Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1926–1930, April 1987
Cell Biology

Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs

(host shutoff/mRNA degradation)

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Communicated by Bernard Roizman, November 14, 1986 (received for review September 24, 1986)

ABSTRACT **The herpes simplex virus virion contains a function that mediates the shutoff of host-protein synthesis and the degradation of host mRNA. Viral mutants affected in this function (*vhs* mutants) have previously been derived. Cells infected with these mutants exhibit a more stable synthesis of host as well as the immediate early (α)-viral proteins. We now show that a function associated with purified virions of the wild-type virus reduces the half-life of host and α mRNAs, whereas purified *vhs-1* mutant virions lack this activity. The functional half-life of many early (β)- and late (γ)-viral mRNAs is also prolonged in mutant virus infections. These studies suggest that the wild-type virion brings into cells a function that indiscriminately reduces the half-life of both host and viral transcripts and that the early translational shutoff of the host is a consequence of this function. This function may facilitate rapid transitions in the expression of groups of genes that are transcriptionally turned on at different times after infection.**

Cells infected with herpes simplex viruses 1 and 2 proceed through the sequential transcription of several coordinately regulated groups of viral genes, including the α (immediate early), β (delayed early), and γ_1 and γ_2 (late) genes (1–3). As suggested originally by Honess and Roizman (1), this transcriptional regulation must also be accompanied by a negative translational-regulatory scheme. Thus, the translation of host mRNA is turned off soon after virus entry into the cells, and the synthesis of α- and β-viral polypeptides decreases at later intervals after infection.

The early shutoff of host-protein synthesis (4) has been shown to be mediated by a virion component (reviewed in ref. 5). Host shutoff occurs in cells that have been infected in the presence of actinomycin D to prevent viral gene expression (6, 7), in cells infected with UV light-irradiated virus, and in infected enucleated cytoplasts (8, 9). The activity of this virion function is associated with the disaggregation of host polyribosomes (refs. 4 and 10; see ref. 5 for review) and with the degradation of host mRNA (refs. 11–13; T. Strom and N.F., unpublished results). A late (secondary) shutoff function completes the inhibition of host-protein synthesis and requires the prior expression of viral genes (9, 14, 15).

Several herpes simplex virus 1 mutants affected in the virion host shutoff function (*vhs* mutants) have been isolated (15). The mutants fail to inhibit host-protein synthesis in the presence of actinomycin D, and recent studies have shown that they fail to degrade preexisting host mRNA (T. Strom and N.F., unpublished results). The *vhs* mutants turn off host-protein synthesis at late times after infection, suggesting that the primary and secondary host shutoff processes are mediated by separate viral functions (15).

Surprisingly, the *vhs* mutants are also altered with respect to a function that is present in the wild-type (wt) virus inoculum, which decreases the synthesis of α proteins from preexisting α mRNA (15). These findings suggest that a single virion-associated function might limit the translation of both host and α mRNAs.

In this paper, we present data concerning the specificity of the host shutoff function. We show that purified virions of the wt virus, but not those of the *vhs-1* mutant virus, carry a function that reduces the half-life of host and α mRNAs. Furthermore, the functional half-life of many β- and γ-viral mRNAs is also prolonged in *vhs-1*-infected cells. These findings suggest that wt virions contain a function that indiscriminately reduces the half-life of the majority of infected-cell mRNAs.

EXPERIMENTAL PROCEDURES

Cells and Viruses. Mouse Ltk$^-$ cells were obtained from B. Roizman (University of Chicago) and Vero monkey cells were obtained from S. Bachenheimer (University of North Carolina). The derivation of the *vhs-1* mutant was described previously (15). Virus stocks representing cell lysates were prepared by three cycles of freeze-thawing of infected cells (16), and virions were purified in dextran T 10 gradients (17, 18).

