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Effects of Herpes Simplex Virus on mRNA Stability

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Herpes simplex virus virions contain one or more functions which mediate shutoff of host protein synthesis, disaggregation of host polyribosomes, and degradation of host mRNA. We studied aspects of the host shutoff mechanism by using herpes simplex virus type 1 mutants deficient in virion-induced shutoff of host protein synthesis (G. S. Read and N. Frenkel, J. Virol. 46:498–512, 1983). Shutoff of host protein synthesis by the wild-type virus was associated with degradation of host mRNAs, including β-actin, α-tubulin, and heat shock protein 70. In contrast, the virion host shutoff (*vhs*) mutants were deficient to various degrees in their ability to induce host mRNA degradation; the extent of mRNA degradation correlated well with the extent of inhibition of host protein synthesis. This finding suggests that inhibition of host protein synthesis and degradation of host mRNA were mediated by the same virion-associated function. Virion-induced degradation of host mRNA was not prevented by inhibitors of ribosome translocation, nor could it be augmented, for mutant *vhs*-1, by drugs which disaggregate polyribosomes. This suggests that mRNA in polyribosomes, as well as nonpolyribosomal mRNA, is susceptible to virion-induced degradation. Finally, the half-life of viral transcripts was also prolonged in cells infected with the *vhs*-1 mutant virus, suggesting that the *vhs* function indiscriminately decreased the half-lives of both host and viral mRNAs. The *vhs* function may thus play a dual role in virus infection. (i) It inhibits host gene expression, and (ii) it enables rapid transitions in the expression of viral genes which are sequentially transcribed as infection progresses.

Infection of cultured cells with herpes simplex virus type 1 or 2 (HSV-1 or HSV-2) results in rapid inhibition of host protein synthesis (45), disaggregation of host polyribosomes (53; reviewed in reference 10), and reduction of host mRNA levels (1, 19, 20, 31, 35–38, 41, 48, 50, 51).

The shutoff process has been divided operationally into two stages: primary shutoff, which is mediated by a virion component(s), and secondary shutoff, which requires prior expression of viral genes (37; reviewed in reference 10). Inhibition of host protein synthesis and disaggregation of host polyribosomes have been shown to occur at least in part in the primary phase in that they were observed in cells infected in the presence of actinomycin D, during infections with UV light-irradiated virus, and in enucleated infected cytoplasts (1, 13, 24a, 37, 38, 43, 48). In some cell types, enhanced degradation of host mRNA was shown to occur only in the secondary phase (20, 38). In Vero cells, however, enhanced degradation of translatable (12) and hybridizable (1, 48) mRNA is caused by HSV virions. It is at present unclear whether one or more virion components are involved and in what way cessation of host protein synthesis, disaggregation of host polyribosomes, and degradation of host mRNA are mechanistically linked.

A number of HSV-1 virion host shutoff (*vhs*) mutants were previously derived in our laboratory on the basis of their altered ability to inhibit host protein synthesis in the presence of actinomycin D (43). At late times postinfection in the absence of drugs, the *vhs* mutants nonetheless inhibited host protein synthesis, indicating that they were not deficient in the secondary shutoff function. The *vhs* mutation is not lethal, and cells infected with the mutants were found to express the normal repertoire of viral genes, although the rate and duration of synthesis of the α (immediate early), β (early), and γ (late) proteins appeared somewhat altered. At least one of the mutants was shown to be altered in a virion function which destabilized α protein synthesis from preformed α mRNA (43). More recent studies have shown that synthesis of β and γ polypeptides from preformed mRNA is also prolonged in *vhs*-1 mutant virus-infected cells (24a).

We report here that *vhs* mutants are also deficient in their ability to cause degradation of host mRNA. Additionally, we address questions regarding the mechanism of the *vhs* function and the specificity of mRNA degradation.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection and S. Bachenheimer (University of North Carolina, Chapel Hill). The cells obtained from the American Type Culture Collection were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. The cells obtained from S. Bachenheimer were maintained in modified autoclavable Eagle minimum essential medium (GIBCO) supplemented with 5% Serum Plus medium supplement (KC Biologicals, Lenexa, Kans.). These Vero cells are designated as Vero-s^+ in the text.

The *vhs* mutants were derived from the HSV-1 strain KOS (43). Working stocks of virus were prepared by infecting HEp-2 cells at 0.01 PFU per cell for 4 to 5 days, followed by three cycles of freeze-thawing (46). For mock infections, cells were either exposed to mock inoculum prepared by harvesting uninfected cells as described above for virus stocks (experiments shown in Fig. 2 and 9), or cells were overlaid with medium alone (experiments in the remaining figures).

Infections in the absence or presence of drugs. Vero cell monolayers in 25-cm^2 or six-well (35-mm diameter) culture dishes were infected in 1 ml of medium 199 (KC Biologicals) supplemented with 1% inactivated calf serum (MA Bioproducts, Walkersville, Md.). Except when noted, infections were done at 37°C with 10 PFU per cell. Adsorption was for 2 h (experiments shown in Fig. 3, 4, and 6) or 1 h

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(remaining experiments), after which the inoculum was removed and replaced with fresh medium. In the case of drug treatment, the cells were preincubated in medium containing the relevant drug for 1 h (phosphonoacetate [PAA]) or 30 min (all other drugs). The virus inoculum and medium overlay contained the drug as specified.

Verrucarin A (used at a final concentration of 12.5 μg/ml) was obtained from Sandoz Ltd. Pactamycin (10^{-5} M) was obtained from R. Keene at The Upjohn Co., Kalamazoo, Mich. Actinomycin D (5 μg/ml), cycloheximide (0.1 mM), puromycin (0.1 mM), and PAA (300 μg/ml) were purchased from Calbiochem-Behringer, La Jolla, Calif. Sodium fluoride (10 mM) was obtained from Fisher Scientific Co., Pittsburgh, Pa., and emetine hydrochloride (0.1 mM) was from Sigma Chemical Co., St. Louis, Mo.

Protein analyses. For protein labeling, cells were incubated in medium 199 containing 5% of the usual unlabeled methionine concentration and 50 μCi of ^{35}S-labeled methionine per ml. Protein samples were prepared and analyzed in sodium dodecyl sulfate (SDS)-polyacrylamide gels (25) as described previously (43). The gels were electrophoresed at 15 mA for about 12 h in the presence of 0.05% Coomassie brilliant blue (3).

