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In Vitro mRNA Degradation System To Study the Virion Host Shutoff Function of Herpes Simplex Virus

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The virion host shutoff (*vhs*) gene of herpes simplex virus encodes a virion polypeptide that induces degradation of host mRNAs at early times and rapid turnover of viral mRNAs throughout infection. To better investigate the *vhs* function, an in vitro mRNA degradation system was developed, consisting of cytoplasmic extracts from HeLa cells infected with wild-type herpes simplex virus type 1 or a mutant encoding a defective *vhs* polypeptide. Host and viral mRNAs were degraded rapidly in extracts from cells productively infected with wild-type herpes simplex virus type 1 but not in extracts from mock-infected cells or cells infected with the mutant *vhs*1. In contrast, 28S rRNA was stable in all three kinds of extract. Accelerated turnover of host mRNAs was also observed in extracts from cells infected with wild-type virus in the presence of dactinomycin, indicating that the activity was induced by a structural component of the infecting virions. The in vitro *vhs* activity was inactivated by heat or proteinase K digestion but was insensitive to brief treatment of the extracts with micrococcal nuclease. It was not inhibited by placental RNase inhibitor, it exhibited a strong dependence upon added Mg^{2+}, it was active at concentrations of K^+ up to 200 mM, and it did not require the components of an energy-generating system. In summary, the in vitro mRNA degradation system appears to accurately reproduce the *vhs*-mediated decay of host and viral mRNAs and should be useful for studies of the mechanism of *vhs* action.

In cells infected with herpes simplex virus (HSV), the shutoff of host macromolecular synthesis and the cascade regulation of viral gene expression are achieved through a complex set of transcriptional and posttranscriptional controls (26, 45, 59, 66, 74, 76, 77). Of the posttranscriptional regulatory mechanisms, the most thoroughly studied to date has been the regulation of host and viral mRNA stabilities by the product of the HSV virion host shutoff (*vhs*) gene. The *vhs* gene encodes a polypeptide that is a structural component of virions and at early times after infection causes the shutoff of most host cell protein synthesis by inducing degradation of cellular mRNAs (16–22, 28, 42, 43, 55, 58, 66, 69, 71, 72). Since copies of the *vhs* protein are present within the infecting virion, virion host shutoff is not dependent upon prior de novo viral protein synthesis (16–22, 42, 43, 55, 66, 69). The activity of the *vhs* protein is not limited, however, to preexisting cellular mRNAs. After the onset of viral transcription, the *vhs* protein induces rapid turnover of viral mRNAs belonging to all kinetic classes (32, 44, 45, 69). Thus, virus mutants that encode a defective *vhs* protein are defective in virion host shutoff and produce viral mRNAs with significantly longer half-lives than those of mRNAs produced by the wild-type virus (32, 44, 45, 69). The *vhs* polypeptide, therefore, appears to provide a nonselective mRNA degradation function that, when coupled with specific transcriptional controls, plays an important role in regulating the steady-state levels and patterns of accumulation of both viral and cellular mRNAs (32, 44, 45).

An ever-increasing body of data has made it clear that control of mRNA stability plays an important role in regulating the expression of many mammalian genes (5, 60). Exposure of cells to a variety of hormones and other stimuli increases the levels of specific mRNAs, at least in part, by increasing their cytoplasmic half-lives. Glucocorticoids enhance the stability of human growth hormone mRNA (46), whereas estrogen specifically stabilizes vitellogenin mRNA (8) and prolactin induces an increase in the half-life of casein message (25). Conversely, stimuli that increase the cytoplasmic concentration of iron destabilize transferrin receptor mRNA, apparently by reducing the affinity of a specific mRNA binding protein for *cis*-acting sequences located within the 3′ untranslated region of the message (40, 41, 64). The stability of β-tubulin mRNA is regulated by the concentration of unpolymerized β-tubulin in the cytoplasm. Thus, treatment of cells with cytochalasin B, which disrupts microtubules and raises the concentration of unpolymerized tubulins, induces rapid degradation of β-tubulin mRNA (23, 78). Similarly, in vivo and in vitro studies suggest that an increase in the level of free histones, which accompanies the cessation of DNA synthesis at the end of the S phase, induces rapid destabilization of histone mRNAs (24, 27, 38, 47, 51, 52, 61–63). Finally, the presence of specific *cis*-acting sequences within the 3′, and sometimes 5′, untranslated regions of the mRNAs encoding a number of lymphokines and proto-oncogene products causes these messages to be very short lived (11, 29, 53, 54, 67, 68, 75). Abrogation of the normal control of the stabilities of these mRNAs can lead to cell transformation, altered growth properties, or abnormal differentiation (1, 10, 12, 13, 15, 36, 37, 50, 67).

