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Isolation of a Herpes Simplex Virus Type 1 Mutant with a Deletion in the Virion Host Shutoff Gene and Identification of Multiple Forms of the *vhs* (UL41) Polypeptide

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The virion host shutoff (*vhs*) gene (UL41) of herpes simplex virus type 1 (HSV-1) encodes a virion component that induces degradation of host mRNAs and the shutoff of most host protein synthesis. Subsequently, the *vhs* protein accelerates the turnover of all kinetic classes of viral mRNA. To identify the *vhs* (UL41) polypeptide within infected cells and virions, antisera raised against a UL41-*lacZ* fusion protein were used to characterize the polypeptides encoded by wild-type HSV-1 and two mutants: *vhs*1, a previously characterized mutant that lacks detectable virion host shutoff activity, and *vhs*-ΔSma, a newly constructed mutant containing a deletion of 196 codons from UL41. Two forms of the *vhs* (UL41) polypeptide were identified in cells infected with the wild-type virus or *vhs*1. Wild-type HSV-1 produced a major 58-kDa polypeptide, as well as a less abundant 59.5-kDa form of the protein, while *vhs*1 produced 57- and 59-kDa polypeptides that were approximately equally abundant. Although for either virus, both forms of the protein were phosphorylated, they differed in the extent of phosphorylation. While both *vhs* polypeptides were found in infected cells, only the faster migrating, less phosphorylated form was incorporated into virions. *vhs*-ΔSma encoded a smaller, 31-kDa polypeptide which, although present in infected cells, was not incorporated into virions. The results identify multiple forms of the *vhs* (UL41) polypeptide and suggest that posttranslational processing affects its packaging into virions, as well as its ability to induce mRNA degradation.

Controls of mRNA stability play an important role in regulating the expression of many mammalian genes (2, 4, 5, 9, 10, 22, 44, 49). During lytic infections with herpes simplex virus type 1 (HSV-1), the half-lives of both viral and cellular mRNAs are negatively regulated by the product of the HSV-1 virion host shutoff (*vhs*) gene (13, 16, 17, 31, 40, 41, 45, 52, 55). At early times after infection, copies of the *vhs* polypeptide that enter the cell as components of the infecting virion induce rapid degradation of cellular mRNAs and the concomitant shutoff of most host protein synthesis (15, 52, 55). Subsequently, after the onset of viral gene expression, the *vhs* protein accelerates turnover of viral mRNAs belonging to all kinetic classes of viral mRNA (30, 31, 40, 41, 45, 55). Thus, mutants encoding a defective *vhs* protein are defective in virion host shutoff and produce viral mRNAs with substantially longer half-lives than those of the wild-type virus (30, 31, 40, 41, 45, 55). The *vhs* function, therefore, appears to accelerate the turnover of most polyadenylated mRNAs, regardless of whether they are of host or viral origin (30, 31, 40, 41, 55). As such, the levels of host and viral mRNAs appear to be the net result of a balance between gene-specific controls of transcription and mRNA processing (38, 51), on the one hand, and the generalized *vhs*-induced turnover of polyadenylated mRNAs on the other.

Despite a number of studies delineating the role of the *vhs* function during lytic infections and the availability of *vhs* mutants, very little is known concerning the *vhs* polypeptide. The published data indicating that the *vhs* function is mediated by a virion component are threefold. (i) Efficient host shutoff is induced by virions that have been inactivated by exposure to UV light (13, 15, 17, 52, 55). (ii) *vhs*-induced destabilization of host mRNAs occurs following infection of cells with wild-type virus in the presence of drugs that block either de novo transcription or translation (52, 55). (iii) HSV infection of enucleated cytoplasts results in rapid inhibition of protein synthesis, although mock-infected cytoplasts continue to synthesize proteins for a prolonged interval (17). Mapping of the mutation carried by mutant *vhs*1 to the UL41 open reading frame allowed identification of the *vhs* gene (33) and the prediction that the primary translation product of the *vhs* mRNA is a 55-kDa polypeptide (36). This prediction was in close agreement with the earlier finding that in vitro translation of an mRNA mapping to what was later shown to be UL41 resulted in production of a polypeptide with an apparent molecular mass of 58 kDa (1, 18). Recently, Smibert and coworkers identified a 58-kDa polypeptide within infected cells that reacts with antiserum prepared against a synthetic peptide corresponding to amino acids 333 through 347 of UL41 (54). However, despite this progress, many questions remain concerning the nature and functioning of the *vhs* (UL41) protein.

In this report, we describe the identification of multiple forms of the *vhs* (UL41) polypeptide by a combination of genetic and immunologic techniques. First, we constructed a mutant, *vhs*-ΔSma, containing a deletion of 196 codons from UL41 which, therefore, encodes a *vhs* polypeptide with increased electrophoretic mobility. Next, antisera raised against a bacterially expressed UL41-*lacZ* fusion protein were used to analyze the *vhs* polypeptides encoded by wild-type HSV-1 and *vhs*-ΔSma, as well as by *vhs*1, a previously characterized mutant that lacks detectable virion host shutoff activity (29, 30, 40, 41, 45). Immunoprecipitation and immunoblotting experiments revealed two forms of the *vhs* (UL41) polypeptide in cells infected with wild-type HSV-1 or *vhs*1. Cells infected with the wild-type virus contained a major 58-kDa polypeptide, as well as a less abundant 59.5-kDa form of the protein, while *vhs*1

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produced 57- and 59-kDa *vhs* (UL41) polypeptides that were approximately equally abundant. For both viruses, both forms of the protein were phosphorylated, although to different extents. Interestingly, although both *vhs* polypeptides were present within infected cells, only the faster migrating, less phosphorylated form was incorporated into progeny virions. *vhs*-ΔSma encoded a smaller, 31-kDa polypeptide which, although readily detectable within infected cells, was not packaged into virions. The results identify at least two forms of the *vhs* (UL41) polypeptide within HSV-1-infected cells and suggest that posttranslational modifications are important for its incorporation into virus and possibly for its ability to induce mRNA degradation.

MATERIALS AND METHODS

Cells and virus. Procedures for the growth and maintenance of Vero cells have been described previously (29, 40, 41). Stocks of the wild-type strain of HSV-1 (KOS) and mutants *vhs*1, *ts*701, and *vhs*-ΔSma were prepared as described in previous reports (29, 40, 41). *vhs*1 exhibits a defective virion host shutoff function and grows well at all temperatures from 34 to 39°C (45). Mutant *ts*701 contains a temperature-sensitive mutation in the UL42 open reading frame (35). It was provided by Priscilla Schaffer.

Plasmids. (i) Plasmids encoding wild-type and mutant *vhs* genes. Plasmid pSG124 contains *Eco*RI fragment A (0.49 to 0.63 map units) of wild-type HSV-1 (KOS) cloned into the *Eco*RI site of pBR325 (19). It was provided by Myron Levine and Rozanne Sandri-Goldin. To construct plasmid pHS, the *Hin*dIII-*Sal*I fragment, spanning map coordinates 0.592 to 0.614, was excised from pSG124 and inserted between the *Hin*dIII and *Sal*I sites of Bluescript KS (Stratagene, La Jolla, Calif.). As is shown in Fig. 1, the insert in pHS contains the entire *vhs* gene (UL41) and portions of flanking open reading frames UL39, UL40, and UL42 (1, 18, 36, 37). In accord with earlier results (35), pHS is able to rescue the temperature-sensitive mutation carried by *ts*701.

pHS contains three *Sma*I sites, two within the *vhs* gene (UL41), separated by 588 bp, and one within the polylinker of the vector. To construct pHS-ΔSma, pHS was partially digested with *Sma*I to remove the 588-bp *Sma*I fragment and then recircularized by treatment with T4 DNA ligase (Fig. 1).

(ii) Plasmid encoding the UL41-*lacZ* fusion protein. Construction of plasmid pBK1, which encodes a UL41-*lacZ* fusion protein, is diagrammed in Fig. 1. As shown in Fig. 1, pN237 contains the synthetic oligonucleotide

5′ AAG CTT AAT TGA ATT CAA TTA AGC TT 3′
3′ TTC GAA TTA ACT TAA GTT AAT TCG AA 5′

inserted into the single *Nru*I site in UL41 of pHS (42). Among other features, the oligonucleotide contains a central *Eco*RI site. To construct pBK1, a 1,737-bp *Eco*RI-*Pst*I fragment that contains a portion of the oligonucleotide plus codons 239 through 489 of wild-type UL41 and 1,026 bp of the 3′ flanking sequence was excised from pN237 and inserted between the *Eco*RI and *Pst*I sites of the vector pWR590-2 (20).

(iii) Plasmids used for hybridization probes. Plasmid pHcGAP contains a 1.2-kb cDNA fragment encoding a portion of human glyceraldehyde-3-phosphate dehydrogenase (GAPD) (56). It was obtained from the American Type Culture Collection (Rockville, Md.). Plasmid pX1r11 contains a 4.6-kb fragment of *Xenopus laevis* rDNA inserted into the *Eco*RI site of colicin E1 (11). The insert in pX1r11 hybridizes with 28S rRNA.

Nucleic acid isolation. Plasmid DNAs were prepared by CsCl density gradient centrifugation as described previously (40, 41). Viral DNA for use in marker transfer experiments was isolated essentially as described by Sandri-Goldin and coworkers (50). Total cytoplasmic RNA was isolated from infected and mock-infected cells as described previously (40, 41, 46).

