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Control of mRNA Stability by the Virion Host Shutoff Function of Herpes Simplex Virus

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***vhs*1 is a mutant of herpes simplex virus type 1 that is defective in the virion host shutoff function responsible for the degradation of cellular mRNAs and the concomitant shutoff of host protein synthesis. In this study, the effect of the *vhs*1 mutation on the metabolism of viral mRNAs was examined by measuring the half-lives and patterns of accumulation of 10 different viral mRNAs representing all kinetic classes. The *vhs*1 mutation had the effect of dramatically lengthening the cytoplasmic half-lives of all 10 mRNAs. In wild-type virus infections, the 10 mRNAs had similar half-lives, suggesting that little, if any, target mRNA selectivity was exhibited by the *vhs* function. The *vhs*1 mutation caused overaccumulation of a number of mRNAs. The effect was most dramatic for the alpha (immediate-early) mRNA for ICP27 and the beta (early) mRNAs encoding thymidine kinase, ICP8, and DNA polymerase. Whereas in wild-type infections these mRNAs increased to peak levels and subsequently declined in abundance, in *vhs*1 infections they continued to accumulate until late times. A significant but less dramatic overaccumulation was observed for several beta-gamma (delayed-early) and gamma (late) mRNAs. The results suggest that the *vhs* protein plays an important role in determining the half-lives of viral mRNAs belonging to all kinetic classes and in so doing is important in the normal downregulation at late times of alpha and beta gene expression.**

In cells infected with herpes simplex virus type 1 (HSV-1), viral gene expression is regulated in a complex cascade fashion (22). For most viral gene products, the rate of synthesis of a protein is directly proportional to the amount of the corresponding mRNA in the cytoplasm (14, 27). Since mRNA levels are necessarily governed by the rate of mRNA degradation as well as the rate of synthesis, factors that control either process will affect gene expression. Many studies of the control of transcription in HSV-1-infected cells have been reported; however, to date few have addressed the factors that govern mRNA stability. The experiments reported in this paper concern the regulation of viral mRNA half-lives by the virion host shutoff (*vhs*) function of HSV-1.

One of the earliest events during lytic HSV-1 infections is the shutoff of most cellular protein synthesis (3–6, 11–13, 16–18, 20, 23, 28–30). This shutoff is initially caused by a structural component of the infecting virion which causes disaggregation of cellular polyribosomes (29, 30) and degradation of host mRNAs (24). Mutant virus containing alterations in the *vhs* gene produce virions that are unable to suppress host protein synthesis or degrade cellular mRNAs (20, 28). Recently, the mutation carried by the mutant *vhs*1 has been mapped to an open reading frame able to encode a protein with a predicted molecular mass of 55 kilodaltons (13, 15). However, the *vhs* protein has yet to be identified within virions or infected cells.

The first demonstration that the *vhs*1 mutation affects the half-lives of viral as well as host mRNAs was provided by the observation that, following reversal of a cycloheximide block present from the time of infection, alpha (immediate-early) viral mRNAs decayed much more rapidly in cells infected with wild-type, HSV-1 than in cells infected with *vhs*1 (18). Subsequently, it was shown that if *vhs*1-infected cells that were already expressing alpha mRNAs were superinfected with wild-type virus in the presence of dactinomycin, a component of the superinfecting wild-type virions destabilized the alpha mRNAs encoded by *vhs*1 (12).

Several studies have suggested that the *vhs*1 gene product may affect the stabilities of beta (early), beta-gamma (delayed early), and gamma (late) viral mRNAs in addition to alpha and host mRNAs. First, the *vhs*1 mutation has been known for some time to affect the accumulation of beta and gamma viral polypeptides within the infected cell (20). Second, at late times after *vhs*1 infection, there are increased amounts of some beta and gamma mRNAs in the cytoplasm (28). Third, Kwong and Frenkel added dactinomycin to infected cultures and then pulse-labeled the cells with [^{35}S]methionine either immediately or after an additional 6 h in the presence of dactinomycin (12). When they compared the proteins being synthesized before and after the 6 h incubation in the presence of dactinomycin, they found that many viral proteins continued to be translated longer in cells infected with *vhs*1 than in cells infected with wild-type virus. While the above data are consistent with the hypothesis that the *vhs* gene product affects the physical stability of beta and gamma mRNAs, they are also consistent with other possibilities.

The present study was undertaken to evaluate the role of the *vhs* gene product in regulating the half-lives of viral mRNAs belonging to different kinetic classes and to assess the importance of controls of mRNA stability in the overall scheme of gene regulation in HSV-1-infected cells. To this end, the half-lives and patterns of accumulation of 10 different viral mRNAs were determined in cells infected with wild-type virus or *vhs*1. The results indicate that the *vhs* gene product significantly affects the stability of viral mRNAs belonging to all kinetic classes. In so doing, it affects the levels and kinetics of accumulation of a number of viral mRNAs. In particular, the *vhs* protein appears to play an important role, in conjunction with transcriptional controls, in the normal downregulation at late times of alpha and beta gene expression.

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MATERIALS AND METHODS

Cells and virus. Vero and HeLa S3 cells were purchased from the American Type Culture Collection and grown in Eagle minimum essential medium (MEM; GIBCO Laboratories) supplemented with antibiotics and 10% (vol/vol) calf serum. Stocks of wild-type HSV-1, strain KOS, and the mutant *vhs*1 were prepared from infected Vero cell monolayers as described previously (20). The *vhs*1 mutant grows well at all temperatures from 34 to 39°C and exhibits a defective virion host shutoff function at all temperatures (20). All experiments were performed at 34°C. In all experiments, virus was allowed to adsorb for 1 h in MEM containing 1% (vol/vol) calf serum. The inocula were then aspirated, and the cells were overlaid with fresh MEM containing 1% calf serum.

Isolation of cytoplasmic RNA. Total cytoplasmic RNA was prepared from monolayer cultures of 1×10^7 to 2×10^7 Vero or HeLa cells as described previously (18). The cells were first scraped into ice-cold phosphate-buffered saline and collected by low-speed centrifugation. They were then suspended in 2.2 ml of resuspension buffer (10 mM Tris [pH 7.9], 0.15 M NaCl, 1.5 mM $MgCl_2$) and lysed by addition of Nonidet P-40 (Calbiochem-Behring) to 0.5%. After 10 min on ice, the nuclei were pelleted by low-speed centrifugation, and the supernatant was removed to a fresh tube. The nuclei were washed with 1.2 ml of resuspension buffer containing 0.65% Nonidet P-40, and the wash was added to the first supernatant. The combined cytoplasmic supernatants were further clarified by centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 10 min and then added to an equal volume of urea buffer containing 10 mM Tris (pH 7.9), 7 M urea, 0.35 M NaCl, 10 mM EDTA, and 1% sodium dodecyl sulfate (SDS). The mixture was extracted twice with phenol-chloroform (1:1) and twice with chloroform, and the RNA was precipitated by addition of 2.5 volumes of ethanol and storage of the sample at −20°C overnight. After precipitation from ethanol a second time, the RNA samples were dissolved in a small volume of 10 mM Tris (pH 7.9)–1 mM EDTA and stored frozen at −90°C.

