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Analysis of herpes simplex virus-induced mRNA destabilizing activity using an *in vitro* mRNA decay system

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ABSTRACT

Most host mRNAs are degraded soon after infection of cells with herpes simplex virus type 1 (HSV-1). This early shutoff or early destabilization response is induced by a virion component, the virion host shutoff (*vhs*) protein. HSV-1 mutants, *vhs1* and *vhs*-ΔSma, which produce defective or inactive *vhs* protein, fail to induce early shutoff. We have used an *in vitro* mRNA decay system to analyze the destabilization process. Polysomes from uninfected human erythroleukemia cells, used as a source of target mRNAs, were mixed with polysomes or with post-polysomal supernatant (S130) from HSV-1- or mock-infected murine erythroleukemia cells. Normally stable γ-globin mRNA was destabilized by approximately 15-fold with S130 from wild-type virus-infected cells but was not destabilized with S130 from mock-infected cells or from cells infected with either of the two HSV mutants. The virus-induced destabilizing activity had no significant effect on the *in vitro* half-lives of two normally unstable mRNAs, histone and c-*myc*. No destabilizing activity was detected in polysomes from infected cells. We conclude that a virus-induced destabilizer activity can function *in vitro*, is located in the S130 of infected cells, and accelerates the decay rates of some, but not all, polysome-associated host mRNAs.

INTRODUCTION

The regulation of mRNA turnover plays an important role in determining the level of expression of many genes. For example, the abundance of some 'housekeeping' mRNAs is determined primarily by their cytoplasmic half-lives, not by their gene transcription rates (1). The half-lives of mRNAs such as histone and tubulin fluctuate with the cell cycle phase, while the half-lives of c-*myc* and c-*fos* mRNAs vary as a function of cell growth rate and stage of differentiation (reviewed in 2–4). Those and related observations have raised the question of how environmental stimuli such as growth factors, hormones, temperature shifts, and viral infection influence mRNA half-lives and why the response to each stimulus varies. Estrogen and herpes simplex virus (HSV), two well-characterized modifiers of mRNA stability, seem to function in very different ways. Estrogen has limited and specific effects, regulating the stabilities of only a few mRNAs in hepatocytes (reviewed in 5). In contrast, when herpes simplex virus type 1 (HSV-1) infects mammalian cells, most host mRNAs are rapidly destabilized in a seemingly non-specific manner. Host mRNAs with half-lives of 20 hours or more (in uninfected cells) are almost completely degraded by two to four hours after viral infection begins (6–14). Rapid destabilization, coupled with virus-induced transcriptional down-regulation (6–15), ensures that host gene expression is virtually silenced, even at early times post-infection.

Host mRNA destabilization occurs in two phases, referred to as early and late shutoff. The early destabilization response, which is the focus of this paper, results, at least in part, from the action of a virion host shutoff (*vhs*) protein (8,14,16–19). This 58 kDa, 489 amino acid protein is associated with the virion tegument and is encoded by the UL41 open reading frame (15, G. S. Read personal communication). Host cell mRNA is rapidly degraded even when infections are carried out in the presence of actinomycin D (8,13), indicating that the *vhs* protein alone is sufficient for early shutoff (destabilization) in the absence of viral protein synthesis. Viral mRNAs also are less stable in the presence than in the absence of *vhs* protein (8,14,17). When cells are infected with HSV-1 mutants defective in the early shutoff function, mRNA half-lives are unchanged at early times after infection (19–21). Only at later times are host mRNAs destabilized, presumably in response to an incompletely characterized late shutoff function that requires viral gene expression and is active in the absence of the *vhs* protein (reviewed in 22). One well-characterized early shutoff mutant virus, *vhs1*, grows more slowly and has a significantly lower burst size than its wild-type counterpart but is viable in tissue culture cells (19). Therefore, although the *vhs* protein is not essential for viral replication, it is required for optimal growth and might play a more prominent role in whole animal infections (17).

Perhaps the most interesting aspect of the HSV-induced mRNA destabilization process is its lack of specificity. The indiscriminate degradation of host mRNAs suggests that the *vhs* protein affects some fundamental aspect(s) of cytoplasmic mRNA metabolism. Primarily for this reason, we are characterizing the early mRNA destabilization process, using an *in vitro* system to compare the effects of extracts from virus-infected and mock-infected cells on mRNA stability (23–25). Our experiments, as well as those published by Krikorian and Read (26), indicate that HSV-1-induced

mRNA destabilization can be reproduced *in vitro*. All of our cell-free reactions contained polysomes from uninfected human erythroleukemia cells (K562) as a source of mRNA. The reactions were then supplemented with post-polysomal supernatant (S130) or with polysomes from HSV-1-infected murine erythroleukemia (MEL) cells as sources of potential mRNA destabilizing factors. Under these conditions, we have detected a virus-induced destabilizing activity in the S130 fraction. The *in vitro* half-lives of several normally stable mRNAs were decreased by at least 15-fold in the presence of S130 from wild-type virus-infected MEL cells. No destabilizing activity was detected in S130's from mock-infected or *vhs* mutant virus-infected cells, indicating that the activity resulted from the *vhs* protein. These data are consistent with the *vhs* protein functioning as a RNase, an activator of host cytoplasmic RNases, or an inactivator of RNase inhibitors. Alternatively, it might lead to modifications in the structure of host mRNAs or their associated proteins, making the mRNAs highly susceptible to attack by cytoplasmic RNases.

MATERIALS AND METHODS

Cells and virus

MEL (clone 745) and human erythroleukemia (K562) cell lines were maintained in exponential growth in RPMI 1640 medium containing 10% calf serum and penicillin-streptomycin. Vero cells were maintained in Dulbecco's Modified Eagles medium containing 10% calf serum and penicillin-streptomycin. HSV-1 strain KOS (wild-type) and HSV-1 mutant viruses, *vhs*1 and *vhs*-ΔSma, were generously supplied by Dr. G. Sullivan Read, University of Missouri-Kansas City. Viral stocks were prepared from infected Vero cells and the virus titer determined (19). For all infections, MEL cells were grown to a density of 5×10^5 cells/ml and concentrated to 10^7 cells/ml in RPMI 1640 medium containing 1% calf serum. Virus was added at a multiplicity of infection = 10 and was allowed to adsorb for 1 h at 37°C. The cells were then pelleted by centrifugation, the medium was aspirated, and the cells were resuspended in RPMI 1640 medium containing 10% calf serum and incubated at 37°C. The time at which virus and cells were mixed is defined here as time 0, and subsequent times are referred to as times post-inoculation. Thus, the time at which the cells are pelleted to remove non-adsorbed virus is 1 hr post-inoculation.