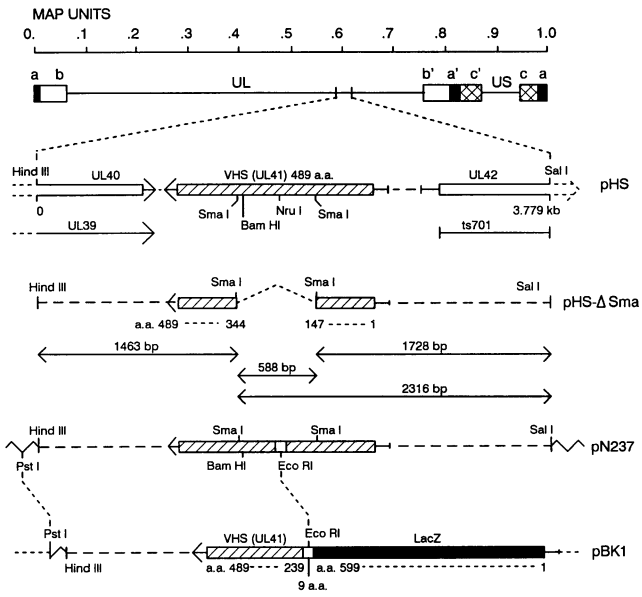

FIG. 1. Structures of plasmids containing wild-type and mutant *vhs* (UL41) genes and of a plasmid encoding a UL41-*lacZ* fusion protein. Beneath a diagram of the prototype orientation of the HSV-1 genome (lines 1 and 2) is an expanded view of the 3,779-bp *Hin*dIII-*Sal*I fragment that spans map coordinates 0.592 to 0.614 on the HSV-1 genome (line 3) and is contained in pHS. The UL41 (*vhs*) open reading frame is represented by a cross-hatched rectangle, and UL40 and UL42 are represented by open rectangles (36). The locations of the mRNAs for UL39, UL40, UL41, and UL42 are indicated by arrows (lines 3 and 4) (1, 18, 36, 37). Short dashed lines indicate that the UL40 and UL42 open reading frames extend to the left of the *Hin*dIII site and the right of the *Sal*I site, respectively. The UL39 and UL40 mRNAs are 3′ coterminal, with 5′ termini located to the left of the *Hin*dIII site. The UL39 open reading frame is located entirely to the left of the *Hin*dIII site. The portion of the *Hin*dIII-*Sal*I fragment that rescues the temperature-sensitive mutation in *ts*701 (35) is shown in line 4. The *Sma*I fragment that is deleted in pHS-ΔSma and in mutant virus *vhs*-ΔSma is indicated in line 5 along with the predicted structure of the mutant *vhs* (UL41) polypeptide. The sizes (in base pairs) of some subfragments of the *Hin*dIII-*Sal*I fragment are shown in lines 6 to 8. As is shown in line 9, UL41 of pN237 is interrupted by insertion, at the *Nru*I site, of an oligonucleotide that contains an *Eco*RI site (represented by an open box). To construct pBK1, the *Eco*RI-*Pst*I fragment from pN237 was inserted between the corresponding sites in the polylinker of pWR590-2 (line 10). The predicted structure of the UL41-*lacZ* fusion protein encoded by pBK1 is shown at the bottom. a.a., amino acids.

Marker transfer. To transfer the pHS-ΔSma deletion into virus, pHS-ΔSma was used to rescue a temperature-sensitive mutation in the neighboring UL42 open reading frame that is carried by *ts*701. Recombinant viruses were then screened by Southern blotting for cotransfer of the deletion in UL41. Briefly, Vero cells (2×10^6/25-cm^2 flask) were transfected with 6 μg of intact *ts*701 DNA, 2 μg of pHS-ΔSma that had been cleaved with *Hin*dIII and *Sal*I to separate the insert and vector sequences, and 6 μg of salmon sperm DNA essentially as described by O'Hare and Hayward (39). The cultures were incubated at 34°C until a generalized cytopathic effect was

observed, at which point the cells were lysed by three cycles of freezing and thawing and the progeny virus was harvested in accordance with standard procedures (40, 41). The progeny virus was plaque purified twice at 39°C. Isolated plaques were then picked, and the progeny was expanded into small virus stocks by infection of Vero cells at 34°C. A portion of each stock was frozen and saved, while infected-cell DNA was prepared from the remainder. Recombinant viruses were then screened by Southern blotting for cotransfer of the pHS-ΔSma deletion. Of 10 recombinants that were screened, 4 were found to contain the deletion. One of these was plaque purified once more at 34°C, and the resulting mutant virus was designated *vhs*-ΔSma.

Southern and Northern (RNA) blot analyses. Methods for electrophoresis of DNA and RNA, transfer of the nucleic acids to Nytran membranes, and hybridization to ^{32}P-labeled probes have been described previously (40, 41, 47). To analyze the structure of the *vhs* gene (UL41) of the recombinant virus, blots of viral DNA were probed with ^{32}P-labeled pHS. To analyze the *vhs*-induced degradation of the host GAPD mRNA, Northern blots were probed with nick-translated pHcGAP, while 28S rRNA was detected with ^{32}P-labeled pX1r11.

Analysis of infected-cell polypeptides (ICPs). Vero cells were infected with 20 PFU per cell and labeled for the intervals described in the text with either [^{35}S]methionine or $^{32}P_i$. To label cells with [^{35}S]methionine, monolayers were washed twice with minimum essential medium (MEM) lacking methionine and overlaid with MEM containing 1/10 of the usual concentration of methionine and supplemented with 2% calf serum and 75 μCi of [^{35}S]methionine per ml. Cells were labeled with $^{32}P_i$ by incubation in MEM lacking sodium phosphate and supplemented with 2% calf serum and 100 μCi of $^{32}P_i$ per ml. At the end of the labeling intervals, cells were scraped into ice-cold phosphate-buffered saline, collected by centrifugation, and then either processed for immunoprecipitation or lysed by boiling for 10 min in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 7.0], 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Immunoprecipitates or whole-cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography as described previously (29, 40).

Virion purification. Wild-type, *vhs*1, and *vhs*-ΔSma virions were collected from the extracellular medium of infected cultures and purified by sedimentation through gradients of 10 to 30% Dextran T10 (Sigma) in MEM containing 0.5% (wt/vol) bovine serum albumin (BSA), essentially as described by Kousoulas et al. (28). Centrifugation was done in a Beckman SW41 Ti rotor for 1 h at 29,000 rpm. Fractions containing the virion peak were pooled and diluted with 2 volumes of MEM containing 0.5% BSA, and the virus was pelleted. Virions were resuspended in a small volume of SDS sample buffer, boiled for 10 min, and analyzed by SDS-PAGE and Western blotting (immunoblotting) as described below.

Preparation of the UL41-*lacZ* fusion protein. The UL41-*lacZ* fusion protein was prepared from lysates of *Escherichia coli* containing pBK1 by preparative SDS-PAGE that was followed by electroelution from the gel slices (21). Samples of the protein were dialyzed against H_2O overnight, lyophilyzed, and resuspended in a small volume of phosphate-buffered saline. Approximately 5 mg of the UL41-*lacZ* fusion protein was routinely obtained from a 500-ml culture of bacteria.

Preparation of antisera. Polyclonal rabbit antisera were prepared against the gel-purified UL41-*lacZ* fusion protein by standard procedures (21).

Immunoprecipitations. Infected Vero cells were labeled from 4 to 20 h after infection with either [^{35}S]methionine or $^{32}P_i$. They were then harvested and lysed in either RIPA or SDS lysis buffer. For RIPA lysis, cells were suspended at 10^7/ml in RIPA buffer (50 mM Tris [pH 7.7], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 mg of BSA per ml and protease and phosphatase inhibitors (2 μg of aprotinin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of leupeptin per ml, 5 mM EDTA, 50 mM NaF, 0.2 mM sodium orthovanadate, 30 mM sodium PP_i) (21, 57). After 30 min on ice, the lysates were spun for 1 h at 40,000 rpm in a Beckman TLA 45 rotor, and the supernatants were stored at −90°C for subsequent immunoprecipitation. For SDS lysis, cell pellets (approximately 10^7 cells) were suspended and then boiled for 10 min in 100 μl of SDS lysis buffer (50 mM Tris [pH 7.5], 2% SDS) supplemented with the protease and phosphatase inhibitors described above. The samples were immediately diluted with 19 volumes of ice-cold RIPA buffer lacking SDS and spun for 1 h at 40,000 rpm in a Beckman TLA 45 rotor, and the supernatants were stored at −90°C.

To initiate the immune precipitations, aliquots of infected-cell lysates were brought to 1 ml by addition of RIPA buffer and incubated with 10 μl of normal rabbit serum (Pierce Chemical Co., Rockford, Ill.) for 1 h at 4°C. Protein A-Trisacryl beads (200 μl of a 50% [vol/vol] suspension in RIPA buffer; Pierce) were added, and incubation was continued for 1 h. After pelleting of the beads, the supernatants were incubated for 1.5 h with 5 to 10 μl of one of the following sera: (i) immune serum raised against the UL41-*lacZ* fusion protein, (ii) preimmune serum, or (iii) immune serum that had been preincubated for 1 h with an excess of gel-purified fusion protein. A 200-μl volume of protein A beads was added, and incubation was continued for an additional 1.5 h. The beads were pelleted and washed five times with RIPA buffer, and the precipitated proteins were eluted by boiling the beads for 10 min in 100 μl of 100 mM Tris [pH 7.0]–4% SDS–20% glycerol–5% β-mercaptoethanol. Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography as described previously (29, 40, 45).

Western blotting. Proteins from purified virions or infected-cell lysates were separated by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes (Amersham) by standard procedures (21). The *vhs* (UL41) polypeptides were detected by probing the filters with immune serum prepared against the UL41-*lacZ* fusion protein. Complexes of the *vhs* (UL41) protein with the primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin G; Amersham) and an ECL Western blotting detection kit (Amersham) in accordance with the instructions of the manufacturer.

RESULTS

To identify and characterize the *vhs* (UL41) polypeptide, we utilized a combination of genetic and immunologic approaches. First, we constructed a *vhs* mutant containing a deletion of a substantial portion of the UL41 open reading frame which should encode a UL41 polypeptide with altered electrophoretic mobility. Next, we raised polyclonal rabbit antisera against a UL41-*lacZ* fusion protein containing sequences shared by both wild-type and mutant forms of the *vhs* polypeptide. The wild-type and mutant forms of the *vhs* polypeptide were then identified on the basis of two criteria: (i) specific reactivity with the antisera and (ii) dependence of the molecular mass of the protein, in a predictable way, upon the *vhs* allele of the infecting virus.

