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Herpes Simplex Virus Mutants Defective in the Virion-Associated Shutoff of Host Polypeptide Synthesis and Exhibiting Abnormal Synthesis of α (Immediate Early) Viral Polypeptides

G. SULLIVAN READ† AND NIZA FRENKEL*

Department of Biology, The University of Chicago, Chicago, Illinois 60637

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Six mutants isolated from herpes simplex virus type 1 were judged to be defective with respect to the virion-associated function acting to rapidly shut off host polypeptide synthesis in herpes simplex virus-infected cells. The mutants were capable of proper entry into the cells, but, unlike the parent wild-type virus, they failed to shut off host polypeptide synthesis in the presence of actinomycin D. They were consequently designated as virion-associated host shutoff (*vhs*) mutants. In the presence of actinomycin D, three of the mutants, *vhs*1, -2, and -3, failed to shut off the host at both 34 and 39°C, whereas *vhs*4, -5, and -6 exhibited a temperature-dependent *vhs* phenotype. Since the mutants were capable of growth at 34°C, it appeared that the *vhs* function was not essential for virus replication in cultured cells. Temperature-shift experiments performed with the *vhs*4 mutant showed that an active *vhs* function was required throughout the shutoff process and that, once established, the translational shutoff could not be reversed. In the absence of actinomycin D, the mutants induced a generalized, secondary shutoff of host translation, which required the synthesis of β (early) or γ (late) viral polypeptide(s). The *vhs* mutants appeared to be defective also with respect to post-transcriptional shutoff of α (immediate early) viral gene expression, since (i) cells infected with mutant viruses overproduced α viral polypeptides, (ii) there was an increased functional stability of α mRNA in the *vhs*1 mutant virus-infected cells, and (iii) superinfection of *vhs*1-infected cells with wild-type virus, in the presence of actinomycin D, resulted in a more pronounced shutoff of α polypeptide synthesis from preformed α mRNA than equivalent superinfection with *vhs*1 virus. The data suggest that the synthesis of α polypeptides in wild-type virus infections is subject to a negative post-transcriptional control involving viral gene product(s) present in infected cell lysates constituting virus stocks. The *vhs*1 mutant and possibly other *vhs* mutants contain a mutation in the gene encoding this function.

The infection of permissive cells with herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) is characterized by the rapid cessation of cellular polypeptide synthesis and the concomitant selective translation of viral mRNA (29). Of relevance to this transition from host to viral gene expression are several previous findings. First, Sydiskis and Roizman (33–35) as well as other investigators (6, 24) have observed that the rapid suppression of host polypeptide synthesis after exposure of cells to HSV coincided with the rapid disaggregation of infected-cell polyribosomes, whereas the subsequent onset of virus-specific polypeptide synthesis was accompanied by the formation of new polyribosomes exhibiting an altered size distribution. The majority, but not all, of the mRNA species associated with polyribosomes at late intervals postinfection were found to be virally encoded (31).

Second, Fenwick and Walker (6) and Nishioka and Silverstein (24) have provided compelling evidence that the initial shutoff of host polypeptide synthesis is mediated by a structural component of the HSV virion. Specifically, the cessation of host polypeptide synthesis has been shown to occur during infections of enucleated cytoplasts (6), after infections of cells with UV light-irradiated virus (6, 24), and during infections of cells in the presence of drugs which precluded the expression of the incoming virus (4, 6, 34). Furthermore, rapid suppression of the

† Present address: Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT 06510.

host has been attained during infections with gradient-purified HSV virions, but not after heat inactivation of the purified virus (24) or after neutralization of crude virus preparations with HSV-specific antiserum (6).

Third, there is evidence to show that the overall shutoff of host polypeptide synthesis during HSV infections is a multistep process (10, 22, 24). Analyses by Nishioka and Silverstein (24) of globin mRNA in infected dimethyl sulfoxide-induced Friend erythroleukemia cells have shown that after the initial disaggregation of host polyribosomes, which was mediated by a virion component, the host mRNA was degraded by a process which required the prior expression of viral genes (22–24). Furthermore, based on their calculations of protein synthetic activity per infected cell polysome, Silverstein and Engelhardt (30) concluded that at late intervals after HSV infection, a substantial fraction of infected Vero cell polyribosomes was not engaged in protein synthetic activity. Thus, the initial shutoff event, involving polyribosome disaggregation, appeared to be followed by secondary perturbations in the protein synthetic apparatus of the infected cell.

Also of possible relevance to these perturbations are recent observations by Fenwick and Walker (7) and by Kennedy et al. (15) that one or two polypeptides associated with the small ribosomal subunit become highly phosphorylated in the course of HSV infections, by a process which depends upon the prior synthesis of infected cell proteins. However, it is not clear at present whether this specific phosphorylation is related to the secondary stages of the host shutoff and whether it reflects a viral gene-mediated activity or a cellular response to the viral infection (15).

Finally, the shutoff of host polypeptide synthesis in HSV-infected cells appears to be determined by the genetic makeup of the infecting virus. Thus, infections of cells with HSV-2 typically result in a more rapid shutoff of the host than equivalent infections with HSV-1 strains (4, 25, 27). Furthermore, using HSV-1 × HSV-2 recombinants, the accelerated shutoff has been mapped to a distinct region of the HSV-2 genome, spanning map coordinates 0.52 to 0.59 (4, 21). The identity of the viral polypeptides encoded within this region and the nature of their function(s) resulting in the shutoff of the host and selective translation of viral gene products are, however, unknown.

In this communication we report the isolation and initial characterization of several HSV mutants which exhibit a defective virion-associated host shutoff (*vhs*) function. Our data show that the expression of the *vhs* function is not essential for virus replication in HEp-2 or Vero cells and that the second stage of shutoff of host polypeptide synthesis can take place in the absence of appreciable virion-associated host shutoff activity and is mediated by a β (early) or γ (late) viral gene product(s). Finally, the data also provide evidence for the existence of a viral activity which regulates the functional stability of viral mRNAs encoding the α (immediate early) viral polypeptides. This activity is present in crude virus preparations and may therefore reflect the function of a structural component of the HSV virion. At least one of the *vhs* mutants appears to be defective with respect to this function, resulting in an increased functional stability of α mRNAs.

MATERIALS AND METHODS

Cells and virus. Vero and HEp-2 cells were obtained from the American Type Culture Collection. The HSV-1 strain KOS, originally isolated by K. O. Smith, Baylor University, Houston, Tex., was obtained from Bernard Roizman, University of Chicago, Chicago, Ill. Wild-type (wt) and mutant viruses were plaque purified three times in Vero cells at 34°C. Virus from the third plaque was propagated into working stocks by three sequential passages at low multiplicity of infection (MOI) (<0.01 PFU per cell).

Isolation of mutant viruses. To prepare the mutagenized virus stocks, Vero cells were infected at 34°C with 0.1 PFU of the wt virus per cell. After virus absorption for 1 h at 34°C, incubation was continued at 34°C for an additional 32 h in the presence of 0.25 or 5.0 μg of 5-bromodeoxyuridine per ml. The addition of the drug at these concentrations reduced infectious virus yields to 1.8 and 0.012%, respectively, of the virus yield in equivalent cultures infected in the absence of 5-bromodeoxyuridine. The resultant mutagenized virus stocks were used to infect Vero cells in 96-well trays (3×10^4 cells per well), at 34°C and at dilutions such that 20 to 80% of the wells became infected. After 3 to 4 days of incubation, the infected cell cultures in the trays were subjected to three cycles of freeze-thawing and were then replica plated at 39°C to screen for temperature-sensitive mutants. This initial screening step was done in the anticipation that inactivation of the virion-associated host shutoff (*vhs*) would be lethal to the virus. Small stocks of candidate virus mutants were prepared in 24-well culture trays (3×10^5 cells per well).

