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The Herpes Simplex Virus Virion Host Shutoff Function

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The virion host shutoff (*vhs*) function of herpes simplex virus (HSV) limits the expression of genes in the infected cells by destabilizing both host and viral mRNAs. *vhs* function mutants have been isolated which are defective in their ability to degrade host mRNA. Furthermore, the half-life of viral mRNAs is significantly longer in cells infected with the *vhs-1* mutant virus than in cells infected with the wild-type (wt) virus. Recent data have shown that the *vhs-1* mutation resides within the open reading frame UL41. We have analyzed the shutoff of host protein synthesis in cells infected with a mixture of the wt HSV-1 (KOS) and the *vhs-1* mutant virus. The results of these experiments revealed that (i) the wt virus shutoff activity requires a threshold level of input virions per cell and (ii) the mutant *vhs-1* virus protein can irreversibly block the wt virus shutoff activity. These results are consistent with a stoichiometric model in which the wt *vhs* protein interacts with a cellular factor which controls the half-life of cell mRNA. This wt virus interaction results in the destabilization of both host and viral mRNAs. In contrast, the mutant *vhs* function interacts with the cellular factor irreversibly, resulting in the increased half-life of both host and viral mRNAs.

Infection of cells with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) results in the shutoff of host protein synthesis and the sequential expression of several coordinately regulated groups of viral genes (13). The inhibition of host macromolecule synthesis in HSV-infected cells is a multiphase process (reviewed in reference 4). A primary phase of the shutoff of host protein synthesis is mediated by a virion component. It occurs in cells infected in the presence of dactinomycin (which prevents viral gene expression) and in cells infected with UV light-irradiated virus (6, 7, 12, 23–25, 28, 34–36). A late (secondary) shutoff function reduces the remaining levels of host protein synthesis and requires the expression of viral genes (5, 24, 28).

HSV-1 and HSV-2 reduce the abundance of host mRNAs in a variety of cell types (1, 6, 14, 15, 18, 21, 22, 24, 25, 27, 30, 32–34). However, the mechanism by which this occurs has yet to be elucidated. In Vero cells, both the shutoff of host protein synthesis and the degradation of host mRNA are observed in the absence of viral gene expression (1, 7, 14, 15, 25, 28, 30, 34). Host mRNA degradation is responsible for the dissociation of host polyribosomes (34).

Several virion host shutoff (*vhs*) mutants were previously isolated in our laboratory (28). The mutants failed to inhibit host protein synthesis in the presence of dactinomycin. Unlike the wild-type (wt) virus, all of these mutants were deficient in their ability to degrade preexisting β-actin and α-tubulin mRNA in the absence of viral gene expression (15, 34). However, *vhs* mutants are not defective in the secondary shutoff function. When viral gene expression is allowed, the synthesis of host proteins is turned off, albeit in a delayed and incomplete manner (15, 28).

Viral protein synthesis begins concomitantly with the shutoff of host protein synthesis. The program of expression involves the sequential turning on of the transcription of several groups of viral genes, including the α (immediate early), β (early), and γ_1 and γ_2 (late) genes (13, 37). As the later viral genes are turned on, the synthesis of earlier viral proteins ceases, implying the existence of a mechanism to turn off the translation of the previously synthesized mRNAs (13, 15). The mechanism of this shutoff is unknown.

HSV encodes a function which indiscriminately reduces the half life of host as well as viral α, β, and γ mRNAs (15, 16, 25, 26, 34). We have proposed that the shutoff of host protein synthesis is a consequence of this function. This hypothesis is based on the finding that both host and viral mRNAs are significantly more stable in cells infected with the *vhs-1* mutant virus than in cells infected with the wt virus. Furthermore, we have mapped the *vhs-1* mutation affecting the shutoff of host protein synthesis, the degradation of host mRNA, and the destabilization of α, β, and γ functional mRNAs within a 265-base-pair (bp) region spanning map coordinates 0.604 to 0.606 of the HSV genome (16). Based on previous transcriptional mapping by Frink et al. (8) and sequence analyses of strain 17 wt virus by McGeoch et al. (19), the *vhs-1* mutation lies within the UL41 open reading frame (16). Recent data (A. D. Kwong, J. Oler, and N. Frenkel, manuscript in preparation) have shown that the *vhs-1* mutant contains a single base mutation in this open reading frame. This protein is homologous to a 61-kilodalton (kDa) protein of HSV-2 which is expressed from a region which has been shown to transform cells in culture (9).