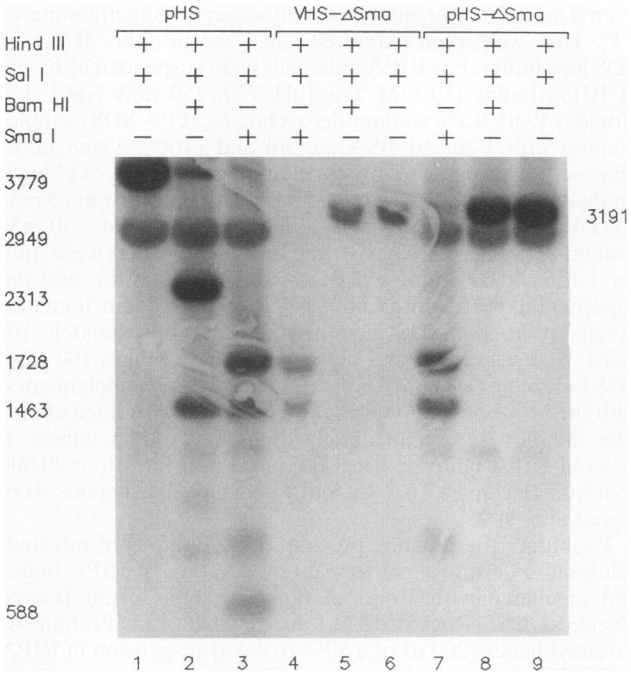

FIG. 2. Southern blot of viral and plasmid DNAs. Plasmids pHS (lanes 1 to 3) and pHS-ΔSma (lanes 7 to 9) and viral DNA from *vhs*-ΔSma (lanes 4 to 6) were cut with the various combinations of *Hin*dIII, *Sal*I, *Bam*HI, and *Sma*I indicated. A plus sign indicates that a DNA sample was cut with an enzyme, and a minus sign indicates that it was not. The DNA fragments were resolved by electrophoresis through an agarose gel, transferred to Nytran filters, and hybridized to ^{32}P-labeled pHS. The approximate sizes (in base pairs) of some of the fragments are shown to the left and right of the autoradiogram.

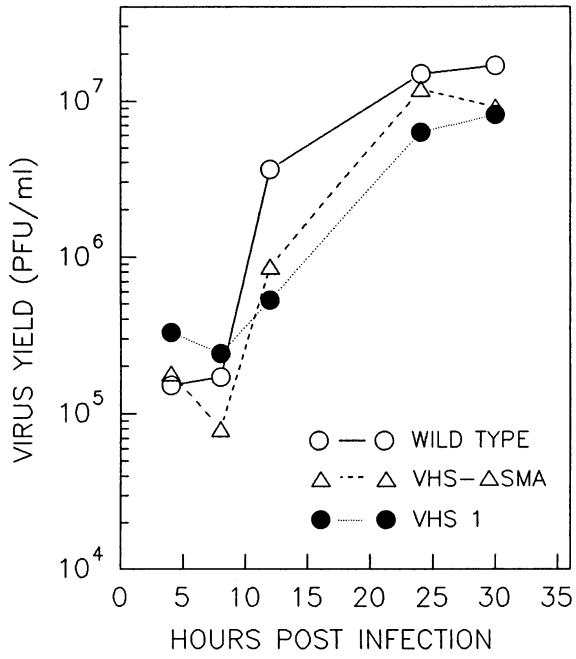

FIG. 3. Single-step growth curves for wild-type HSV-1, *vhs*1, and *vhs*-ΔSma. Cultures of Vero cells were infected with 5 PFU of wild-type HSV-1 (○), *vhs*1 (●), or *vhs*-ΔSma (△) per cell. At various times after infection, the cells were disrupted by three cycles of freezing and thawing, and the virus concentration in the lysates were determined by titration on Vero cells.

Construction of a *vhs* deletion mutant. Plasmid pHS contains a 3,779-bp *Hin*dIII-*Sal*I fragment containing UL41 from wild-type HSV-1 (KOS) (Fig. 1). Examination of the nucleotide sequence indicates that wild-type UL41 should encode a *vhs* polypeptide of 489 amino acids (36). Plasmid pHS-ΔSma was constructed by excising a 588-bp *Sma*I fragment from the middle of UL41 (Fig. 1). Mutant UL41 should encode a 293-amino-acid *vhs* polypeptide in which the first 147 amino acids of the wild-type protein are fused in frame to amino acids 344 through 489.

To introduce the deletion into a virus, pHS-ΔSma was used to rescue a temperature-sensitive mutation in the neighboring UL42 open reading frame, and viruses that grew at the nonpermissive temperature were screened by Southern blotting for cotransfer of the deletion in UL41. DNA from the mutant virus, *vhs*-ΔSma, is compared in Fig. 2 to plasmids pHS and pHS-ΔSma. Cleavage of pHS with *Hin*dIII and *Sal*I excised the insert from the vector, resulting in a vector fragment of 2,949 bp and a 3,779-bp insert containing wild-type UL41 (Fig. 2, lane 1). In contrast, cleavage of *vhs*-ΔSma viral DNA with the same two enzymes resulted in a *Hin*dIII-*Sal*I fragment that had been shortened to approximately 3,191 bp (Fig. 2, lane 6), the same size as the insert fragment that was observed in plasmid pHS-ΔSma (Fig. 2, lane 9). A fragment of this size would be predicted if the 588-bp *Sma*I fragment had been deleted from the middle of the *vhs* gene. Since *Bam*HI cuts the wild-type *vhs* gene at a site 3 bp to the right of the left-hand *Sma*I site (Fig. 1) (36), restriction of pHS with *Bam*HI in addition to *Hin*dIII and *Sal*I divided the wild-type *Hin*dIII-*Sal*I insert into two fragments of 2,313 and 1,466 bp (Fig. 2, lane 2). Deletion of the 588-bp *Sma*I fragment from UL41 should eliminate this *Bam*HI site. As predicted, the 3,191-bp *Hin*dIII-*Sal*I fragment of *vhs*-ΔSma viral DNA was resistant to *Bam*HI (Fig. 2, lane 5), as was the *Hin*dIII-*Sal*I insert carried by pHS-ΔSma (Fig. 2, lane 8). Cutting of pHS with *Sma*I in addition to *Hin*dIII and *Sal*I divided the wild-type *Hin*dIII-*Sal*I insert into three fragments of 1,728, 1,463, and 588 bp (Fig. 2, lane 3). As expected, cutting of *vhs*-ΔSma viral DNA or pHS-ΔSma with the same three enzymes resulted in bands of 1,728 and 1,463 bp; however, no 588-bp fragment was observed (Fig. 2, lanes 4 and 7).

Phenotype of *vhs*-ΔSma. (i) Growth properties. Mutant *vhs*1 exhibits a slight, but reproducible, growth deficit. Thus, *vhs*1 produces smaller plaques than does the wild-type virus, has a burst size that is reduced two- to fivefold, and is rapidly outgrown by the wild-type virus upon repeated passage of the progeny from mixed infections (33, 45). To examine the effect of the *vhs*-ΔSma deletion upon virus growth, single-step growth curves for wild-type HSV-1, *vhs*1, and *vhs*-ΔSma were compared (Fig. 3). As was the case for *vhs*1, the rise in titer of *vhs*-ΔSma was delayed somewhat relative to that of the wild-type virus; however, by 24 h postinfection, the yield was very similar. Thus, the *vhs*-ΔSma deletion had, at most, a marginal effect upon virus growth in culture.

(ii) *vhs* activity. To analyze the virion host shutoff activity of *vhs*-ΔSma, Vero cells were infected in the presence of 5 μg of dactinomycin per ml with either the wild-type virus of *vhs*-ΔSma and the decay of the cellular mRNA for GAPD was analyzed by Northern blotting (Fig. 4). This cellular mRNA was relatively stable in mock-infected cells (Fig. 4, lanes 1 to 4) but decayed rapidly in cells infected with the wild-type virus (Fig. 4, lanes 5 to 8). GAPD mRNA was every bit as stable in

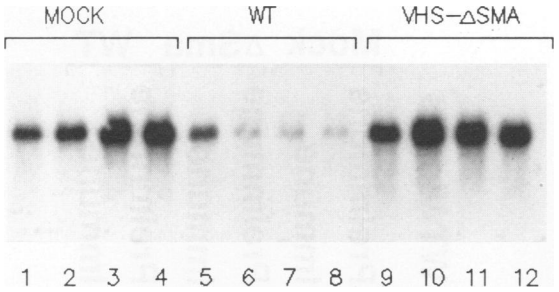

FIG. 4. Comparison of the virion host shutoff activities of wild-type HSV-1 (WT) and *vhs*-ΔSma. Vero cells were mock infected (lanes 1 to 4) or infected with 50 PFU of wild-type HSV-1 (lanes 5 to 8) or *vhs*-ΔSma (lanes 9 to 12) per cell in the presence of 5 μg of dactinomycin per ml. Total cytoplasmic RNAs were prepared at 2 h (lanes 1, 5, and 9), 4 h (lanes 2, 6, and 10), 6 h (lanes 3, 7, and 11), and 8 h (lanes 4, 8, and 12) after infection. After electrophoresis through an agarose gel and transfer to Nytran, the cellular mRNA encoding GAPD was detected with ^{32}P-labeled pHcGAP.

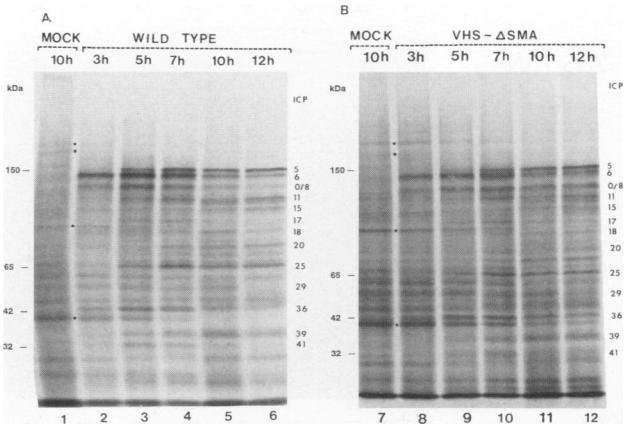

FIG. 5. Kinetics of viral gene expression by wild-type HSV-1 and *vhs*-ΔSma. Vero cells were mock infected (lanes 1 and 7) or infected with 20 PFU of wild-type HSV-1 (lanes 2 to 6) or *vhs*-ΔSma (lanes 8 to 12) per cell. The cultures were labeled with [^{35}S]methionine for 1-h intervals beginning at the postinfection times indicated above the lanes. At the end of the labeling intervals, whole-cell lysates were prepared and the labeled polypeptides were analyzed by SDS-PAGE and autoradiography. ICPs are labeled to the right of lanes 6 and 12. The following ICPs are the products of the indicated viral open reading frames: ICP5 (UL19), ICP6 (UL39), ICP8 (UL29), ICP25 (UL48), ICP29 (US3), and ICP36 (UL23) (12, 48). Scales of molecular masses are shown to the left of lanes 1 and 7. The dots to the right of lanes 1 and 7 indicate selected cellular polypeptides for which secondary shutoff of host protein synthesis (45) was particularly evident in the *vhs*-ΔSma infections.

cells infected with *vhs*-ΔSma as it was in mock-infected cells (Fig. 4, lanes 1 to 4 and 9 to 12), indicating that *vhs*-ΔSma lacks detectable virion host shutoff activity.