Preparation and analyses of RNA. The cell monolayers were rinsed three times in ice-cold 0.15 M NaCl and then lysed and incubated for 12 to 15 h at 37°C in 1 ml of lysis buffer. Lysis buffer contained 0.1 M NaCl, 10 mM Tris (pH 8.0), 10 mM disodium EDTA, 0.8% SDS (Bio-Rad Laboratories, Richmond, Calif.; electrophoresis grade), 0.5 mg of proteinase K (E.M. Reagents) per ml, and 10 mM aurintricarboxylic acid (Sigma; see reference 14). The lysate was then extracted repeatedly with 1 volume of redistilled phenol saturated with TE (10 mM Tris, 1 mM EDTA, pH 7.4) and 1 volume of chloroform–2% isoamyl alcohol. The final extraction was with chloroform–2% isoamyl alcohol alone, followed by precipitation with 3 volumes of cold 95% ethanol. The samples were suspended in sterile water, treated with pancreatic DNase I (56), precipitated with ethanol, and suspended in a minimum volume of 75% deionized formamide–25% TE. In a few cases, as noted in the figure legends, DNase treatment was omitted. The RNAs were electrophoresed at 3 to 4 V/cm in 1.2% agarose gels containing 2.2 M formaldehyde, as described by Maniatis et al. (29). Northern blots were prepared by electrotransfer to Gene Screen (New England Nuclear Corp., Boston, Mass.). The membranes were dried for several hours at room temperature and then baked for 2 h at 80°C. The membranes were prehybridized overnight in hybridization solution containing twice the concentration of Denhardt solution (7). Hybridization took place in sealed bags at 42°C (except where noted) in 0.075 ml of hybridization solution per cm^2. Hybridization solution consisted of 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% SDS, 200 μg of denatured calf thymus DNA (Sigma) per ml, 1× Denhardt solution, and 0.6×10^6 to 2.5×10^6 cpm/ml. After 24 to 40 h of hybridization, the membranes were incubated several times for 2-h intervals at 70°C in 2× SSC–1% SDS. They were then rinsed in 0.2× SSC, air dried, and autoradiographed with intensifying screens (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Within each experiment, equivalent fractions of the RNA preparations (representing equal numbers of cells) were loaded on the gels. Because of the presence of aurintricarboxylic acid in the RNA samples, it was impossible to estimate sample losses by UV absorbance of the RNA. The amount of RNA present in each lane was estimated by hybridization of a 28S rRNA probe to the Northern blot, ethidium bromide staining of the gel before electrotransfer, or both methods.

Hybridization probes. cDNA clones of human cytoplasmic actin (15) and human keratinocyte α-tubulin (clone kα1; reference 5) were obtained from E. Fuchs (The University of Chicago). The human *hsp*70 plasmid (clone pH2.3; reference 18) was obtained from R. Morimoto (Northwestern University, Evanston, Ill.). The HSV-1 *Bam*HI-P plasmid (clone pRB145; reference 34) was obtained from B. Roizman (The University of Chicago). pJD101 (33) was derived in our laboratory by L. P. Deiss. The human 28S rRNA probe pA_{BE} (9) was obtained from J. Sylvester (University of Pennsylvania, Philadelphia). ^{32}P-labeled probes were prepared by nick translation followed by spermine precipitation (17). The probes were denatured in sealed micropipettes in an ethylene glycol bath at 107°C for 7 min just before the start of hybridization.

RESULTS

HSV virions enhance degradation of actin and tubulin mRNAs. To study the effect of the *vhs* mutation on host mRNA stability, Vero cells were mock infected or infected with HSV-1 strain KOS, HSV-2 strain G, or the mutant *vhs*-1. To test for a virion-associated function (s), the infections were done in the presence or absence of actinomycin D, which inhibits viral gene expression. Nucleic acids were harvested at 3 h postinfection, and Northern blots were prepared. To characterize the extent of inhibition of host protein synthesis, infected-cell proteins were labeled with [^{35}S]methionine in parallel cultures and analyzed in SDS-polyacrylamide gels.

The results of two such experiments are shown in Fig. 1 and 2. Lanes 1 to 3 of Fig. 1 show that wild-type (wt) HSV-1 (KOS) inhibited host protein synthesis early postinfection, while the *vhs*-1 mutant did not. Lanes 4 to 7 show that HSV-1 (KOS) had the same inhibitory effect on host protein synthesis in the absence of viral gene expression. HSV-2 (G) effected even more pronounced reduction under these conditions, while the *vhs*-1 mutant had no effect. These results are consistent with previous studies which showed that HSV-2 inhibited host protein synthesis more rapidly than did HSV-1 (11, 16, 42) and that the *vhs*-1 mutant was defective in the virion-associated shutoff function (43).

Lanes 8 to 14 of Fig. 1 show a Northern blot from this experiment, probed with a human α-tubulin cDNA-containing plasmid (5). Infection with HSV-1, and more so that with HSV-2, resulted in pronounced loss of α-tubulin mRNA, whereas no loss of this host mRNA was observed in cells infected with the *vhs*-1 mutant. Loss of α-tubulin mRNA in wt virus-infected cells also occurred in the presence of actinomycin D. This idicates that a factor(s) associated with virions of HSV-1 and HSV-2 caused a reduction in the level of this mRNA species and that this reduction was due to an enhanced rate of degradation. In accordance with the effects of these viruses on protein synthesis, mRNA degradation was more pronounced in HSV-2-infected cells than in their HSV-1-infected counterparts, and no degradation was observed with the *vhs*-1 mutant.

To test whether degradation of α-tubulin mRNA occurred during mRNA extraction, a mixing experiment was done in which RNAs from infected and uninfected cells were prepared in two ways. Lane 15 of Fig. 1 contained RNAs extracted from infected and uninfected cells which were mixed before RNA preparation. Lane 16 contained a mixture

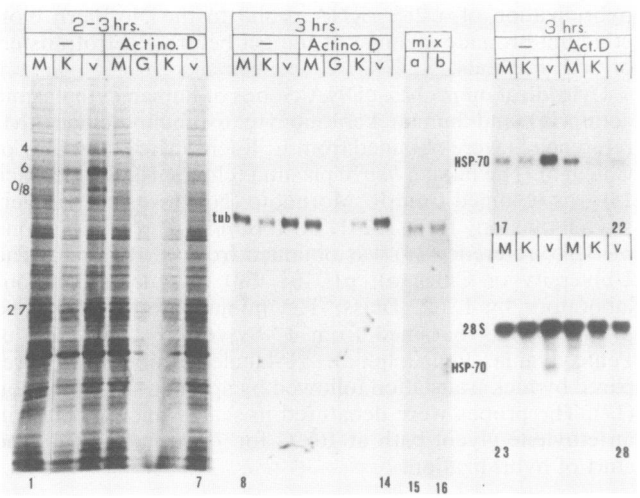

FIG. 1. The stability of α-tubulin (tub) and *hsp*70 mRNAs in cells infected with the wt and *vhs*-1 mutant viruses. Vero cells (25-cm^2 cultures) were mock infected (M) or infected with 10 PFU of HSV-1 strain KOS (K), HSV-2 strain G (G), or the *vhs*-1 mutant (v) per cell in the presence or absence of actinomycin D (Act. D; Actino. D; 5 μg/ml, starting 30 min before infection). One set of cultures was labeled with [^{35}S]methionine from 2 to 3 h postinfection. Another set of cultures was harvested for RNA at 3 h postinfection. Lanes: 1 to 7, 9.25% SDS-polyacrylamide gel of the labeled proteins; 8 to 16, a Northern blot containing the RNA samples (1/10 of the 25-cm^2 culture preparation) and probed with the human α-tubulin cDNA probe (5); 15 and 16, KOS-infected cells and uninfected cells scraped off the dish at 3 h postinfection (In mix a, the cells were mixed before lysis and nucleic acid preparation. In mix b, the nucleic acids were processed separately and mixed just before loading on the agarose-formaldehyde gel); 17 to 22, a second Northern blot probed with the human *hsp*70 probe (18); 23 to 28, the same Northern blot used for lanes 17 to 22 probed with both the *hsp*70 probe and the 28S rRNA probe (9) to quantitate the amount of total RNA loaded. Numbers to the left of lane 1 denote ICPs.