To ensure that infection was successful, 2 million infected MEL cells were incubated for 30 min at various times post-inoculation in 2 ml of methionine-free RPMI 1640 medium containing 10% calf serum and 2 μCi of trans [^{35}S]-label™ methionine and cysteine (ICN Biomedicals, Inc., Irvine, CA). Total cell lysates were prepared, and the proteins were separated in a 9% polyacrylamide SDS gel. The gel was fixed, soaked in Amplify (Amersham Corporation, Arlington Heights, IL), dried under vacuum, and autoradiography was performed.

Northern blot analysis

Total cytoplasmic RNA, 20 μg, was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and the RNA was transferred to a Zeta-probe blotting membrane (Bio-Rad Laboratories, Richmond, CA). Following prehybridization, the blot was hybridized to a [^{32}P]-labeled DNA probe prepared by the random priming method (27) for 18 h at 42°C (28). The filter was washed two times in 0.2×SSC (1×SSC=0.15 M sodium chloride, 0.015 M sodium citrate) and 0.1% SDS at 60°C.

The following [^{32}P]-DNA probes were used: The Hind III-BamH I fragment from plasmid pLG2, containing exons 1 and 2 of the mouse β-globin gene; the BamH I-Hind III fragment from pM104BH, containing the coding region from mouse c-*myc* mRNA (29); the Nco I-Ssp I fragment from pHU73, containing the coding region and a portion of the 3-untranslated region of human poly(A) binding protein (PABP) mRNA (30); the full-length cDNA for rat glyceraldehyde phosphate dehydrogenase (GAPDH; Pst I fragment) (31); the Sal I-EcoR I fragment from plasmid p1.53sr, containing approximately the 5′ one-half of mouse H3.2 histone mRNA (32).

Preparation of polysomes and S130

MEL and logarithmically growing K562 cells were harvested, washed three times in cold (4°C) serum-free F12 medium, resuspended to approximately 10^8 cells/ml in low salt buffer (10 mM Tris-hydrochloride [pH 7.6], 12 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol), and lysed by homogenization (24). The nuclei were removed by low speed centrifugation, and the post-nuclear supernatant was layered over a 1.5 ml cushion of 30% sucrose dissolved in low salt buffer. The tube was centrifuged at 130,000×g in a SW60 rotor at 2°C for 2.5 h (24). The S130 fraction above the cushion was removed, the polysome pellet was washed and resuspended in low salt buffer, and both fractions were stored at −80°C.

In vitro mRNA decay reactions

A previously described *in vitro* mRNA decay system (24) was used but was modified in several ways, because cellular mRNAs are rapidly degraded in HSV-1 infected MEL cells (11,12; Fig. 1). Polysomes from uninfected human K562 cells were mixed with polysomes or S130 from mock- or virus-infected mouse cells (MEL). The goal of this approach was to determine whether extracts from the infected mouse cells would accelerate the degradation of polysome-associated human (target) mRNAs. Reactions contained (in a final volume of 25 μl) 65 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 6.5 mM creatine phosphate, 0.3 μg of creatine kinase, 0.65 mM ATP, 0.26 mM GTP, 0.07 mM spermidine, 5 units of human placental RNase inhibitor (RNasin, Promega, Madison, WI), 3.3 mM Tris-HCl, pH 7.6, and equal amounts of uninfected K562 cell polysomes (from approximately 4×10^6 cells). It is important to note that the potassium acetate concentration in these experiments was lower than the 100 mM concentration used in our previously described reactions (24). The lower potassium concentration was necessary for optimal activity of the viral-induced mRNA destabilizer (unpublished observations). Where noted, an equal number of cell equivalents of S130 or polysomes from mock or HSV-infected MEL cells was added to the reactions containing uninfected K562 polysomes in a 1:1 or 2.5:1 cell equivalent ratio, respectively. Reactions were incubated at 37°C for various times. At each time point 5 μg of total RNA was assayed as described below.

Analysis of polysome-associated mRNA

S1 nuclease mapping was performed as previously described (24). The human γ-globin probe was the 3′-EcoR I fragment from the γ-globin gene (33) that protects the 3′-terminal 167 nucleotides (nt) of the mRNA. The human H4 histone mRNA probe was a 300 bp Nco I-Hind III fragment from the structural gene clone pHh4A (34) that protects the 3′-terminal 104 nt of histone mRNA. RNase protection analysis of human c-*myc* mRNA was performed

with a uniformly labeled Ssp I-EcoR I fragment from the human c-*myc* gene, as previously described (23). These human probes do not cross-hybridize with their mouse counterparts under these conditions (unpublished observations). To quantitate mRNA decay rates, autoradiograms developed without intensifying screens were analyzed by laser densitometry using an LKB Ultroscan XL densitometer and GelScan XL software (LKB Pharmacia Biotechnology).

RESULTS

HSV-induced mRNA destabilization in MEL cells

Within an hour following inoculation of permissive cells with HSV-1, most host mRNAs are destabilized and begin to disappear (6–14). The *vhs* function is responsible for mRNA destabilization, for the rapid (early) shutoff of host protein synthesis (6,7,11,12), and for disaggregation of polysomes (12,14). This function allows HSV-1 to seize the cell's translation machinery for its own use, by eliminating competition with cellular mRNAs.