In this study, we report observations which suggest that the wt virus *vhs* protein interacts with a cellular factor, resulting in decreased mRNA stability. In contrast, the mutant *vhs-1* virus protein appears to protect host cell mRNA from degradation by the wt virus *vhs* function. The implications of these observations for the mechanism of action of the *vhs* function are discussed.

MATERIALS AND METHODS

Cells and virus. Rabbit skin cells and mouse Ltk$^-$ cells were obtained from B. Roizman (University of Chicago). Vero monkey cells were obtained from S. Bachenheimer (University of North Carolina) and human epidermoid 2 (HEp-2) cells were obtained from the American Type Culture Collection. The *vhs-1* mutant was derived by bromodeoxyuridine mutagenesis of HSV-1 (KOS), as described pre-

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viously (28). The KOS strain of HSV-1 was obtained from P. A. Schaffer (Harvard Medical School).

Assay for the host shutoff function. Cultures of Vero cells in 24-well plates were preincubated for 30 min with medium 199 plus dactinomycin. This medium consists of medium 199 (KC Biologicals, Lenexa, Kans.) containing 1% heat-inactivated calf serum and 5 μg of dactinomycin (CalBiochem) per ml. The cells were infected with the test virus in medium 199 plus dactinomycin. After 2 h of virus adsorption at 37°C, the cells were rinsed three times and incubated in medium 199 plus dactinomycin. Before being labeled with [^{35}S] methionine, the cells were washed three times with medium 199 lacking methionine and containing 1% dialyzed calf serum and 5 μg of dactinomycin per ml. The cells were then labeled for 2 h in labeling medium containing 5 μg of dactinomycin per ml. Labeling medium consists of medium 199 with 1/20 the normal concentration of unlabeled methionine, 1% dialyzed calf serum, and 50 μCi of [^{35}S]methionine (New England Nuclear Corp.) per ml. The protein samples were prepared as described previously (28).

RESULTS

Dependence of shutoff on the amount of input wt HSV-1 (KOS). Initially, we predicted that the degree of virion-associated host shutoff would depend on the input number of virus particles per cell. Thus, an experiment was designed to investigate the relationship between the degree of host shutoff and the multiplicity of infection (MOI) with the wt virus stock used in the experiments described below. Ltk$^-$ cells were mock infected or infected with 1, 2, 10, or 50 PFU of the wt HSV-1 (KOS) per cell in the presence of dactinomycin (5 μg/ml). The infected-cell polypeptides were labeled with [^{35}S]methionine from 10 to 12 h postinfection in the presence of dactinomycin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1, there was essentially no difference in the amount of host proteins synthesized in mock-infected cells (lane 1) compared with cells infected with 1 or 2 PFU of wt HSV-1 (KOS) virus per cell. The lack of host shutoff at an input MOI of 2 PFU of KOS virus (in the presence of dactinomycin) per cell was also observed in additional experiments in which the level of host cell protein synthesis in mock- and KOS-infected cells was estimated by trichloroacetic acid (TCA) precipitation of [^{35}S]methionine-labeled mock-infected and infected host cell proteins (data not shown). In contrast, there was a dramatic shutoff in the amount of host proteins synthesized in cells infected with 10 PFU/cell (lane 3). Furthermore, the amount of shutoff obtained with 10 PFU of wt HSV-1 (KOS) per cell was not significantly increased when the amount of virus in the inoculum was increased to 50 PFU/cell (lane 5). Thus, there was a sharp transition in the degree of host shutoff observed when the wt HSV-1 (KOS) inoculum increased between 2 and 10 PFU/cell.