Drug Treatment and Analyses of Proteins. For cycloheximide reversal in the presence of actinomycin D (1), the cells were incubated for 30 min in the presence of 50 μg of cycloheximide (Sigma) per ml and then infected in the presence of the drug. Five and one-half hours after infection, actinomycin D (Calbiochem) was added to the medium at a final concentration of 5 μg/ml. After a 30 min incubation, the cycloheximide block was reversed by three washes with medium containing actinomycin D, and incubation was continued in the presence of actinomycin D. The superinfections were done using virus inoculum containing actinomycin D. Prior to protein labeling, the infected cells were washed three times with medium 199 lacking methionine and containing 1% dialyzed calf serum. Proteins were labeled by the addition of 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (New England Nuclear) per ml of medium 199 (KC Biological, Lenexa, KS) containing 1/20th the normal concentration of unlabeled methionine and 1% dialyzed calf serum. Where indicated, actinomycin D was present in the washing and labeling media. Preparation of the protein lysates and gel electrophoresis were done as previously described (15).

Analyses of Infected-Cell RNA. The procedures used in RNA preparation and RNA-blot hybridization will be detailed elsewhere (T. Strom and N.F., unpublished work). Briefly, the cells were lysed in lysis buffer containing aurin tricarboxylic acid and proteinase K, extracted with phenol/chloroform (1:1), and precipitated with ethanol. The samples were suspended in buffer containing 75% (vol/vol) formam-

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Abbreviations: *vhs*, virion host shutoff; wt, wild type; pfu, plaque-forming unit(s); ICP, infected-cell protein.

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ide and electrophoresed in 1.2% agarose gels in 2.2 M formaldehyde/Mops/acetate buffer (19). Blots were prepared by electrotransfer to Nytran (Schleicher & Schuell) in electrotransfer buffer (12 mM Tris, pH 7.8/10 mM dibasic sodium phosphate/0.33 mM EDTA), baked for 2 hr at 80°C, and prehybridized at 42°C in hybridization buffer [50% (vol/vol) formamide/6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate)/2× Denhardt's solution (20)/1% $NaDodSO_4$/salmon sperm or calf thymus carrier DNA (0.2 mg/ml)]. Hybridization was done at 42°C in hybridization buffer containing 10^6 cpm of denatured nick-translated probe per ml. After 24–36 hr, the blots were rinsed three times at room temperature with 2× NaCl/Cit, once (for 5–6 hr) at 70°C in 2× NaCl/Cit and 0.1% $NaDodSO_4$, and three times in 0.2× NaCl/Cit at room temperature.

RESULTS

The Functional Stability of α mRNA Is Affected by a Component of the Virion. Our previous studies revealed that a function present in the wt virus inoculum destabilized the synthesis of host and α proteins (15). In these earlier studies, the virus inoculum was prepared by freeze-thawing the total infected-cell lysates. Therefore, the present study was designed to determine whether the destabilizing effect on α-protein synthesis was mediated by a protein present in the cell lysates or by a component of the virion. Infected cells containing α mRNA were superinfected with purified wt or mutant virions, and the effect of the superinfecting virions on the translation of the preexisting α mRNA was measured. Specifically, replicate Vero cell cultures were mock-infected or were infected with 10 plaque-forming units (pfu) of wt (KOS) virus or *vhs-1* mutant virus per cell. This infection was done in the presence of cycloheximide, which allows the synthesis of α mRNA, but prevents the transcription of the β and γ genes because expression of these genes requires the prior synthesis of α proteins (ref. 1 and reviewed in ref. 3). Six hours after infection, the cycloheximide block was reversed, and the cells were mock-infected or superinfected with 100 pfu of wt or *vhs-1* mutant virus per cell, respectively. Both crude cell lysates and dextran T 10 gradient-purified virions were used as the superinfection inoculum. The superinfection was done in the presence of actinomycin D to prevent the expression of the incoming virus. One set of cultures was then labeled with [^{35}S]methionine from 0 to 2 hr after the reversal of cycloheximide without superinfection. Another set of cultures was labeled 4–6 hr after superinfection in the continued presence of actinomycin D. Comparison of the rates of protein synthesis in cells that were superinfected with crude lysates and purified wt and *vhs-1* virions allowed us to determine whether the superinfecting virions affected the translational stability of preexisting host and α mRNA.