of RNA samples prepared separately from infected and uninfected cells and mixed just before loading on the gel. If enhanced degradation had occurred during preparation of the RNA, one would expect it to have affected the mRNA in lane 15 substantially more than that in lane 16. As this was not the case, mRNA degradation must have occurred within the intact infected cells.

Figure 2 shows the results of a similar experiment in which a human β-actin cDNA-containing plasmid (15) was used as the hybridization probe. In agreement with the α-tubulin results, the amount of β-actin mRNA was greatly reduced in the wt KOS virus infections in the presence or absence of actinomycin D. In contrast, no degradation of β-actin mRNA was observed in *vhs*-1 mutant-infected cells. In fact, levels of actin mRNA were somewhat higher in these cells than in their mock-infected counterparts, even in the presence of actinomycin D (compare lanes 6 and 4 and lanes 9 and 7), suggesting possible protection from normal degradative processes.

***hsp*70 mRNA in wt and *vhs*-1 mutant virus-infected cells.** A number of viruses, including HSV, have been shown to induce transient expression of stress genes (21, 22, 24, 26, 39). In the case of HSV, heat shock gene transcription was shown to be induced by an α gene product(s) (39; R. Robinson, personal communication). It was of interest, therefore, to determine whether heat shock mRNA was susceptible to *vhs*-mediated mRNA degradation. To investigate this question, Northern blots containing RNA samples from the experiments shown in Fig. 1 and 2 were probed with a plasmid containing a human *hsp*70 cDNA (18, 59). The two hybridization tests yielded similar results, and only one of the blots is shown in lanes 17 to 22 of Fig. 1. To control for RNA loading variations, the same blot was also hybridized with a mixture of *hsp*70 and 28S rRNA probes, with the results shown in lanes 23 to 28.

Several composite effects are apparent from the data. (i) *hsp*70 mRNA was detected in cells which were mock infected for 3 h. This could represent constitutive levels of *hsp*70 mRNA in nonstressed cells (59). Alternatively, *hsp*70 mRNA synthesis or stabilization could have been induced during overlaying of the cells with the 199 medium containing 1% serum, since heat shock proteins are known to be cell cycle dependent (21).

(ii) *hsp*70 mRNA was more abundant in *vhs*-1 mutant virus-infected cells than in their wt virus-infected counterparts. This difference was observed in the absence of actinomycin D (compare lanes 18 and 19), as well as in the presence of the drug (compare lanes 22 and 21; lane 22 was underloaded as seen from the 28S probe hybridization, lane 28). It can thus be concluded that *hsp*70 mRNA was susceptible to degradation induced by the wt *vhs* function.

(iii) Infection with the *vhs*-1 mutant in the presence of actinomycin D resulted in a significant loss of *hsp*70 mRNA (compare lanes 22 and 20; there was about a 50% loss after

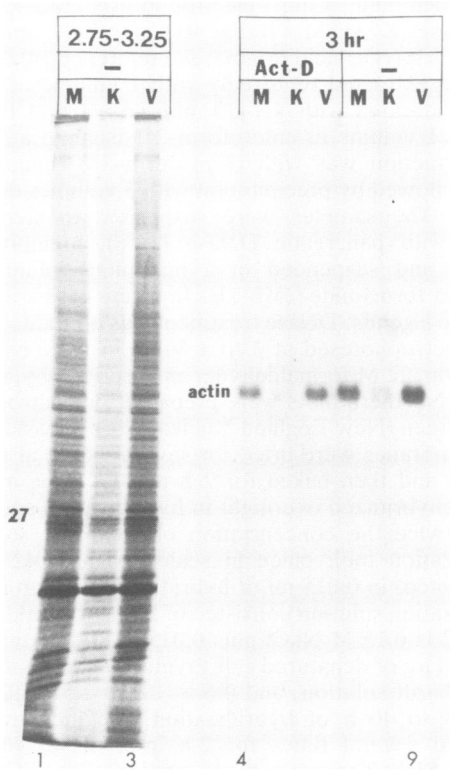

FIG. 2. The stability of actin mRNA in cells infected with the wt and *vhs*-1 mutant viruses. Vero-s$^+$ cells (in six-well dishes) were mock infected (M) or infected with 10 PFU of the wt KOS (K) or *vhs*-1 mutant (v) per cell in the presence or absence of actinomycin D (Act-D). Lanes: 1 to 3, proteins from cells labeled with [^{35}S]methionine from 2.75 to 3.25 h postinfection; 4 to 9, cells harvested for RNA at 3 h postinfection. The Northern blot was hybridized with the human β-actin cDNA probe (15). The position of ICP27 is indicated on the left.

RNA loading variations were taken into account). This suggested that the mutant *vhs*-1 was not entirely disabled in its ability to enhance HSP70 mRNA degradation. This instability is unique to HSP70 mRNA since, as already discussed, actin and tubulin mRNAs were stable in the same *vhs*-1-infected cells.

(iv) Infection with *vhs*-1 in the absence of actinomycin D resulted in a significant increase in the level of HSP70 mRNA compared with mock-infected cells (compare lanes 17 and 19). This observation suggests that HSV increases the rate of HSP70 mRNA synthesis. This conclusion is strengthened by the observation that the level of HSP70 mRNA was not significantly reduced by infection with wt virus in the absence of actinomycin D (compare lanes 17 and 18), although the degradation rate of this mRNA was presumably enhanced (compare lanes 20 and 21).

Additional *vhs* mutants are defective with respect to degradation of host mRNA. The finding that the *vhs*-1 mutant virus was simultaneously deficient in inhibition of host protein synthesis and degradation of host mRNA suggested that the two phenotypes were consequences of a mutation in a single viral gene. However, the *vhs*-1 mutant was selected from a bromodeoxyuridine-mutagenized virus stock, and it could conceivably contain multiple mutations affecting host gene expression. Given the additional fact that not all host cytoplasmic mRNA is actively involved in protein synthesis (8, 47), it remained possible that reduction of host mRNA levels and reduction in the rate of host protein synthesis were not mechanistically linked. If they were not, one might expect one or more independently derived *vhs* mutants to exhibit a substantially greater effect on one than on the other. The mutants *vhs*-2, -5, and -6 were derived from independently mutagenized virus stocks and selected on the basis of their inability to reduce host protein synthesis in the presence of actinomycin D (43). These mutants were studied in the experiment described in Fig. 3.