Host shutoff in single and mixed infections with wt and *vhs-1* mutant viruses. A second series of experiments were designed to investigate more thoroughly the transition zone between 2 and 10 PFU/cell and to determine whether the wt virion host shutoff (*vhs*) function was a dominant trait. A typical example of these experiments is shown in Fig. 2. Replicate cultures of Ltk$^-$ cells were infected with wt HSV-1 (KOS) at 2, 4, 6, 10, 25, and 100 PFU/cell in the presence of dactinomycin (5 μg/ml). Parallel cultures of cells were infected with wt HSV-1 (KOS) alone or coinfected with a ratio of *vhs-1* mutant virus to wt virus of 1:1, 1:2, 1:4, 1:10, and 1:20 in the presence of dactinomycin. The infected-cell proteins were labeled with [^{35}S]methionine from 12.5 to 18.5 h after infection in the presence of dactinomycin and analyzed by SDS-PAGE and TCA precipitations to quantitate the degree of shutoff of host protein synthesis.

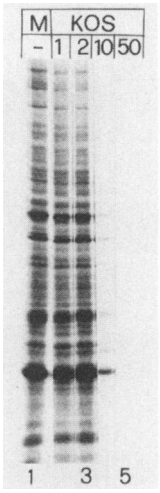

FIG. 1. Dependence of the virion-associated host shutoff function on the MOI of wt virus. Proteins were from lysates of mock-infected Ltk$^-$ cells (M) or cells infected with 1, 2, 10, or 50 PFU of HSV-1 (KOS) virus per cell. The infections were performed in the presence of dactinomycin (5 μg/ml), and the infected-cell proteins were labeled with 50 μCi of [^{35}S]methionine per ml from 10 to 12 h postinfection in the presence of dactinomycin.

The effect of the MOI of the wt virus alone on the degree of shutoff of host protein synthesis will be discussed first (based on lanes 1, 7, 13, 19, 25, and 31 of Fig. 2). As observed in the experiment shown in Fig. 1, there was essentially no shutoff of host protein synthesis (Fig. 2, lane 1) in cells infected with 2 PFU of wt HSV-1 (KOS) per cell. In contrast, wt virus infections of 4 PFU/cell and above resulted in a shutoff of host protein synthesis (lane 7). This sharp transition, which is apparent in the autoradiogram of the polyacrylamide gel and quantitated in the TCA assays shown in Fig. 3B, suggests that a critical threshold of input wt virions is required for the shutoff to occur. The sharp transition in the curve of host shutoff versus input virus was reproduced in multiple experiments (data not shown). The actual concentration of the amount of infectious KOS virus around which the transition occurred changed with different virus stocks. This is probably due to differences in the PFU-to-virus particle ratio characteristic of each virus stock. These results are consistent with the association of the host shutoff function with a structural component of the virion.

Figure 3A shows the same data plotted on a logarithmic scale so as to allow more detailed examination of the region above 4 PFU/cell. The curve can be divided into three parts. In the first part of the curve, as seen in the experiment shown in Fig. 1, there was no effect on host protein synthesis when 2 PFU/cell or less of input HSV-1 (KOS) virus was used. In the second part of the curve, extending from 4 to 25 PFU/cell, there was a sharp increase in the amount of shutoff of host protein synthesis corresponding to a sharp drop in TCA-precipitable counts of host proteins. Finally, in the third part of the curve, between 25 and 100 PFU/cell input, complete shutoff of host protein synthesis was attained.

The simplest interpretation of these results is that shutoff requires the association of a virion factor with a critical cellular component. Below the threshold MOI of 2 PFU/cell,