As a baseline for the *in vitro* experiments described below, the steady-state levels of five MEL cell mRNAs were measured following infection by HSV-1. Each mRNA decreased after virus inoculation, albeit at different rates and to different extents, consistent with previous observations (7,10,11,13). β-globin and GAPDH mRNAs, which are normally stable (35,36), decreased by ten-fold or more by 60 min post-inoculation (Fig. 1, panels A and B). c-*myc* mRNA, which is normally very unstable, also disappeared rapidly (panel C). Histone mRNA decreased by a maximum of only 45% during the first 180 min post-inoculation but disappeared more rapidly thereafter, consistent with some previous reports (compare ref. 10 with ref. 13). These results confirmed that HSV-1 infection rapidly depleted MEL cells of some polyadenylated mRNAs, even those (β-globin, GAPDH) with relatively long half-lives in uninfected cells. One exception was the polyadenylated PABP mRNA (panel E), the level of which decreased to only 50% by 360 min post-inoculation. We do not yet know why or how this mRNA is spared from early shutoff in infected cells (see Discussion).

Analysis of HSV-induced mRNA destabilization in a cell-free system

We asked whether extracts from HSV-infected MEL cells accelerated the degradation of K562 polysomal mRNAs. It seemed essential to use polysome-associated rather than free mRNAs as the targets in these assays, because some mRNA stability-regulating factors analyzed in our laboratory were detected using polysomes but not free mRNA, implying that polysome structure is essential for proper regulation of mRNA stability (23,25,37,38, and unpublished observations). Uninfected K562 cell polysomal mRNAs were used as targets, rather than host (MEL) mRNAs, because MEL mRNAs begin to be degraded soon after virus inoculation (see above). Therefore, the K562 polysomes provided reproducible and uniform mRNA substrates which had not been subjected to prior abuse by HSV-1.

Preliminary experiments indicated that crude cytoplasmic extracts from infected MEL cells destabilized some K562 mRNAs (data not shown). To determine the source of the destabilizing activity, polysomal and post-polysomal supernatant (S130) fractions were isolated from infected MEL cells. Each fraction was then mixed in separate reactions with K562 polysomes, and the decay of human γ-globin mRNA was monitored. The mRNA was very stable (half-life >500 min) in reactions containing S130 from mock-infected cells (Fig. 2A, top, and Fig. 2C, squares). This result is consistent with previous measurements of very long half-

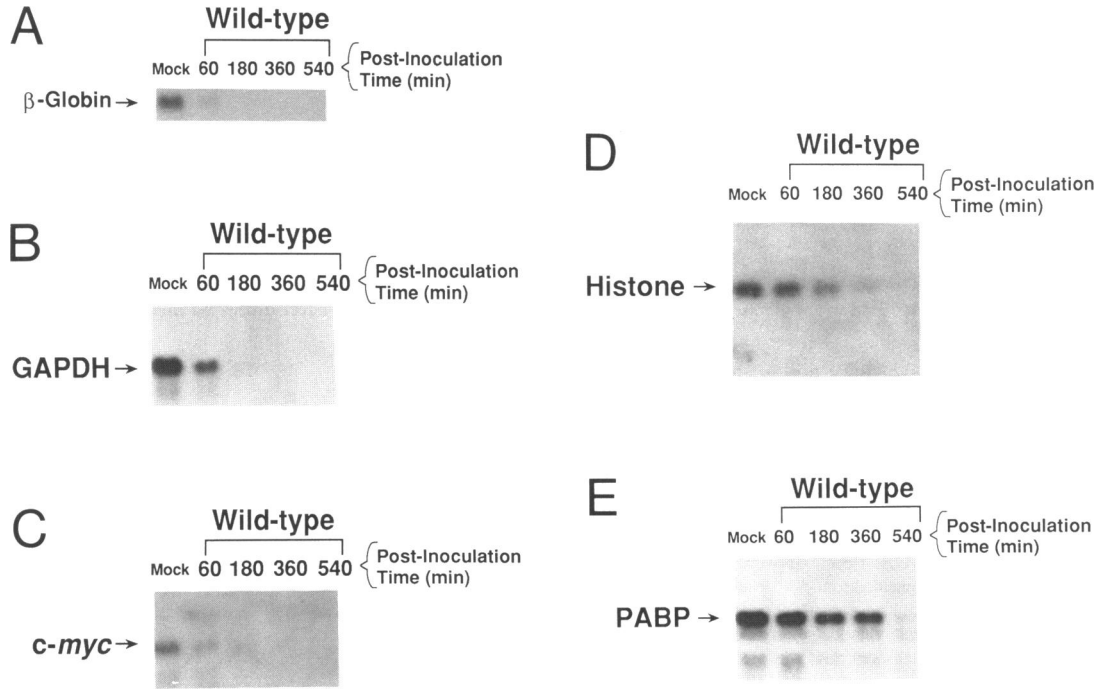

Fig. 1. Analysis of steady-state mRNA levels following infection by wild-type HSV-1. Total cytoplasmic RNA (20 μg) extracted from mock or wild-type virus-infected MEL cells (60–540 min post-inoculation of virus) was analyzed by Northern blot analysis. The same blot was stripped and reprobed for each mRNA: (A) β-globin (B) GAPDH (C) c-*myc* (D) H3.2 histone (E) PABP.

lives for γ-globin mRNA in cells and *in vitro* (24,36). In contrast, the mRNA was at least 15-fold less stable (half-life 15−30 min) in reactions containing S130 from wild-type HSV-1-infected MEL cells (Fig. 2B, bottom, and Fig. 2C, circles). Similar results were observed with GAPDH mRNA (Fig. 3). These results indicate that the post-ribosomal supernatant (S130) from HSV-infected cells contains an activity capable of destabilizing two polysome-associated mRNAs that are normally very stable.

For γ-globin mRNA, the extent of *in vitro* destabilization varied with the time post-inoculation. S130's from cells infected for 360 min were more effective than those from 60 to 180 min post-inoculation (Fig. 2C). One possible explanation for this difference is that two destabilization activities, corresponding to the early and late host shutoff functions, were being detected in these assays. Perhaps the early (*vhs*) shutoff activity was labile *in vitro* and was inactivated after 20 to 30 min at 37°C, while the late shutoff activity (in S130 from 360 min post-inoculation) remained active until 100% of the mRNA had been degraded.