The results of this study (Fig. 1) revealed the following: (*i*) As expected, cells infected with the wt virus in the absence of superinfecting virus exhibit a pronounced shutoff of host protein synthesis and only a transient synthesis of the α-infected cell proteins (ICPs) 4, 0, 22, and 27 (Fig. 1, lanes 3 and 4). In contrast, the synthesis of host and α proteins was stable in the *vhs-1*-infected cells, as seen by their abundant synthesis at both the early and late pulses after cycloheximide reversal (lanes 11 and 12). ICP6 was also expressed as an α polypeptide under these conditions. (*ii*) There was a pronounced shutoff of host- and α-protein synthesis in the cells, which were first infected with *vhs-1* mutant virus and then superinfected with the crude KOS wt virus lysate (lane 14) or with the purified KOS virions (lane 17). In contrast, there was no significant change in the rate of synthesis of host and α proteins after superinfection with the *vhs-1* mutant virus (lanes 15 and 18). (*iii*) The cells that were infected first with the wt KOS virus exhibited a pronounced shutoff of host-protein synthesis. However, the synthesis of host proteins could still be detected during the late pulse in the absence of superinfecting virus (lanes 4 and 5), reflecting, most likely, the lack of expression of the secondary host shutoff function due to the presence of the drugs (15). Superinfection of the wt virus-infected cells with the crude lysate or purified virions of wt virus resulted in a further decline in host- and α-protein synthesis (lanes 6 and 9). In contrast, the level of both host- and α-protein synthesis remained stable in those cells that were superinfected with the *vhs-1* virus (compare lanes 7 and 10 to lane 3). Taken together, these experiments demonstrate that a function present in the wt virions causes the unstable expression of