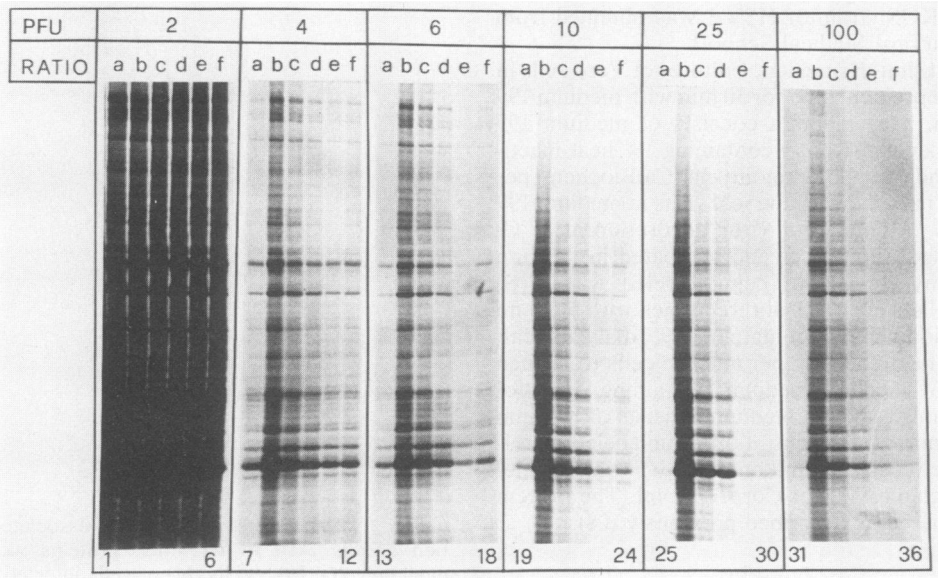

FIG. 2. Host shutoff in single and mixed infections with wt HSV-1 (KOS) and *vhs-1* mutant virus. Infected-cell proteins from lysates of cells infected with 2, 4, 6, 10, 25, or 100 PFU of wt HSV-1 (KOS) per cell and coinfected with *vhs-1* mutant in the following ratios of *vhs-1* to KOS (PFU per cell) in the inoculum: a, no *vhs-1*; b, 1:1; c, 1:2; d, 1:4; e, 1:10; f, 1:20. The infections were done in the presence of dactinomycin (5 μg/ml), and the infected-cell polypeptides were labeled with 50 μCi of [^{35}S]methionine per ml from 12.5 to 18.5 h postinfection in the presence of dactinomycin.

the concentration of the virion factor per cellular component was not high enough to cause detectable shutoff. In contrast, the range of 4 to 25 PFU/cell represents the approach to saturation. Finally, the existence of the plateau when the MOI was greater than 25 PFU/cell suggests that the virion shutoff function has saturated the cellular component. Furthermore, the lack of linearity in the degree of shutoff of host protein synthesis observed compared with the amount of wt virus present in the inoculum is consistent with a multimeric (and perhaps cooperative) form of the virion host shutoff factor in its interaction with a cellular factor.

Shutoff during coinfections with wt and *vhs-1* mutant viruses. The remaining lanes of Fig. 2 represent coinfections with wt virus and the *vhs-1* mutant virus in the presence of dactinomycin. The degree of host shutoff in these coinfections depended on the ratio of wt virus to *vhs-1* mutant virus

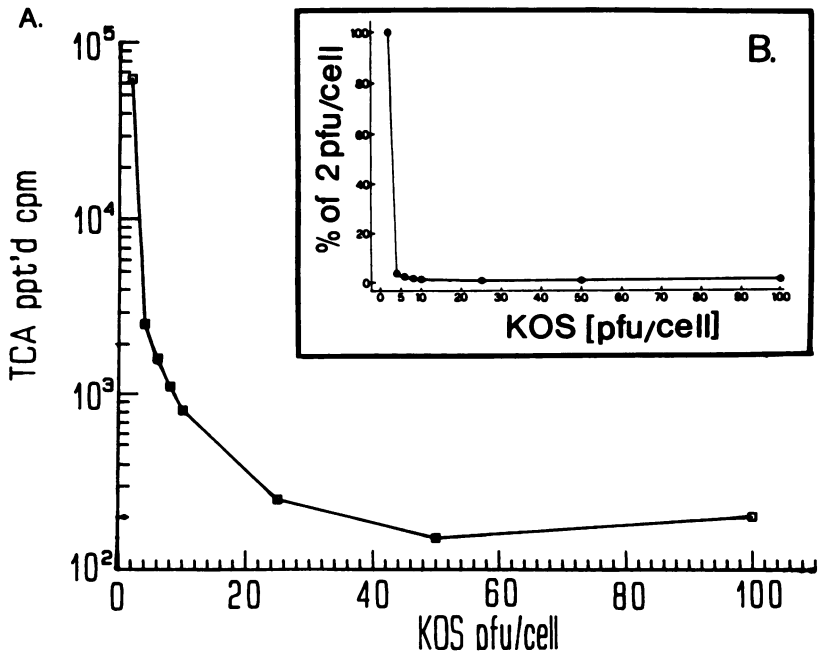

FIG. 3. Shutoff of host protein synthesis as a function of the MOI of wt virus. Duplicate samples of the protein lysates from the experiment described in Fig. 2 were precipitated with TCA to quantitate the degree of host shutoff. (A) Graph of the log of TCA-precipitated (ppt'd) cpm versus MOI of wt HSV-1 (KOS) (PFU per cell). (B) Linear plot of the data. Shown is the amount of TCA-precipitated cpm expressed as a percentage of the value at 2 PFU/cell, plotted as a function of the MOI of wt HSV-1 (KOS) virus.