In the second step of the screening procedure, we employed the assay described by Fenwick et al. (4) to identify potential mutants exhibiting reduced *vhs* capacity. Specifically, half of each of the small (24-well) virus stocks was used to infect 3×10^5 Vero cells at 39°C in the presence of 5 μg of actinomycin D per ml. Similar well cultures were also infected with wt virus or were mock infected. After 5 h of incubation, the infected cell cultures were labeled for 1 h with ^{14}C-amino acids, and the total amount of radioactivity incorporated in trichloroacetic acid (TCA)-precipitable material was determined. In cultures infected with wt virus, the rate of amino acid incorporation was approximately 25% of that in mock-infected cultures. Mutant viruses, for which the amount of TCA-precipitable radioactivity was approximately equal to the

mock-infected level, were selected for further analyses. Each of these candidate mutants was retested for *vhs* activity during infections performed at an MOI of 40 PFU per cell, at both 34 and 39°C. The virus mutants, which in these tests exhibited a reduced *vhs* capacity, were plaque purified and propagated into working stocks as described above. Mutant *vhs*1 was isolated from a stock of virus which survived mutagenesis with 5 μg of 5-bromodeoxyuridine per ml. Mutant *vhs*6 represented 1 of 254 surviving progeny of a stock mutagenized with 0.25 μg of the drug per ml. Mutants *vhs*2, -3, -4, and -5 were all isolated from a third mutagenized stock treated with 0.25 μg of 5-bromodeoxyuridine per ml and represented 4 out of 323 surviving progeny which were tested.

Analyses of proteins synthesized in infected and mock-infected cells. For protein analysis, infected Vero cell cultures (3×10^5 cells per culture) were incubated in 199 maintenance medium (supplemented with 1% calf serum) up to the time of labeling. Before labelling, the cell monolayers were rinsed twice with maintenance medium containing 1/10 the usual concentration of isoleucine, leucine, and valine, and then labeled by incubation in medium containing 2 to 8 μCi each of ^{14}C-labeled isoleucine, leucine, and valine per ml. At the end of the labeling period, the cells were harvested by a rapid rinse of the cell monolayers with ice-cold phosphate-buffered saline, followed by the addition of lysis buffer (50 mM Tris, pH 7.0, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 3% sucrose). The resultant cell extracts were sonicated for 30 s and heated to 100°C for 2 min, and portions were either precipitated with TCA or loaded onto SDS-polyacrylamide gels. When indicated, actinomycin D was used at 5 μg/ml, cycloheximide at 50 μg/ml, canavanine at 500 μg/ml, and phosphonoacetate (PAA) at 300 μg/ml. For reversal of a cycloheximide block, the infected cells were washed three times and then overlaid with maintenance medium lacking cycloheximide. In experiments in which the cycloheximide reversal was done in the presence of actinomycin D and in the superinfection experiment, actinomycin D (5 μg/ml) was added to the cultures 20 min before the removal of the cycloheximide. SDS-polyacrylamide gel electrophoresis was done as described by Morse et al. (21) except that the separation gel consisted of 9.25% acrylamide cross-linked with *N*,*N*-diallyltartardiamide, whereas the stacker gel contained 3% acrylamide cross-linked with *N*,*N*′-methylenebisacrylamide. In both stacker and separation gels, the ratio of cross-linker to acrylamide was 1:37.5 (wt/wt).

RESULTS

Inhibition of host polypeptide synthesis during wt virus infections. The screening assay which we used to identify mutants exhibiting a defective virion-associated host shutoff function was based upon the observation by Fenwick et al. (4) that infection of cells with HSV in the presence of actinomycin D resulted in rapid suppression of amino acid incorporation into acid-insoluble material. To test the features of this assay, replicate Vero cell cultures were either exposed to uninfected cell lysates (mock infected) or were infected with different MOI of the HSV-1 (KOS) virus. The cells were incubated from the onset of infection at either 34 or 39°C in the presence of 5 μg of actinomycin D per ml. Figure 1 shows the relative incorporation of ^{14}C-labeled amino acids into TCA-precipitable material during 1-h pulses of infected and mock-infected cells. As seen in the figure (insert), the mock-infected cells continued to synthesize proteins for several hours after the addition of the drug, albeit at decreasing rates. In contrast, exposure of the cells to wt virus resulted in the rapid and pronounced suppression of amino acid incorporation into TCA-precipitable material at both 34 and 39°C, with the observed shutoff being somewhat more complete in the course of

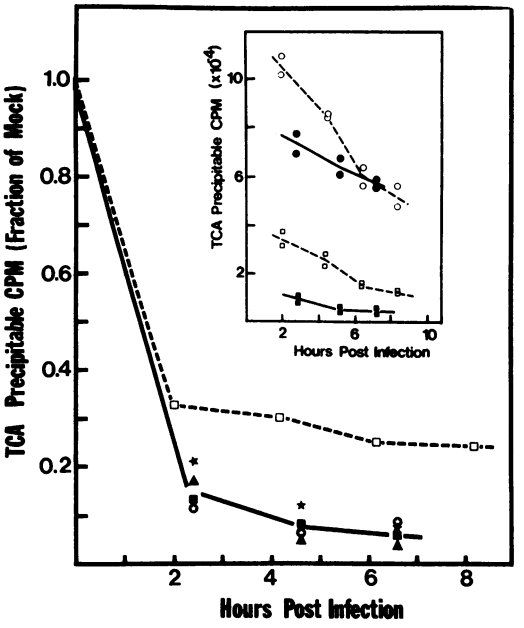

FIG. 1. Virion-associated shutoff of host polypeptide synthesis by wt HSV-1. Replicate Vero cell cultures (3×10^5 cells per culture) were infected with wt virus in the presence of 5 μg of actinomycin D per ml or were mock infected in the presence of the drug by using a lysate from uninfected Vero cells. At various intervals postinfection, duplicate cultures were labeled with ^{14}C-amino acids for 1 h, harvested, and processed for TCA precipitation. Cells were infected with 110 PFU per cell at 34°C (✪), 55 PFU per cell at 34°C (■), 25 PFU per cell at 34°C (▲), 10 PFU per cell at 34°C (★), or 55 PFU per cell at 39°C (□). Data points denote the ratios between the TCA-precipitable counts in the infected cell cultures and the corresponding mock-infected cell cultures. The points are plotted at the midpoints of the labeling intervals. Each point represents the average value of measurements of two separate cultures. The inset shows the total TCA-precipitable counts per minute for the cultures which were mock infected at 39 (○) or 34°C (●) or infected with 55 PFU of wt virus per cell at 39 (□) or 34°C (■).

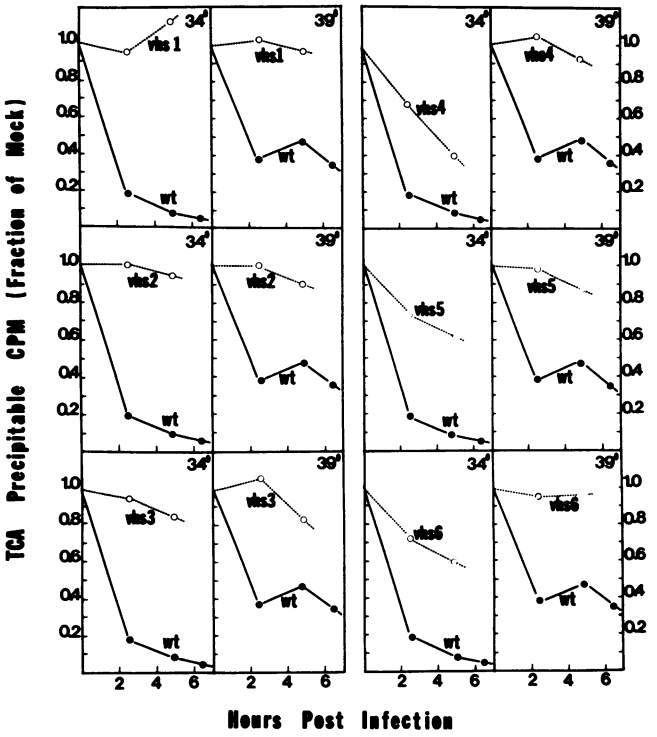

FIG. 2. Virion-associated host shutoff by the mutants. Replicate Vero cell cultures were mock-infected or infected with 40 PFU of the wt and *vhs* mutant viruses per cell, in the presence of 5 μg of actinomycin D per ml. After incubation at 34 and 39°C, duplicate cultures were labeled with ^{14}C-amino acids for 1-h intervals, harvested, and processed for TCA precipitation. Data are expressed as in Fig. 1. Each point is based on measurements of two separate cultures.

the 34°C infections. Both the rate and extent of suppression of polypeptide synthesis appeared to be independent of the MOI used in the range of 10 to 110 PFU per cell. Because no viral polypeptide synthesis could be detected in cells infected in the presence of 5 μg of actinomycin D per ml (see below), the rapid decline in the rate of cellular protein synthesis could be attributed to a structural component of the infecting virions, in accordance with previously published reports (6, 24).