In reactions supplemented with polysomes from HSV-infected cells, γ-globin mRNA was not significantly destabilized at a ratio of 2.5:1 (infected:target) cell equivalents of polysomes (Fig. 4). Similar results were observed at a ratio of 7.5:1 infected:target polysomes (data not shown). Therefore, an mRNA destabilizing activity is not detected in infected cell polysomes under these *in vitro* conditions.

To determine whether the destabilizing activity appeared even earlier than 60 min post-inoculation, S130 was prepared from MEL cells immediately after adding virus (0 min) and at intervals thereafter and was assayed *in vitro*. For the first time point (time 0), virus and cells were mixed, and the cells were immediately washed three times and lysed, as described in Materials and Methods. The total elapsed time between mixing cells with virus and lysing cells was 20 to 30 min. γ-Globin mRNA was clearly destabilized with this S130, having a half-life of

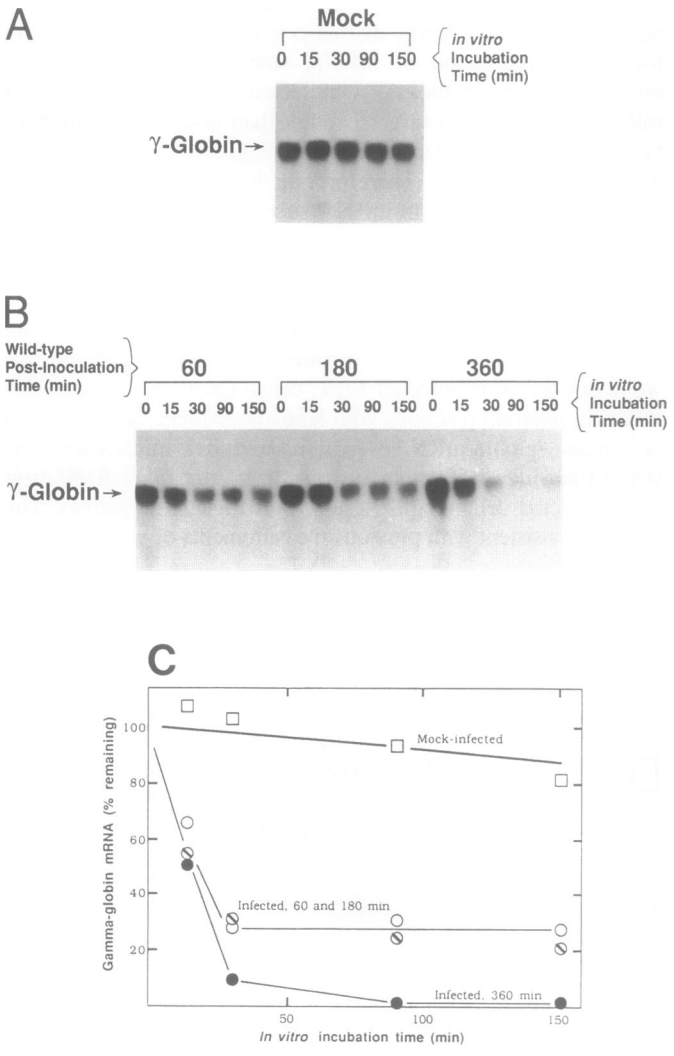

Fig. 2. Destabilization of γ-globin mRNA in cell-free mRNA decay reactions containing S130 from wild-type virus-infected MEL cells. S130 from (A) mock or (B) wild-type virus-infected MEL cells was mixed in a 1:1 cell equivalent ratio with polysomes from uninfected K562 cells. Reactions were incubated at 37°C for the indicated times, and RNA was annealed to a 3′-^{32}P-labeled EcoR I-EcoR I probe for human γ-globin mRNA. S1 nuclease-resistant products were analyzed by electrophoresis in a 6% denaturing polyacrylamide gel. These data were analyzed by laser densitometry (Materials and Methods), and the percentage of γ-globin mRNA remaining in mRNA decay reactions containing S130 from mock or wild-type virus-infected cells is plotted as a function of the *in vitro* incubation time.
□-□ S130 from mock-infected cells.
○-○ S130 from HSV-infected cells: 60 min post-inoculation.
⊘-⊘ S130 from HSV-infected cells: 180 min post-inoculation.
●-● S130 from HSV-infected cells: 360 min post-inoculation.

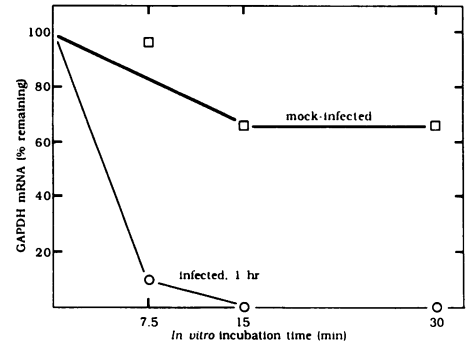

Fig. 3. The percentage of GAPDH mRNA in decay reactions containing S130 from mock- or virus-infected cells (60 min post-inoculation) is plotted as a function of the *in vitro* incubation time. S130 was mixed in a 1:1 cell equivalent ratio with K562 polysomes. RNA (5 μg) from each time point was analyzed by Northern blot analysis, using a GADPH mRNA probe. The data were obtained by laser densitometry (Materials and Methods).
□-□ S130 from mock-infected cells.
○-○ S130 from HSV-infected cells 60 min post-inoculation.