A number of studies suggest that the degradation of mRNAs normally occurs by 3′-to-5′ exonuclease digestion. Studies from J. Ross's laboratory with extracts from uninfected K562 cells have shown that the in vitro degradation of c-*myc* mRNA involves progressive shortening of the poly(A) tail, followed by 3′-to-5′ degradation of the body of the message (6, 7). That this mechanism is generally followed for

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the decay of polyadenylated messages in vivo is suggested by the finding of Wilson and Treisman that sequences located in the 3′ untranslated region of c-*fos* mRNA, which accelerate its rate of turnover, also cause more rapid shortening of the poly(A) tails of c-*fos* messages (75). 3′-to-5′ degradation also occurs for nonpolyadenylated messages. Ross and co-workers have shown, both in vitro and in vivo, that the decay of nonpolyadenylated histone mRNAs proceeds by cleavage of the last 4 to 13 nucleotides from the 3′ end of the mRNA, followed by rapid 3′-to-5′ degradation of the remainder of the message (51, 52, 61–63).

At present, only a limited amount has been reported concerning the development of in vitro mRNA degradation systems. The most extensive studies to date have come from Ross's laboratory involving extracts from uninfected K562 erythroleukemia cells (3, 4, 6, 7, 51, 52, 60–63). In this system, in vitro degradation of histone mRNAs is mediated by a polysome-bound exonuclease that can be isolated in an active, soluble form by washing the polysomes with buffers containing 0.3 M KCl (61, 62). Although the RNase that degrades histone messages is normally polysome associated, in vitro regulation of the rate of histone mRNA decay by the concentration of free histones also requires a factor that is present in the S130 high-speed supernatant obtained after pelleting the polysomes from a cytoplasmic extract (51). Similar results have been obtained for the decay of some polyadenylated messages. In vitro poly(A) shortening and 3′-to-5′ degradation of c-*myc* message can be observed upon incubation of isolated polysomes, indicating that the relevant nucleases are polysome associated (6). However, c-*myc* decay is greatly accelerated by a labile factor that is found in the S130 postpolysomal supernatant fraction from cytoplasmic extracts (7). This factor specifically accelerates the decay rates of c-*myc* and c-*myb* mRNAs and is inactivated by treatment with micrococcal nuclease, suggesting that it may be a ribonucleoprotein (7). However, in spite of this impressive progress, as yet little is known concerning the identity of the nucleases involved in mRNA turnover and the factors that control their activities.

The *vhs*-mediated control of mRNA stabilities in HSV-infected cells provides a particularly attractive model system for studying the factors that control message half-lives in mammalian cells. In particular, the *vhs* protein is one of the few *trans*-acting regulators of mRNA stability that have been identified to date. In addition, mutants encoding defective *vhs* proteins have been isolated and characterized (17, 55), allowing a combination of genetic and biochemical techniques to be applied to the problem. Mapping of the mutation carried by the mutant *vhs*1 to the UL41 open reading frame of HSV type 1 (HSV-1) has allowed identification of the *vhs* gene (34, 39), and recent characterization of the structural polypeptides encoded by a *vhs* deletion mutant has allowed identification of the *vhs* polypeptide within purified virions (55a). Clearly, elucidation of the mechanism of *vhs*-mediated control of mRNA half-lives would be greatly facilitated by the availability of an in vitro mRNA degradation system that accurately reflects the *vhs* activity observed in vivo. In this paper we report the development and preliminary characterization of such a system from HSV-1-infected HeLa cells.