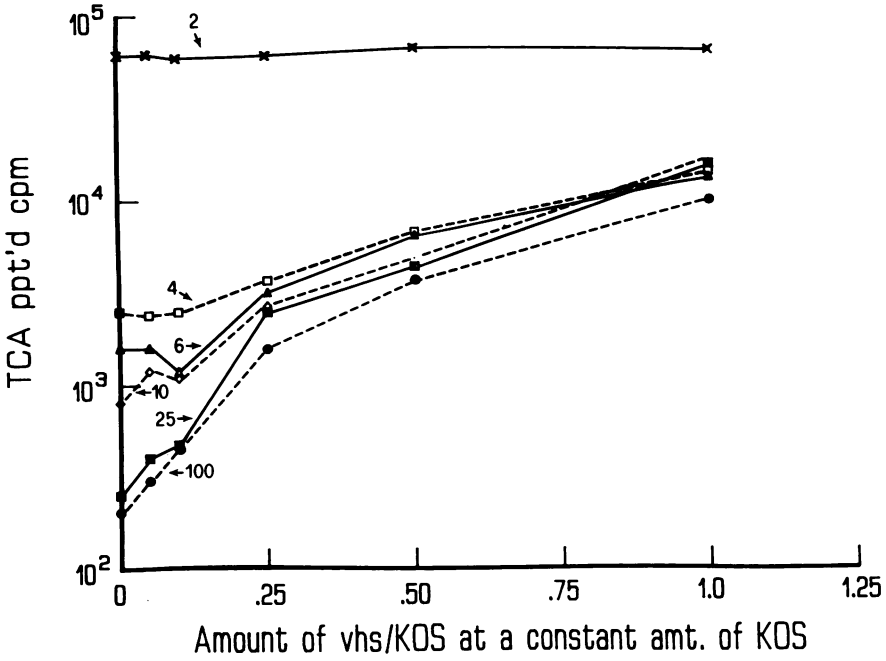

FIG. 4. Shutoff of host protein synthesis as a function of the ratio of *vhs-1* to wt HSV-1 (KOS) in the inoculum. Duplicate samples of the protein lysates from the coinfection experiment described in Fig. 2 were precipitated with TCA to quantitate the host shutoff. The log of the amount of host protein synthesis (in TCA-precipitated [ppt'd] cpm) is plotted as a function of the ratio of *vhs-1* to wt HSV-1 (KOS) virus in the inoculum at a constant MOI of the wt HSV-1 (KOS) for each line. The MOI of the wt virus for each plotted line is labeled on the graph by an arrow.

in the inoculum. To quantitate the results, the amount of TCA-precipitable, [^{35}S]methionine-labeled counts was determined for each of the samples. The results are graphically presented in Fig. 4 and 5. In Fig. 4, the TCA-precipitable counts of host protein synthesis are plotted versus the ratio of *vhs-1* to wt HSV-1 (KOS) virus when 4, 6, 10, 25, or 100 PFU of wt virus per cell was added to the inoculum. In Fig. 5, each curve represents a different ratio of wt to *vhs-1* mutant virus used in the coinfection.

As apparent from Fig. 4, no shutoff was observed below the threshold level of 2 PFU of wt virus per cell, consistent with the concept of a critical threshold of the wt virion factor to the cell component. Furthermore, the rate at which a given level of shutoff was attained depended on the input wt HSV-1 (KOS). As apparent from Fig. 5, the level of shutoff at the high input of wt virus depended on the ratio of KOS to *vhs-1* mutant virus. The dependence of the amount of shutoff of host protein synthesis on the ratio of wt to *vhs-1* mutant virus is supportive of the hypothesis that the *vhs* function interacts irreversibly with a cellular component. If the process were reversible, one would expect that the KOS virus in the inoculum would eventually interact with more and more cellular components, leading to the irreversible shutoff of host protein synthesis (via mRNA degradation). Instead, the *vhs-1* mutant irreversibly protected cell protein synthesis from shutoff by the wt virus.