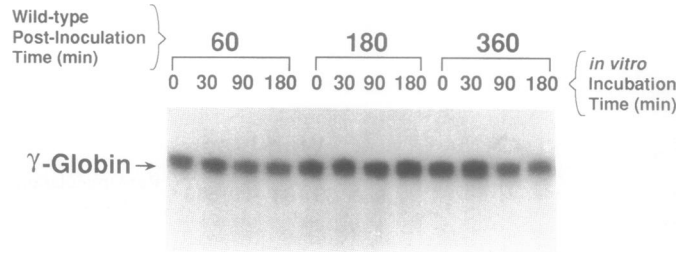

Fig. 4. Stability of γ-globin mRNA in cell-free reactions containing polysomes from wild-type virus-infected MEL cells. Polysomes from uninfected K562 cells and from infected MEL cells were mixed in a 1:2.5 cell equivalent ratio, respectively, and incubated in cell-free mRNA decay reactions for the indicated times. RNA was extracted and analyzed by S1 nuclease mapping, as per Figure 2.

approximately 30 min (Fig. 5, time 0). Its half-life was 15 to 30 min with S130 from cells exposed to virus for only 15 to 30 min (Fig. 5). Therefore, the onset of the destabilization response occurred very rapidly following virus adsorption, consistent with it being induced by a virion protein (see below). γ-Globin mRNA was not destabilized when intact, purified virions were added directly to the cell-free reactions (data not shown), implying that virus adsorbtion was required to induce or activate the destabilization process. Moreover, the destabilizing activity of infected cell S130 was not simply a secondary effect resulting from virus-induced inhibition of translation, since S130 prepared from

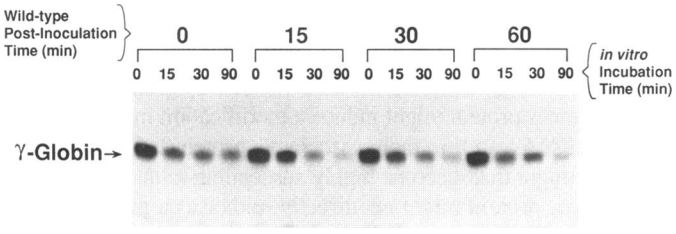

Fig. 5. Destabilizing activity of S130 prepared from wild-type virus-infected MEL cells during the first hour following virus inoculation. S130's were prepared from MEL cells during the hour that the virus was allowed to absorb. Each S130 was mixed with uninfected K562 polysomes in a 1:1 cell equivalent ratio, and cell-free reactions were incubated for the indicated times. To prepare S130 for the time 0 point (first 4 lanes), virus and MEL cells were mixed, and the cells were then immediately harvested and processed. We estimate that the total time between virus-cell mixing and homogenization of cells, most of which was carried out at 4°C, was 20–30 min (see Materials and Methods). γ-Globin mRNA was analyzed by S1 nuclease mapping, as per Figure 2.

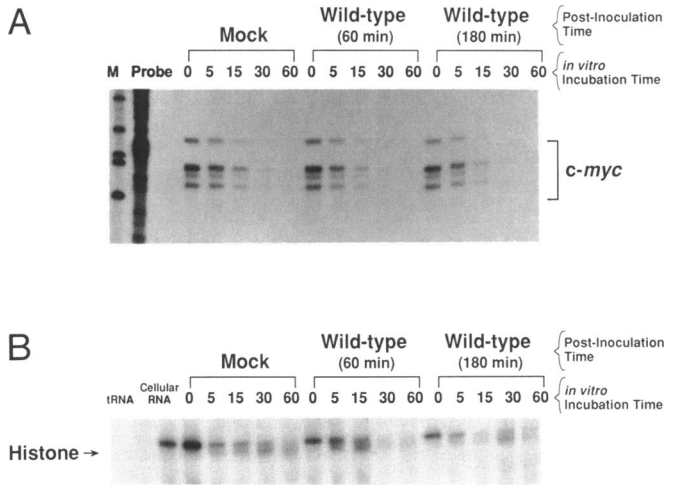

Fig. 6. Lack of effect of S130 from wild-type virus-infected MEL cells on c-*myc* and histone mRNA stability. S130 from wild-type virus-infected MEL cells was mixed with polysomes from uninfected K562 cells in a 1:1 cell equivalent ratio, and cell-free reactions were incubated for the times indicated. (A) RNA (5 μg) from each reaction was annealed to a uniformly ^{32}P-labeled 620 nt RNA probe from the 3′ region of c-*myc* mRNA (23). The samples were treated with RNases P1 and T1, and protected fragments were analyzed by electrophoresis in a denaturing 6% polyacrylamide gel. The marker (M) was pBR322 ^{32}P-DNA cleaved with *Hae* III. From top to bottom, the marker bands are 234, 213, 192, 184, 124 nt. (B) RNA (5 μg) from each reaction was annealed to a 3′-^{32}P-labeled human H4 histone DNA probe (34). S1 nuclease mapping was performed, and the nuclease-resistant products were analyzed in a denaturing 10% polyacrylamide gel. K562 cellular RNA and tRNA were used as positive and negative controls, respectively.

pactamycin or cycloheximide-incubated MEL cells did not destabilize γ-globin mRNA *in vitro* (data not shown).

To determine whether the destabilizer activity also affected normally short-lived mRNAs, the *in vitro* stabilities of c-*myc* and histone mRNAs were monitored. In an RNase protection assay with a c-*myc* probe, four bands were observed with unincubated polysomes (Fig. 6A, lanes 0). These bands correspond to intact c-*myc* mRNAs that differ only in their poly(A) addition sites (23). The intensity of each band diminished at similar rates in reactions containing S130 from mock-infected and infected cells (Fig. 6A). Histone mRNA decay also was unaffected by S130 from HSV-1-infected cells (Fig. 6B). One interpretation of these results is that the half-lives of c-*myc* and histone mRNAs were at their maximums under these conditions and could not be further influenced by the S130 (1,25,37,38). Presumably, transcriptional down-regulation in infected cells accounts for the rapid depletion of some inherently unstable mRNAs like c-*myc* (Fig. 1C). The regulation of histone mRNA levels in infected cells is not well understood (see Discussion).