Gel electrophoresis and Northern (RNA) blot transfer of RNAs. Samples (5 μg) of total cytoplasmic RNA were denatured with glyoxal and electrophoresed through 1.2% agarose gels cast in 10 mM sodium phosphate, pH 6.8, as described previously (21). The RNAs were then electroblotted onto GeneScreen Plus membranes (Du Pont NEN Products) with a transblot apparatus (Bio-Rad Laboratories) and a transfer buffer of 12 mM Tris–6 mM sodium acetate–0.3 mM EDTA, pH 7.5. Following electrophoretic transfer of the RNA, the membranes were rinsed for 30 s in 50 mM NaOH to remove the glyoxal and then neutralized by a 30-s rinse in 0.2 M Tris (pH 7.5)–1 × SSC (0.15 M NaCl, 0.015 M sodium citrate). The filters were then air dried at room temperature.

DNA probes. DNA probes for use in Northern analysis or pulse-chase experiments were derived from recombinant pBR325 plasmids or M13 phages containing as inserts appropriate fragments of the HSV-1 genome. The plasmids pSG28 and pSG87, which were used for Northern blot hybridization, were kindly provided by Myron Levine and Rozanne Sandri-Goldin (8). pSG28 was used as a probe to detect the mRNAs for ICP4, ICP0, and ICP27, while pSG87 was used to detect thymidine kinase and glycoprotein H (gH) mRNAs. Both plasmids were maintained in *Escherichia coli* HB101. The plasmid pX1r11 contained a 4.6-kilobase (kb) fragment of *Xenopus laevis* rDNA inserted into the *Eco*RI site of colicin E1 (2). The cloned fragment contained most of the 28S rRNA coding region and a small portion of the 18S rRNA region. pX1r11 was kindly provided by Jeff Doering. Plasmid DNAs were prepared by standard protocols (1).

The probes that were used for Northern analysis of ICP8, DNA polymerase, glycoprotein B (gB), ICP5, and glycoprotein C (gC) mRNAs were prepared from recombinant M13 phages containing as inserts intragenic fragments of the viral genes. These phages were kindly provided by Steven Weinheimer and have been described previously (31). The phages contained the following inserts of HSV-1 DNA: ICP8, a 1.6-kb *Sal*I-*Bam*HI fragment; DNA polymerase, a 0.6-kb *Bgl*II-*Eco*RI fragment; gB, a 0.6-kb *Pst*I-*Sal*I fragment; ICP5, a 0.6-kb *Bam*HI-*Hin*dIII fragment; and gC, a 1.6-kb *Eco*RI-*Bam*HI fragment.

To prepare probes for Northern blot analysis, the inserts were excised from appropriate plasmids or M13 replicative forms. DNA fragments were separated by agarose gel electrophoresis, and the desired inserts were purified from low-melting-temperature agarose (21). The probes were then labeled with ^{32}P by nick translation with a nick translation kit from Bethesda Research Laboratories (Rockville, Md.).

For preparation of probes to be used in the pulse-chase experiment, we found it convenient to reclone the inserts from recombinant M13 phages into the equivalent sites of the polylinker of the plasmid Bluescript SK (Stratagene). The recombinant phages that were used for the pulse-chase experiment contained the following inserts: for ICP0, a 1.6-kb *Sst*I-*Bam*HI fragment; for TK, a 1.1-kb *Bgl*II-*Hin*dIII fragment from the 3′ *tk* deletion mutant Δ3′-1.6 (31). These phage were also provided by Steven Weinheimer.

Hybridizations. Membranes containing transferred RNAs were hybridized to nick-translated probes by the protocol of Graham and co-workers (9). The filters were first prehybridized at 42°C for 24 h in prehybridization buffer containing 50% formamide (Mallinkrodt, Inc.), 10% dextran sulfate (Pharmacia Inc.), 10% Denhardt solution (1), 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM sodium phosphate [pH 6.8], 1 mM EDTA), 1% sodium lauryl sulfate, and 250 μg of denatured salmon sperm DNA per ml. Following prehybridization, the probe was denatured by being placed in boiling water for 10 min and was then added to the prehybridization buffer. The final probe concentration was generally 5×10^7 to 10×10^7 cpm/ml. Hybridization was then continued at 42°C for 24 h. At the end of hybridization, the blots were washed twice for 45 min each at room temperature in 2× SSPE–0.4% SDS and then twice more for 15 min each in 0.1× SSPE at 60°C. The still moist blots were then sealed inside freezer bags and exposed to Kodak XAR-5 film for autoradiography.

For quantitation of mRNA levels, appropriate exposures of the autoradiograms were scanned with a Gilford-Response spectrophotometer equipped with a gel-scanning apparatus, and the areas under the peaks were integrated. In Fig. 2 through 5, showing the kinetics of mRNA accumulation, the amount of any given mRNA is expressed in arbitrary units. The specific activities of the probes for different mRNAs varied, and exposures of different length were scanned for different mRNAs. Thus, while the data allowed comparison of the levels of a particular mRNA in mutant and wild-type infections or the levels at different times after infection, it was not possible to directly compare the level of one type of mRNA with that of another.

After appropriate exposures of the autoradiogram had been obtained, the probe was stripped from the filters by boiling the membranes for 20 to 30 min in 10 mM Tris (pH

7.9)–1 mM EDTA–0.1% SDS. Complete stripping of the probe was monitored by autoradiography. Following stripping, the filters were ready for hybridization to another probe. This procedure allowed at least five rounds of hybridization to be performed with each filter.

Measurement of mRNA half-lives with a dactinomycin chase. Vero cell monolayers were infected with 20 PFU of wild-type virus or *vhs*1 per cell. At 6 h postinfection 5 μg of dactinomycin per ml was added to the culture medium, and incubation was continued. Total cytoplasmic RNA was extracted at 45-min intervals, and the decay of specific mRNAs in the presence of dactinomycin was analyzed by Northern hybridization. To control for small variations in the amount of mRNA that was loaded onto different gel lanes, the results were normalized to the amount of 28S rRNA detected by hybridization to the plasmid pX1r11.

Measurement of mRNA half-lives with a pulse-chase protocol. Measurement of mRNA half-lives by pulse-chase analysis was performed as described by Pilder and co-workers (19) with minor modifications. HeLa cells infected with 20 PFU of wild-type virus per cell were pulse-labeled with MEM containing 200 μCi of [^{3}H]uridine per ml for 30 min beginning at 5.5 h postinfection. The label-containing medium was then removed, and the cells were washed and then overlaid with MEM containing 5 mM each cold uridine and cytidine and 35 mM glucosamine. Total cytoplasmic RNAs were prepared at 45-min intervals and then subjected to exhaustive digestion with RQ1 DNase (RNase free; Promega Biotec), followed by phenol-chloroform extraction and ethanol precipitation. The samples of labeled RNA were then hybridized to specific fragments of the viral genome which had been fixed to nitrocellulose filter disks. For pulse-chase experiments, HeLa cells were used rather than Vero cells because we were able to obtain a more complete chase of the label with HeLa cells. Nevertheless, the patterns of viral gene expression are very similar in HSV-1-infected HeLa and Vero cells (data not shown).