Virion host shutoff function is irreversible and progressive. A third set of experiments were based on the finding that the *vhs-1* virus protected the cell from shutoff by the wt virus. A time course experiment was performed to determine the extent of reversibility of the shutoff process. Specifically, replicate cultures of Ltk$^-$ cells were mock infected or infected with 10 PFU of KOS virus per cell in the presence of dactinomycin (5 μg/ml). Parallel cultures of KOS-infected cells were superinfected with 100 PFU of *vhs-1* virus per cell at 0 (coinfection), 0.5, 1.0, 2.0, and 6.0 h after the initial KOS wt virus infection. The infected-cell proteins were labeled with [^{35}S]methionine from 8 to 10 h after the initial KOS wt virus infection in the presence of dactinomycin and processed by SDS-PAGE.

As shown in Fig. 6, the ability of coinfection with *vhs-1* virus to "protect" host protein synthesis in KOS-infected cells progressively diminished when the *vhs-1* virus was added at increasingly longer intervals after the initial KOS virus infection. These results suggest that the wt virion host shutoff function acts in a progressive, irreversible manner.

DISCUSSION

Two conclusions can be drawn from the experiments discussed in this paper concerning the *vhs* mechanism. (i) The wt virus *vhs* function interacts irreversibly with a cellular component, causing a progressive and irreversible shutoff of host protein synthesis. (ii) The mutated *vhs* function interacts irreversibly with a cellular component in a manner which allows protein synthesis to occur but prevents the wt *vhs* function from inducing shutoff. It is noteworthy that Hill et al. (12) observed that a virion component of HSV-1 (KOS) could interfere with the rapid shutoff induced by HSV-2 strain 186.

In the remaining discussion of this paper, an attempt was made to derive some models for the shutoff of host protein synthesis by the *vhs* function. In these models, we tried to take into consideration the points listed above and some pertinent results from previous studies which have shown that (i) the wt *vhs* function is required continuously in order for shutoff to occur and the *vhs-1* mutation does not prevent secondary shutoff (28); (ii) host polyribosomes are disaggregated by a virion-associated function (4, 35, 36) (however, this reaction is not inhibited by cycloheximide, a drug which affects the elongation reaction of protein synthesis [7]); and (iii) the *vhs-1* mutant is defective in a virion function which