***vhs* activity of the mutants.** Using the screening assays described above, we identified six mutants which were derived from three separate mutagenized virus stocks as candidate *vhs* mutants and selected these for additional study. The patterns of ^{14}C-labeled amino acid incorporation during 1-h pulses of cells infected with these mutants at 34 and 39°C in the presence of actinomycin D are shown in Fig. 2. Three of the mutants, namely *vhs*1, -2, and -3, showed reduced ability to shut off host polypeptide synthesis at both temperatures. The other three mutants, *vhs*4, -5, and -6, exhibited a temperature-dependent host shutoff phenotype in that they induced minimal shutoff at 39°C and variable degrees of host polypeptide shutoff at 34°C.

To determine whether the variable *vhs* phenotypes reflected differential effects on the synthesis of specific subsets of host polypeptides, replicate cell cultures were either mock infected or were infected with 40 PFU per cell of the wt or *vhs*1, -4, -5, and -6 mutant viruses. After 6.5 h of incubation at 34 or 39°C in the presence of 5 μg of actinomycin D per ml, the cells were pulse-labeled for 1 h with ^{14}C-labeled amino acids, and the resultant labeled polypeptides were analyzed by electrophoresis in SDS-polyacrylamide gels. These analyses are shown in Fig. 3 and can be summarized as follows. (i) In accordance with the results of the TCA precipitation assays (Fig. 1, insert), the addition of actinomycin D to the mock-infected cultures resulted in a partial reduction in the rate of protein synthesis. With the exception of two polypeptides, 92 × 10^3 and 72 × 10^3 in molecular weight, the addition of the drug affected various cell proteins to approximately the same extent. The 92,000- and 72,000-molecular-weight polypeptides resembled previously described heat-

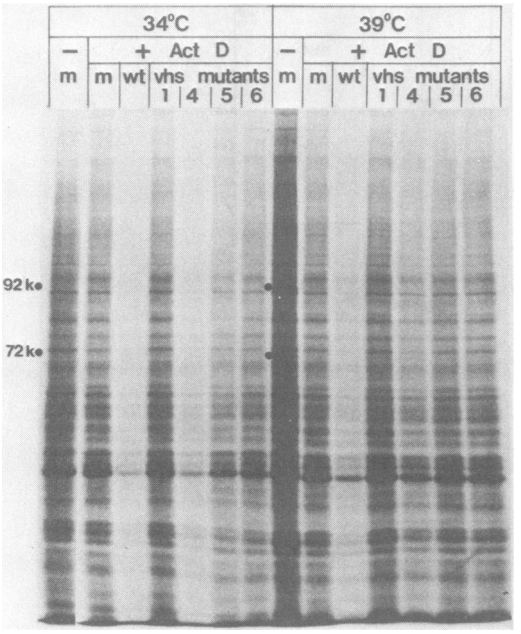

FIG. 3. Virion-associated host shutoff of the wt and mutant viruses. Vero cells were mock infected (m) in the presence (+Act D) or absence (−Act D) of actinomycin D or infected in the presence of the drug with the wt virus or the *vhs*1, -4, -5, or -6 mutant viruses. Infections were at 40 PFU per cell. After incubation at 34 or 39°C, the cells were labeled with ^{14}C amino acids for 1 h, harvested, and processed for electrophoresis in SDS-polyacrylamide gels. The labeled proteins were visualized by autoradiography. The two polypeptides, 92 × 10^3 and 72 × 10^3 in molecular weight (92k and 72k, respectively), were induced in response to the high-temperature incubation.

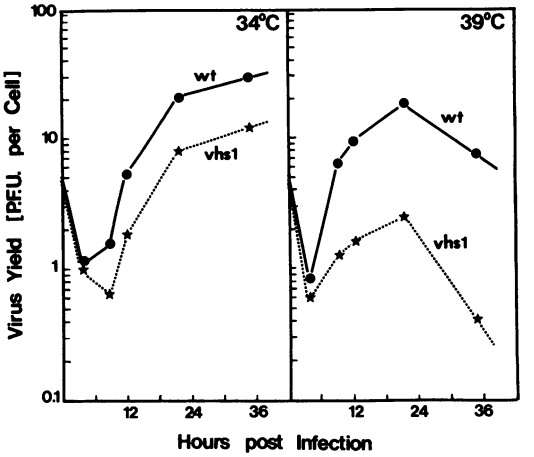

FIG. 4. Single-step growth curves for the wt virus and *vhs*1. Replicate Vero cell cultures were infected with 5 PFU of wt or *vhs*1 virus at 34 or 39°C. At various times postinfection, the infected cells were harvested and the progeny virus was titered.

shock proteins (1, 14) in that they appeared to be induced at the elevated temperature, and their synthesis was dependent upon de novo transcription after incubation of the cells at 39°C. (ii) No viral polypeptides could be detected in either wt or mutant virus-infected cells. (iii) Infection of the cells with wt virus in the presence of actinomycin D resulted in the marked suppression of host polypeptide synthesis, with no apparent selectivity in the shutoff of particular cellular proteins. In contrast, the cells which were infected with the *vhs*1 mutant virus at either temperature continued to synthesize host polypeptides at rates similar to those of mock-infected cells. The other *vhs* mutants exhibited, to various degrees, a temperature-sensitive *vhs* phenotype, with *vhs*4 showing a strong temperature dependence in its ability to shut off the host.

Growth properties of the mutants. The finding of HSV mutants which exhibited a defective *vhs* function at 34°C suggested that the virion-associated host shutoff function was not essential for virus replication in HEp-2 and Vero cells in culture, because all of the mutants yielded relatively high-titer working stocks at that temperature. We therefore examined more closely the growth properties of the mutant viruses.

Single-step growth curves for the wt and the *vhs*1 mutant viruses at 34 and 39°C are compared in Fig. 4. As was noted above, in the presence of

TABLE 1. Growth properties of the *vhs* mutants

Virus	Expression of *vhs* at:		Relative EOP (39°C/34°C)[a]	Relative adsorption (39°C/34°C)[b]
	34°C	39°C		
wt	+	+	1.00	1.05
*vhs*1	−	−	0.56	1.03
*vhs*2	−	−	$<5.5 \times 10^{-8}$	0.92
*vhs*3	−	−	3.4×10^{-3}	ND[c]
*vhs*4	+	−	4.3×10^{-6}	0.75
*vhs*5	Partial	−	1.12	1.27
*vhs*6	Partial	−	2×10^{-4}	1.00

[a] Determined from virus titrations on Vero cells at 34 and 39°C. Relative EOP denotes the number of plaques at 39°C divided by the number of plaques at 34°C.

[b] Virus adsorption for 1 h at 39 or 34°C was followed by the washing of cells with medium containing human gamma globulin and subsequent incubation of the infected cells at 34°C, in the presence of human gamma globulin, until plaques developed. The relative adsorption is defined as the number of plaques after adsorption at 39°C divided by the number of plaques after adsorption at 34°C. Control experiments involving the addition of human gamma globulin at the onset (0 time) of 34°C infections with the wt and *vhs* mutants resulted in 84 to 91% reduction in the number of plaques.

[c] ND, Not determined.