To fix the recombinant plasmid DNAs to nitrocellulose filters, the plasmids were linearized by restriction enzyme digestion, and 15-μg portions were denatured and fixed to 25-mm nitrocellulose filter disks as described previously (1). Labeled RNA samples from approximately 5×10^6 cells were hybridized to the filter disks as described by Zhang and co-workers (32). Briefly, the disks were prehybridized overnight at 68°C in 2× SSC (1× SSC is 0.15 NaCl and 0.015 M trisodium citrate) containing 5× Denhardt solution. The labeled RNA samples were then added to the prehybridization buffer, and hybridization was continued at 68°C for 48 h. The filters were then washed twice for 30 min each at room temperature with 2× SET (1× SET is 0.15 M NaCl, 20 mM Tris [pH 7.8], 1 mM EDTA) containing 0.1% SDS and twice for 30 min each at 60°C with 0.1× SET containing 0.1% SDS. They were then digested for 30 min with 5 μg of RNase A (Sigma Chemical Co.) per ml and then washed twice more for 30 min each at 60°C with 0.1× SET–0.1% SDS. The filters were then air dried and counted with a toluene-based fluor.

RESULTS

Effect of *vhs*1 mutation on accumulation of different viral mRNAs. In an earlier study, we showed that, following reversal of a cycloheximide block present from the time of infection and addition of dactinomycin, alpha mRNAs decayed more rapidly in cells infected with wild-type HSV-1 than in cells infected with the *vhs*1 mutant (18). This suggested that, at least under the conditions of a cycloheximide reversal experiment, the *vhs* gene product negatively regulates the stability of alpha mRNAs. If the *vhs* gene product also regulates the half-lives of alpha mRNAs during lytic infections in the absence of drugs, one would expect the *vhs*1 mutation to affect the accumulation of alpha mRNAs within infected cells. To test this prediction, Vero cells were infected with 20 PFU of either wild-type or *vhs*1 virus per cell and cytoplasmic mRNAs were prepared at 2-h intervals. The levels of selected alpha mRNAs were then analyzed by Northern blotting and quantitated by densitometric scanning of appropriately exposed autoradiograms. At the same time, the accumulation kinetics of selected beta, beta-gamma, and gamma mRNAs were also determined. The results shown in Fig. 1 through 5 indicate that the *vhs*1 mutation affected the accumulation kinetics of a number of different viral mRNAs belonging to different kinetic classes.

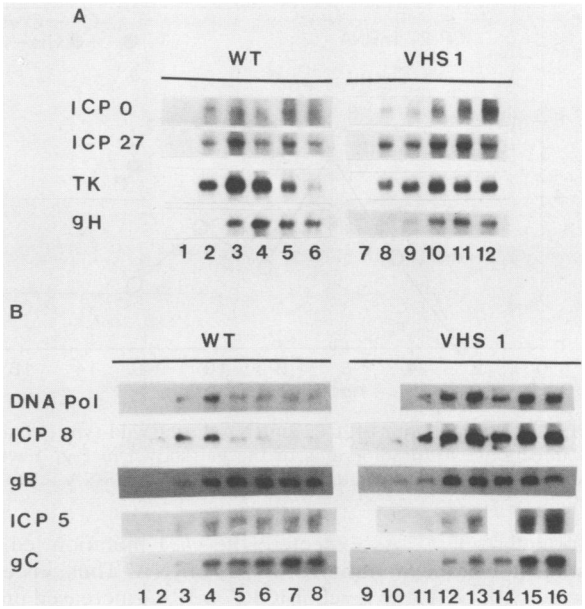

FIG. 1. Accumulation of HSV-1 mRNAs during wild-type (WT) and *vhs*1 infections. (A) Vero cells infected with 20 PFU of either wild-type virus (lanes 1 to 6) or *vhs*1 (lanes 7 to 12) per cell. Total cytoplasmic RNA was extracted at 2.5 h (lanes 1 and 7), 4.5 h (lanes 2 and 8), 6.5 h (lanes 3 and 9), 8.5 h (lanes 4 and 10), 10.5 (lanes 5 and 11), and 12.5 h (lanes 6 and 12). (B) Vero cells were infected with 20 PFU of either wild-type virus (lanes 1 to 8) or *vhs*1 (lanes 9 to 16) per cell. Total cytoplasmic RNA was extracted at 2 h (lanes 1 and 9), 4 h (lanes 2 and 10), 6 h (lanes 3 and 11), 8.25 h (lanes 4 and 12), 10 h (lanes 5 and 13), 12 h (lanes 6 and 14), 14 h (lanes 7 and 15), and 16 h (lanes 7 and 16). Samples (5 μg) of RNA were analyzed by Northern blotting. DNA Pol, DNA polymerase.

Alpha mRNAs. The *vhs*1 mutation affected the accumulation kinetics of the alpha mRNAs for both ICP27 and ICP0. In cells infected with wild-type virus, ICP27 mRNA appeared early in infection, peaked in abundance at about 6 h, and then declined (Fig. 1 and 2). By late times, only 40 to 50% of the peak amount of mRNA was still present in the cytoplasm. Similar accumulation profiles have been reported previously for the alpha mRNAs encoding ICP22 and ICP47 (31) and ICP4 (10, 31) with the exception that in these studies the time of peak alpha mRNA accumulation was slightly earlier than was observed in our experiments. It is likely that this difference was due to the fact that the present experiments were performed at 34°C while the earlier studies were

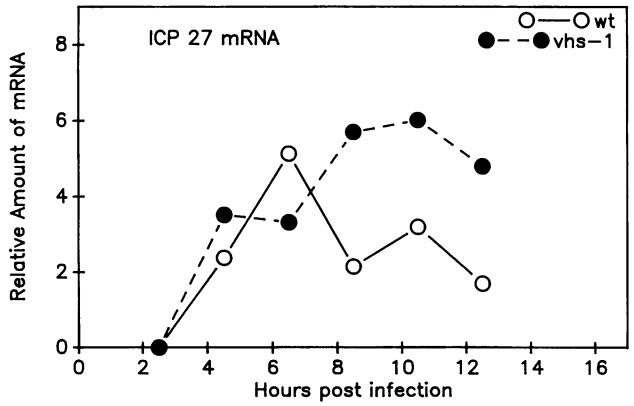

FIG. 2. Accumulation of ICP27 mRNA during wild-type (wt) and *vhs*1 infections. Autoradiograms of the gel shown in Fig. 1 were scanned in order to quantitate the levels of ICP27 mRNA.

performed at 37°C. As expected, the *vhs*1 mutation led to significant overaccumulation of ICP27 mRNA. Thus, in cells infected with *vhs*1, the level of ICP27 mRNA increased until 8 h and then remained high until at least 12.5 h after infection. At late times there was at least twice as much ICP27 mRNA in cells infected with *vhs*1 as in cells infected with wild-type virus.

In wild-type infections ICP0 mRNA appeared in the cytoplasm at the same time as ICP27 mRNA and initially increased in abundance with similar kinetics (Fig. 1). However, although the diffuse nature of the band for ICP0 mRNA made precise quantitation difficult, it is apparent that at late times the level of ICP0 mRNA did not decline to the same extent as did the level of ICP27 mRNA. Similarly, both Harris-Hamilton and Bachenheimer (10) and Weinheimer (31) have reported that at late times ICP0 mRNA did not decline in abundance as much as did the mRNAs encoding ICP4, (10, 31), ICP22, and ICP47 (31). In contrast to wild-type infections, in cells infected with *vhs*1 the level of ICP0 mRNA continued to increase throughout the first 12.5 h of infection. Thus, at late times there was two to three times as much ICP0 mRNA in *vhs*1-infected cells as in cells infected with wild-type virus.