MATERIALS AND METHODS

Cells and virus. HeLa S3 and Vero cells were purchased from the American Type Culture Collection and grown at 37°C in Eagle minimum essential medium (MEM; GIBCO) supplemented with antibiotics and 10% (vol/vol) calf serum (45). Stocks of wild-type HSV-1 strain KOS and the mutant *vhs*1 were prepared by infection of Vero cell monolayers as previously described (45). *vhs*1 grows well at all temperatures from 34 to 39°C and exhibits a defective virion host shutoff function at all temperatures (55). In these studies, all infections were performed at 34°C. HeLa S3 cells were used for the preparation of all in vitro mRNA degradation extracts and were infected and maintained as described previously for Vero cells (31, 45). In all experiments, virus was allowed to adsorb for 1 h in MEM containing 5% (vol/vol) calf serum. The inocula were then aspirated, and the cells were overlaid with fresh MEM plus 2% calf serum. Mock-infected cells were treated in the same way as infected cells, except that they were exposed to lysates of uninfected Vero cells prepared in the same way as the virus stocks were prepared from infected cells.

Plasmids. The plasmid pHcGAP contains a 1.2-kb cDNA insert encoding a portion of human glyceraldehyde-3-phosphate dehydrogenase (GAPD) (73) and was obtained from the American Type Culture Collection. The plasmid, pHSV106, which contains a 3.4-kb *Bam*HI fragment encoding the HSV-1 thymidine kinase, was purchased from Bethesda Research Laboratories. The plasmid pXlr11 contains a 4.6-kb fragment of *Xenopus laevis* rDNA inserted into the *Eco*RI site of colicin E1 (14) and was provided by Jeff Doering. The inserted fragment in pXlr11 hybridizes with 28S rRNA. pXlr11 was maintained in *Escherichia coli* HB101, whereas pHcGAP and pHSV106 were maintained in *E. coli* DH5 alpha (Bethesda Research Laboratories). Plasmid DNAs were prepared by CsCl density gradient centrifugation as described previously (45, 56, 57).

In vitro mRNA degradation extracts. Cytoplasmic extracts for studying in vitro mRNA degradation were prepared from infected or mock-infected HeLa S3 cells by a modification of the procedure of Brown and colleagues for the preparation of in vitro translation extracts (9). At various times after infection or mock infection, HeLa cells were washed twice with ice-cold wash buffer consisting of 0.15 M sucrose, 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate [$Mg(OAc)_2$], and 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4). The cells were then permeabilized by the addition of 300 μg of lysolecithin (L-α-lysophosphatidyl choline; Sigma) per ml in wash buffer directly to the monolayers for 60 s. After the permeabilization buffer was removed, the cells from one 100-mm dish were scraped into 200 μl of standard reaction buffer [0.1 M HEPES (pH 7.4), 0.2 M NH_4Cl, 20 mM $Mg(OAc)_2$, 7 mM KCl, 1 mM dithiothreitol, 1 mM ATP (dipotassium salt), 1 mM GTP (sodium salt), 40 μM each of the 20 amino acids, 0.1 mM *S*-adenosylmethionine, 1 mM spermidine, 10 mM creatine phosphate (dipotassium salt), 40 U of creatine kinase per ml, and 100 U of placental RNase inhibitor (RNasin; Promega Corp, Madison, Wis.)]. The cells were then disrupted by 10 passages through a 25-gauge needle. The nuclei were removed by low-speed centrifugation, and the supernatant was stored on ice.