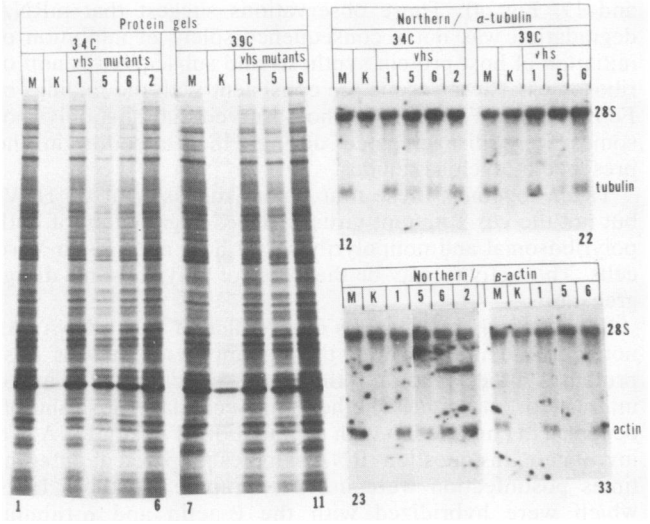

FIG. 3. Vero cells (six-well cultures) were mock infected (M) or infected with 40 PFU of the wt KOS (K) virus or the different *vhs* mutants shown (*vhs*-1, -2, -5, and -6) per cell. The cells were infected in the presence of actinomycin D, at 34 or 39°C. After 2 h of adsorption, the cells were shifted to 95% methionine-free medium with (for the protein samples) or without (for the nucleic acid samples) [^{35}S]methionine. Cells were left at 34 or 39°C for 2 h more and then harvested for proteins or total nucleic acids. Lanes: 1 to 11, labeled proteins separated on a 9.25% SDS-polyacrylamide gel; 12 to 22, Northern blot probed with a mixture of the α-tubulin and 28S rRNA probes; 23 to 33, Northern blot probed with a mixture of the actin and 28S rRNA probes.

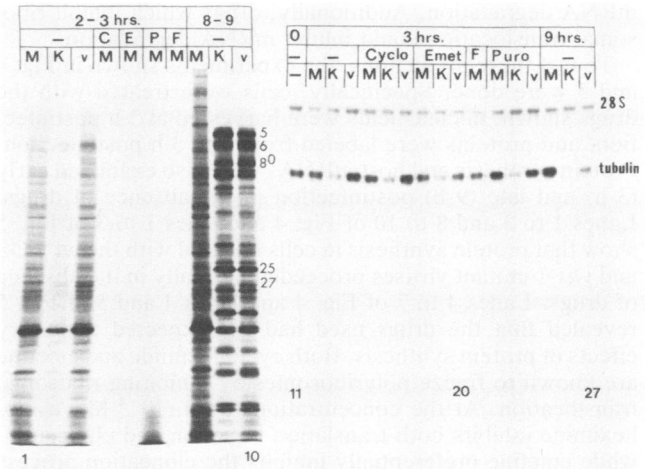

FIG. 4. Effects of metabolic inhibitors on HSV-induced mRNA degradation. Vero cells (six-well cultures) were mock infected (M) or infected with the wt KOS (K) or *vhs*-1 mutant (v) virus in the absence or presence of the drugs shown. At 1 h before harvest, cells were shifted into 95% methionine-free medium with or without the appropriate drug. Cell cultures used for protein analyses were then labeled with [^{35}S]methionine. Cells were harvested for proteins or total RNA at 3 or 9 h postinfection. Lanes: 1 to 10, 9.25% SDS-polyacrylamide gel of labeled proteins (the positions of ICPs are indicated on the right); 11, RNA from untreated uninfected cells; 11 to 27, a Northern blot hybridized with a mixture of the α-tubulin probe and the 28S rRNA probe (0.5% of total counts). Abbreviations: cyclo (C), cycloheximide; emet (E), emetine; puro (P), puromycin; F, sodium fluoride.

Cells were infected at 34 or 39°C with wt or mutant virus in the presence of actinomycin D. Proteins were labeled, and β-actin and α-tubulin mRNAs were analyzed as in Fig. 1 and 2. The protein profiles (lanes 1 to 11) showed that the mutants *vhs*-1, -2, -5, and -6 exhibited various degrees of virion-associated host shutoff at the two temperatures, in accordance with the previously observed phenotypes of these mutants (43). The patterns of hybridizations (lanes 12 to 33) revealed that each of these mutants was also defective in its ability to induce degradation of host mRNA. Furthermore, the ability of the different mutants to inhibit host protein synthesis correlated well with their ability to degrade host mRNA at that temperature. There was, however, a lack of good correlation between the two phenomena for the mutant *vhs*-5. In this case, inhibition of host protein synthesis was stronger at 34°C than at 39°C, whereas mRNA degradation was more pronounced at the higher temperature. Further studies are needed to better quantitate and explain the behavior of this mutant, which was clearly leaky with regard to its virion-associated shutoff function.

Host mRNA is degraded in the presence of drugs which inhibit translation. As noted in the introduction, HSV causes rapid disaggregation of host polyribosomes. It was of interest to ask whether host mRNA degradation was a cause or a consequence of polyribosome disaggregation. Thus, degradation of polyribosomal mRNA could lead to dissociation of polyribosomes. Alternatively, polyribosome dissociation could release the mRNA into a pool which could turn over more rapidly. If this were the case, it could be predicted that drugs which disaggregate polyribosomes would enhance

mRNA degradation. Additionally, drugs which inhibit ribosomal translocation could inhibit mRNA degradation.

To test these predictions, the experiments shown in Fig. 4 and 5 were done. Specifically, cells were treated with the drugs shown, nucleic acids were harvested at 3 h postinfection, and proteins were labeled from 2 to 3 h postinfection. Protein synthesis and host mRNAs were also examined early (3 h) and late (9 h) postinfection in the absence of drugs. Lanes 1 to 3 and 8 to 10 of Fig. 4 and lanes 1 to 3 of Fig. 5 show that protein synthesis in cells infected with the wt KOS and *vhs*-1 mutant viruses proceeded normally in the absence of drugs. Lanes 4 to 7 of Fig. 4 and lanes 4 and 5 of Fig. 5 revealed that the drugs used had the expected inhibitory effects of protein synthesis. Both cycloheximide and emetine are known to freeze polyribosomes by inhibiting ribosomal translocation. At the concentrations used (10^{-4} M), cycloheximide inhibits both translation initiation and elongation, while emetine preferentially inhibits the elongation process (40, 58). Sodium fluoride disaggregates polyribosomes by reducing nucleotide triphosphate levels in the cells (28, 30), by directly inhibiting 60S ribosomal subunit attachment to the initiation complex, or by both processes (57). Puromycin causes premature termination of translation (57), as also evident by the presence of the truncated peptides seen near the bottom of lane 6 of Fig. 4. Pactamycin inhibits initiation by interaction with the 40S, as well as the 60S, subunit (6, 23, 54). Finally, verrucarin A, unlike puromycin, blocks only the first few rounds of peptide bond formation (57).