Beta mRNAs. In wild-type virus infections, the beta mRNAs encoding ICP8, thymidine kinase (TK), and DNA polymerase all accumulated with similar kinetics (Fig. 1 and 3). Each appeared slightly later than the alpha mRNAs and increased to peak levels by 6 to 8 h after infection. mRNA levels then declined sharply, so that by 12.5 h very little mRNA remained in the cytoplasm. In our hands the decline in abundance of the beta mRNAs was noticeably sharper and more complete than it was for ICP27 mRNA. This pattern of accumulation was similar to those reported previously for various beta mRNAs by other investigators (10, 25, 31).

The *vhs*1 mutation had a striking effect on the accumulation of all three beta mRNAs (Fig. 1 and 3). ICP8, TK, and DNA polymerase mRNAs all overaccumulated in mutant infections. In contrast to the wild-type accumulation patterns, characterized by a peak and subsequent decline, in *vhs*1 infections the beta mRNAs reached peak levels at 10 to 12 h after infection and then leveled to a plateau rather than declining. Thus, *vhs*1 is defective in a function that plays an important role in the normal turning-down of beta gene expression.

Beta-gamma mRNAs. In wild-type virus infections, the beta-gamma mRNAs encoding ICP5, glycoprotein B (gB),

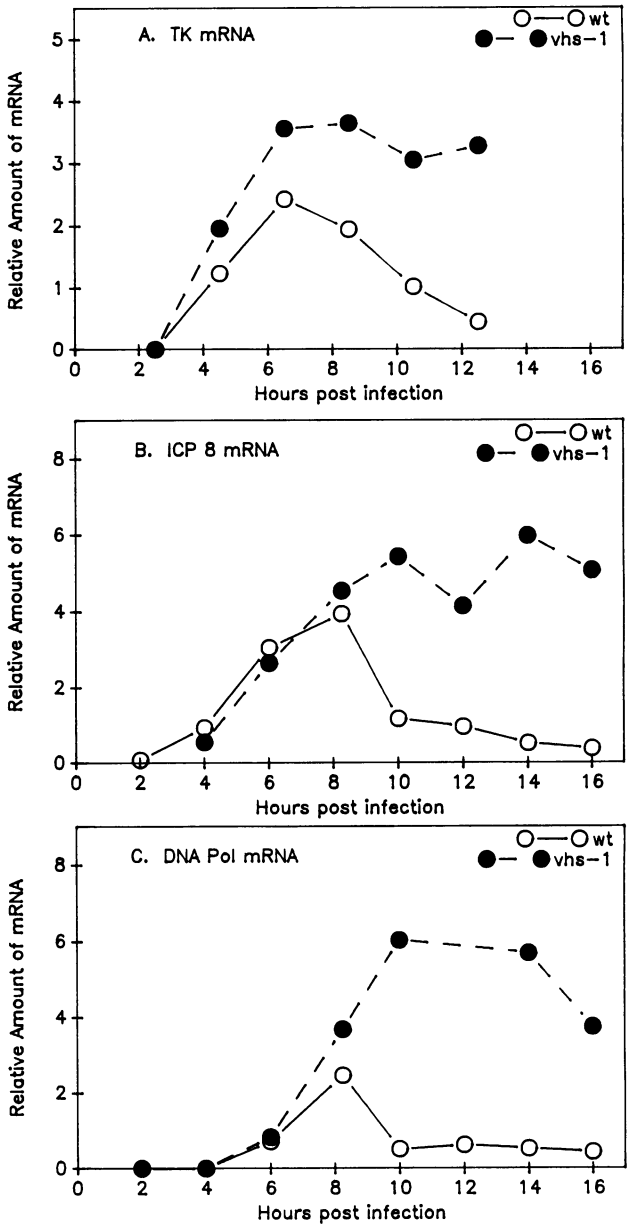

FIG. 3. Accumulation of beta mRNAs during wild-type (wt) and *vhs*1 infections. Autoradiograms of the gels shown in Fig. 1 were scanned in order to quantitate the levels of beta mRNAs. (A) TK; (B) ICP 8; (C) DNA polymerase.

and glycoprotein H (gH) all appeared in the cytoplasm at about the same time as the beta mRNAs (Fig. 1, 3, and 4). However, unlike the beta mRNAs, the beta-gamma mRNAs did not decline in abundance at late times. Instead, significant amounts of these mRNAs remained in the cytoplasm as late as 12 to 16 h after infection.

The *vhs*1 mutation affected the accumulation of the three beta-gamma mRNAs to different extents (Fig. 1 and 4). However, in no instance was the overaccumulation as dramatic as that observed for the beta mRNAs. At late times, ICP5 mRNA was overproduced to the greatest extent of the three mRNAs. In wild-type infections, the levels of ICP5 mRNA remained relatively constant at 8 h postinfection. In contrast, in *vhs*1 infections the level of ICP5 mRNA contin-

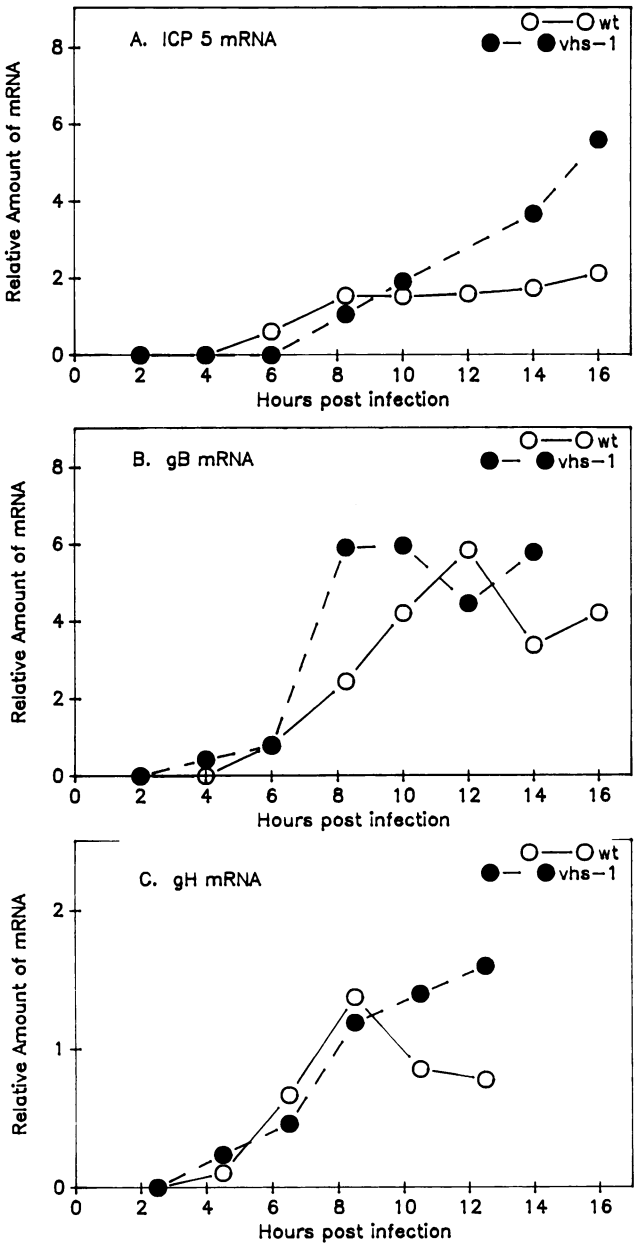

FIG. 4. Accumulation of beta-gamma mRNAs during wild-type (wt) and *vhs*1 infections. Autoradiograms of the gels shown in Fig. 1 were scanned in order to quantitate the levels of beta-gamma mRNAs. (A) ICP5; (B) gB; (C) gH.

ued to increase until 16 h, by which time there was at least 2.5 times as much mRNA present as in wild-type infections. In addition, although the amounts of ICP5 mRNA in *vhs*1-infected cells eventually surpassed the wild-type levels, at early times accumulation of ICP5 mRNA in *vhs*1-infected cells actually lagged slightly behind that observed in cells infected with wild-type virus.