To initiate mRNA degradation reactions, cytoplasmic supernatants were transferred to a 30°C water bath. At various times after the start of incubation, samples were removed, added to an equal volume of urea buffer (7 M urea, 10 mM Tris hydrochloride [pH 7.9], 0.35 M NaCl, 10 mM EDTA, and 1% sodium dodecyl sulfate), extracted twice with phenol-chloroform (1:1, vol/vol) and twice with chloroform, and precipitated from ethanol, all as described previously (45, 56, 57). Samples taken immediately before transferring the reaction mixtures from 4°C to 30°C served as 0-h

time points. The samples were subsequently analyzed for in vitro decay of host and viral mRNAs and 28S rRNA by Northern RNA blotting and hybridization as described below.

In experiments to optimize the concentration of Mg^{2+} ion needed for efficient in vitro mRNA degradation, extracts were prepared in the standard reaction buffer modified to contain 2.5 mM $Mg(OAc)_2$ and then supplemented with concentrated $Mg(OAc)_2$ to bring the Mg^{2+} concentration to the desired values. To optimize the K^+ ion concentration, standard extracts were prepared containing 7 mM KCl and then supplemented with concentrated KCl to bring the K^+ concentration to the values described in the figure legends. To test the requirement for energy-generating components, extracts were prepared in standard reaction buffer lacking ATP, GTP, creatine phosphate, and creatine kinase. The extracts were all incubated and analyzed as described above.

Pretreatment of extracts with heat, proteinase K, or micrococcal nuclease. To test the sensitivity of the extracts to brief heat treatment, standard in vitro degradation reactions were heated at 90°C for 10 min, cooled to 4°C, and then analyzed for in vitro decay of mRNAs according to the standard protocol. To test the sensitivity of the extracts to pretreatment with protease, standard extracts were supplemented with proteinase K (Sigma; molecular biology grade) to a concentration of 1 mg/ml and incubated at 30°C for 30 min. The extracts were cooled briefly to 4°C and then incubated at 30°C and analyzed for in vitro mRNA degradation according to the standard protocol.

To test the effect of pretreating the extracts with micrococcal nuclease, standard in vitro degradation extracts were supplemented with micrococcal nuclease (Pharmacia) to 1,000 U/ml and $CaCl_2$ to 1 mM and then incubated at 30°C for 10 min. Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) was added to 2 mM, and the extracts were chilled on ice for 10 min. To each reaction mixture was added deproteinized total cytoplasmic RNA from an equivalent number of uninfected HeLa cells, and the mixtures were incubated at 30°C. Samples were taken at various times and analyzed by Northern blotting for the in vitro decay of host mRNAs and 28S rRNA.

Agarose gel electrophoresis, Northern blotting, and hybridization. RNA samples were denatured with glyoxal, electrophoresed through 1% agarose gels cast in 10 mM sodium phosphate (pH 7.0), and transferred to Nytran membranes (Schleicher and Schuell) by capillary blotting, all as described previously (45). Filters containing immobilized RNAs were prehybridized for 1 to 2 h and then hybridized to nick-translated probes as described previously (44, 45). In some experiments probes were stripped from the filters, and the membranes were rehybridized with a second probe as previously described (45). Nick-translated pHcGAP and pHSV106 were used as probes to detect the cellular GAPD and the HSV-1 thymidine kinase mRNAs, respectively. Nick-translated pX1r11 was used to detect 28S rRNA.

Quantitation of mRNA levels. To quantitate the levels of host and viral mRNAs and 28S rRNA, autoradiograms from the Northern blots were scanned with a Hoeffer model GS300 scanning densitometer. Since 28S rRNA proved to be stable in all types of extracts, the levels of specific mRNAs were normalized to the amount of 28S rRNA in each sample before mRNA decay curves were plotted in all experiments except that shown in Fig. 7. In Fig. 7 the relative amounts of 28S rRNA and GAPD mRNA were plotted without prior normalization. Nick-translated pHcGAP hybridized to GAPD mRNA; it also cross-hybridized at a low level to 28S rRNA. Equivalent results were obtained for the decay curves of GAPD mRNA regardless of whether the amount of GAPD mRNA was normalized to the level of 28S rRNA detected by cross-hybridization with pHcGAP or by stripping the pHcGAP probe from the blot and then rehybridizing with nick-translated pX1r11.