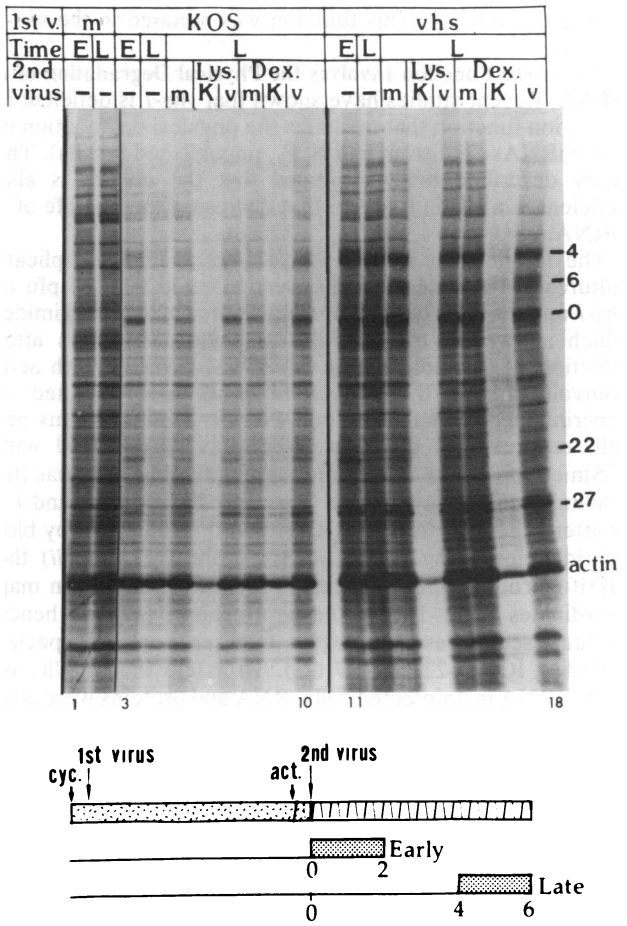

FIG. 1. The functional stability of α mRNA is affected by a component of the virion. (*Upper*) Autoradiogram of a gel containing protein samples of cells which were mock-infected (lanes 1 and 2), or infected with either the wt KOS virus (lanes 3–10) or the *vhs-1* mutant virus (lanes 11–18), in the presence of cycloheximide. Six hours after infection, the cycloheximide block was reversed, and the cells were mock-superinfected (m) or were superinfected with wt KOS (K) or the *vhs-1* (v) mutant virus. Superinfection was with crude lysates (Lys.) or dextran T 10-purified virions (Dex.) in the presence of actinomycin D. The proteins were labeled either early (E, 0–2 hr after the cycloheximide reversal) or late (L, 4–6 hr after the cycloheximide reversal). Lanes 1–4, 11, and 12: no superinfection. Lanes 5 and 13: mock-superinfection with medium. Lanes 6 and 14: superinfection with 100 pfu of KOS crude lysates per cell. Lanes 7 and 15: superinfection with 100 pfu of *vhs-1* crude lysates per cell. Lanes 8 and 16: mock superinfection with the buffer used to resuspend the purified virions. Lanes 9 and 17: superinfection with 100 pfu of dextran T 10 gradient-purified KOS virions per cell. Lanes 10 and 18: superinfection with 100 pfu of *vhs-1* virions per cell. The positions of actin and ICP4, -6, -0, -22, and -27 are indicated. (*Lower*) Schematic outline of the experimental design. Cycloheximide (cyc.) was added 30 min before the first virus infection, and actinomycin D (act.) was added 30 min before the second virus infection.

host and α mRNA. This function was mutated in the *vhs-1* virions.

The α-*vhs* Function Involves the Physical Degradation of α mRNA. Recent studies have shown that *vhs-1* is deficient in the virion function that mediates the physical degradation of host mRNAs (T. Strom and N.F., unpublished results). The study described below revealed that the mutant is also deficient in a virion function that decreases the half-life of α mRNA.

The design of the study is diagrammed in Fig. 2. Replicate cultures of mouse Ltk$^-$ cells were infected with 15 pfu of *vhs-1* mutant virus per cell in the presence of cycloheximide, which allowed α transcription to occur. Six hours after infection, the cycloheximide block was reversed with actinomycin D, and the cells were either mock-infected or superinfected with 150 pfu of wt or *vhs-1* mutant virus per cell, respectively. One set of cultures was labeled with [^{35}S]methionine for 4–5 hr after superinfection, whereas the remaining cultures were used to prepare RNA at 1, 3, and 4.5 hr after superinfection. The RNA was then analyzed by blot hybridization using (*i*) an α-actin probe (21) and (*ii*) the pJD101 probe, which contains sequences arising from map coordinates 0.945–1.00 of the viral genome (22) and hence contains sequences homologous to three α-mRNA species including ICP4, -22 and -47 (23). To assess the stability of actin mRNA in uninfected cells, RNA and proteins were also analyzed in mock-infected cultures that were similarly treated with cycloheximide (for 6 hr) and then actinomycin D.

The results of this experiment are shown in Fig. 2. In the mock-infected cells, host-protein synthesis continued at undiminished rates and host (α-actin) mRNA was stable during the 4-hr actinomycin D chase (compare lanes 1 and 2 and also lanes 6 and 7). In the *vhs-1* virus-infected cells, the synthesis of host and α proteins was stable after superinfection with *vhs-1* virus, but it was markedly reduced after superinfection with the wt virus (lanes 3–5). Furthermore, host and α mRNAs were stable following mock-infection or superinfection with *vhs-1*, but they were significantly degraded after superinfection with wt virus (lanes 8–16 and 19–27). The degradation of the actin and α mRNAs after superinfection with wt virus appeared to follow similar kinetics. One hour after superinfection, both types of mRNAs were partially degraded, and 3 hr after superinfection, they were significantly degraded. This lag in RNA degradation may reflect events in viral entry and uncoating and/or a multistep process that results in the destabilization of the mRNAs. We conclude from this study that the wt virus contains a function that decreases the half-life of both host and α mRNAs. This function is mutated in the *vhs-1* virus.

The Functional Stability of β and γ mRNAs. To determine the effect of the *vhs* mutation on the translational stability of β and γ mRNAs, Vero cell cultures were infected with 15 pfu of the wt or *vhs-1* mutant virus per cell. The infection was done in the absence of drugs to allow the progressive transcription of early- and late-viral genes. At 3, 5, 10, and 16 hr after infection, actinomycin D was added to the cultures to inhibit further transcription. The rate of synthesis of ICPs was then determined at 0–1 hr and 6–7 hr following the addition of actinomycin D.

