the region necessary for binding to VP16 (36). The positions of the various linker insertion mutations examined in this study are indicated. Mutations that inactivate *vhs* activity are placed on top, and mutations that inactivate function are placed on the bottom. Asterisks designate mutations that were assayed following transfer into the viral genome.

One approach to elucidating the mechanism of *vhs* action is to identify functional subdomains within the vhs polypeptide. Our mutational analysis represents an advance in that direction and has identified a minimum of three mutationally sensitive regions separated by two regions that tolerate linker insertions. It is of considerable interest to compare this functional map with the distribution of conserved and nonconserved amino acid residues in the vhs homologs of other alphaherpesviruses. Berthomme and coworkers (1) compared the UL41 homologs from five alphaherpesviruses and identified four regions that display ≥25% amino acid sequence invariance among all five proteins (Fig. 8, homology boxes I to IV). If one relaxes the criterion to 11% invariance (thereby including all of remaining invariant residues), a fifth region, extending from residue 381 to 462 in the HSV-1 protein, becomes evident (Fig. 8, box A). Berthomme et al. (1) also noted that large spans of two of the relatively nonconserved regions (encompassing residues 100 to 178 and 275 to 367 in the HSV-1 protein) are completely absent from some vhs homologs. Rather strikingly, the four mutations which had no effect on vhs activity (N138, N166, N331, and S344) map within these two regions (Fig. 8). Moreover, with the exception of P374, each of the inactivating linker insertion mutations lies within one of the conserved regions described above, and P374 maps just six residues upstream of box A (Fig. 8). Taken together, these data lend further credence to the notion that the transfection assay measures a biologically relevant function of *vhs* and support the hypothesis that the conserved regions correspond to functionally important domains of the vhs polypeptide.

It seems likely that the mutational map and biological reagents developed during the present study will facilitate efforts to uncover the mechanism of *vhs* action.

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