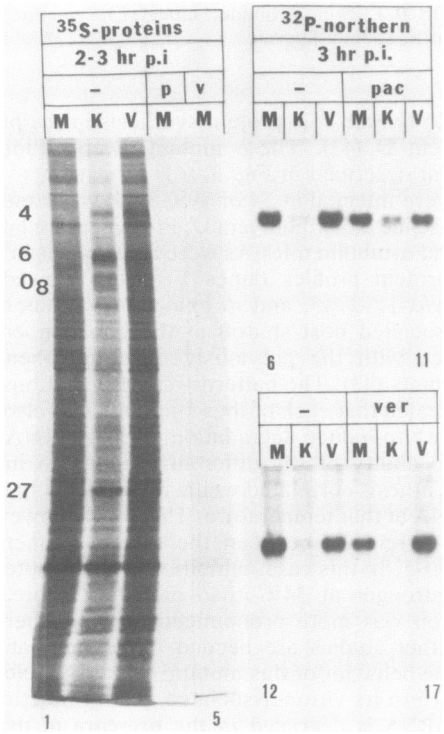

FIG. 5. Effects of pactamycin and verrucarin A on HSV-induced mRNA degradation. Vero-s$^+$ cells (six-well cultures) were infected with HSV-1 strain KOS (K) or the *vhs*-1 mutant virus (V) in the presence or absence of pactamycin (pac) or verrucarin A (ver). At 2 h postinfection (p.i.), the cells were shifted into 95% methionine-free medium 199 with or without [^{35}S]methionine. At 3 h p.i., cells were harvested. Lanes: 1 to 5, ^{35}S-labeled proteins separated on a 9.25% SDS-polyacrylamide gel; 6 to 17, Northern blots hybridized with the α-tubulin cDNA probe. The positions of ICPs are indicated on the left.

Hybridization of the α-tubulin probe to Northern blots, shown in Fig. 4 and 5 revealed the following. (i) In mock-infected cells, none of the drugs known to disaggregate polyribosome (puromycin, sodium fluoride, pactamycin, and verrucarin A) caused a reduction in the level of α-tubulin mRNA comparable to that caused by infection with wt HSV-1 (KOS). This finding extends an earlier observation by Nishioka and Silverstein (38) on Friend erythroleukemia cells and suggests that polyribosomal disaggregation by itself cannot explain the reduction in mRNA levels induced by HSV.

(ii) Puromycin and verrucarin A had no significant effect on degradation of α-tubulin mRNA during infections with the wt virus, whereas pactamycin had a slight inhibitory effect (Fig. 5, lane 10).The effect of sodium fluoride on HSV-induced mRNA degradation could not be precisely determined because the drug most likely inhibits virus penetration into the cells (data not shown). These results indicate that mRNA, which was not present in polyribosomes, was nevertheless susceptible to wt virus-induced degradation.

(iii) The defect in the *vhs*-1 virus could not be complemented by puromycin, pactamycin, or verrucarin A. If polyribosome disaggregation were the cause of mRNA degradation and *vhs*-1 were deficient in this function, one might expect that *vhs*-1 would induce mRNA degradation in cells treated with the drugs used. Lanes 22 to 24 of Fig. 4 and lanes 9 to 11 and 15 to 17 of Fig. 5 show that this was not the case.

(iv) Neither cycloheximide nor emetine, which interfere with ribosome translocation, significantly inhibited degradation of α-tubulin mRNA induced by the wt virus (lanes 16 and 19, Fig. 4). These observations suggest that mRNA degradation was not a consequence solely of inhibition of initiation of host protein synthesis and subsequent runoff of ribosomes. These results are consistent with the findings of Fenwick and Walker (13), who observed that host polyribosomes were disaggregated during HSV infection in the presence of cycloheximide.

Taken together, these four points suggest that wt HSV, but not the *vhs*-1 mutant virus, induces degradation of both polyribosomal and nonpolyribosomal host mRNAs in Vero cells. This activity may be the cause of polyribosome disaggregation.

Secondary shutoff involves degradation of host mRNA. As noted in the introduction, the mutant *vhs*-1 inhibits host protein synthesis at late times postinfection. It was of interest to determine whether this secondary host shutoff occurred in conjunction with degradation of host mRNA. To investigate this question, RNA samples prepared at different times postinfection were used to prepare Northern blots which were hybridized with the β-actin and α-tubulin probes. Proteins were labeled in parallel with [^{35}S]methionine.

The protein patterns (Fig. 6) revealed a difference between the wt and the *vhs*-1 mutant in the degree of inhibition of host protein synthesis. However, the *vhs*-1 mutant was able to shut off host protein synthesis at late times postinfection, in accordance with previous results (43). The pattern of synthesis of the viral proteins will be discussed below.

The hybridization patterns (Fig. 7) revealed two major points. (i) The *vhs*-1 mutant was able to reduce host mRNA levels at late times postinfection, in parallel with its effect on

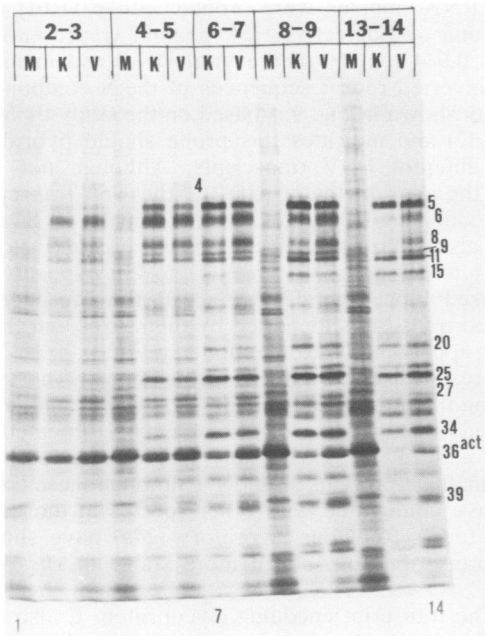

FIG. 6. Time course of protein synthesis in cells infected with the wt and *vhs*-1 viruses. The proteins shown were labeled during the experiment shown in Fig. 7 and 9. See the legend to Fig. 7. act, Actin. The numbers on the right indicate ICPs.

host protein synthesis. (ii) The *vhs*-1 mutant did not reduce host mRNA levels to the same extent as did the wt virus. Thus, the late viral function(s) which contributed to the loss of host mRNA did not fully compensate for the *vhs* mutation.