(iii) Effect of the *vhs*-ΔSma deletion upon viral gene expression. Although the mutation carried by *vhs*1 is not lethal, it does have a significant effect upon the normal cascade regulation of viral gene expression (29–31, 33, 40, 41, 45, 55). Thus, in addition to being defective in virion host shutoff, *vhs*1 produces viral mRNAs that are significantly more stable than those encoded by the wild-type virus (40, 41). The most obvious effect of this is that at late times, *vhs*1 overproduces many immediate-early and early polypeptides (31, 40, 41, 45, 55).

To determine whether the *vhs*-ΔSma deletion has a similar effect upon viral gene expression, Vero cells were pulse-labeled with [^{35}S]methionine at various times after infection with the wild-type virus or *vhs*-ΔSma and the labeled polypeptides were examined by SDS-PAGE and autoradiography (Fig. 5). While in wild-type infections synthesis of immediate-early (ICP0) and early (ICP6, ICP8, ICP17, ICP36, and ICP41) polypeptides increased to peak rates at early times and subsequently declined, synthesis of these polypeptides continued at high levels for a prolonged period following infection with *vhs*-ΔSma. Thus, the effect upon viral gene expression of the *vhs*-ΔSma mutation was indistinguishable from that previously reported for *vhs*1.

(iv) Dominance relationships between wild-type HSV-1, *vhs*-ΔSma, and *vhs*1. Some HSV-1 strains that exhibit weak or slow virion host shutoff inhibit the shutoff activity of fast-shutoff strains during mixed infections (14, 23, 32). Of particular interest is the report of Kwong and Frenkel that *vhs*1 inhibits the shutoff activity of wild-type HSV-1 (KOS) (32). To confirm this finding and to test the dominance relationship between *vhs*-ΔSma and the wild-type virus, Vero cells were infected in the presence of dactinomycin with various amounts of wild-type HSV-1 (KOS) or with mixtures containing different ratios of the wild-type virus and *vhs*1 or the wild-type virus and *vhs*-ΔSma (Fig. 6). Total cytoplasmic RNAs were prepared at 6 h after infection and analyzed by Northern blotting to detect the cellular mRNA for GAPD. Probing of the same blot for 28S rRNA provided an internal control for the relative amounts of RNA loaded onto the different lanes of the gel.

In accord with earlier results (52, 55), infection of cells with 20, 50, or 100 PFU of the wild-type virus per cell resulted in the degradation of all detectable GAPD mRNA (Fig. 6, lanes 2 to 4). Similarly, efficient degradation of GAPD mRNA was observed when cells were coinfected with 80 PFU of the wild-type virus per cell and 20 PFU of *vhs*1 per cell (Fig. 6, lane 5). However, infection with a mixture containing 50 PFU of the wild-type virus per cell and 50 PFU of *vhs*1 per cell resulted in significantly less degradation of GAPD mRNA than did infection with 50 or 100 PFU of the wild-type virus alone per cell (Fig. 6, lane 6). In contrast, coinfection of cells with 50 PFU of wild-type HSV-1 per cell and 50 PFU of *vhs*-ΔSma per cell resulted in efficient *vhs*-induced degradation of host mRNAs (Fig. 6, lane 8). Thus, while both the *vhs*1 and *vhs*-ΔSma mutations abolished virion host shutoff activity, the *vhs*1 allele was codominant with the wild type in a mixed infection and the *vhs*-ΔSma allele was recessive.

Identification of multiple forms of the *vhs* (UL41) polypeptide. (i) Production of antisera. To identify the *vhs* polypeptide, polyclonal antisera were raised against a bacterially expressed UL41-*lacZ* fusion protein and used in immunoprecipitation and Western blotting experiments to characterize the proteins encoded by wild-type HSV-1 and mutants *vhs*-ΔSma and *vhs*1. On the basis of its DNA sequence, pBK1 should encode a fusion protein containing the first 599 amino acids of β-galactosidase, followed by the nine-amino-acid (GRARIQLSF) peptide encoded by linker sequences from pWR590-2 and pN237, and amino acids 239 through 489 of the *vhs* polypeptide from HSV-1 (KOS) (Fig. 1). In accord with this prediction, the pBK1 fusion protein exhibited an apparent molecular mass on SDS-PAGE of approximately 100 kDa (data not shown).

(ii) Immunoprecipitation of the *vhs* polypeptide from infected cells. The antisera were first used to immunoprecipitate ^{35}S-labeled proteins from extracts of infected or mock-infected

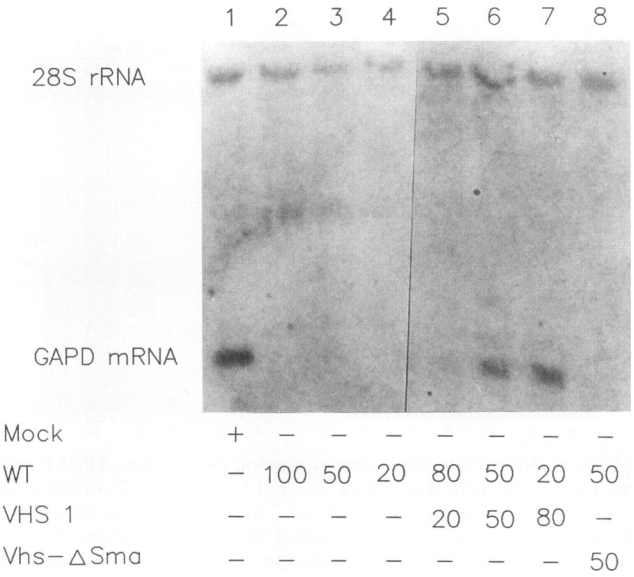

FIG. 6. Dominance relationships between *vhs* mutants and wild-type HSV-1 (WT). Vero cells were mock infected or infected with various combinations of wild-type HSV-1, *vhs*1, or *vhs*-ΔSma, all in the presence of 5 μg of dactinomycin per ml. The number of PFU of each virus per cell is indicated below each lane. All cultures were harvested at 6 h after infection. Whole cytoplasmic RNAs were prepared and analyzed by Northern blotting to detect the cellular mRNA encoding GAPD, as well as 28S rRNA.

cells lysed in RIPA buffer (Fig. 7 and 8, lanes 1 to 3). The immune serum precipitated a 58-kDa polypeptide from cells infected with wild-type HSV-1 (indicated by closed circles to the right of Fig. 7, lane 7, and to the left of Fig. 8, lane 3). The apparent molecular mass of this protein was in close agreement with the value of 55 kDa predicted for the wild-type UL41 polypeptide from the HSV-1 nucleotide sequence (36) and identical to that reported by Wagner and coworkers for the in vitro translation product of mRNA mapping to what is now known to be UL41 (1, 18). As would be predicted if it were a product of wild-type UL41, the 58-kDa polypeptide was not detected in extracts of cells infected with *vhs*-ΔSma; instead, a 31-kDa polypeptide was precipitated (indicated by an arrow to the left of Fig. 7, lane 5). As expected, the apparent molecular mass of 31 kDa was very similar to the value of 33 kDa predicted from the DNA sequence. Neither the 58-kDa nor the 31-kDa protein was precipitated by preimmune serum from infected-cell lysates (Fig. 7, lanes 4 and 6; Fig. 8, lane 2) or by immune serum from lysates of mock-infected cells (Fig. 7, lane 3). In addition, preincubation of the immune serum with an excess of the unlabeled UL41-*lacZ* fusion protein significantly reduced the amount of the labeled 58-kDa polypeptide that could be subsequently precipitated from wild-type infected-cell lysates (Fig. 8, lane 1). This combination of immunologic and genetic results identified the 58- and 31-kDa polypeptides as products of the *vhs* genes (UL41) of wild-type HSV-1 and *vhs*-ΔSma, respectively.

Interestingly, the immune serum precipitated significantly less of the 31-kDa polypeptide from cells infected with *vhs*-ΔSma than it did of the wild-type 58-kDa polypeptide. This can be seen in Fig. 7, where a longer autoradiographic exposure is shown for immunoprecipitates from mock-infected and *vhs*-ΔSma-infected cells (Fig. 7, lanes 2 to 5) than for those from cells infected with the wild-type virus (Fig. 7, lanes 6 and 7).