The mRNA encoding glycoprotein H accumulated with similar kinetics in *vhs*1 and wild-type infections for the first 8.5 h of infection (Fig. 1 and 4). However, after that time the levels of gH mRNA in wild-type infections either plateaued or decreased slightly, while they continued to gradually increase in *vhs*1 infections. By 12.5 h after infection, there

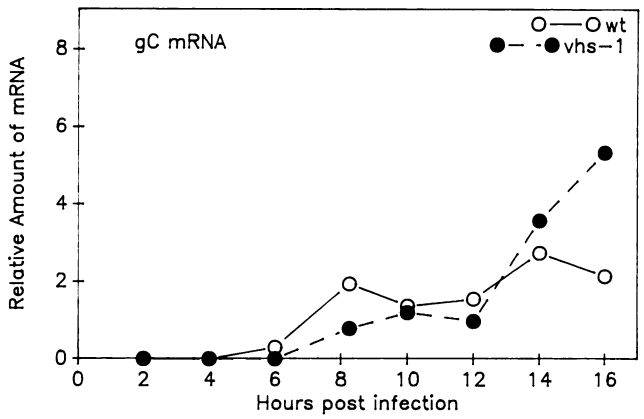

FIG. 5. Accumulation of the gamma mRNA encoding glycoprotein C during wild-type (wt) and *vhs*1 infections. Autoradiograms of the gels shown in Fig. 1 were scanned in order to quantitate the levels of gC mRNA.

was approximately twice as much gH mRNA in cells infected with *vhs*1 as in cells infected with wild-type virus. The *vhs*1 mutation had the least effect on the accumulation of the mRNA for glycoprotein B, causing only a marginal increase in the gB mRNA levels at intermediate and late times after infection (Fig. 1 and 4).

Gamma mRNAs. In cells infected with wild-type virus, the gamma mRNA for glycoprotein C began to accumulate by 6 h after infection (Fig. 2 and 5). It had reached high levels by 8 h, and large amounts of gC mRNA persisted in the cytoplasm until at least 16 h after infection.

The *vhs*1 mutation led to some overaccumulation of gC mRNA, but only at late times after infection. By 16 h there was more than twice as much gC mRNA in *vhs*1-infected cells as in cells infected with wild-type virus. However, prior to 12 h, the levels of gC mRNA in *vhs*1 infections lagged behind the levels in cells infected with wild-type virus.

Measurement of mRNA half-lives with a dactinomycin chase. We next turned our attention to the specificity of the *vhs* gene product; that is, are some mRNAs intrinsically more sensitive or resistant to *vhs*-induced degradation than others? In cells infected with wild-type virus, beta-gamma and gamma mRNAs continued to accumulate at late times when alpha and beta mRNAs were declining in abundance. One possible explanation was that beta-gamma and gamma mRNAs are intrinsically more resistant to the effects of the *vhs* gene product than are alpha and beta mRNAs. Alternatively, all viral mRNAs may be equally sensitive to the *vhs* protein, but some may persist at high levels because they are continuously replenished by new transcription, while transcription of other genes is turned off. As was discussed in the preceding section, the *vhs* mutation affected the accumulation of different viral mRNAs to different extents. Thus, while the beta mRNAs for ICP8, TK, and DNA polymerase were affected dramatically, accumulation of some beta-gamma mRNAs, such as gB mRNA, were affected to a much lesser extent. This may reflect differential sensitivities of different mRNAs to *vhs*-induced degradation.

To resolve these questions, the half-lives of 10 different viral mRNAs were measured beginning at 6 h after infection. We decided that the best way to determine the question of differential target sensitivity was to measure the half-lives of different mRNAs exposed to the same cytoplasmic factors at the same time. We therefore chose a time at which members of all kinetic classes of mRNA were present in the cytoplasm

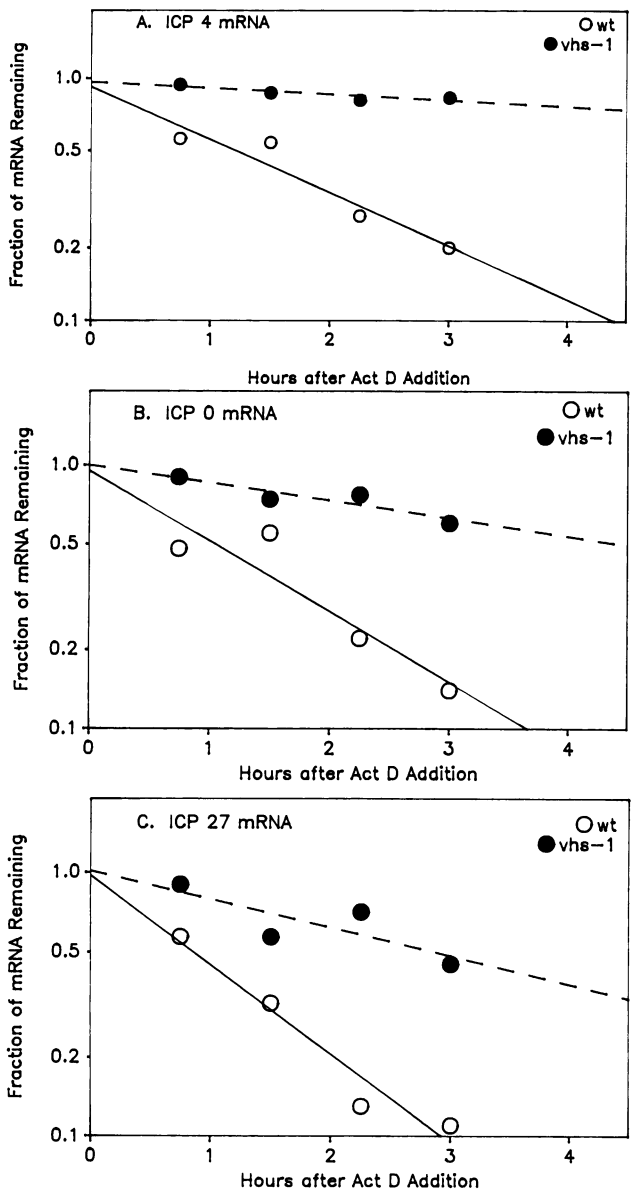

FIG. 6. Decay of alpha mRNAs. Vero cells were infected with 20 PFU of either wild-type (wt) virus or *vhs*1 per cell. At 6 h after infection, 5 μg of dactinomycin (Act D) per ml was added to the medium, and incubation was continued. Total cytoplasmic mRNAs were isolated at 45-min intervals, and 5-μg samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The 28S rRNA was used as a normalization standard to ensure that equal amounts of total cytoplasmic RNA were loaded onto each lane of the gel. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of specific mRNAs. (A) ICP4; (B) ICP0; (C) ICP27.