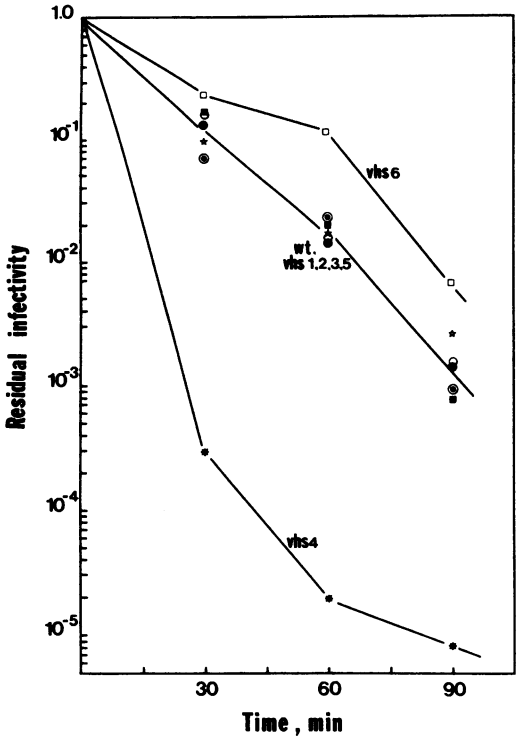

FIG. 5. Heat stability of wt and mutant viruses. Infected-cell lysates constituting stocks of wt virus (●), *vhs*1 (○), *vhs*2 (☆), *vhs*3 (◉), *vhs*4 (✱), *vhs*5 (■), and *vhs*6 (□) were diluted to equal concentrations (plaque-forming units per milliliter) and incubated at 45°C. After different incubation periods, the residual infectivity was determined by titration on Vero cells. Residual infectivity denotes plaque-forming units per milliliter incubation divided by the plaque-forming units before incubation.

actinomycin D this mutant failed to suppress host polypeptide synthesis at either temperature. As can be seen in the figure, although replication of the mutant virus at 34°C was delayed relative to wt virus replication, the yield of *vhs*1 virus, as measured at 34 h postinfection, was only threefold lower than that from the corresponding wt virus-infected cells. Mutant virus replication was also found to be delayed in the course of the 39°C infections. However, final (34 h) mutant virus recovery was significantly (20-fold) lower than that in the corresponding wt virus infections.

The relative plaquing efficiencies of *vhs*1 and the other *vhs* mutants at 34 and 39°C are shown in Table 1. As can be seen in the table, although four of the mutants were temperature sensitive in their abilities to form plaques, the temperature-dependent growth phenotypes did not correlate with the temperature dependence of the *vhs* function. Furthermore, as can be seen in Table 1, in no case did the temperature-sensitive plaquing efficiency reflect an inability of the mutant viruses to properly adsorb to cells at the elevated temperatures.

Finally, because the *vhs* function has been attributed to a structural component of the HSV virion, we assayed the effect of the *vhs* mutations upon virion stability during incubation of the infecting virus inoculum at 45°C before infection of the cells. Five of the six *vhs* mutants exhibited a virion lability at 45°C which was similar to that of wt virus, whereas the sixth mutant, *vhs*4, was more rapidly inactivated (Fig. 5). Taken together, the experiments described above failed to reveal any consistent and significant effect of the *vhs* mutations upon various growth parameters of the *vhs* mutants.

Reversibility of the virion-associated shutoff. The availability of the *vhs*4 mutant, which exhibited a temperature-dependent *vhs* phenotype, made possible temperature-shift experiments designed to test the reversibility of the virion-

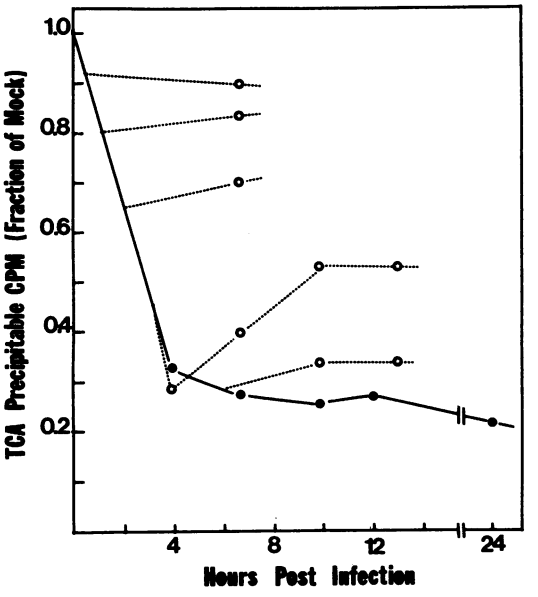

FIG. 6. Temperature-shift experiments with *vhs*4. Vero cells were mock infected or infected with 30 PFU of *vhs*4 per cell in the presence of 5 μg of actinomycin D per ml. Cultures were either incubated continuously at 34°C (●) or were incubated at 34°C for various lengths of time and then shifted to 39°C (✪). For each time point, duplicate cultures were labeled with ^{14}C-amino acids for 1 h, harvested, and processed for TCA precipitation. Data are expressed as the ratios of TCA-precipitable counts in infected cultures to those in mock-infected cultures subjected to similar temperature shifts. Data points are plotted at the midpoints of the labeling intervals. Dashed lines are connected to the solid 34°C curve at the times corresponding to the temperature shift.

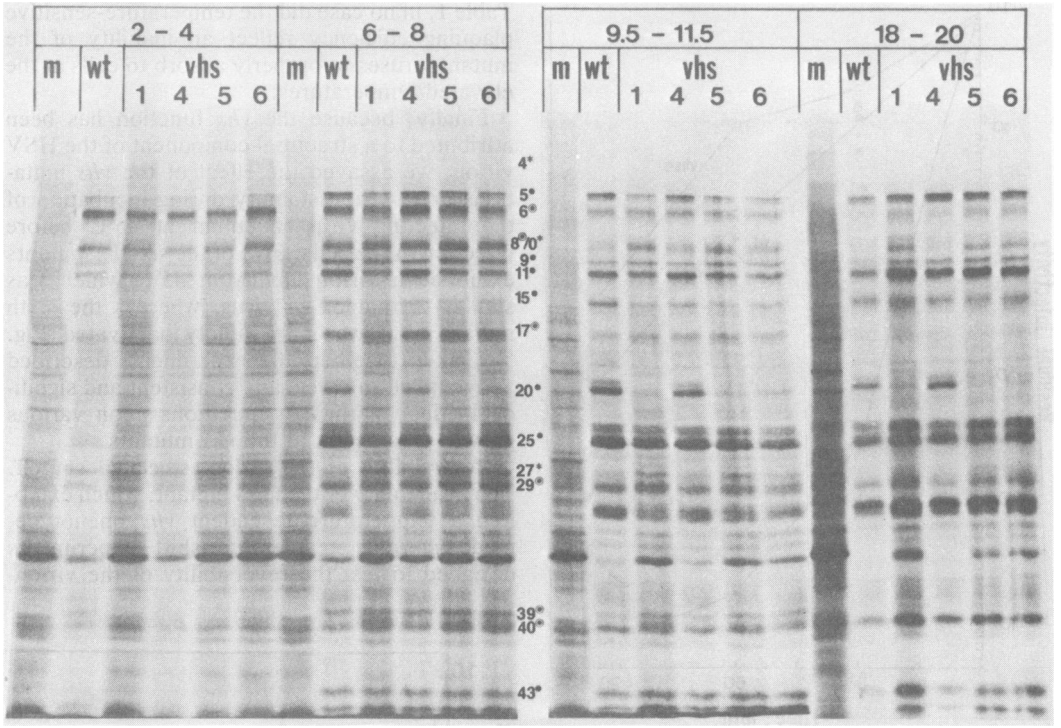

FIG. 7. Polypeptides synthesized during 34°C infections with wt and mutant viruses. Vero cells were mock infected (m) or infected with wt virus of *vhs*1, -4, -5, or -6. Infections were at 40 PFU per cell. The cultures were incubated at 34°C and labeled with ^{14}C-amino acids at the times shown (top row, in hours). Lysates from equal numbers of cells were electrophoresed in SDS-polyacrylamide gels, and the labeled polypeptides were visualized by autoradiography. Viral ICPs are designated (center) as α (✱), β (◉), or γ (●), according to Morse et al. (21).

associated shutoff of host polypeptide synthesis. Specifically, Vero cell cultures were infected with *vhs*4 in the presence of actinomycin D and incubated at 34°C. At different intervals postinfection, a number of the infected cell cultures were transferred from 34 to 39°C and were further incubated for various lengths of time at the elevated temperature. A parallel set of control mock-infected cells was similarly incubated at the corresponding temperatures. After a ^{14}C-labeled amino acid pulse during the last hour of incubation, matching sets of mock-infected and infected cell cultures were harvested and processed for TCA precipitation. The results of these experiments are shown in Fig. 6 and revealed that, in each case, after transfer to 39°C, the *vhs*4-infected cells exhibited approximately the same level of shutoff as was attained in the cells before the temperature shift. Because a brief incubation of the *vhs*4-infected cells at 34°C was not sufficient to fully overcome the block in host shutoff activity, it can be concluded that the gene product affected by the *vhs*4 mutation is continuously required to establish a complete shutoff. It thus appears that this gene product is directly involved in the shutoff process. Furthermore, after the temperature shift (in the presence of actinomycin D), there was no immediate appreciable recovery of host polypeptide synthesis. It can thus be concluded that the function affected in *vhs*4 mediates a process which (in the absence of new RNA synthesis) results in the irreversible inactivation of the ability of the cells to translate host mRNA.