Correlation between *in vitro* destabilizing activity and the *vhs* protein

To determine whether the HSV-1-induced S130 destabilizing activity correlated with functional *vhs* protein, S130 was prepared from MEL cells infected with either of two HSV-1 mutants, *vhs*1 or *vhs*-ΔSma. Both mutants are defective in the early shutoff function, do not induce mRNA destabilization during the early stages of infection, but do induce destabilization late in infection (14, G.Sullivan Read, personal communication). The *vhs*1 virus contains a point mutation (thr to ile at amino acid 214) in the UL41 *vhs* gene (16); the *vhs*-ΔSma virus contains a 588 bp deletion (deleting 196 amino acids, from 148 to 343) in UL41 (G.Sullivan Read, personal communication). As determined by densitometry of the autoradiogram shown in Figure 7, S130 from wild-type virus-infected cells reduced the half-life of γ-globin mRNA by at least 15-fold (compare wild-type and mock lanes). In contrast, S130 from *vhs*1- or *vhs*-ΔSma-infected cells did not induce significant destabilization. These data are consistent with the *in vivo* phenotype of the mutant viruses and with *in vitro* results published by Krikorian and Read (14,19,26, G.Sullivan Read, personal communication).

Two additional control experiments were performed to assess the apparent correlation between *vhs* function and *in vitro* mRNA destabilization. First, as determined by labeling infected cells with

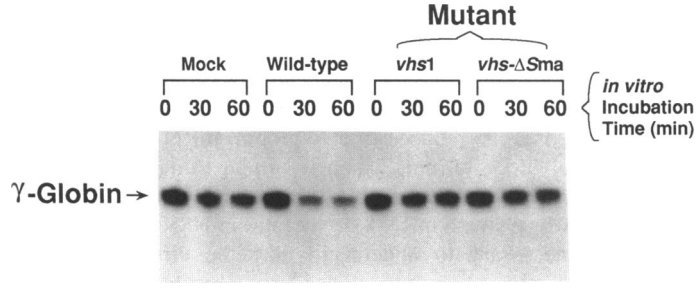

Fig. 7. Stability of γ-globin mRNA in reactions containing S130 from mutant virus-infected MEL cells. MEL cells were either mock-infected or infected for 60 min with wild type or mutant viruses. S130's were prepared from each set of cells and were mixed with polysomes from uninfected K562 cells in a 1:1 cell equivalent ratio. Reactions were incubated for the indicated times, and S1 nuclease digestion was performed as per Figure 2.

^{35}S-methionine, both mutant viruses were competent to infect cells and to induce late shutoff of host protein synthesis (unpublished observations). The second experiment was performed to determine whether blocking viral gene expression at early times affected the *in vitro* results. The *vhs* protein is a virion component, and viral gene expression is not required for early shutoff (8,12,13). Therefore, if the *vhs* function were responsible for *in vitro* destabilization, S130 from cells infected with wild-type HSV-1 should induce mRNA destabilization, even if viral gene expression were blocked. MEL cells were cultured with actinomycin D (5 μg per ml) for 30 min prior to inoculation and were then infected with wild-type virus for 60 min, still in the presence of inhibitor. S130 was prepared and assayed *in vitro* and behaved virtually identically with S130 from cells infected without inhibitor (unpublished observations). This result is consistent with the fact that the *vhs* protein is carried into the cell with HSV-1 virions and is active in the absence of immediate-early viral gene products.

DISCUSSION

The data presented here indicate that HSV-1-induced mRNA destabilization can be reproduced in our *in vitro* system. mRNAs such as γ-globin and GAPDH, which have relatively long half-lives under our standard *in vitro* conditions, are degraded rapidly in reactions supplemented with S130 from wild-type virus-infected MEL cells. There is general agreement that the *vhs* protein product of the HSV UL41 gene induces mRNA degradation in a more or less indiscriminate fashion, affecting most or all cellular and viral mRNAs. Infection with mutant viruses containing defective *vhs* protein fails to cause early shutoff in cells, and cell-free extracts from these infections are deficient in destabilizing activity. If the *vhs* protein itself is not a ribonuclease, then it must interact with cellular factors to destabilize mRNAs. Since our ultimate goal is to purify these factors, we have designed our *in vitro* system to permit us to analyze regulatory factors in *trans*, by mixing human polysomes with extracts from HSV-infected mouse cells. Under these conditions, the destabilizing activity appears as early as 15 min post-inoculation in the S130 (Fig. 2) but not in the polysomes (Fig. 4).

Krikorian and Read observed that viral mRNAs are also unstable in extracts from infected cells (26), implying that mRNA destabilization is, for the most part, non-specific. Therefore, our observation that S130 from wild-type virus-infected cells did not destabilize c-*myc* and histone mRNAs was unexpected (Fig. 6). Perhaps these mRNAs, being inherently short-lived, were already being degraded at or near their maximum rates. HSV-induced destabilizing activity could then exert little or no additional effect. In support of this notion, we observed that S130 from wild-type virus-infected cells reduced the γ-globin mRNA half-life to approximately 15−30 min, which is similar to the normal half-lives of c-*myc* and histone mRNAs. In order to correlate steady-state mRNA levels with mRNA decay rates in HSV-infected cells, it will be necessary to understand how the virus affects the transcription rates of specific genes [e.g., histone, c-*myc*, and PABP (see Fig. 1, panel E)] throughout the infectious cycle. Since histone mRNA lacks poly(A) and is apparently maintained at an unusually high level in some (ref. 10 and Fig. 1, panel D) but not all cells (13), it will also be important to determine whether the HSV destabilizer affects polyadenylated and non-polyadenylated mRNAs in different ways.