The results of these studies are shown in Fig. 3, and they reveal significant differences in the translational stability of viral mRNAs in the wt versus *vhs-1* virus-infected cells. Two criteria were used to classify the viral ICPs: First, the kinetics of synthesis of individual proteins throughout the infectious cycle was established by the pattern of their synthesis during the hourly pulses at 3, 5, 10, and 16 hr. Second, the functional stability of the mRNAs was evaluated by comparing the synthesis of individual proteins at 0–1 and 6–7 hr following the addition of actinomycin D.

The first class of viral ICPs included the α proteins. In both

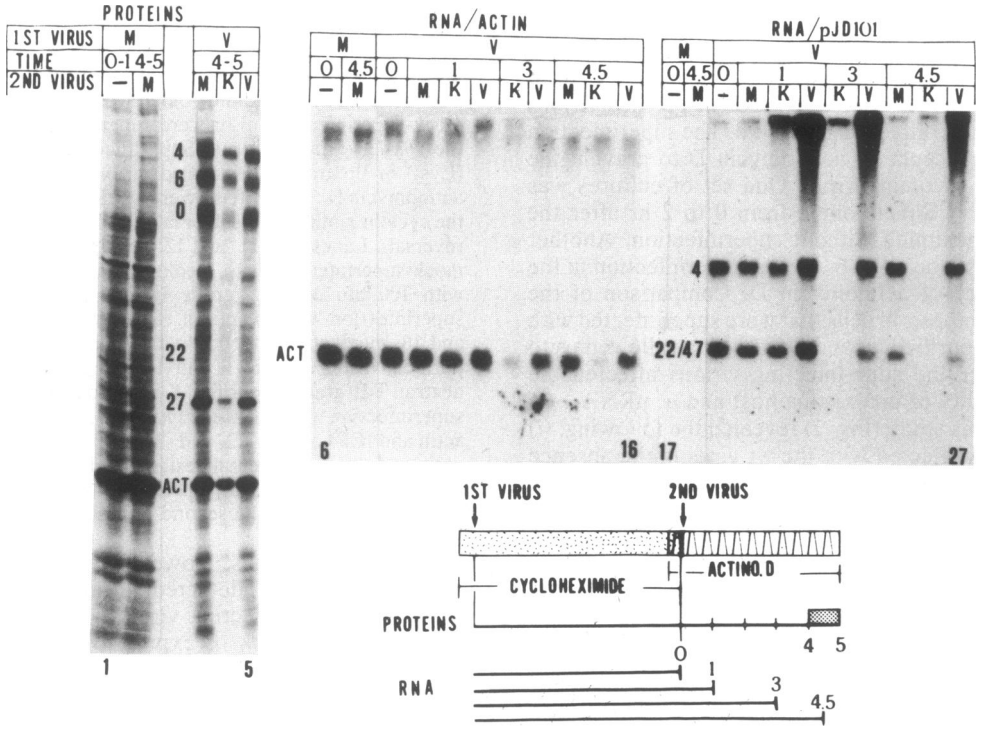

FIG. 2. The *vhs* function affects the stability of host and α mRNAs. Autoradiograms and design of an experiment in which cells were first infected in the presence of cycloheximide and then superinfected in the presence of actinomycin D as described in the legend to Fig. 1. Proteins were labeled from 4–5 hr after the cycloheximide reversal. RNA was harvested at 0, 1, 3, and 4.5 hr after cycloheximide reversal. Lanes 1–5: patterns of protein synthesis in the *vhs-1*-infected cells that were mock-superinfected (M) or infected with wt KOS virus (K) or the *vhs-1* mutant virus (v). Lanes 6–16: blot hybridization of the RNA samples hybridized with an α-actin probe (21). Lanes 17–27: replicate RNA blot that was hybridized with the pJD101 probe (22). The positions of the mRNAs for actin (ACT), α4, and α22 and 47 (22/47), which comigrate, are indicated.