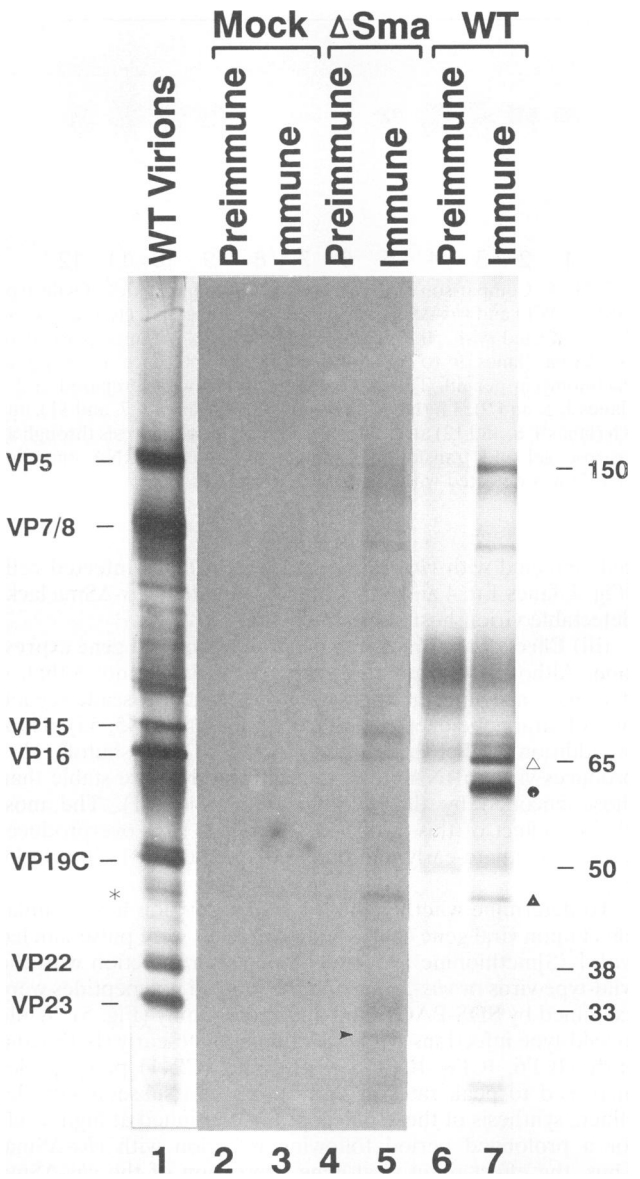

FIG. 7. Immunoprecipitation of the *vhs* (UL41) protein from RIPA lysates of infected cells. Vero cells were labeled with [^{35}S]methionine from 4 to 20 h after infection with wild-type (WT) HSV-1 (lanes 6 and 7) or *vhs*-ΔSma (lanes 4 and 5) or after mock infection (lanes 2 and 3). Lysates were prepared in RIPA buffer, and immunoprecipitates were prepared by using either immune (lanes 3, 5, and 7) or preimmune (lanes 2, 4, and 6) sera. The 58-kDa *vhs* (UL41) polypeptide that was present in immune precipitates from wild-type lysates is indicated by the closed circle to the right of lane 7. The 65- and 43-kDa polypeptides that coprecipitated with the *vhs* polypeptide are indicated by the open and closed triangles, respectively. The 31-kDa *vhs* (UL41) polypeptide encoded by *vhs*-ΔSma is indicated by the arrowhead to the left of lane 5. Total proteins contained in purified wild-type HSV-1 virions are shown in lane 1. The designations of selected virion polypeptides are shown to the left of lane 1. The molecular masses (in kilodaltons) of virion proteins VP5 (UL19), VP16 (UL48), VP19C (UL38), VP22 (UL49), and VP23 (UL18) are shown in the scale to the right of lane 7. The asterisk to the left of lane 1 indicates a minor virion polypeptide that comigrated with the 43-kDa polypeptide that coprecipitated with the *vhs* polypeptide from infected cell lysates.

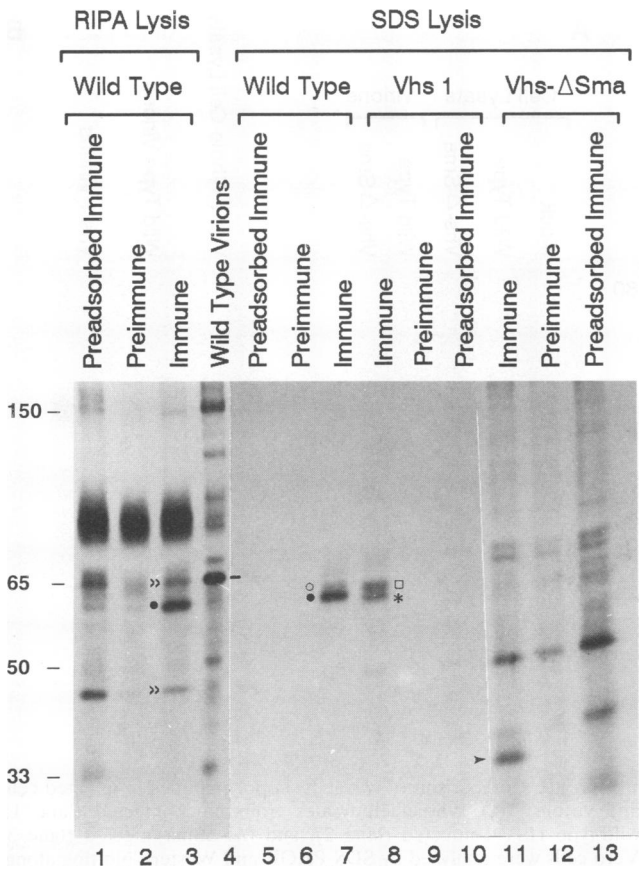

FIG. 8. Immunoprecipitation of *vhs* (UL41) polypeptides from infected cells lysed in RIPA or SDS lysis buffer. Vero cells were labeled with [^{35}S]methionine from 4 to 20 h after infection with wild-type HSV-1 (lanes 1 to 3 and 5 to 7), *vhs*1 (lanes 8 to 10), or *vhs*-ΔSma (lanes 11 to 13). Infected cells were lysed in either RIPA buffer (lanes 1 to 3) or by boiling in SDS lysis buffer (lanes 5 to 13). Immunoprecipitates were prepared with immune serum (lanes 3, 7, 8, and 11), preimmune serum (lanes 2, 6, 9, and 12), or immune serum that had been preincubated for 1 h with an excess of the UL41-*lacZ* fusion protein (lanes 1, 5, 10, and 13). The two forms of the *vhs* (UL41) polypeptide immunoprecipitated from SDS lysates of cells infected with the wild-type virus are indicated by the closed and open circles to the left of lane 7, while the 58-kDa *vhs* polypeptide immunoprecipitated from RIPA lysates is indicated by the closed circle to the left of lane 3. The two forms of the *vhs* (UL41) protein observed in SDS lysates of *vhs*1-infected cells are indicated by the asterisk and open square to the right of lane 8, while the 31-kDa *vhs* polypeptide encoded by *vhs*-ΔSma is indicated by the arrowhead to the left of lane 11. Total proteins contained in purified wild-type HSV-1 virions are shown in lane 4, and 65-kDa virion polypeptide VP16 (UL48) is indicated by a line to the right of lane 4. A scale of molecular masses (in kilodaltons) is shown to the left of lane 1. The double arrowheads between lanes 2 and 3 indicate the 65- and 43-kDa polypeptides that coprecipitated with the *vhs* polypeptide from RIPA lysates of infected cells.

Thus, background bands that are barely visible in lanes 6 and 7 are readily apparent in lanes 2 through 5. Reduced amounts of the 31-kDa polypeptide were precipitated, even though control experiments indicated that an excess of immune serum had been used in the immunoprecipitations (data not shown). Reduced amounts of the mutant *vhs* (UL41) polypeptide also were observed by immunoblotting ICPs following separation by SDS-PAGE, as well as by immunoprecipitation of infected-cell lysates that had been previously denatured by boiling in SDS (Fig. 8; see Fig. 9). Whether the reduced abundance of the *vhs*-ΔSma polypeptide was due to a decreased rate of synthesis or more rapid turnover of the protein is under investigation.

In addition to the 58-kDa wild-type *vhs* (UL41) polypeptide, the immune serum precipitated several labeled polypeptides that comigrated with structural polypeptides of purified HSV-1 virions (Fig. 7, compare lanes 1 and 7; Fig. 8, compare lanes 3 and 4). Reproducibly, the most prominent of these were a 65-kDa polypeptide that comigrated with virion transcriptional transactivator VP16 (UL48) and a 43-kDa polypeptide that comigrated with a minor virion component. Neither the 65-kDa nor the 43-kDa polypeptide was precipitated by immune serum from lysates of mock-infected cells (Fig. 7, lane 3) or by preimmune serum from lysates of cells infected with wild-type HSV-1 or *vhs*-ΔSma (Fig. 7, lanes 4 and 6; Fig. 8, lane 2). However, it is of the most significance for the present study that small amounts of both the 65- and 43-kDa polypeptides were precipitated by immune serum from lysates of cells infected with *vhs*-ΔSma (Fig. 7, lane 5), indicating that they were not products of UL41. In addition, partial V8 proteolysis of the 58- and 65-kDa proteins immunoprecipitated from cells infected with the wild-type virus resulted in very different patterns of digestion, providing further evidence that the 65-kDa polypeptide was not encoded by UL41 (43).

In addition to the 58- and 31-kDa versions of the *vhs* (UL41) polypeptide and the 65- and 43-kDa proteins, all of which were precipitated by immune but not preimmune serum, proteins forming a broad band ranging from 75 to 90 kDa were nonspecifically precipitated from infected-cell lysates by immune, preimmune, or preadsorbed immune serum (Fig. 7, lanes 6 and 7; Fig. 8, lanes 1 to 3). These proteins were observed only when a source of serum was included in the precipitation reaction, ruling out the possibility that they interacted directly with the protein A beads (data not shown). The intensity of the band was dependent upon the extent to which the lysates had been precleared by incubation with normal rabbit serum and protein A beads prior to initiation of the immune precipitation. HSV-1 glycoproteins gE and gI have been shown to form a complex that has Fc receptor activity (3, 24, 25). In view of this, it is likely that the band of nonspecifically precipitated proteins was due to viral glycoproteins precipitated because they bound to the Fc domains of immunoglobulin G molecules that simultaneously interacted with the protein A beads.

(iii) An additional form of the *vhs* polypeptide identified by immunoprecipitation and immunoblotting of denatured polypeptides. Since the immune serum was raised against samples of the UL41-*lacZ* fusion protein that had been denatured and purified by SDS-PAGE, it was of interest to determine whether it would recognize the authentic *vhs* polypeptide following denaturation. This was tested in two ways. (i) Infected cells were lysed by boiling in 2% SDS, after which the lysates were chilled and diluted with 20 volumes of RIPA buffer lacking SDS, and the *vhs* (UL41) polypeptide was immunoprecipitated (Fig. 8, lanes 5 through 13). (ii) Following lysis of the cells by boiling in 2% SDS and 5% β-mercaptoethanol, the ICPs were resolved by SDS-PAGE and analyzed by Western blotting (Fig. 9).