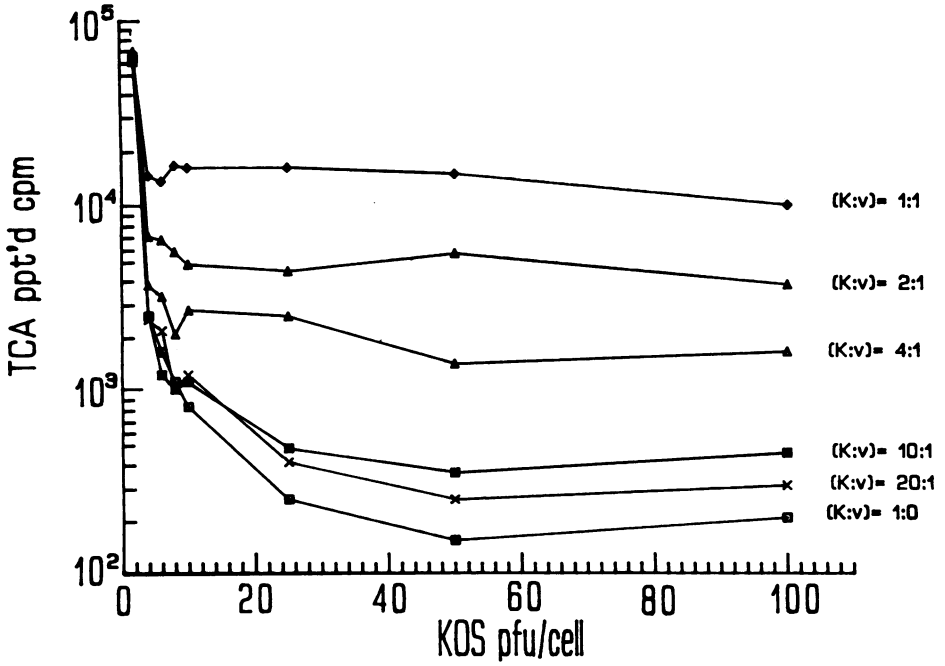

FIG. 5. Shutoff of host protein synthesis as a function of the MOI of wt virus in the mixed infections. Data are from the experiment shown in Fig. 2. The log of the TCA-precipitated (ppt'd) cpm is plotted versus the MOI of wt HSV-1 (KOS) virus (PFU per cell) for each of the different cotransfection HSV-1 (KOS)–*vhs-1* ratios (K:v), which are labeled on the graph.

is associated with the degradation of host and viral mRNA but not rRNA in Vero cells (15, 34). Furthermore, mRNA degradation also occurs in cells infected with wt virus in the presence of puromycin (which disaggregates polyribosomes), as well as cycloheximide (which freezes polyribosomes). This suggests that both free and polyribosome-bound mRNA can serve as a target for the *vhs* function (34).

The observations enumerated above are consistent with at least three models for the mechanism of action of the *vhs* function. Each model postulates the interaction of a virion component with a cellular factor and activation of a function which directly or indirectly mediates the degradation of infected-cell mRNAs.

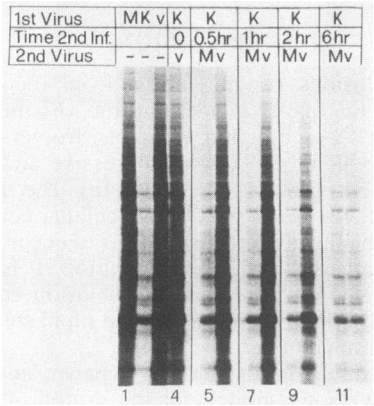

FIG. 6. Degree of host protein synthesis in cells superinfected with *vhs-1* mutant virus at different times after infection with wt virus. Proteins were from lysates of mock-infected Ltk$^-$ cells (M) or from lysates of cells infected with 10 PFU of KOS (K) or *vhs-1* virus (v) per cell. At 0 (coinfection), 0.5, 1, 2, or 6 h after infection with KOS virus, the cells were superinfected with 100 PFU of *vhs-1* virus per cell (lanes 4 to 12). The control infections in lanes 1 to 3 were not superinfected. All infections were done in the presence of dactinomycin, and the infected-cell proteins were labeled with [^{35}S]methionine in the presence of dactinomycin from 8 to 10 h after infection with the first virus.

In the first model, the virion component is a multisubunit RNase which interacts irreversibly with viral and cellular mRNAs, resulting in the irreversible shutoff of protein synthesis in the absence of new transcription. Because the wt HSV-1 (KOS)-induced shutoff is inhibited by the addition of *vhs-1* mutant virions, it can be postulated that the RNase is a multisubunit enzyme and that *vhs-1* multimers as well as mixed KOS–*vhs-1* multimers are inactive. They bind irreversibly to the mRNA without inhibiting the translation of the bound mRNA. The putative bound, inactive multimers can prevent the degradation of the mRNA by preventing its interaction with active, wt RNase.

In the second model, the *vhs* virion component activates a cellular RNase by interacting with the RNase in an irreversible manner. As in the first model, the virion component may be a multimer, and putative *vhs-1* or mixed KOS–*vhs-1* multimers cannot activate the cellular RNase. This model is consistent with a *vhs* component which interacts with a cellular component in a stoichiometric fashion, not in an enzymatic fashion.

In the third model, the *vhs* virion component binds irreversibly to the host translational machinery or to ribonucleoprotein components protecting cellular and viral mRNA or otherwise affecting the half-life of the mRNA. Binding of the wt virus virion component modifies the host translational machinery so that the mRNA stability is reduced and the translational efficiency of host and viral mRNA declines. For example, if the mRNA is not protected by being part of a polyribosome complex, it could be more susceptible to degradation by endogenous cellular RNases. This model is also consistent with the concept that the virion component interacts with a cellular component in a stoichiometric

fashion. As in the first and second models, the active form of the *vhs* protein may be a multimer.

Experiments testing these models involving in vitro translation approaches are in progress.

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