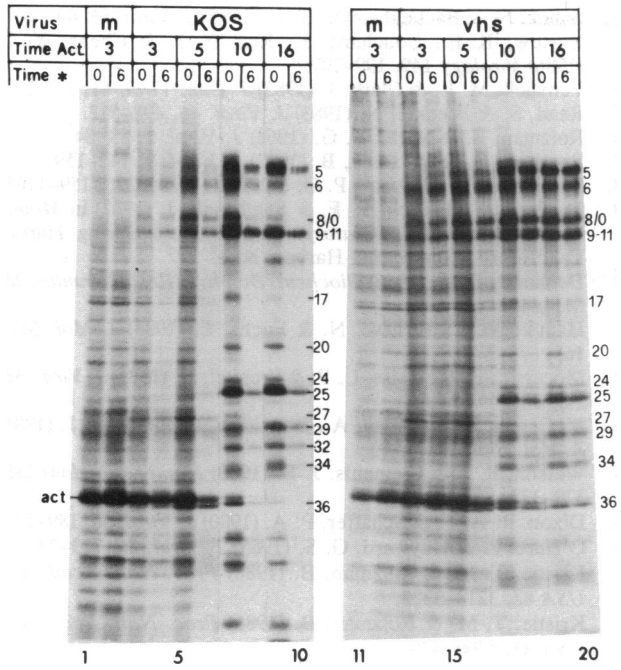

FIG. 3. Functional stability of β and γ mRNAs in wt and *vhs-1*-infected cells. Vero cells were mock-infected (m) or infected with the wt KOS or *vhs-1* mutant virus in the absence of drugs for 3, 5, 10, or 16 hr. At these points actinomycin D was added to the medium, and the cells were labeled for 1 hr either immediately (0) or 6 hr (6) after the addition of actinomycin D. The positions of the actin and viral proteins are indicated.

wt and mutant virus infections, the synthesis of the α proteins increased up to 5 hr after infection and then declined. The α mRNA was functionally unstable throughout the infection with wt virus as evidenced by comparing the rate of synthesis of ICP27 at 0 and 6 hr after the addition of actinomycin D (lanes 3–6). In contrast, α-protein synthesis was relatively stable at the early interval (3 hr) after infection with *vhs-1* virus (lanes 13 and 14) and became more labile in the later intervals of the *vhs-1* infections (lanes 15–18). The eventual destabilization of α-protein synthesis in the *vhs-1*-infected cells resembled the late (secondary) shutoff of host-protein synthesis, which was mediated by the nonvirion host shutoff function. As previously shown, this function was not mutated in the *vhs-1* virus (ref. 15; Fig. 3).

The second class of viral proteins is exemplified by β ICP6, -8, and -36. In wt virus infections, synthesis of these polypeptides had increased at 5 hr, had peaked at 10 hr, and had subsided at 16 hr. In contrast, these β ICPs were synthesized at relatively high rates by 3 hr after *vhs-1* infection. Synthesis then continued throughout the infection and had only partly declined by 16 hr. In cells infected with wt virus, the β mRNAs were functionally unstable, as seen from the decline in the rate of protein synthesis 6 hr after the addition of actinomycin D (Fig. 3, lanes 3–10). In contrast, the rate of protein synthesis in the *vhs-1*-infected cells remained rather stable during each of the actinomycin D chases (lanes 13–20). We conclude from these surprising results that the functional stability of the β mRNAs was lower in wt virus-infected cells than in their *vhs-1* virus-infected counterparts.

The third class of viral proteins included the β/γ and γ polypeptides (ICP5, -9, -10, -11, -14, -25, -29, -32, and -34). The synthesis of these proteins increased at later times after infection and continued at undiminished rates at 16 hr. The mRNAs encoding these polypeptides were functionally more stable in the *vhs-1* virus-infected cells. It is noteworthy that despite this increased functional stability, the rate of the β/γ- and γ-protein synthesis was generally lower in the mutant virus-infected cells than in the wt virus-infected cells. This may reflect overburdening of the cell translational machinery by continued β-protein synthesis.

The last class of viral polypeptides is exemplified by ICP17, -20, and -24. The synthesis of these polypeptides was equally unstable in both wt and *vhs-1* infections. These polypeptides thus appeared to be encoded by unstable mRNAs, the half-life of which was not further decreased by the *vhs* function.