The immune serum precipitated a 58-kDa polypeptide from SDS lysates of cells infected with wild-type HSV-1 (Fig. 8, closed circle to the left of lane 7) that comigrated with the 58-kDa *vhs* polypeptide precipitated from RIPA lysates (Fig. 8, closed circle to the left of lane 3). In addition, denaturation of the ICPs prior to immunoprecipitation virtually abolished coprecipitation of the 65- and 43-kDa polypeptides (Fig. 8,

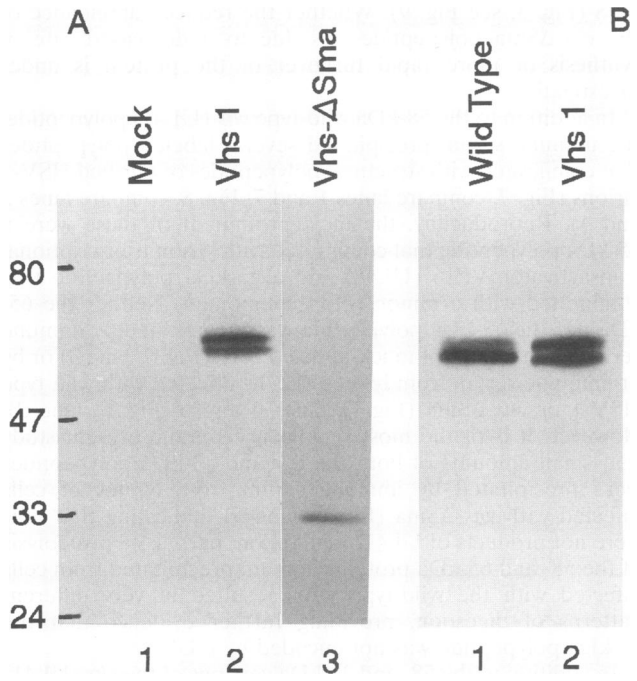

FIG. 9. Western blot analysis of *vhs* (UL41) polypeptides. Vero cells were infected with 20 PFU of wild-type HSV-1 (panel B, lane 1), *vhs*1 (panel A, lane 2, and panel B, lane 2), or *vhs*-ΔSma (panel A, lane 3) per ml or were mock infected (panel A, lane 1). The cells were lysed at 20 h after infection or mock infection by boiling in SDS lysis buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blotting with antiserum prepared against the UL41-*lacZ* fusion protein. A scale of molecular masses (in kilodaltons) is shown to the left of lane 1.

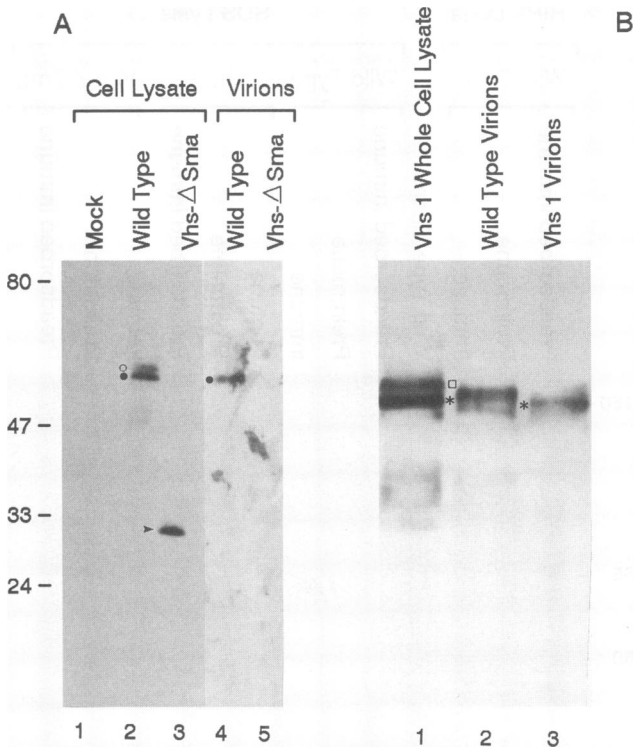

FIG. 10. Comparison of *vhs* (UL41) polypeptides in infected cells and virions. (A) Whole-cell lysates from mock-infected (lane 1), wild-type HSV-1-infected (lane 2), and *vhs*-ΔSma-infected (lane 3) Vero cells were analyzed by SDS-PAGE and Western blotting alongside wild-type (lane 4) and *vhs*-ΔSma (lane 5) virions. The two forms of the wild-type protein that were present in infected cells are indicated by the closed and open circles to the left of lane 2, while the single form that was incorporated into wild-type virions is indicated by the closed circle to the left of lane 4. The form of the *vhs* polypeptide that was present in cells infected with *vhs*-ΔSma but not incorporated into virions is shown by the arrowhead to the left of lane 3. A scale of molecular masses (in kilodaltons) is shown to the left of lane 1. (B) Western blot analysis of a whole-cell lysate from *vhs*1-infected cells (lane 1), wild-type virions (lane 2), and *vhs*1 virions (lane 3). The open square and asterisk to the right of lane 1 indicate the two forms of the *vhs* polypeptide that were present in cells infected with *vhs*1; the asterisk to the left of lane 3 indicates the *vhs* polypeptide present in *vhs*1 virions.

compare lanes 3 and 7), a finding consistent with the possibility that the 65- and 43-kDa polypeptides were coprecipitated with the *vhs* protein from RIPA lysates because they formed a physical complex with it. Surprisingly, although the 58-kDa polypeptide was the major protein precipitated from SDS lysates of infected cells, the immune serum also precipitated another polypeptide with an apparent molecular mass of 59.5 kDa (Fig. 8, open circle to the left of lane 7). Densitometric scanning of appropriate exposures of this gel and others indicated that the band formed by the 59.5-kDa polypeptide was at least threefold less intense than that of the 58-kDa protein. Similar results were obtained by Western blot analysis of ICPs (Fig. 9); namely, a doublet consisting of a major 58-kDa polypeptide and a less abundant 59.5-kDa protein was detected in extracts of cells infected with the wild-type virus, while the 65- and 43-kDa polypeptides were not. The 58- and 59.5-kDa polypeptides were both judged to be products of the UL41 open reading frame on the basis of three findings. (i) Neither polypeptide was precipitated by preimmune serum from infected-cell extracts (Fig. 8, lane 6) or detected with the immune serum used to probe immunoblots of mock-infected-cell lysates (Fig. 9A, lane 1; Fig. 10A, lane 1). (ii) Preincubation of the immune serum with an excess of the pBK1 fusion protein completely abolished subsequent immunoprecipitation of both the 58- and 59.5-kDa proteins from lysates of infected cells (Fig. 8, lane 5). (iii) Neither polypeptide was detected by immunoprecipitation (Fig. 8, lane 11) or immunoblotting (Fig. 9A, lane 3) in extracts of cells infected with *vhs*-ΔSma. Instead, in this case, we observed a 31-kDa polypeptide (Fig. 8, lane 11; Fig. 9A, lane 3) whose detection could be abolished by preincubation of the immune serum with the fusion protein (Fig. 8, lane 12). Therefore, the wild-type 59.5-kDa polypeptide represents an additional form of the *vhs* (UL41) protein that was detected by the immune serum only following denaturation.

Two *vhs* (UL41) polypeptides were also observed in cells infected with mutant *vhs*1 (Fig. 8, lane 8; Fig. 9A, lane 2; Fig. 9B, lane 2; Fig. 10B, lane 1). However, the polypeptides encoded by *vhs*1 differed from their wild-type counterparts in two important respects. (i) The two *vhs*1 polypeptides exhibited altered mobilities on SDS-PAGE, migrating with apparent molecular masses of 57 and 59 kDa as opposed to 58 and 59.5 kDa for the wild-type polypeptides. (ii) While the 59.5-kDa form of the wild-type *vhs* polypeptide was significantly less abundant than the 58-kDa form, the 57- and 59-kDa polypeptides encoded by *vhs*1 were approximately equally abundant. The fact that the electrophoretic mobilities and relative abun-

dances of the polypeptides were dependent upon the *vhs* allele of the infecting virus provides further evidence that for both the wild-type virus and *vhs*1, the two proteins were both products of UL41.

Structural polypeptides of wild-type and mutant virions. The finding that two forms of the *vhs* polypeptide were present in cells infected with wild-type HSV-1 or *vhs*1 raised the question of whether both forms of the protein were incorporated into virions, as well as whether the shortened 31-kDa polypeptide encoded by *vhs*-ΔSma was packaged into the virus. To answer these questions, the polypeptides of purified wild-type, *vhs*1, and *vhs*-ΔSma virions were compared by SDS-PAGE and immunoblotting to the proteins present in lysates of whole infected cells.

The results shown in Fig. 10 allowed two important conclusions. (i) Although two forms of the *vhs* (UL41) polypeptide were present in cells infected with the wild-type virus (Fig. 10A, lane 2) or *vhs*1 (Fig. 10B, lane 1), only the faster migrating form was incorporated into virions (Fig. 10A, lane 4; Fig. 10B, lanes 2 and 3). (ii) While the 58-kDa wild-type and 57-kDa *vhs*1 polypeptides were easily detected within virions (Fig. 10A, lane 4), the 31-kDa protein encoded by *vhs*-ΔSma was not (Fig. 10A, lane 5). To ensure that the latter result was not simply due to unequal loading of the gel, we utilized ^{35}S-labeled virions and analyzed the filter by autoradiography to obtain profiles of total virion polypeptides. Comparison of the wild-type and *vhs*-ΔSma virions immunoblotted in Fig. 10A revealed similar intensities for structural proteins other than *vhs* (data not shown), indicating that approximately equal numbers of wild-type and mutant virions had been loaded onto the gel and transferred to the nitrocellulose. Thus, although the 31-kDa protein encoded by *vhs*-ΔSma was present within infected cells, it was not packaged into virions.

Alternate forms of the *vhs* polypeptide differ in the extent of phosphorylation. Smibert and coworkers have reported that a 58-kDa polypeptide that reacts with antiserum raised against a synthetic peptide from UL41 is phosphorylated (54). Since numerous proteins have been shown to exist in alternate forms that differ in the state of phosphorylation (6–8, 27, 57), we undertook to determine (i) whether both forms of the wild-type and *vhs*1 polypeptides were phosphorylated and (ii) if so, whether they differed in the extent of phosphorylation.