Analyses of polypeptides produced in cells infected with mutant viruses in the absence of actinomycin D. To examine the pattern of viral polypeptide synthesis in cells infected with the *vhs* mutants in the absence of actinomycin D, replicate Vero cell cultures were either mock infected or were infected with 40 PFU of the wt virus and four of the *vhs* mutant viruses (1, 4, 5, and 6) per cell. At different times postinfection at 34 and 39°C, the infected cells were labeled for 2 h with ^{14}C-amino acids, and the labeled polypeptides were analyzed by electrophoresis on SDS-polyacrylamide gels. The results of these analyses (Fig. 7 and 8) can be summarized as follows. (i) The cells which were infected at 34 and 39°C with the mutant viruses synthesized

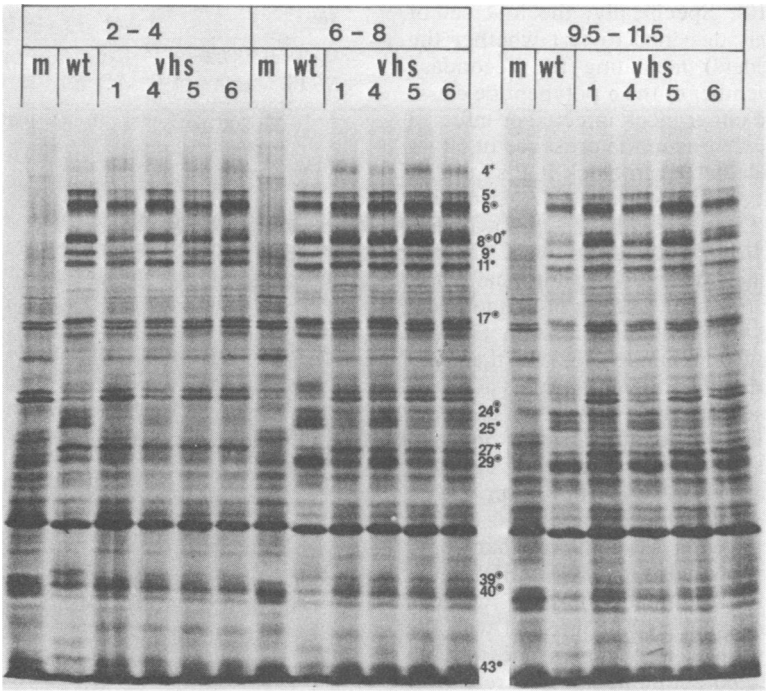

FIG. 8. Polypeptides synthesized during 39°C infections with wt and mutant viruses. Vero cells were infected, labeled, and processed as described in the legend to Fig. 7, except that incubation was at 39°C.

most, if not all, of the polypeptides characteristic of HSV-infected cells. This result shows that all of the mutants were capable of proper entry into the cells and sufficient uncoating to allow transcription of viral DNA. (ii) At 34°C, the overall scheme of viral gene expression in cells infected with *vhs*1, -5, and -6 (all of which exhibited reduced *vhs* activity in the presence of actinomycin D) was somewhat delayed relative to that in infections with wt virus. The synthesis of the α (immediate early) viral polypeptides (in particular, infected-cell polypeptides [ICPs] 0 and 27) continued for a longer time after infection, whereas the production of the β (early) and γ (late) viral polypeptides was slightly delayed. In contrast, the pattern of viral gene expression in cells infected at 34°C with the *vhs*4 mutant (which at 34°C exhibited wt *vhs* activity in the presence of actinomycin D) was indistinguishable from that in wt virus-infected cells. (iii) Infections of cells with all the *vhs* mutants (including *vhs*4) at 39°C resulted in a pronounced overproduction of the α polypeptides, and cells infected with mutant viruses continued to synthesize substantial amounts of these viral proteins (in particular, ICPs 4, 0, and 27) as late as 11 h postinfection, despite ongoing synthesis of the later groups of the viral proteins. The delay in β and γ polypeptide synthesis in the mutant virus 39°C infections appeared to be only transient (it was apparent in the 2- to 4-h labeling interval) and was not as pronounced as in the 34°C infections. (iv) Although all four mutants were defective in their *vhs* function, in the absence of actinomycin D they all exhibit generalized, albeit delayed, shutoff of host polypeptide synthesis. Mutants *vhs*1, -5, and -6 exhibited partial shutoff in the course of both the 34 and 39°C infections, whereas *vhs*4 induced wt levels of host shutoff at 34°C but only delayed and incomplete host shutoff at 39°C. Because, as is shown below, shutoff induced by the mutant viruses was dependent upon polypeptide synthesis after the infection, we shall refer to it as the secondary shutoff.

Requirements for secondary shutoff of host polypeptide synthesis. In the experiments described below, we employed the *vhs*1 mutant to test the requirements for the observed (secondary) suppression of host polypeptide synthesis during mutant virus infections in the absence of actinomycin D. As was shown above, in the presence of the drug, *vhs*1 exhibited no detectable virion-associated shutoff. The experimental protocols employed in this series of studies were based on previous findings regarding the requirements for the synthesis of the three coordinately regulated classes of viral polypeptides α,

β, and γ (9, 10). Specifically, the first set of experiments was designed to test whether the viral polypeptide(s) mediating the secondary shutoff was a member of the α polypeptide class. Vero cells were either mock infected or infected with wt and *vhs*1 virus in the presence of 50 μg of cycloheximide per ml to block ICP synthesis but allow the accumulation of mRNA specifying the α proteins (2, 9, 12, 16, 17, 32, 37). At 5 h postinfection, the drug was removed, and the cultures were incubated for an additional 8 h in the absence or presence of 5 μg of actinomycin D per ml. As was previously shown (9), the reversal of a cycloheximide block in the presence of actinomycin D prevents expression of β and γ viral genes, whereas cells reversed from the cycloheximide block in the absence of added drugs proceed through the timely regulated scheme of viral gene expression. After a ^{14}C-labeled amino acid pulse from 8 to 10 h after cycloheximide reversal, the cells were harvested and processed for electrophoresis in SDS-polyacrylamide gels. Additional Vero cell cultures were either mock infected or infected with the wt and *vhs*1 viruses in the presence of both cycloheximide and actinomycin D to retest for the absence of *vhs* activity in the mutant virus-infected cells. The results of this experiment (Fig. 9) can be summarized as follows. (i) As predicted, no viral polypeptides could be detected in the control cultures infected with either wt or the *vhs*1 mutant virus in the presence of both cycloheximide and actinomycin D. Under these conditions, the wt virus-infected cells exhibited a complete shutoff of cellular protein synthesis (by the *vhs* function), whereas no suppression of host polypeptide synthesis was apparent in the *vhs*1-infected cells. (ii) Both wt and *vhs*1 virus-infected cells synthesized the complete set of viral polypeptides after reversal of the cycloheximide in the absence of actinomycin D. Under these conditions, the *vhs*1-infected cells exhibited secondary shutoff of host polypeptide synthesis. (iii) Only α polypeptides could be detected in the *vhs*1-infected cells, which were incubated in the presence of actinomycin D after the reversal of cycloheximide. At the same time, there was no apparent shutoff of host polypeptide synthesis. Thus, the secondary shutoff was not an α gene function. As predicted, the shutoff of host polypeptides under these conditions was pronounced in the wt virus-infected cells. (iv) Unexpectedly, the *vhs*1 and wt virus infections which were reversed from the cycloheximide block in the presence of actinomycin D differed also with respect to the synthesis of the α proteins. Thus, whereas the *vhs*1-infected cells were efficiently synthesizing α polypeptides 8 h after cycloheximide reversal, the wt virus-infected cells did not produce any detectable viral