Taken together, these studies reveal that a herpes simplex virus function that is mutated in *vhs-1* modulates the functional half-life of the majority of infected-cell mRNAs.

DISCUSSION

The Host and α-*vhs* Functions Are Most Likely Mediated by the Same Gene Product. We have used the *vhs-1* mutant of herpes simplex virus 1 to investigate the specificity of the virion-associated host shutoff function. These studies extended our previous observation regarding the α-destabilization activity (15) and revealed that the translational stability of β and γ mRNAs is also altered in *vhs-1*-infected cells.

Although it is formally possible that the *vhs-1* mutant virus contains multiple mutations, evidence strongly supports the hypothesis that the host- and viral-mRNA shutoff phenotypes are mediated by the same viral product. Thus, the host- and α-mRNA degradation functions are associated with the structural virion, and several additional *vhs* mutants, which were derived from several independently mutagenized virus stocks (*vhs-2* through *vhs-6*), were previously found to exhibit altered α-gene expression. Moreover, one of the mutants (*vhs-4*) is temperature sensitive with respect to both the host- and α-shutoff phenotypes. Finally, it has been found (J. Kruper, A.D.K., and N.F., unpublished results) that the altered host, α, and β/γ phenotypes all map within a 260-base-pair region of the *vhs-1* mutant virus DNA. Taken together, these results suggest that the wt virus encodes a single, virion-associated function, which decreases the half-life of most infected-cell mRNAs. However, at present, we cannot rule out other explanations. For example, the destabilization of β- and γ-protein synthesis could be mediated by a function that depends on prior alterations of the translational machinery of the infected cells by the virion-associated shutoff function.

The Possible Role of the Host and Viral Shutoff Functions. The indiscriminate nature of the host and viral translational shutoff function(s) raises the interesting possibility that the host shutoff function may be part of a mechanism that the virus has evolved to regulate the expression of its own genes. According to this model, a virion function that is brought into the cell at the time of infection allows rapid adjustments in the pattern of protein synthesis in response to alterations in transcription. As transcriptional activation turns on different classes of viral genes, only the newly transcribed viral mRNAs will be translated, whereas mRNA transcribed at earlier intervals after infection will be functionally deactivated. An optimal level of viral gene expression can thus be attained by means of three mechanisms including (*i*) transcriptional activation; (*ii*) transcriptional shutoff—e.g., by means of autoregulation by ICP4 (24–26); and (*iii*) mRNA destabilization, by means of the *vhs* function.

Because the *vhs-1* mutant grows to relatively high titers, which are at most 10 times lower than those of the wt virus, we have previously concluded that the *vhs* function was not absolutely essential for virus growth in cultured cells (15). However, the timely shutoff of host-, α-, and β-protein synthesis might be advantageous under limiting conditions, which might exist *in vivo*. It may allow an efficient and sequential expression of specific viral gene products at the time when they are required for the progression of viral

replication. For example, limiting the expression of α and β genes at the time when γ-gene transcription is turned on, may allow maximal synthesis of the β/γ- and γ-structural proteins when virion maturation begins. Indeed, as is apparent in Fig. 3, the synthesis of γ proteins in *vhs-1* virus-infected cells is somewhat delayed and reduced.

Limiting the expression of α and β genes may be advantageous in another respect. Thus, the α proteins play a role in the transcriptional activation of later viral genes and, as recently shown, may act by binding to specific promoter sequences (27–29). Synthesis of excessive amounts of these proteins could lead to altered transcriptional specificity (30) and to the turning on of host genes, which can be activated at some efficiency by the α4 function (31–33). Similarly, the β genes have been assigned functions related to nucleotide metabolism and viral DNA synthesis. An imbalance, or excess, of these activities may lead to wastage and altered specificity (34–37).

We thank Dr. R. Camilleri for help in the preparation of this manuscript. These studies were supported by U.S. Public Health Service Research Grants AI 15488 and CA 19264. A.D.K. was a predoctoral trainee supported by Training Grant PHS AI 07182.

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