Cells infected with wild-type HSV-1 or *vhs*1 were labeled with [^{35}S]methionine or $^{32}P_i$ and lysed by boiling in SDS, and immunoprecipitates were prepared and analyzed as described above. The results shown in Fig. 11 allowed two important conclusions. (i) Both forms of the wild-type and *vhs*1 UL41 polypeptides were phosphorylated (Fig. 11, lanes 3 and 7). (ii) The relative intensities of the bands formed by the two wild-type and the two *vhs*1 polypeptides depended upon whether the cells had been labeled with ^{35}S or ^{32}P. Thus, densitometric scanning of the autoradiogram revealed that for [^{35}S]methionine-labeled immunoprecipitates, the band formed by the 59.5-kDa wild-type polypeptide was at least threefold less intense than that of the 58-kDa virion-associated form (Fig. 11, lane 2). In contrast, analysis of ^{32}P-labeled immunoprecipitates indicated that the two wild-type bands were approximately equally intense (Fig. 11, lane 3). A qualitatively similar result was observed for the two UL41 polypeptides encoded by *vhs*1. While the intensities for the two ^{35}S-labeled polypeptides were approximately equal, the band formed by the more slowly migrating ^{32}P-labeled polypeptide was approximately five times more intense than that of the faster 57-kDa form. Therefore, for both the wild-type virus and *vhs*1, the more slowly migrating *vhs* (UL41) polypeptide was more highly phosphorylated than the faster migrating virion-associated form.

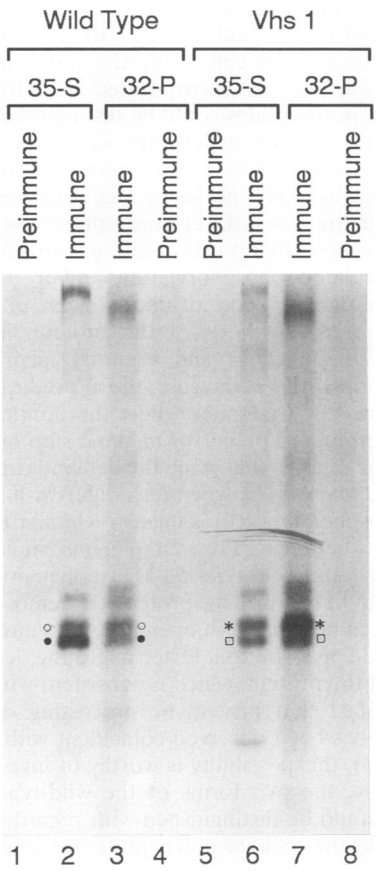

FIG. 11. Alternate forms of the *vhs* (UL41) polypeptide differ in the extent of phosphorylation. Vero cells were infected with 20 PFU of wild-type HSV-1 (lanes 1 to 4) or *vhs*1 (lanes 5 to 8) per cell. The cells were labeled from 4 to 20 h postinfection with either [^{35}S]methionine (lanes 1, 2, 5, and 6) or $^{32}P_i$ (lanes 3, 4, 7, and 8) and then lysed by boiling in SDS lysis buffer. Immune and preimmune precipitates were prepared and analyzed by SDS-PAGE and autoradiography. The two forms of the wild-type *vhs* (UL41) polypeptide are indicated by the closed and open circles to the left of lane 2 and the right of lane 3, while the two UL41 polypeptides produced by *vhs*1 are indicated by the asterisk and open box to the left of lane 6 and the right of lane 7.

DISCUSSION

The primary contributions of this work are the isolation of an HSV-1 mutant containing a deletion in the UL41 open reading frame and the identification of multiple forms of the *vhs* (UL41) polypeptide. In a recent study, Smibert and coworkers identified a 58-kDa polypeptide within HSV-1-infected cells and virions that reacted with antiserum raised against a synthetic peptide corresponding to amino acids 333 through 347 of UL41 (54). The present study confirms this result and extends it in several important respects.

Most significantly, whereas Smibert and coworkers observed a single *vhs* (UL41) polypeptide, we identified two electrophoretically distinguishable forms of the protein in cells infected with either wild-type HSV-1 or mutant *vhs*1. Characterization of the alternative *vhs* (UL41) polypeptides indicated that they differed in several important ways. (i) For both the

wild-type virus and *vhs*1, the two forms of the protein were phosphorylated to different extents. (ii) Although both were present within infected cells, only the faster migrating, less phosphorylated form was incorporated into virions. (iii) The two wild-type polypeptides could be distinguished with regard to the conditions under which they were reactive with antiserum raised against a UL41-*lacZ* fusion protein.

The finding that only the faster migrating, less phosphorylated form of the *vhs* (UL41) polypeptide was incorporated into virions suggests that posttranslational processing (possibly involving regulated phosphorylation and/or dephosphorylation) is important in one or more steps of the pathway involving de novo synthesis of the protein, its subsequent transport to the nucleus, and eventual incorporation into progeny virions. If this is the case, the altered ratio of the two forms produced by *vhs*1 may reflect the accumulation of an inefficiently processed precursor at some step in the pathway. One should emphasize that while the data indicate that the two wild-type and two *vhs*1 polypeptides differ with respect to the extent of phosphorylation, it is unclear whether this is the only difference between them. For example, one cannot exclude the possibility that the wild-type 58-kDa polypeptide is derived from the 59.5-kDa form by proteolytic removal of a small phosphorylated peptide from one end of the molecule. Such a cleavage would give rise to a faster migrating, less phosphorylated form of the protein, which is consistent with the data. In view of the fact that proteolytic processing of other HSV polypeptides has been observed coincident with the assembly of capsids (34), this possibility is worthy of investigation.

Interestingly, the two forms of the wild-type *vhs* (UL41) polypeptide could be distinguished with regard to immunoreactivity. While the 58-kDa polypeptide was efficiently immunoprecipitated from cell lysates prepared either in RIPA buffer or by boiling cells in SDS, the 59.5-kDa polypeptide was precipitated only following denaturation. This difference could reflect differences in the conformation of the proteins, their state of posttranslational modification, or their state of multimerization or association with other polypeptides. For example, in RIPA buffer, the 59.5-kDa form of the protein may exist as a multimer or as part of a complex with other polypeptides, the result of which is to mask epitopes so that they are not available for antibody binding. Disruption of the multimers or complexes by boiling in SDS would render the epitopes accessible to antibodies and allow immunoprecipitation of the protein. Alternatively, even if it exists as a monomer, in its native form, the 59.5-kDa polypeptide may lack all epitopes that are recognized on the 58-kDa protein. Denaturation of the protein by boiling in SDS might expose denaturation-specific epitopes that are recognized by antibodies present in the immune serum. Whether these differences in immunoreactivity of the 58- and 59.5-kDa polypeptides reflect functionally important differences in their structures or states of aggregation remains to be determined.

Characterization of *vhs*-ΔSma revealed that it was similar to *vhs*1 in many respects. Both viruses lacked any detectable virion host shutoff activity, and both carried mutations that had similar effects upon viral gene expression. The major difference was that *vhs*1 was codominant with the wild-type virus in a mixed infection, while *vhs*-ΔSma was recessive. This difference could be explained by the absence of the *vhs*-ΔSma protein from mutant virions. The fact that the UL41 polypeptide encoded by *vhs*-ΔSma was not packaged even though it was present within infected cells suggests that it either is misfolded or lacks a domain required for transport to the nucleus or for packaging into virions. Studies are under way to define any nuclear localization or packaging signals by site-directed mutagenesis.

Mutants encoding UL41 polypeptides that are not packaged should fall into two groups: those for which the mutant protein would be able to induce mRNA degradation, if it was present, and those for which the mutant protein lacks mRNA-degradative activity. Preliminary data suggest that *vhs*-ΔSma falls into the latter category. Cotransfection of cells with a reporter gene encoding chloramphenicol acetyltransferase (CAT) and a wild-type *vhs* gene results in significantly less CAT activity than does cotransfection with the CAT gene and a control plasmid from which the *vhs* gene has been deleted, presumably because expression of the *vhs* polypeptide causes destabilization of CAT mRNA (42). However, cotransfection of cells with the CAT gene and a plasmid containing the *vhs*-ΔSma mutation results in production of just as much CAT activity as does cotransfection with the CAT gene and the control plasmid. In the future, it will be important to distinguish mutations that affect packaging of the *vhs* polypeptide from those that affect its mRNA-degradative activity.

The findings that *vhs*1 is codominant in a mixed infection and that some slow-shutoff strains of HSV inhibit the host shutoff activity of other fast-shutoff strains (14, 23, 32) have important implications with regard to the mechanism of *vhs* action, suggesting that the *vhs* protein is multimeric or that it interacts with cellular proteins that are present in limiting amounts. Recent studies of the native *vhs* polypeptide by velocity sedimentation and gel filtration indicate that it has an apparent molecular mass of approximately 120 kDa; this finding is consistent with either of these possibilities (26).

Of potential importance was the finding that in RIPA buffer, the 58-kDa *vhs* polypeptide was immunoprecipitated along with the 65- and 43-kDa polypeptides that comigrated on SDS-PAGE with major tegument protein VP16 and another minor virion polypeptide. The identities of these coprecipitated proteins remain to be definitively determined. However, the facts that they were labeled with [^{35}S]methionine during an interval when, in wild-type infections, there was little ongoing host protein synthesis and that they comigrated with two known virion structural proteins suggest that they were, in fact, virus encoded. Coprecipitation of the 65- and 43-kDa polypeptides with the *vhs* (UL41) protein could have resulted from several alternative mechanisms: (i) nonspecific sticking of the proteins to immune complexes or protein A beads; (ii) specific recognition of the proteins by antibodies in the immune serum, or (iii) coprecipitation of the proteins with the *vhs* polypeptide because they formed a stable complex with it. The fact that neither the 65-kDa nor the 43-kDa protein was precipitated by preimmune serum argues against the first possibility. Although it is not possible to rule out either of the last two alternatives, several considerations favor the third. (i) Reduced amounts of the 65- and 43-kDa polypeptides were precipitated from cells infected with *vhs*-ΔSma, paralleling the reduced amounts of the mutant *vhs* polypeptide that were detected. (ii) The 65-kDa, 43-kDa, and *vhs* polypeptides were not immunoprecipitated from lysates of cells infected with a nonsense mutant encoding a *vhs* polypeptide consisting of the first 237 amino acids of UL41 (42). This result was expected for the mutant *vhs* polypeptide, since it lacked sequences in common with the UL41-*lacZ* fusion protein against which the immune serum was prepared. However, the absence of the 65- and 43-kDa polypeptides from immune precipitates from cells infected with the nonsense mutant and the reduced amounts that were precipitated from cells infected with *vhs*-ΔSma are difficult to explain if the 65- and 43-kDa polypeptides reacted directly with antibodies in the immune serum. Smibert et al. have

recently reported that the 58-kDa *vhs* (UL41) polypeptide forms a specific complex with VP16 (UL48) in vitro (53), strongly suggesting that the 65-kDa protein that coprecipitated with the *vhs* polypeptide in our studies was, in fact, VP16.