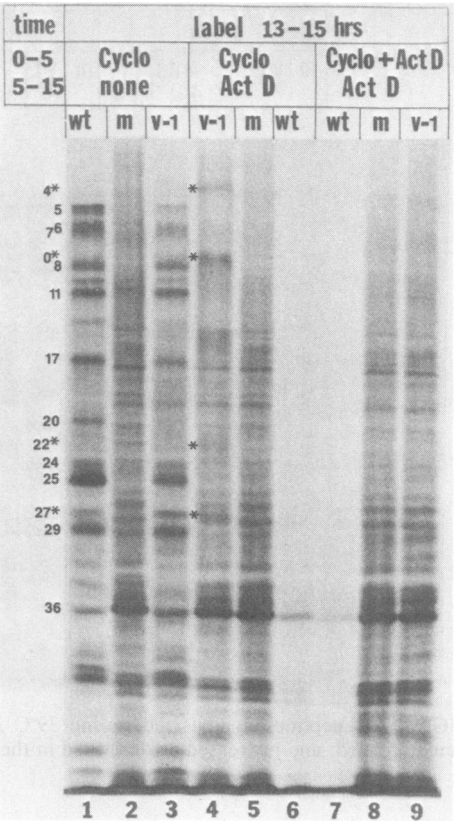

FIG. 9. Polypeptides synthesized in wt and *vhs*1 virus-infected cells after reversal of a cycloheximide block. Vero cells were mock infected (m) or infected with 40 PFU of wt or *vhs*1 (v-1) virus per cell. Incubation was at 34°C in the presence of 50 μg of cycloheximide per ml (lanes 1 through 6) or in the presence of 50 μg of cycloheximide and 5 μg of actinomycin D per ml (lanes 7 through 9). At 5 h postinfection, the cycloheximide was washed out, and incubation was continued for an additional 10 h in the absence (lanes 1 through 3) or presence (lanes 4 through 9) of actinomycin D. All cultures were labeled from 8 to 10 h after the cycloheximide reversal (i.e., from 13 to 15 h postinfection). Lysates from equal numbers of cells were electrophoresed in SDS-polyacrylamide gel, and labeled polypeptides were visualized by autoradiography. ICP designations (left) follow Morse et al. (21). The α polypeptides are marked with asterisks.

polypeptides. This occurred even though wt virus-infected cells synthesized the entire spectrum of viral polypeptides if the cycloheximide was reversed in the absence of actinomycin D. This observation will be pursued in more detail below.

In a second series of experiments, replicate Vero cell cultures were mock infected or were infected with wt or the *vhs*1 mutant in the

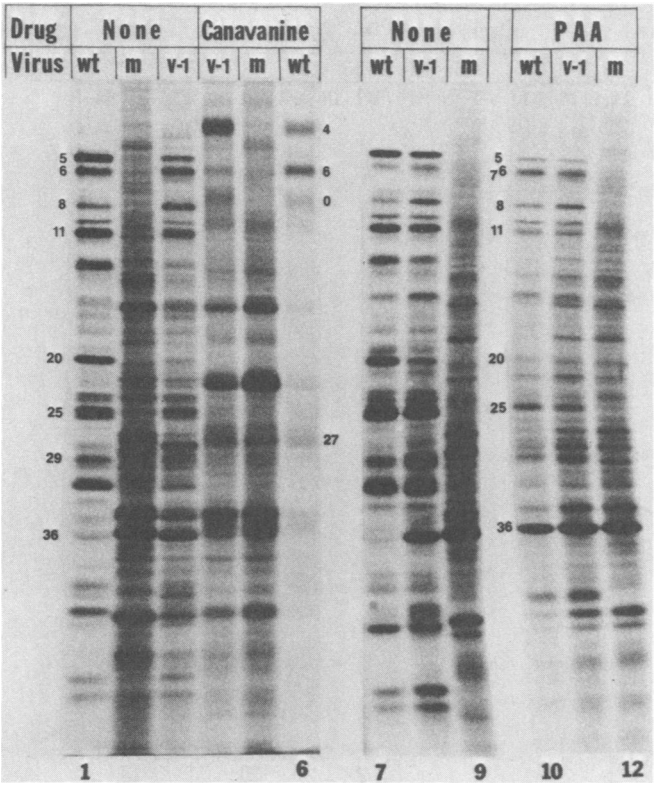

FIG. 10. Polypeptides synthesized during wt and *vhs*1 infections in the presence of canavanine and PAA. (Lanes 1 through 6) HEp-2 cells were mock infected (m) or infected with 20 PFU of wt or *vhs*1 (v-1) virus per cell. Incubation was at 34°C in the absence or presence of 500 μg of canavanine per ml. Cultures were labeled with ^{14}C-amino acids from 12 to 13 h postinfection, harvested, and processed for electrophoresis in SDS-polyacrylamide gel. (Lanes 7 through 12) Vero cells were mock infected or infected with 30 PFU of wt or *vhs*1 virus per cell. Incubation was at 34°C, in the absence or presence of 300 μg of PAA per ml. Cultures were labeled with ^{14}C-amino acids from 13 to 15 h postinfection.

absence of added drugs or in the presence of 500 μg of the arginine analog canavanine per ml. The addition of canavanine to infected cells has been shown to result in the inhibition of the synthesis of many β and γ polypeptides, most likely due to incorporation of this amino acid analog into the immediate early viral polypeptide(s) responsible for turning on synthesis of the later ICPs (10, 25). Additional cultures were mock infected or were infected with the wt and *vhs*1 viruses in the presence of PAA at a concentration (300 μg/ml) known to inhibit viral DNA replication (20). As was previously shown, the addition of PAA or other inhibitors of viral DNA replication results in the reduced synthesis of the majority of γ viral polypeptides and in the complete block in the synthesis of a small subset (γ_2) of the late ICPs (8, 11, 13, 28, 36).

Both canavanine and PAA appeared to inhibit the secondary shutoff, although low levels of shutoff were apparent in both cases (Fig. 10). We conclude on the basis of these experiments that the secondary shutoff of host protein synthesis required the expression of a viral polypeptide(s) whose synthesis was turned on before the synthesis of viral DNA, but which increased after viral DNA replication. Furthermore, in line with the cycloheximide reversal experiment described above, the cells infected with the *vhs*1 virus in the presence of canavanine synthesized substantially higher amounts of the α polypeptides than their wt virus-infected counterparts.

Control of α polypeptide synthesis in wt and *vhs*1 virus-infected cells. The experiments shown in Fig. 7 through 10 revealed unexpected differences between the patterns of α polypeptide synthesis in wt and *vhs*1 virus-infected cells. To examine these differences more closely, we compared the functional stability of mRNA in wt and *vhs*1 virus-infected cells. Specifically, Vero cells were either mock infected or were infected with wt or *vhs*1 virus in the presence of cyclo-