Perhaps the major question raised by these observations is how the viral-encoded *vhs* (destabilizer) function induces rapid degradation of so many mRNAs. There are at least four possible mechanisms. One involves direct activation of a latent cellular RNase, similar to RNase L activation in interferon-treated cells (39,40). HSV-1-induced destabilization is unlikely to involve RNase L, however, since it begins very soon after virus adsorption and does not require *de novo* cellular or viral gene expression. A second mechanism involves inactivation of RNase inhibitors followed by activation of 'non-specific' RNases. Little is known about the function of RNase inhibitors in mammalian cells, but the best characterized one is apparently ubiquitous and, by implication, important for RNA metabolism (41). We have not observed any apparent interaction between the HSV destabilizer activity and the major RNase inhibitor, because addition of a 16-fold excess of RNasin (82 units vs. the usual 5 units) to *in vitro* reactions failed to inhibit the destabilizing activity (data not shown). Third, the *vhs* protein might induce a modification in the structure of the mRNAs and/or their associated proteins. The modified mRNP's might then become highly susceptible to nuclease attack. Finally, *vhs* protein might act directly, either as a nuclease or an mRNA-binding protein. Although it has no strong homology to most sequenced cellular proteins, as determined by the FastA and TFastA programs (University of Wisconsin Genetics Computer Group; 42,43) it does have some homology to the RNP 1 and 2 consensus domains of some RNA-binding proteins, as defined by Bandziulis et al. (44). The *vhs* sequence LFCIRV (amino acids 177 to 182) is most homologous to the RNP 1 sequence LFVARV of the human 70 kDa U1 snRNA binding protein (44,45; see Fig. 8). The *vhs* sequence LGYAYIN is very similar to 7 of the 8 RNP 2 consensus amino acids of poly(A) binding proteins from various species (Fig. 8). Although the same *vhs* sequence (LGYAYIN) is present in the UL41 gene of HSV-2 (46) and is deleted in the *vhs*-ΔSma mutant, the significance, if any, of these homologies needs to be determined. For example, it will be important to assess whether *vhs* protein functions stoichiometrically or catalytically. Each virion contains approximately 200 molecules of *vhs* protein (personal communication, G.Sullivan Read), and host cell mRNA is rapidly degraded at a multiplicity of infection as low as 4 (47). If the particle to pfu ratio were 100, then 80,000 molecules of *vhs* protein (4×100×200) would destabilize approximately 2×10^5 molecules of host cell mRNA. Therefore, it is not unreasonable to suggest that the *vhs* protein functions stoichiometrically. Moreover, it might be active as dimers or multimers, since dominant negative effects are observed in mixed

```
        vhs protein  AHLFCIRV------LRALGYAYINSGQ-LEADDACANL
                     :.:::|||       |.||||||:| .|  :|: | ..:
human, Xenopus PABP  GPILSIRVCRDMITRRSLGYAYVNFQQPADAERALDTM

        vhs protein  AHLF----------CIRVLR------ALGYAYINSGQLEAD-DACANL
                     |||:          :||| |       .||||||:| .: ||: .|...|
         yeast PABP  AHLYDIFSPIGSVSSIRVCRDAITKTSLGYAYVNFNDHEAGRKAIEQL

        vhs protein  AHLFCIRV------LRALGYAYINSGQ-LEADDACANL
                       :::|||       |.||||||:| .|  :|: | ..:
   Drosophila PABP   --VLSIRVCRDVITRRSLGYAYVNFQQPADAERALDTM
```

Fig. 8. Regions of homology among the 489 amino acid HSV-1 *vhs* protein and the RNP 2 consensus sequences of poly(A) binding proteins (PABP) (2,9). The *vhs* sequence begins at amino acid number 170 (A) and ends at 200 (L). The human, Xenopus, yeast, and Drosophila PABP sequences begin at 35, 35, 52, and 28, respectively, and end at 72, 72, 99, and 63, respectively. Identities and strong or weak similarities are indicated by lines and double or single dots, respectively. The 7 *vhs* amino acids homologous to the RNP 2 consensus are underlined.

infections with HSV-1 and HSV-2 or with wild-type HSV-1 and *vhs* mutants (47,48).

Another major question concerns the pathway by which host mRNAs are degraded. We have not detected discrete mRNA degradation products in infected cells, presumably because they are so labile. Nevertheless, we favor a pathway involving endonucleolytic cleavage of polysome-associated mRNAs, because functional inactivation and degradation of host mRNAs occur so rapidly following viral adsorption. Endonucleolytic cleavage within the mRNA coding region would block translation in a single step, whereas exonucleolytic cleavage would necessitate multiple hits to block translation. We also suggest that the putative mRNase is ribosome-associated, which could account for the apparent sparing of some host mRNAs (Fig. 1E). That is, poorly translated mRNAs would be exposed less frequently than efficiently translated ones to a ribosome-associated nuclease.

At least three other viruses, vaccinia, adenovirus, and influenza, also induce host mRNA destabilization during their replication (49–52), but the role of destabilization in the viral life cycle is not clear. In the case of HSV-1, switching from immediate-early to early to late gene expression is controlled to a large extent transcriptionally (22,53). If rapid mRNA turnover is coupled with transcriptional and translational regulation to ensure efficient mRNA class switching and orderly virion assembly, then the *vhs* protein might have two major functions: to degrade host mRNAs, thereby liberating cellular ribosomes for viral mRNA translation; to degrade immediate-early and other viral mRNAs, thereby facilitating rapid transitions in viral gene expression. If so, there might be some interesting functional similarities between the *vhs* protein and the recently described ORF61.9 gene product of bacteriophage T4 (54). ORF 61.9 encodes or induces an endonuclease that cleaves between G and A in Shine-Dalgarno sequences. Ruckman et al. (54) suggest that T4 immediate-early mRNAs have especially strong Shine-Dalgarno sequences which permit a rapid burst of expression at early times. These same sequences also ensure rapid mRNA degradation via the ORF61.9 gene product, thereby facilitating the immediate-early to early switch. It will be most interesting to know if the first step in *vhs* protein-mediated mRNA decay also involves cleavages at a limited number of sites.