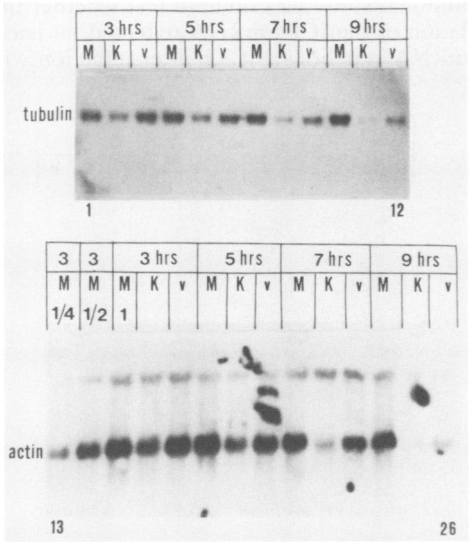

FIG. 7. Time course study of HSV-induced mRNA degradation. Vero cells (six-well cultures) were mock infected (M) or infected with HSV-1 strain KOS (K) or *vhs*-1 (v). At 1 h before harvest for either nucleic acids or proteins, cells were shifted into 95% methionine-free medium, and those to be harvested for proteins (shown in Fig. 6) were labeled with [^{35}S]methionine. Northern blots containing RNAs prepared at the indicated times were hybridized with the α-tubulin probe (lanes 1 to 12) or a mixture of the actin and the human 28S rRNA probes (lanes 13 to 26). Lanes 13 and 14 contained 1/4 and 1/2 (respectively) of the RNA sample loaded in lane 15.

Levels of host mRNA in the presence of PAA. The role of late viral gene products in host shutoff was studied further with PAA, which inhibits viral DNA replication (2) and does not allow normal expression of late (γ) viral genes (reviewed in reference 44). RNA and [^{35}S]methionine-labeled proteins were analyzed at different times after wt KOS virus infection in the presence or absence of the drug. The results are shown in Fig. 8 (lanes 13 to 37). As expected, in the presence of the drug there was delayed shutoff of the β polypeptides (e.g., infected-cell proteins 6 and 8 [ICP6 and ICP8]) and reduced synthesis of γ proteins (e.g., ICP15).

The Northern blot in Fig. 8 (lanes 13 to 37) was probed with the β-actin plasmid. The results yielded two conclusions: (i) PAA did not inhibit early (presumably virion-induced) degradation of actin mRNA, and a rapid decline in the level of actin mRNA was attained by 3 h postinfection in the presence of the drug (lane 27). (ii) The level of β-actin mRNA transiently increased at late times postinfection in the presence of PAA, as seen most clearly at the 9-h time point (lane 33, Fig. 8). At these late time points the level of β-actin mRNA was higher than that in cells infected in the absence of PAA. The observed increase was not due to variations in the amount of total RNA loaded onto lanes 26 to 37, as judged by scanning of the negative of the ethidium bromide-stained gel photograph. Increased abundance of α-tubulin mRNA was observed at late times postinfection in an additional experiment involving PAA (data not shown).

Furthermore, the *vhs*-1 mutant showed a similar increase in α-tubulin, as well as β-actin, mRNA, although the minimum levels were higher than the corresponding levels for wt virus (data not shown). Therefore, transcription of these two

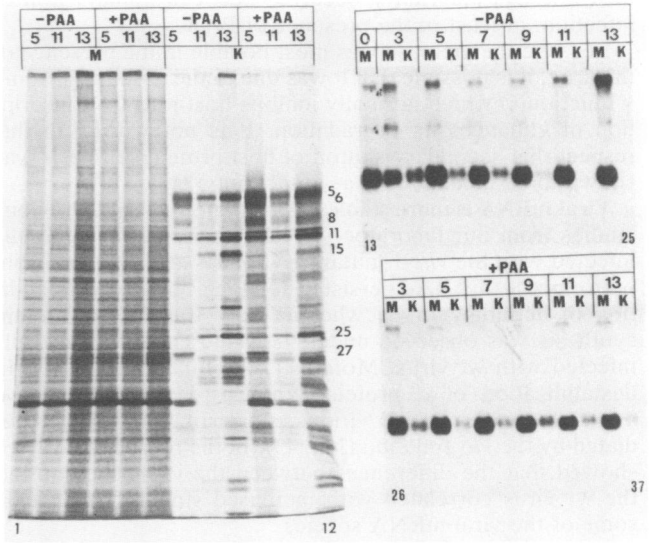

FIG. 8. Effect of PAA on the levels of host mRNAs after infection. Vero-s$^+$ cells (six-well cultures) were mock infected (M) or infected with HSV-1 strain KOS (K) in the presence or absence of PAA. Lanes 1 to 12, cells labeled with [^{35}S]methionine that received the label 15 min before the times indicated in the figure and were labeled for 30 min (the numbers on the right indicate ICPs); 13 to 37, Northern blot prepared from total nucleic acids (not treated with DNase during preparation) and hybridized with the actin cDNA probe. Lanes 13 to 25 and 26 to 37 are portions of the same blot. Ethidium bromide staining revealed that lane 14 was significantly underloaded and lane 24 was somewhat overloaded relative to the others.

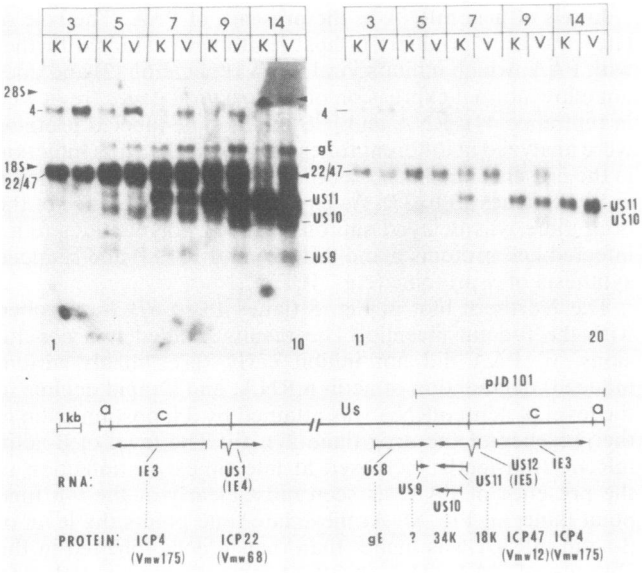

FIG. 9. Time course study of viral mRNA levels for wt KOS and mutant *vhs*-1. RNA samples from the experiment described in Fig. 6 were used to prepare a Northern blot which was hybridized with the plasmid pJD101. Lanes 11 to 20 show a lighter exposure of lanes 1 to 10. The map shows the S component of the HSV-1 genome, the location of the pJD101 probe, the relevant transcripts, and their known protein products (32). The positions of 18S and 28S rRNAs (heavy arrows) seen in the ethidium bromide-stained gel are shown. Abbreviations: gE, glycoprotein E; kb, kilobases; Vmw, viral protein molecular weight; IE, immediate early; K, kilodaltons.

host mRNAs must have occurred between 5 and 9 h after infection, at least in the presence of PAA.