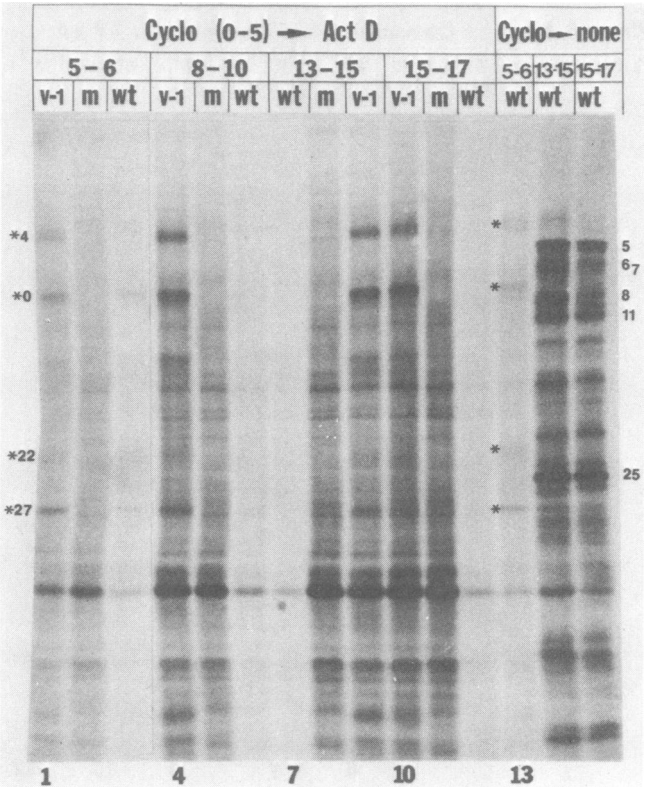

FIG. 11. Functional stability of α mRNA in wt and *vhs*1 infection. Vero cells were mock infected (m) or infected with 40 PFU of wt or *vhs*1 (v-1) virus per cell. Incubation was at 34°C in the presence of cycloheximide. At 5 h postinfection, the cycloheximide was removed from the cultures, and incubation was continued in the presence (lanes 1 through 12) or absence (lanes 13 through 15) of actinomycin D. Cells were labeled with ^{14}C-amino acids during the indicated intervals postinfection. After the radioactive pulse, the cells were harvested, and lysates from equal numbers of cells were electrophoresed in SDS-polyacrylamide gels. The α polypeptides are marked with asterisks.

heximide. At 5 h postinfection, the cycloheximide block was reversed, and the cells were further incubated in the presence of actinomycin D for different lengths of time. Replicate cultures treated in this manner were labeled with ^{14}C-amino acids at 5 to 6, 8 to 10, 13 to 15, and 15 to 17 h postinfection (i.e., 0 to 1, 3 to 5, 8 to 10, and 10 to 12 h after cycloheximide reversal, respectively). Control wt virus-infected cells in which the cycloheximide was reversed in the absence of actinomycin D were similarly labeled from 5 to 6, 13 to 15, and 15 to 17 h postinfection (i.e., 0 to 1, 8 to 10, and 10 to 12 h postreversal, respectively). At the end of these pulses the infected cells were harvested and processed for electrophoresis in SDS-polyacrylamide gels. The results of these analyses are shown in Fig. 11. As expected, when the cycloheximide block was reversed in the presence of actinomycin D, no shutoff of host polypeptide synthesis was apparent in the *vhs*1-infected cell cultures, whereas wt virus infections resulted in complete shutoff of the host. Although both the *vhs*1 and wt virus-infected cells synthesized α polypeptides from 0 to 1 h after cycloheximide reversal, the level of α ICP synthesis was significantly higher in the mutant virus infections. Furthermore, *vhs*1-infected cells continued to synthesize high levels of both α and host polypeptides for at least 10 h after the reversal of the drug. In contrast, by 3 h after reversal of the cycloheximide block, wt virus-infected cells had virtually ceased to make detectable amounts of α proteins. Because in these experiments the synthesis of new α RNA was blocked by the addition of actinomycin D (at the point of cycloheximide reversal), the residual protein synthetic activity at different times after cycloheximide reversal must reflect the functional stability of the α mRNA transcribed before the reversal of the drug. These results, therefore, suggest that the functional stability of α mRNA is controlled, at

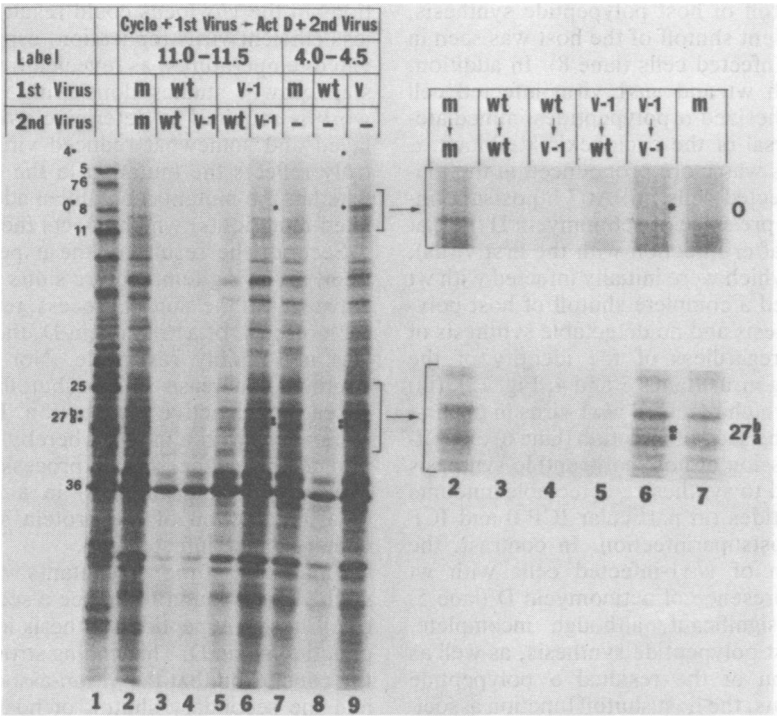

FIG. 12. Functional stability of α mRNA after superinfection with wt and *vhs*1 viruses. HEp-2-cells were mock infected (lanes 2 and 7), infected with 30 PFU of wt virus per cell (lanes 3, 4, and 8), or infected with 30 PFU of *vhs*1 virus per cell (lanes 5, 6, and 9). After 4 h of incubation at 34°C in the presence of cycloheximide, the inocula were removed, and the cells were either labeled with ^{14}C-amino acids in the presence of actinomycin D from 4.0 to 4.5 h postinfection (lanes 7 through 9) or mock infected (lane 2) or superinfected with 30 PFU of wt virus (lanes 3 and 5) or *vhs*1 virus (lanes 4 and 6) per cell. All superinfections were in the presence of actinomycin D. The superinfected cultures were labeled with ^{14}C-amino acids from 7 to 7.5 h after infection (i.e., 11 to 11.5 h after the first infection). After the radioactive pulses, the cells were harvested, and lysates from equal numbers of cells were electrophoresed in SDS-polyacrylamide gels. Lane 1 contains lysate from an unrelated infected cell culture serving as a gel marker. The gel portions to the right show the marked areas of the autoradiograms magnified and printed at adequate intensities to more clearly show the ICP 0 and the a and b forms of ICP 27 (21).

least in part, by either an α gene product or by a component present in the virus inoculum and that *vhs*1 contains a mutation in the gene encoding this protein.

The experiment shown in Fig. 12 was designed to further clarify this point. Specifically, to test whether superinfecting wt virus preparations contained a function which destabilized the expression of preformed α mRNA, three HEp-2 cell cultures were infected in the presence of cycloheximide with 30 PFU of wt virus per cell, and three additional cultures were similarly infected with *vhs*1 virus. At 4 h postinfection, the cycloheximide was withdrawn from all of the infected cell cultures. One of the wt virus-infected cell cultures was superinfected in the presence of actinomycin D with 30 PFU per cell of wt virus, whereas a second wt virus-infected culture was superinfected at the same MOI and in the presence of actinomycin D with *vhs*1 virus. The third culture was not exposed to added virus but was labeled with ^{14}C-amino acids in the presence of actinomycin D from 0 to 0.5 h after cycloheximide reversal. Similarly, of the three *vhs*1 virus-infected cell cultures, one was labeled from 0 to 0.5 h after cycloheximide reversal in the presence of actinomycin D, whereas the second and third cultures were superinfected in the presence of actinomycin D with wt and *vhs*1 viruses, respectively. All of the superinfected cell cultures were labeled with ^{14}C-amino acids from 7 to 7.5 h after superinfection in the presence of actinomycin D. A control mock-infected culture was similarly prepared in the presence of the respective drugs. The results of these analyses are shown in Fig. 12 and revealed the following. (i) As expected, the *vhs*1-infected cells which were labeled from 0 to 0.5 h after cycloheximide reversal (i.e., at 4 to 4.5 h postinfection; lane 9, Fig. 12) exhibited no