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REFERENCES

1. Carneiro, M., and Schibler, U. (1984) *J. Mol. Biol.*, **17,** 869–880.
2. Ross, J. (1988) *Mol. Biol. Med.*, **5,** 1–14.
3. Brawerman, G. (1989) *Cell*, **57,** 9–10.
4. Cleveland, D. W., and Yen, T. J. (1989) *New Biol.*, **1,** 121–126.
5. Shapiro, D. J., Blume, J. E., and Nielsen, D. A. (1987) *BioEssays*, **6,** 221–226.
6. Fenwick, M. L., and Clark, J. (1982) *J. Gen. Virol.*, **61,** 121–125.
7. Ingles, S. C. (1982) *Molec. Cell. Biol.*, **2,** 1644–1648.
8. Kwong, A. D., and Frenkel, N. (1987) *Proc. Natl. Acad. Sci.*, **84,** 1926–1930.
9. London, F. S., Brinker, J. M. Ziaie, Z., and Kefalides, N. A. (1990) *Lab Invest.*, **62,** 189–195.
10. Mayman, B. A., and Nishioka, Y. (1985) *J. Virol.*, **53,** 1–6.
11. Nishioka, Y., and Silverstein, S. (1977) *Proc. Natl. Acad. Sci., USA*, **74,** 2370–2374.
12. Nishioka, Y., and Silverstein, S. (1978) *J. Virol.*, **27,** 619–627.
13. Schek, N., and Bachenheimer, S. L. (1985) *J. Virol.*, **55,** 601–610.
14. Strom, T., and Frenkel, N. (1987) *J. Virol.*, **61,** 2198–2207.
15. Fenwick, M. L. (1984) *In:* Comprehensive Virology (eds.) Fraenkel-Conrat, H., and Wagner, R. R. Plenum Press, New York, pp. 359–390.
16. Kwong, A. D., Kruper, J. A., and Frenkel, N. (1988) *J. Virol.*, **62,** 912–921.
17. Oroskar, A. A., and Read, G. S. (1987) *J. Virol.*, **61,** 604–606.
18. Oroskar, A. A., and Read, G. S. (1989) *J. Virol.*, **63,** 1897–1906.
19. Read, G. S., and Frenkel, N. (1983) *J. Virol.*, **46,** 498–512.
20. Fenwick, M. L., and Everett, R. D. (1990) *J. Gen. Virol.*, **71,** 2961–2967.
21. Smibert, C. A., and Smiley, J. R. (1990) *J. Virol.*, **64,** 3882–3894.
22. Roizman, B., and Sears, A. E. (1990) *In*: Virology (eds) Fields, B. N., and Knipe, D. M. et al. Raven Press, Ltd., New York.
23. Brewer, G., and Ross, J. (1988) *Mol. Cell. Biol.*, **8,** 1697–1708.
24. Ross, J., and Kobs, G. (1986) *J. Mol. Biol.*, **188,** 579–593.
25. Ross, J., Kobs, G., Brewer, G., and Peltz, S. W. (1987) *J. Biol. Chem.*, **262,** 9374–9381.
26. Krikorian, C. R., and Read, G. S. (1991) *J. Virol.*, **65,** 112–122.
27. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.*, **132,** 6–13.
28. Thomas P. S. (1980) *Proc. Natl. Acad. Sci. USA*, **77,** 5201–5205.
29. Shen-Ong, G. L. C., Keath, E. J., Piccoli, S. P., and Cole, M. D. (1982) *Cell* **31,** 443–452.
30. Grange, T., Martins de Sa, C., Oddos, J., and Pictet, R. (1987) *Nucleic Acid Res.*, **15,** 4771–4787.
31. Fort, Ph., Marty, L., Piechaczyk, M., Sabrouty, S. El., Dani, Ch., Jeanteur, Ph., and Blanchard, J. M. (1985) *Nucleic Acid Res.*, **13,** 1431–1442.
32. Sittman, D. B., Graves, R. A., and Marzluff, W. F. (1983) *Nucleic Acid Res.*, **11,** 6679–6697.
33. Shen, S.-H., Slightom, J. L., and Smithies, O. (1981) *Cell*, **26,** 191–203.
34. Heintz, N., Zernik, M., and Roeder, R. G. (1981) *Cell*, **24,** 661–668.
35. Dani, C., Piechaczyk, M., Audigier, Y., El Sabouty, S., Cathala, G., Marty, L., Fort, P., Blanchard, J. M., and Jeanteur, P. (1984) *Eur. J. Biochem.*, **165,** 299–304.
36. Ross, J., and Pizarro, A. (1983) *J. Mol. Biol.*, **167,** 607–617.
37. Peltz, S. W., Brewer, G., Groppi, V., and Ross, J. (1989) *Mol. Biol. Med.*, **6,** 227–238.
38. Peltz, S. W., Brewer, G., Kobs, G., and Ross, J. (1987) *J. Biol. Chem.*, **262,** 9382–9388.
39. Baglioni, C., deBenedetti, A., and Williams, G. J. (1984) *J. Virol.*, **52,** 865–871.
40. Pacha, R. F., Meis, R. J., and Condit, R. C. (1990) *J. Virol.*, **64,** 3853–3863.
41. Blackburn, P., Wilson, G., and Moore, S. (1977) *J. Biol. Chem.*, **252,** 5904–5910.
42. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.*, **12,** 387–395.
43. Pearson, W. R., and Lipman, D. J. (1988) *PNAS*, **85,** 2444–2448.
44. Bandziulis, R. J., Swanson, M. S., and Dreyfuss, G. (1989) *Genes and Develop.*, **3,** 431–437.
45. McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E., and Taylor, P. (1988) *J. Gen. Virol.*, **69,** 1531–1574.
46. Everett, R. D., and Fenwick, M. L. (1990) *J. Gen. Virol.*, **71,** 1387–1390.
47. Kwong, A. D., and Frenkel, N. (1989) *J. Virol.*, **63,** 4834–4839.
48. Hill, T. M., Sadler, J. R., and Betz, J. L. (1985) *J. Virol.*, **56,** 312–316.
49. Herz, C., Staunezer, E., Krug, R. M., and Gurney, T. (1981) *Cell*, **26,** 391–400.
50. Lazaridis, I., Babich, A., and Nevins, J. R. (1988) *Virology*, **165,** 438–445.
51. Plotch, S. J., Bouloy, M., Ulmanen, I., and Krug, R. M. (1981) *Cell*, **23,** 847–858.
52. Rice, A. P., and Roberts, B. E. (1983) *J. Virol.*, **47,** 529–539.
53. Dixon, R. A., and Schaffer, P. A. (1980) *J. Virol.*, **36,** 189–203.
54. Ruckman, J., Parma, D., Tuerk, C., Hall, D. H., and Gold, L. (1989) *New Biol.*, **1,** 54–65.