Because the increase was most notable in the presence of the drug, it is possible that it was due to the absence of some γ function(s) which normally inhibits host mRNA transcription or enhances its degradation. It is noteworthy in this respect that secondary shutoff of host protein synthesis was shown to be mediated by a γ function(s) (43).

Viral mRNA is more stable in *vhs*-1-infected cells. Previous studies from our laboratory (43) have shown that, in cells infected with the *vhs*-1 mutant, synthesis of α proteins from preformed α mRNAs persisted for long periods after addition of actinomycin D, whereas only transient α protein synthesis was observed under the same conditions in cells infected with wt virus. Moreover, the function involved in destabilization of α protein synthesis was shown to be associated with purified virions, suggesting that it was mediated by the *vhs* function (24a, 43). In the present study, we showed that the differences between the *vhs*-1 mutant and the wt virus correlated with increased stability of at least some of the viral mRNA species.

RNA samples from the time course experiment shown in Fig. 7 were used to prepare another Northern blot, and a viral DNA probe was used to detect several viral mRNAs. The labeled proteins from this experiment are shown in Fig. 6. In accordance with previous results (43), several differences between the *vhs*-1 mutant and the wt virus were noted. Specifically, for the *vhs*-1 mutant, expression of α proteins was prolonged (particularly that of ICP27), only partial shutoff of β protein synthesis was observed even by 13 h postinfection (e.g., ICP6 and ICP8), and some delay in the synthesis of γ proteins was noted (e.g., ICP15).

The RNA samples were probed with pJD101, a cloned repeat unit of a defective HSV genome, representing coordinates 0.944 to 1.00 of the viral DNA and including the entire inverted repeat sequences of the S component (33). The map shown in Fig. 9 is based on the study by McGeoch et al. (32) and indicates this probe should hybridize with seven different HSV transcripts. Although not mapping within the coordinates of pJD101, the US1 transcript contains sequences from the inverted repeat of S and thus should also hybridize with this probe. Figure 9 shows dark (lanes 1 to 10) and soft (lanes 11 to 20) exposures of the hybridized blot. The data revealed the presence of the expected transcripts identified by their sizes and the timing of their appearance postinfection.

The results yielded three conclusions. (i) The transcript encoding the α 4 protein was present at elevated levels and persisted for longer periods of time in *vhs*-1-infected cells. A similar pattern was most likely true also for the mRNAs encoding the α proteins 22 and 47, although these transcripts may have comigrated with other species in the gel. Additional studies from our laboratory (24a) have shown that these transcripts are indeed more stable in *vhs*-1-infected cells.

(ii) The transcript encoding glycoprotein E also accumulated to a higher level and persisted for a longer time in *vhs*-1-infected cells. Thus, this γ_1 transcript was affected by the *vhs*-1 mutation in the same way as the α transcripts. The same conclusion can be made regarding the US10 transcript.

(iii) The third point relates to the transcript which increased only at later times postinfection in wt virus-infected cells and which was tentatively identified as US11 mRNA on the basis of its mobility. This mRNA accumulated with a slight delay during *vhs*-1 mutant virus infection (see the 5- and 7-h lanes in the dark and soft exposures), but it too accumulated to higher levels by late times postinfection (soft exposure, lane 20).

An additional study was done to test whether the greater accumulation of the ICP4 mRNA reflected, at least in part, altered mRNA stabilities. At 3 h after infection with the wt

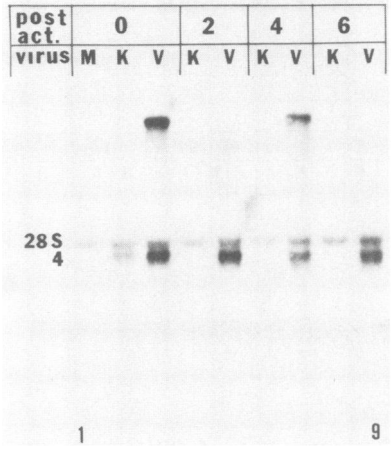

FIG. 10. Actinomycin D chase study of decay rate for ICP4 mRNA in cells infected with HSV-1 strain KOS and *vhs*-1. The experiment described in Fig. 2 was continued as follows. At 3 h postinfection, cells infected with KOS (K) and *vhs*-1 (V) were shifted to medium containing actinomycin D (act.) and incubation was continued for an additional 2, 4, or 6 h. The Northern blot from this experiment was hybridized at 48°C with a plasmid containing the HSV-1 *Bam*HI P fragment (34). The positions of the 28S mRNA probe and ICP4 mRNA are indicated on the left.

or mutant *vhs*-1 virus, cells were either harvested or treated with actinomycin D for 2, 4, or 6 h so as to measure mRNA decay. Parallel cultures labeled with [^{35}S]methionine from 2.75 to 3.25 h postinfection are shown in Fig. 2 and have already been discussed.

The Northern blot shown in Fig. 10 was probed with a plasmid containing the HSV-1 *Bam*HI P fragment (34). This fragment spans the 3.1-kilobase rightmost sequences of HSV-1 (F) DNA and was expected to hybridize only to the ICP4 transcript (denoted as 4 in Fig. 10). However, it is noteworthy that two additional species were seen in the blot. The first, just above the ICP4 mRNA band, appeared in all of the lanes, including the mock-infected sample, and most likely represented weak hybridization to the 28S rRNA species. Such nonspecific hybridization was also observed with the pJD101 probe when hybridized at the same temperature (48°C). A second high-molecular-weight species can be seen in two of the lanes. The nature of this band and the reproducibility of this observation are unclear.

The hybridization patterns revealed the following. (i) At the point of actinomycin D addition (lanes 1 to 3), the level of ICP4 mRNA was significantly higher in *vhs*-1-infected cells than in their wt virus-infected counterparts. (ii) ICP4 mRNA persisted longer after actinomycin D treatment in *vhs*-1-infected cells than in wt virus-infected cells. The somewhat elevated level of ICP4 mRNA seen in lane 9 reflected higher amounts of total RNA loaded on the gel. These results suggest that the *vhs* function causes indiscriminate degradation of both host and viral mRNAs.

DISCUSSION

Induction of host mRNA degradation by a virion function. Several independently derived mutants altered in their ability to cause primary shutoff of host protein synthesis (*vhs* mutants) were found to be deficient to various degrees in the ability to induce host mRNA degradation. The extent of mRNA degradation correlated well with the extent of inhibition of host protein synthesis. This finding suggests that inhibition of host protein synthesis and degradation of host mRNA are mediated by the same virion-associated function.