apparent shutoff of host polypeptide synthesis, whereas efficient shutoff of the host was seen in the wt virus-infected cells (lane 8). In addition, although both wt and *vhs*1 virus-infected cell cultures synthesized α polypeptides immediately after reversal of the cycloheximide block, α ICP synthesis was more pronounced in the mutant virus-infected cells. (ii) At 7 h postsuperinfection in the presence of actinomycin D (i.e., at 11 to 11.5 h after infection with the first virus), the cultures which were initially infected with wt virus exhibited a complete shutoff of host polypeptide synthesis and no detectable synthesis of α proteins, regardless of the identity of the superinfecting virus (lanes 3 and 4, Fig. 12). (iii) The culture which received *vhs*1 virus in the first as well as in the second infection (lane 6) exhibited no suppression of host polypeptide synthesis and continued to synthesize detectable amounts of α polypeptides (in particular ICP 0 and ICP 27) at 7 h postsuperinfection. In contrast, the superinfection of *vhs*1-infected cells with wt virus in the presence of actinomycin D (lane 5) resulted in significant, although incomplete, shutoff of host polypeptide synthesis, as well as in suppression of the residual α polypeptide synthesis. Thus, the host shutoff function associated with the superinfecting wt virions appeared to be a dominant trait. Furthermore, in the presence of actinomycin D, the superinfecting wt virus was capable of destabilizing the expression of preformed α mRNA, whereas the superinfecting *vhs*1 mutant virus appeared to be defective with respect to this capacity.

DISCUSSION

Shutoff of host polypeptide synthesis. We have described in this paper the isolation and initial characterization of six mutants derived from the KOS strain of HSV-1. We have concluded that these mutants were defective with respect to the virion-associated host shutoff function because, unlike their wt parent virus, they failed to suppress the synthesis of host polypeptides during infections in the presence of actinomycin D. This failure to rapidly turn off the translation of host mRNA did not reflect a decreased ability to either adsorb or penetrate into the cells because, in the absence of the drug, all of the mutants induced the synthesis of viral polypeptides.

There are several conclusions which can be drawn from our studies regarding the HSV-encoded host shutoff function. First, because our studies have failed to reveal a correlation between the *vhs* phenotype and the ability of the mutants to form plaques, it can be concluded that the virion-associated host shutoff is not an obligatory requirement for virus replication in cultured HEp-2 and Vero cells. However, mutations in the *vhs* locus could result in somewhat less efficient virus replication, especially at elevated temperatures, as revealed from the single-step growth studies done with *vhs*1. Further work is required to determine whether the delayed and somewhat reduced virus replication truly reflects the mutation in the *vhs* locus, or whether the mutants contain an additional unrelated mutation(s) which affects their growth.

Second, the results of the experiments with *vhs*4, involving temperature shifts during different stages of the shutoff process, revealed that in the presence of actinomycin D, the *vhs* process was not rapidly reversible. Nor was there a continued increase in the shutoff level in the absence of an active *vhs* function. This pattern is compatible with a model whereby the *vhs* function mediates the shutoff process by inducing irreversible modification(s) in a gradually increasing fraction of the protein synthetic machinery of the infected cell.

Finally, all of the *vhs* mutants which we have studied were found to induce a secondary shutoff of host polypeptide synthesis in the absence of actinomycin D. This finding strongly supports the conclusion that the virion-associated shutoff and the secondary shutoff of host polypeptide synthesis are mediated by distinct viral gene products.

Shutoff of α polypeptide synthesis. Our studies have unexpectedly revealed differences between the wt and *vhs* mutant viruses with respect to α polypeptide synthesis. Specifically, the α polypeptides were overproduced in cells infected with the *vhs* mutants in the absence of drugs (most notably at 39°C); after cycloheximide reversal in the presence of actinomycin D, the synthesis of the α polypeptides persisted longer in *vhs*1-infected cells than in their wt virus-infected counterparts. Furthermore, α polypeptides were overproduced in cells infected with *vhs*1 virus in the presence of canavanine. Finally, the decay of α polypeptide translation from preformed mRNA was more pronounced after superinfection of *vhs*1-infected cells with wt virus, in the presence of actinomycin D, than after similar superinfection with the *vhs*1 mutant.

Taken together, these results led us to conclude that (i) the translation of α mRNA was negatively regulated by an activity which was associated with crude infected-cell lysates constituting virus stocks, and (ii) the *vhs*1 mutant was defective with respect to this post-transcriptional regulatory activity.

Although there is currently no direct evidence to show that the *vhs* function and the destabilization of α mRNA translation are both mediated by the same virion-associated polypeptide, it is attractive to propose that the two functions

indeed may reflect the activity of a single viral gene product. First, both functions appear to act by destabilizing the translation of preformed mRNA. Second, both functions appear to be associated with the virus inoculum. Third, four different mutants, selected from three separate mutagenized stocks on the basis of their inability to turn off host polypeptide synthesis in the presence of actinomycin D, exhibited abnormal patterns of α polypeptide synthesis in the absence of the drug. Furthermore, the *vhs*4 mutant possessed a temperature-dependent phenotype in both respects. Finally, previous reports by other investigators have revealed that HSV-1 and HSV-2 exhibit parallel differences with respect to both activities. Thus, HSV-2 infections have been reported to result in an accelerated host shutoff (4, 21, 25, 27), as well as a reduced functional stability of mRNA during cycloheximide reversal (27) or during canavanine treatment (25).

Although further work is required to unambiguously determine the relationship between the virion-associated host shutoff and the destabilization of α mRNA translation, it is of interest to examine their relatedness as part of a more generalized model, based upon previous observations regarding the control of α gene expression in HSV-infected cells. Specifically, by this model the HSV virions contain both positive and negative regulatory effectors operating in the expression of α genes. The positive effector acts to induce α gene transcription. Its existence in infected-cell lysates constituting virus stocks has recently been proposed by Post et al. (26) and Mackem and Roizman (18, 19) on the basis of their analyses of thymidine kinase induction after superinfection of transformed cells carrying a modified thymidine kinase gene constructed to contain the α regulatory promoter sequences. The negative effector might correspond to the *vhs* function which, by the model, can interact with both host and α mRNA (or polyribosomes) to suppress their translational activity. Due to the presence of limiting amounts of the *vhs* function in HSV-1-infected cells or due to a relatively low rate constant for the interaction of the *vhs* protein with polyribosomal complexes, this suppressive interaction could be gradual, directed initially at the most abundant, i.e., host, mRNA, thus allowing a transient phase of α polypeptide translation. After the accumulation of sufficient amounts of α mRNA and α polypeptides, the shutoff of α gene expression would begin as the composite result of three activities: (i) autoregulation of α gene transcription by threshold levels of ICP 4, as suggested by Dixon and Schaffer (3) on the basis of temperature-shift experiments with temperature-sensitive mutants of ICP 4 (members of the 1-2 complementation group); (ii) the interaction of the *vhs* function with α mRNA, which at this point of infection occupies sufficiently high proportions of the infected cell polysomes, and (iii) the activity of a newly made viral polypeptide(s) acting similar to *vhs* function to reduce α translation. This activity was originally proposed by Honess and Roizman (9, 10) on the basis of their observations that the decay of α polypeptide synthesis was more rapid in cells which, after cycloheximide reversal, were allowed to proceed through the synthesis of β and γ polypeptides. Furthermore, early cytoplasmic switch off of α (and host) gene expression has also been proposed by Fenwick and Roizman (5) on the basis of the pattern of protein synthesis in enucleated infected cells. It remains to be seen whether the proposed β gene product, acting to repress α protein translation, is in fact the *vhs* protein synthesized de novo as a structural component of the progeny virus. Finally, the virion-associated positive transcriptional effector discussed above must also be synthesized at late intervals postinfection as a structural component of progeny virus. However, at late times, the activity of this polypeptide in turning on α gene expression could be counteracted by the negative autoregulatory effect of ICP 4 on gene transcription or by the *vhs*- or β gene-mediated destabilization of α mRNA (or both).

The data presented in this paper, taken together with the results of previous studies, clearly suggest that herpes simplex viruses have evolved a complex regulatory scheme to control the expression of their α genes. Further studies involving *vhs* mutants should thus be fruitful in helping to elucidate some of the mechanisms underlying the early stages in the cascade regulation of host and viral gene expression in HSV-infected cells.

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