RESULTS

Strategy. The HSV virion host shutoff protein is known to induce rapid degradation of host and viral mRNAs in the cytoplasm (32, 44, 45, 66, 69). In an effort to develop an in vitro mRNA degradation system to study the *vhs* function, we decided to compare the rates of mRNA degradation in in vitro translation extracts from mock-infected HeLa cells and extracts from cells infected with either wild-type HSV-1 or the mutant *vhs*1, which has been shown to encode a defective *vhs* polypeptide (55). HeLa cells were chosen because they are readily infected with HSV and because numerous studies have proven them to be a particularly suitable cell line for the preparation of in vitro translation extracts. Although in vivo studies indicated that *vhs*-induced mRNA degradation does not require efficient ongoing translation of the mRNAs (66, 69), we decided to prepare in vitro translation extracts because we reasoned that they would be the best initial approximation to a functional cytoplasm.

Extracts were prepared by a procedure shown by Brown and co-workers to be suitable for the preparation of highly active in vitro translation extracts from a variety of cultured cells (9). This procedure involves briefly exposing the monolayers to lysolecithin to permeabilize the cells, harvesting the cells directly into buffer containing the components required for in vitro translation, disrupting the cells by repeated passage through a 25-gauge needle, and removing the nuclei by low-speed centrifugation. This protocol was chosen because it is simple and rapid, making it possible to prepare and analyze the activity of extracts on the same day. This enabled us to perform all experiments with freshly prepared lysates and eliminated the need to freeze in vitro degradation extracts before analysis, an obvious advantage in view of the possible detrimental effects that freezing and thawing could have upon the activities of as yet uncharacterized proteins.

To optimize the concentration of lysolecithin needed to permeabilize HeLa cells and to verify that this procedure yielded active in vitro translation extracts, standard reaction mixtures were prepared by using a variety of lysolecithin concentrations in the permeabilization buffer. Unlabeled methionine was omitted from the reaction buffer and [^{35}S]methionine was added to a concentration of 75 μCi/ml. At various times after the start of incubation, samples were withdrawn and the amount of [^{35}S]methionine incorporated into trichloroacetic acid-precipitable material was determined as described previously (55). Exposure of the HeLa cells to 300 μg of lysolecithin per ml was found to be optimal for in vitro translation (data not shown). For extracts prepared with this concentration of lysolecithin, efficient in vitro translation continued for approximately 40 min, after which the amount of incorporated label leveled to a plateau (Fig. 1).

In vitro degradation of host mRNAs. The *vhs* function was originally identified on the basis of its ability to induce rapid degradation of host mRNAs and the concomitant shutoff of host polypeptide synthesis (16, 17, 19, 20, 55, 66). To determine whether HeLa cell lysates would be suitable for in vitro studies of the *vhs* function, standard in vitro translation

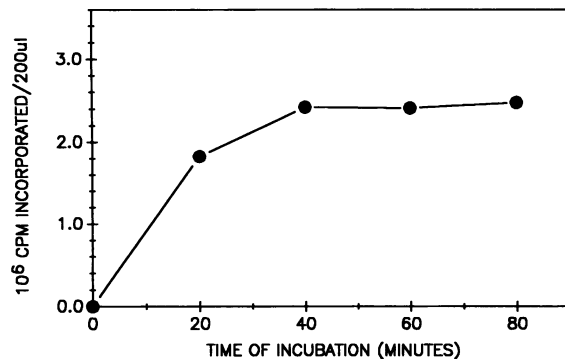

FIG. 1. In vitro translation by HeLa cell extracts. An in vitro mRNA degradation extract was prepared from mock-infected HeLa cells in standard reaction buffer containing 2.5 mM $Mg(OAc)_2$, lacking unlabeled methionine, and supplemented with $[^{35}S]$methionine to 75 μCi/ml. The reaction mixture was incubated at 30°C. At various times samples were withdrawn, and the amount of radioactivity incorporated into trichloroacetic acid-precipitable material was determined as described previously (55).