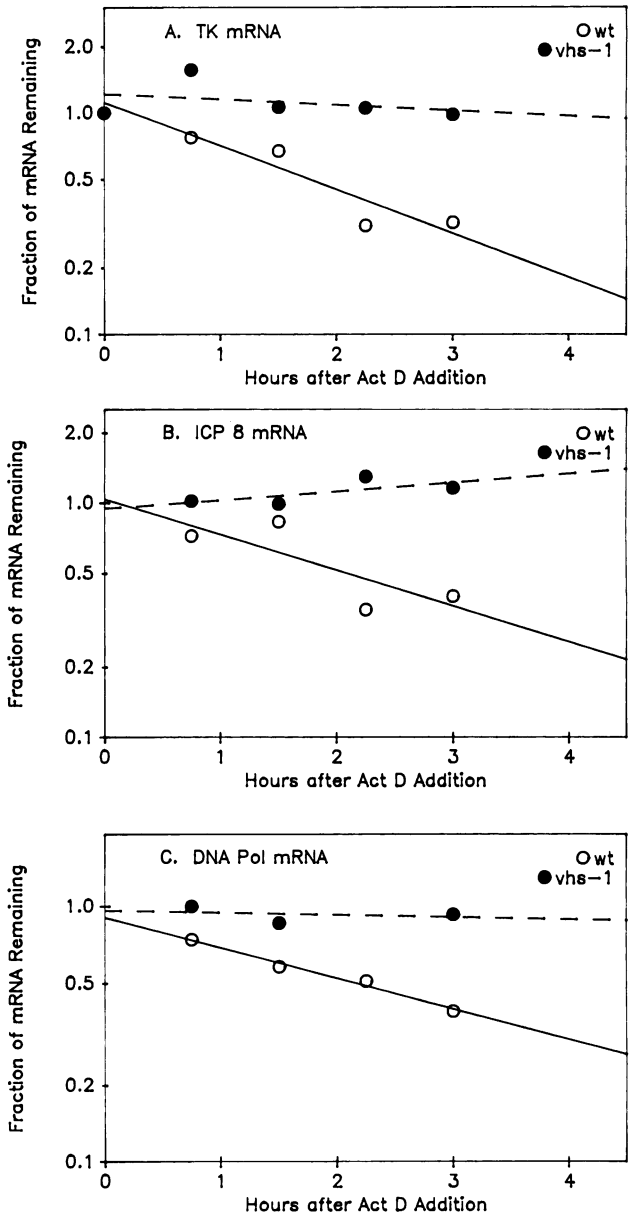

FIG. 7. Decay of beta mRNAs. Vero cells were infected with 20 PFU of either wild-type (wt) virus or *vhs*1 per cell. At 6 h after infection, 5 μg of dactinomycin (Act D) per ml was added to the medium, and incubation was continued. Total cytoplasmic mRNAs were isolated at 45-min intervals, and 5-μg samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The 28S rRNA was used as a normalization standard to ensure that equal amounts of total cytoplasmic RNA were loaded onto each lane of the gel. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of specific mRNAs. (A) TK; (B) ICP8; (C) DNA polymerase.

to an appreciable extent. Cells were infected with 20 PFU of either wild-type or *vhs* 1 virus per cell. At 6 h postinfection, dactinomycin was added to the cultures, and total cytoplasmic mRNAs were prepared at 45-min intervals. The levels of different viral mRNAs were analyzed by Northern blotting and quantitated by densitometric scanning of the autoradiograms. Following the analysis of viral mRNAs, the blots were stripped and then rehybridized to a probe specific for 28S rRNA. This allowed us to normalize the results to control for small differences in the amounts of cytoplasmic RNA that were loaded onto the different lanes of the gel.

The results of this experiment and a second set of half-life determinations are shown in Fig. 6 through 9 and summarized in Table 1. The *vhs*1 mutation had a dramatic effect on the half-lives of all mRNAs examined. The half-life of each mRNA was at least severalfold greater in cells infected with

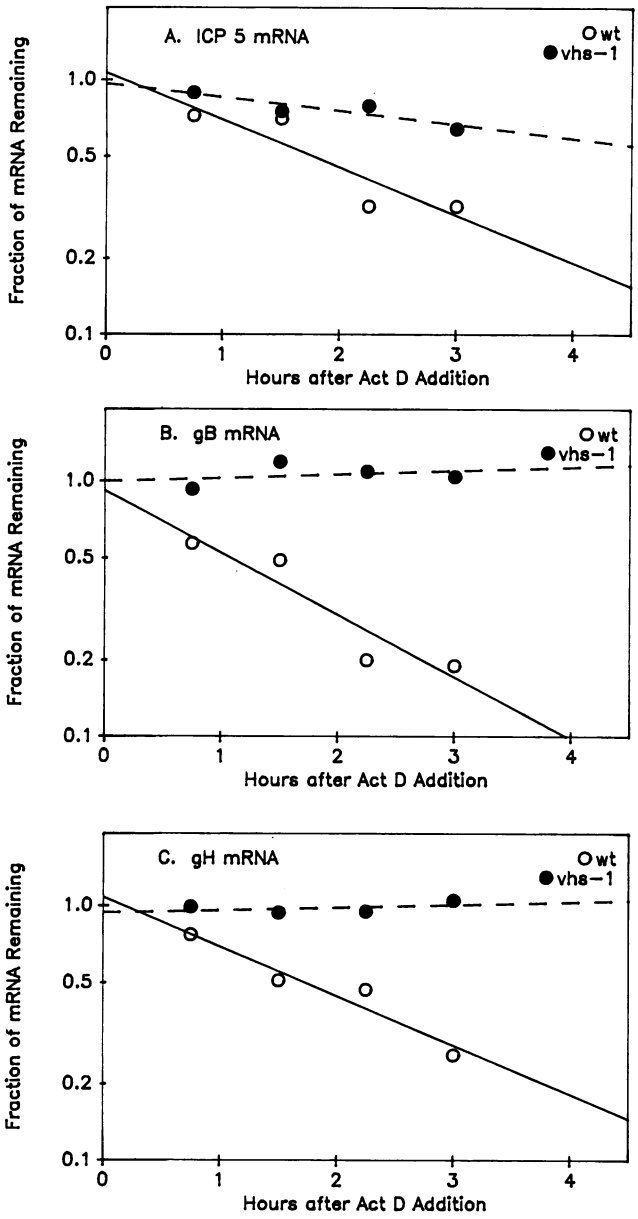

FIG. 8. Decay of beta-gamma mRNAs. Vero cells were infected with 20 PFU of either wild-type virus or *vhs*1 per cell. At 6 h after infection, 5 μg of dactinomycin (Act D) per ml was added to the medium, and incubation was continued. Total cytoplasmic mRNAs were isolated at 45-min intervals, and 5-μg samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The 28S rRNA was used as a normalization standard to ensure that equal amounts of total cytoplasmic RNA were loaded onto each lane of the gel. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of specific mRNAs. (A) ICP5; (B) gB; (C) gH.