Two of the most important questions raised by this study are how many forms of the *vhs* protein are there, and what are their functions? While two forms of the protein were identified by one-dimensional SDS-PAGE, it is possible, if not likely, that additional forms will be resolved by electrophoresis on two-dimensional gels. Our finding that only the faster migrating, less phosphorylated polypeptide was incorporated into virions revealed one important functional difference between the alternative forms of the protein. Another may involve their relative abilities to induce mRNA degradation. The *vhs* protein has been shown to accelerate degradation of both host and viral mRNAs nonspecifically (30, 40, 41, 52, 55). As such, a small amount of the protein apparently is beneficial to the virus because it facilitates host shutoff and the sequential transition between expressions of different classes of viral genes. However, in view of its lack of specificity, de novo synthesis of large amounts of the *vhs* protein might be expected to shutoff all viral gene expression unless its activity was controlled in some way. In this light, modification of the protein by phosphorylation or in some other way may be a means of inhibiting the mRNA-degradative activity of newly synthesized copies of the protein prior to their packaging into progeny virions. Alternatively, since the *vhs* protein degrades mRNAs in the cytoplasm while virion assembly occurs in the nucleus, any modifications required for transport of the protein to the nucleus and/or packaging may also be important because they lower the cytoplasmic concentration of the protein and, therefore, the amount available for mRNA degradation. Efforts are under way to test these and other models with in vitro mRNA degradation systems.

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REFERENCES

1. **Anderson, K. P., R. J. Frink, G. B. Devi, B. H. Gaylord, R. H. Costa, and E. K. Wagner.** 1981. Detailed characterization of the mRNA mapping in the *Hin*dIII fragment K region of the herpes simplex virus type 1 genome. J. Virol. **37:**1011–1027.
2. **Atwater, J. A., R. Wisdom, and I. Verma.** 1990. Regulated mRNA stability. Annu. Rev. Genet. **24:**519–541.
3. **Bell, S., M. Cranage, L. Borysiewicz, and T. Minson.** 1990. Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1. J. Virol. **64:**2181–2186.
4. **Bernstein, P., and J. Ross.** 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. **14:**373–377.
5. **Brawerman, G.** 1989. mRNA decay: finding the right targets. Cell **57:**9–10.
6. **Buchkovich, K., L. A. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
7. **Chackalaparampil, I., and D. Shalloway.** 1988. Altered phosphorylation and activation of pp60$^{c\text{-}src}$ during fibroblast mitosis. Cell **52:**801–810.
8. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the Rb protein. Cell **65:**1053–1061.
9. **Cleveland, D. W.** 1988. Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. Trends Biochem. Sci. **13:**339–343.
10. **Cleveland, D. W., and T. J. Yen.** 1989. Multiple determinants of eukaryotic mRNA stability. New Biol. **1:**121–126.
11. **Dawid, I. B., and P. K. Wellauer.** 1976. A reinvestigation of the 5′ to 3′ polarity in 40S ribosomal RNA precursor of Xenopus laevis. Cell **8:**443–448.
12. **Elliott, G. D., and D. M. Meredith.** 1992. The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. J. Gen. Virol. **73:**723–726.
13. **Fenwick, M. L., and J. Clark.** 1982. Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. J. Gen. Virol. **61:**121–125.
14. **Fenwick, M. L., and R. D. Everett.** 1990. Transfer of UL41, the gene controlling virion-associated cell shutoff, between strains of herpes simplex virus. J. Gen. Virol. **71:**411–418.
15. **Fenwick, M. L., and M. M. McMenamin.** 1984. Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. J. Gen. Virol. **65:**1225–1228.
16. **Fenwick, M. L., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting rapid inhibition of host DNA and protein synthesis. J. Virol. **29:**825–827.
17. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41:**37–51.
18. **Frink, R. J., K. P. Anderson, and E. K. Wagner.** 1981. Herpes simplex virus type 1 *Hin*dIII fragment L encodes spliced and complementary mRNA species. J. Virol. **39:**559–572.
19. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38:**50–58.
20. **Guo, L.-H., R. P. Stepien, J. Y. Rso, R. Brousseau, S. Narang, D. Y. Thomas, and R. Wu.** 1984. Synthesis of human insulin gene. VIII. Construction of expression vectors for fused proinsulin production in Escherichia coli. Gene **29:**251–254.
21. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Hentze, M. W.** 1991. Determinants and regulation of cytoplasmic mRNA stability in eukaryotic cells. Biochim. Biophys. Acta **1090:** 281–292.
23. **Hill, T. M., J. R. Sadler, and J. L. Betz.** 1985. Virion component of herpes simplex virus type 1 KOS interferes with early shutoff of host protein synthesis induced by herpes simplex virus type 2 186. J. Virol. **56:**312–316.
24. **Johnson, D. C., and V. Feenstra.** 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. J. Virol. **61:**2208–2216.
25. **Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow.** 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62:**1347–1354.
26. **Karr, B. M., and G. S. Read.** 1993. Unpublished data.
27. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60$^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.
28. **Kousoulas, K. G., D. J. Bzik, N. A. DeLuca, and S. Person.** 1983. The effect of ammonium chloride and tunicamycin on the glycoprotein content and infectivity of herpes simplex virus type 1. Virology **125:**468–474.
29. **Krikorian, C. R., and G. S. Read.** 1989. Proteins associated with mRNA in cells infected with herpes simplex virus. Biochem. Biophys. Res. Commun. **164:**355–361.
30. **Krikorian, C. R., and G. S. Read.** 1991. An in vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. J. Virol. **65:**112–122.
31. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral

mRNAs. Proc. Natl. Acad. Sci. USA **84:**1926–1930.
32. **Kwong, A. D., and N. Frenkel.** 1989. The herpes simplex virus virion host shutoff function. J. Virol. **63:**4834–4839.
33. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912–921.
34. **Liu, F., and B. Roizman.** 1993. Characterization of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus 1 U_L26 protein. J. Virol. **67:**1300–1309.
35. **Marchetti, M. E., C. A. Smith, and P. A. Schaffer.** 1988. A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. J. Virol. **62:**715–721.
36. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
37. **McLauchlan, J., and J. B. Clements.** 1983. Organization of the herpes simplex virus type 1 transcription unit encoding two early proteins with molecular weights of 140,000 and 40,000. J. Gen. Virol. **64:**997–1006.
38. **McLauchlan, J., A. Phelan, C. Loney, R. M. Sandri-Goldin, and J. B. Clements.** 1992. Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3′ processing. J. Virol. **66:**6939–6945.
39. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans* activation by herpesvirus infection. J. Virol. **52:**522–531.
40. **Oroskar, A. A., and G. S. Read.** 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (*alpha*) mRNAs. J. Virol. **61:**604–606.
41. **Oroskar, A. A., and G. S. Read.** 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J. Virol. **63:**1897–1906.
42. **Pak, A. S., K. Knight, and G. S. Read.** 1993. Unpublished data.
43. **Patterson, M., and G. S. Read.** 1993. Unpublished data.
44. **Peltz, S. W., G. Brewer, P. Bernstein, P. A. Hart, and J. Ross.** 1991. Regulation of mRNA turnover in eukaryotic cells. Crit. Rev. Eukaryotic Gene Expression **1:**99–126.
45. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of α (immediate early) viral polypeptides. J. Virol. **46:**498–512.
46. **Read, G. S., J. A. Sharp, and W. C. Summers.** 1984. In vitro and in vivo transcription initiation sites on the TK-encoding BamHI Q fragment of HSV-1 DNA. Virology **138:**368–372.
47. **Read, G. S., and W. C. Summers.** 1982. In vitro transcription of the thymidine kinase gene of herpes simplex virus. Proc. Natl. Acad. Sci. USA **79:**5215–5219.
48. **Roizman, B., and A. E. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields and D. M. Knipe (ed.), Fields virology. Raven Press, New York.
49. **Ross, J.** 1988. Messenger RNA turnover in eukaryotic cells. Mol. Biol. Med. **5:**1–14.
50. **Sandri-Goldin, R. M., A. L. Goldin, M. Levine, and J. C. Glorioso.** 1981. Method for induction of mutations in physically defined regions of the herpes simplex virus genome. J. Virol. **38:**41–49.
51. **Sandri-Goldin, R. M., and G. E. Mendoza.** 1992. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes Dev. **6:**848–863.
52. **Schek, N., and S. L. Bachenheimer.** 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. **55:**601–610.
53. **Smibert, C. A., J. Danna, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1992. The HSV1 virion host shutoff protein (vhs) forms a complex with VMW65(VP16), abstr. p76. Abstr. 17th Int. Herpesvirus Workshop 1992. Glasgow, Scotland.
54. **Smibert, C. A., D. C. Johnson, and J. R. Smiley.** 1992. Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. J. Gen. Virol. **73:**467–470.
55. **Strom, T., and N. Frenkel.** 1987. Effects of herpes simplex virus on mRNA stability. J. Virol. **61:**2198–2207.
56. **Tso, J. Y., X.-H. Sun, T.-H. Kao, K. S. Knight, and R. Wu.** 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res. **13:**2485–2502.
57. **Yaciuk, P., and E. Moran.** 1991. Analysis with specific polyclonal antiserum indicates that the E1A-associated 300-kDa product is a stable nuclear phosphoprotein that undergoes cell cycle phase-specific modification. Mol. Cell. Biol. **11:**5389–5397.