HSV has been shown to reduce the level of host mRNAs in a variety of cell types (1, 12, 19, 20, 31, 35–38, 48, 50, 51). However, contradictory results have been reported regarding the requirement of viral gene expression for this process. Nishioka and Silverstein (38) and Nishioka et al. (36) showed that, in dimethyl sulfoxide-induced Friend erythroleukemia cells, degradation of globin mRNA required viral gene expression. A similar observation was also made by Isom et al. (20) regarding shutoff of α fetoprotein synthesis in rat hepatoma cells. In contrast, Fenwick and McMenamin (12) showed that an HSV virion component reduced the level of host mRNA in Vero cells, as assayed in an in vitro translation system. Schek and Bachenheimer (48) showed that degradation of host mRNA in Vero cells was induced by a virion component. Bastow et al. (1) reached a similar conclusion regarding dihydrofolate reductase mRNA degradation in dihydrofolate reductase-transformed human cells.

It is possible that these apparently conflicting conclusions reflect the use of different assays to quantitate the mRNAs in the studies cited above. Alternatively, they may reflect differences in the cell types or in the pathways of degradation of the different mRNAs which were studied. Because mRNA degradation may involve a specific interaction(s) of a virally encoded factor(s) with its cellular target(s), the outcome of this interaction may well vary with different virus-cell combinations.

The mechanism of host shutoff. We found additionally that (i) mRNA degradation was not prevented by cycloheximide and emetine, both of which inhibit ribosome translocation, (ii) treatment of mock-infected cells with puromycin, sodium fluoride, pactamycin, or verrucarin-A, all of which disaggregate polyribosomes, did not by itself lead to rapid degradation of host mRNA, (iii) drug-induced polyribosome disaggregation did not complement the function mutated in *vhs*-1, nor did it prevent mRNA degradation by the wt virus. Taken together, these results suggest that HSV-induced mRNA degradation is not merely a consequence of polyribosome disaggregation. Rather, they are consistent with a mechanism involving a direct ribonuclease attack on polyribosomal (as well as nonpolyribosomal) mRNA.

Such a mechanism has been considered by several investigators (1, 4, 13, 19, 48), although direct nuclease attack on polyribosomes has not been demonstrated; in fact, indirect evidence appears not to support such activity. Thus, Fenwick and Walker (13) have shown that monosomes from infected cells were dissociated into the large and small ribosomal subunits by high salt, unlike monosomes generated by RNase treatment of cell lysates in vitro. It is possible, however, that monosomes generated in vivo may have run off the fragmented mRNA. In addition, polyribosome disaggregation was observed in Friend erythroleukemia cells in the absence of viral gene expression, whereas mRNA degradation was not (38). However, it is noteworthy that globin mRNA was quantitated in these studies by liquid hybridization kinetics, and degradation products of the primary shutoff process may have been sufficiently large for hybridization to occur. Also, globin mRNA is known to be very stable (58) and therefore may have been uniquely protected from *vhs*-induced degradation.

Whether or not mRNA in polyribosomes is susceptible, it is clear from our data that a virion component(s) enhances mRNA degradation, at least in Vero cells. This may occur through at least three mechansims. (i) An RNase could enter the cells as part of the virion, (ii) a virion component could activate a pre-existing cellular degradation pathway, or (iii) a virion component could disable a cellular mechanism that acts to protect mRNA from degradation, such as an endogenous ribonuclease inhibitor.

The mRNA degradation process presumably involves recognition of one or more signals within the mRNAs. It is noteworthy in this respect that recent studies have established that specific signals in the 3′ untranslated regions of mRNAs encoding certain oncogenes and growth factors contain signals which determine their stability in cells (49). We predict, however, that in the case of HSV the signal specifying reduced stability of the infected-cell mRNA is shared by a variety of host, as well as viral, transcripts. Likely candidates for this role are the signal specifying mRNA polyadenylation or the poly(A) tail itself. In this regard, it is of interest to note that Mayman and Nishioka (31) have recently observed that histone H3 mRNA was more stable than other species in HSV-infected Friend erythroleukemia cells and suggested that lack of the poly(A) tail was the reason for this relative stability. In addition, HSV infection was reported to result in an increased poly(A)$^-$ RNA pool and a concomitant decrease in the abundance of poly(A)$^+$ mRNA (35, 52). These changes suggest that poly(A)$^-$ RNA is not susceptible to HSV-induced mRNA degradation.

Two other mechanisms of cell mRNA degradation do not appear to be central to the HSV-induced process. (i) As already noted, recent studies have revealed the existence of

pathways effecting rapid turnover of mRNAs encoding certain proto-oncogenes and growth factors (27, 49, 55). However, these transcripts are stabilized by cycloheximide treatment, whereas, as shown in this paper, cycloheximide has no effect on *vhs*-induced mRNA degradation. (ii) Cayley et al. (4) have shown that infection of human Chang cells with HSV-1 or HSV-2 did not induce the ppp(A2′p)$_n$A-dependent RNase. Furthermore, in cells which were infected following interferon treatment, the activity of this enzyme increased only at late times postinfection. Thus, if a cellular RNase is indeed induced by the *vhs*-1 function, it is unlikely to be the enzyme which is involved in the interferon response system.

The role of the *vhs*-1 function in virus replication. As already noted, the half-life of viral mRNA was also prolonged in cells infected with the *vhs*-1 mutant. Although the effects on host and viral mRNAs could be mediated by two separate viral functions, both mutated in *vhs*-1, other studies suggest that this is not the case. Thus, the α proteins are overproduced in cells infected with any of the independently derived *vhs* mutants (43). More recent studies (24a) have shown that the stability of α mRNA was indeed reduced by a component of the infecting wt virions. Also, the duration of synthesis of β and γ proteins following actinomycin D addition was increased in *vhs*-1 virus-infected cells (24a). Finally, a mutation(s) which affects the functional stability of the α, β, and γ mRNAs has been mapped within a 6-kilobase-pair segment of the virus genome (A. D. Kwong, J. Kruper, and N. Frenkel, manuscript in preparation). Taken together, these data suggest that the wt virions contain a single gene product which indiscriminately destabilizes most, if not all, infected-cell mRNAs.

The *vhs* function could play a versatile role in viral replication. First, shutoff of expression of host genes may allow better utilization of the translational machinery of the infected cell for expression of newly synthesized viral mRNAs. Furthermore, it could be advantageous to the virus to limit induction of stress proteins, such as HSP70, which may constitute a response mounted by the cells to virus infection. Finally, the decreased stability of most infected-cell mRNAs may permit rapid transitions in the expression of specific groups of viral genes, the transcription of which is sequentially activated as infection progresses. Although the *vhs* function is not essential for virus growth in culture (43), recent studies have shown that wt virus possesses a growth advantage over the *vhs*-1 mutant (Kwong et al., in preparation). Further studies are needed, however, to assess the role of the *vhs* function during infections in the human host.

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