*vhs*1 than in cells infected with wild-type virus. In fact, many mRNAs decayed so slowly in *vhs*1-infected cells that precise determination of the half-life was difficult.

In cells infected with wild-type virus, the mRNA half-lives ranged from approximately 1 to 2.5 h. While it is possible that small actual differences in stability did exist between different mRNAs, the most striking result was that the half-lives of all 10 mRNAs were remarkably similar. The

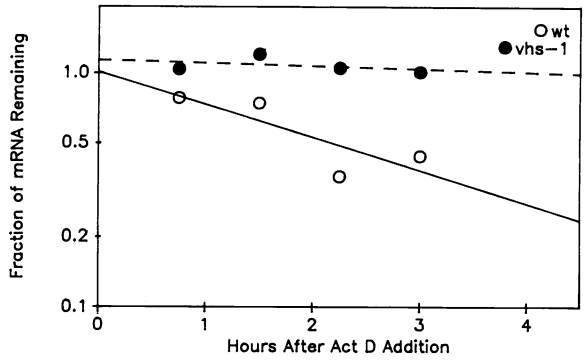

FIG. 9. Decay of the gamma mRNA encoding glycoprotein C. Vero cells were infected with 20 PFU of either wild-type (wt) virus or *vhs*1 per cell. At 6 h after infection, 5 μg of dactinomycin (Act D) per ml was added to the medium, and incubation was continued. Total cytoplasmic mRNAs were isolated at 45-min intervals, and 5-μg samples were analyzed by Northern blotting. The blots were probed for viral mRNAs and for 28S rRNA. The 28S rRNA was used as a normalization standard to ensure that equal amounts of total cytoplasmic RNA were loaded onto each lane of the gel. Autoradiograms of the resulting gels were scanned in order to quantitate the levels of gC mRNA.

correlation coefficient relating the fit of the data to a curve with the calculated half-life was determined for each of the decay curves summarized in Table 1. For ICP8 and gC mRNAs in experiment 1, the correlation coefficients were 0.804 and 0.83, respectively. For ICP27 mRNA in experiment 2, the correlation coefficient was 0.84. The correlation coefficients for all of the other decay curves exceeded 0.90. In addition, the half-lives of five of the mRNAs were measured in two separate experiments. The average values for the resulting half-lives were: ICP27, 1.4 h; TK, 1.7 h; gB, 1.6 h; gH, 1.4 h; and gC, 1.95 h. In each case the half-life values in each experiment varied from the average by at most 0.5 h. This gives an indication of the variability in half-life values that were obtained when half-life measurements were performed on different days with different batches of cells and virus.

In spite of small differences that were observed in the half-lives for different mRNAs in wild-type infections, it is clear that the differences between the accumulation kinetics of, for example, DNA polymerase and ICP8 mRNAs on the

TABLE 1. Half-lives of HSV-1 mRNAs in the presence of dactinomycin[a]

mRNA	Half-life (h)			
	Wild-type virus		*vhs*1	
	Expt 1	Expt 2	Expt 1	Expt 2
ICP4	1.4		S[b]	
ICP0	1.1		4.4	
ICP27	0.9	1.9	3.3	4.4
TK	1.5	1.9	S	4.4
DNA polymerase	2.5		S	
ICP8	2.0		S	
ICP5	1.6		5.5	
gB	1.3	1.9	S	S
gH	1.6	1.2	S	S
gC	2.1	1.8	S	S

[a] Determined from data in Fig. 6 through 9.
[b] S, Half-life greater than 8 h.

one hand and gB or gC mRNAs on the other cannot be explained by differential mRNA half-lives. The data indicate that the *vhs* gene product causes degradation of many different viral mRNAs in a rather nonselective fashion. Differences in the kinetics of accumulation of different mRNAs must therefore be primarily due to differences in the rates of transcription or processing and transport to the cytoplasm.

Measurement of mRNA half-lives with a pulse-chase protocol. The procedure of determining mRNA half-lives by measuring their decay in the presence of dactinomycin has been used widely and produced meaningful results for many different mRNAs. However, the drug does have secondary effects on processes other than transcription, and these in some instances may complicate half-life measurements. Furthermore, in some instances, turning off transcription may affect the half-lives of mRNAs that already exist in the cytoplasm. Therefore, to confirm the results of our half-life measurements with dactinomycin, we determined the half-lives of selected viral mRNAs with a pulse-chase protocol.

Cells were infected with 20 PFU of wild-type virus per cell and incubated until 5.5 h postinfection. At this time the infected cells were pulsed for 30 min with [^{3}H]uridine. The label was then removed, and the cells were washed and then incubated in medium containing an excess of unlabeled uridine as well as cytidine and glucosamine. Cytoplasmic RNA samples were prepared at 45-min intervals after removal of the label, and the amount of label remaining in specific viral mRNAs was determined by hybridization to filter disks containing an excess of specific viral DNA fragments. The decay curves for ICP0 and TK mRNAs are shown in Fig. 10. The resulting half-lives were: ICP0, 2.4 h; TK, 1.5 h. These values, obtained with the pulse-chase procedure, were very similar to those obtained by measuring mRNA decay in the presence of dactinomycin and therefore confirm the earlier results.

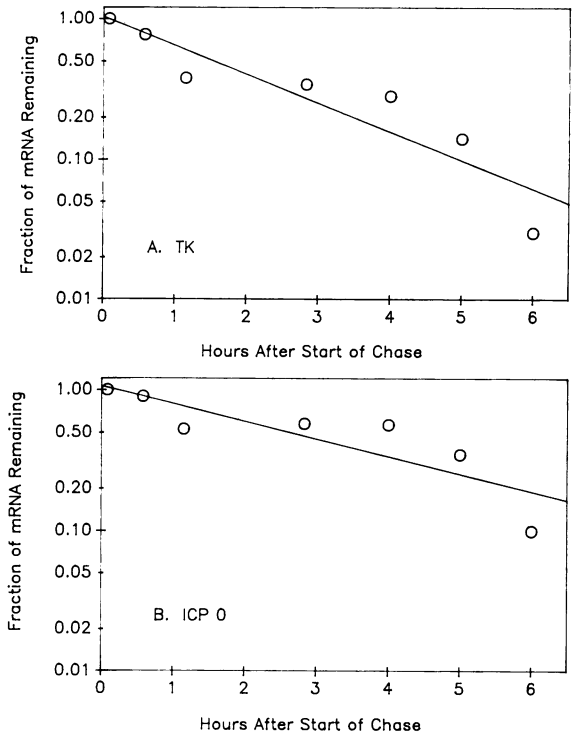

FIG. 10. Measurement of mRNA half-lives by pulse-chase analysis. HeLa cells were infected with 20 PFU of wild-type virus. At 5.5 h postinfection, they were pulse-labeled for 30 min with [^{3}H]uridine, after which the label was removed and the cells were incubated in medium containing an excess of cold uridine, cytidine, and glucosamine. Total cytoplasmic RNA was extracted at various times after the start of the chase, and the amount of labeled ICP0 (B) or TK (A) mRNA was determined by hybridization to an excess of specific viral DNA fragments immobilized on nitrocellulose filter disks.

DISCUSSION

The experiments reported here demonstrate that the *vhs*1 mutation dramatically affects the physical stabilities of many viral mRNAs representing all kinetic classes. The data suggest that the *vhs* protein plays an important role, in conjunction with transcriptional controls, in determining the abundance levels and kinetics of accumulation of a number of viral mRNAs. These studies constitute the first systematic measurement, in either wild-type or mutant infections, of the half-lives of HSV-1 mRNAs belonging to all kinetic classes. The *vhs* protein is responsible for the degradation of cellular mRNAs and the concomitant shutoff of host polypeptide synthesis that occurs early during lytic infections. The present experiments suggest that another important role of the protein is the regulation of viral mRNA stability.

A central question addressed by these studies was the specificity or selectivity of the *vhs* gene product. Immediately after infection, copies of the *vhs* protein that enter the cell as components of the infecting virion cause degradation of a variety of preexisting cellular mRNAs at the same time that viral mRNAs begin to accumulate (24, 28). However, cellular mRNAs are not necessarily intrinsically more sensitive to *vhs*-induced degradation than are viral mRNAs, as shown by the data of Smiley and co-workers (26). These investigators found that when a rabbit β-globin gene was inserted into the viral genome, accumulation of the resulting β-globin mRNA was regulated in a fashion similar to that of members of the beta class of viral mRNAs. In the present studies, the *vhs*1 mutation was found to dramatically affect the half-lives of all viral mRNAs that were examined, and in cells infected with wild-type virus the *vhs*-regulated half-lives of all 10 viral mRNAs were very similar. Thus, the accumulated data strongly suggest that the *vhs* protein negatively regulates the stability of many, if not all, mRNAs with little or no target selectivity.

As would be expected if it lengthened mRNA half-lives, the *vhs*1 mutation affected the abundance levels and accumulation kinetics of a number of viral mRNAs belonging to different kinetic classes. The effect was most dramatic for the alpha mRNA encoding ICP27 and the beta mRNAs for ICP8, TK, and DNA polymerase. Thus, these mRNAs accumulated to higher levels in *vhs*1-infected cells than in cells infected with wild-type virus. In wild-type infections the mRNAs encoding ICP27, ICP8, TK, and DNA polymerase all appeared at early times, increased to peak levels, and then declined in abundance. The decline at late times was sharper and more complete for the beta mRNAs than for ICP27 mRNA. In contrast, in *vhs*1 infections the levels of these four mRNAs failed to decline after reaching peak values and instead leveled to a plateau. An attractive hypothesis to explain these accumulation curves is that mRNA levels continued to increase for as long as the rates of transcription, processing, and transport of the mRNA to the cytoplasm remained high and that, following a decline in any or all of these rates, the levels of alpha and beta mRNAs

declined in wild-type infections as a result of *vhs*-induced mRNA turnover.

Whether the normal decline in the rate of appearance of alpha and beta mRNAs in the cytoplasm results exclusively from transcriptional controls is unclear. In studies in which transcription rates were measured by pulse-labeling cells in vivo with [^{3}H]uridine, Zhang and co-workers observed that the rate of transcription of the beta gene for dUTPase increased from 1 to 2 h after infection and then declined (32). If the transcription patterns for TK, ICP8, and DNA polymerase are similar, then the accumulation curves for these three mRNAs could be explained as the result of specific transcriptional controls acting in conjunction with the *vhs*-mediated turnover of the cytoplasmic mRNAs. However, in nuclear runoff measurements of the transcription rates of the TK, ICP8, and DNA polymerase genes, Weinheimer and McKnight were unable to determine whether the decline in the steady-state cytoplasmic levels of the mRNAs resulted from transcriptional or posttranscriptional controls (31). Interpretation of their results was hampered by the observation that significant amounts of symmetric, promoter-independent transcription of these genes was observed beginning at 4 h after infection and increased thereafter. In other studies utilizing the nuclear runoff technique, Godowski and Knipe obtained similar results for the rate of ICP8 transcription (7). Thus, at present, one cannot exclude the possibility that the normal shutoff of beta gene expression may involve control at the level of processing and transport of the mRNA to the cytoplasm as well as regulation of transcription rates and the cytoplasmic stabilities of the mRNAs.

The *vhs*1 mutation led to overaccumulation of some beta-gamma and gamma mRNAs, but not to nearly the extent that was observed for the beta mRNAs. The differences that were observed in wild-type infections between the accumulation patterns of the beta mRNAs on the one hand and the beta-gamma and gamma mRNAs on the other were particularly striking in view of the fact that the half-lives of the mRNAs were very similar. The data therefore indicate that these differences in accumulation kinetics were primarily due to controls at the levels of transcription, processing, or mRNA transport rather than differential mRNA stabilities.

The effect of the *vhs*1 mutation on the accumulation of some mRNAs could not be explained by simply postulating that the mutation altered mRNA half-lives while leaving the rates of transcription, processing, and transport unchanged. This was especially true for the mRNA for glycoprotein C. In *vhs*1 infections, accumulation of gC mRNA actually lagged behind that observed in wild-type infections until 12 h, at which point the *vhs*1 mRNA levels finally surpassed those in wild-type infections. The reason for this behavior is not known. However, overexpression of one or more earlier gene products may delay or reduce transcription or transport of some mRNAs that are normally expressed later in the virus cycle.

The experiments presented here suggest that in wild-type virus infections, the *vhs* gene product causes rapid turnover of many different viral and cellular mRNAs. The net result of such a wild-scale decrease in mRNA half-lives would be to accentuate the importance and flexibility of transcriptional controls. It is interesting that HSV apparently encodes a protein which is involved in the regulation of mRNA stabilities and does not rely on unmodified cellular functions for determining the rates of mRNA turnover. A similar observation has recently been made by Kwong and co-workers (12).

The importance to virus growth of the *vhs*-mediated regulation of mRNA levels is unclear. The fact that *vhs*1 is a viable, nonconditionally lethal mutation indicates that a certain amount of overexpression of many gene products can be tolerated, at least in cell culture. Nevertheless, the *vhs* protein is probably important to optimal virus growth, as evidenced by the facts that the *vhs*1 mutation resulted in a severalfold reduction in virus burst size in single-step growth experiments (20) and wild-type virus rapidly outgrows *vhs*1 in mixed infections (13). Furthermore, fine tuning of mRNA levels may be more critical to growth of the virus in animals than it is in cell culture.

Finally, the mechanism of *vhs*-induced mRNA turnover is unknown. The *vhs* protein may be a virus-encoded RNase or, alternatively, may activate a preexisting cellular enzyme. Recent data from our laboratory suggest that at early times after infection, changes in the structure of messenger ribonucleoprotein particles correlate with a wild-type *vhs* function (C. R. Krikorian and G. S. Read, unpublished data). Thus, by inducing changes in messenger ribonucleoprotein particle structure, the *vhs* product might render mRNAs more susceptible to digestion by RNases that are constitutively present within the cell. Elucidation of the exact mechanism of *vhs*-induced mRNA turnover will await experiments with in vitro mRNA degradation systems.

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