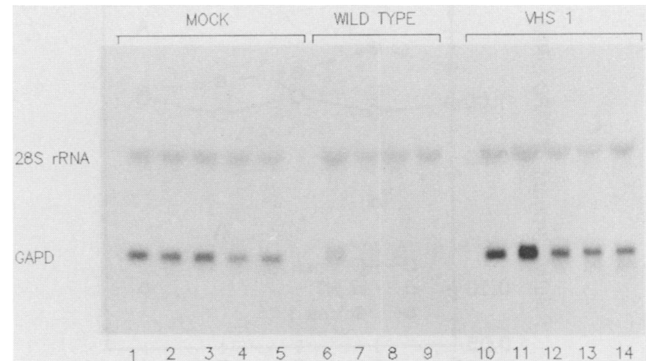

FIG. 2. In vitro degradation of host mRNAs. Standard in vitro mRNA degradation extracts were prepared from HeLa cells 5 h after mock infection (lanes 1 through 5), or infection with 20 PFU of wild-type HSV-1 (lanes 6 through 9) or *vhs*1 (lanes 10 through 14) per cell. Samples were withdrawn from the reactions at 0 h (lanes 1, 6, and 10), 1 h (lanes 2, 7, and 11), 2 h (lanes 3 and 12), 3 h (lanes 4, 8, and 13), or 4 h (lanes 5, 9, and 14). The samples were extracted twice with phenol-chloroform and twice with chloroform, and the RNAs were precipitated from ethanol. Samples of total cytoplasmic RNA were denatured with glyoxal, electrophoresed through 1% agarose gels, and transferred to Nytran membranes by capillary blotting as described in the text. The membranes were then probed to detect GAPD mRNA and 28S rRNA as described in the text.

extracts were prepared from mock-infected cells and from cells 5 h after infection with 20 PFU of either wild-type HSV-1 or the mutant *vhs*1 per cell. The extracts were incubated at 30°C; at various times samples were withdrawn and extracted with phenol and chloroform, and the decay of specific cellular mRNAs was analyzed by Northern blotting. To control for the total amount of cytoplasmic RNA loaded onto each lane of the gel, the blots were also probed for 28S rRNA, and the amount of mRNA was normalized to the amount of 28S rRNA before the mRNA decay curve was plotted.

In these initial experiments we decided to study the decay of endogenous cellular mRNAs rather than that of added exogenous mRNAs because we reasoned that the structure of messenger ribonucleoprotein particles (mRNPs) reconstituted on exogenous mRNAs might differ from that of endogenous mRNPs. mRNP structure could easily affect mRNA stability; in fact, HSV infection has been shown to induce changes in mRNP structure that correlate with a wild-type virion host shutoff function (31). Focusing upon the decay of endogenous mRNAs should, therefore, remove one potential variable from the experiments. In addition, the results involving decay of endogenous mRNAs should provide a base line for later attempts to study the degradation of exogenous messages.

The decay of the cellular mRNA encoding GAPD is shown in Fig. 2 and 3. GAPD mRNA was chosen for study because it has a long in vivo half-life in uninfected cells (73). Thus, any *vhs*-induced reduction in message stability should be more easily detected for this mRNA than for a message that is inherently unstable. GAPD mRNA was relatively stable for at least 4 h in extracts from mock-infected cells (Fig. 2, lanes 1 through 5; Fig. 3). In contrast, it decayed rapidly in extracts from cells infected with wild-type HSV-1, so that little detectable mRNA remained by 1 h after the start of incubation (Fig. 2, lanes 6 through 9; Fig. 3). That considerable *vhs*-induced degradation had occurred in vivo in wild-type infections before the preparation of extracts is indicated by the fact that the intensity of the band formed by GAPD mRNA at 0 h of incubation for wild-type extracts was reduced considerably relative to that observed at 0 h for mock or *vhs*1 infections (compare Fig. 2, lanes 1, 6, and 10, with Fig. 3A). In contrast to the case for wild type-infected cell extracts, in extracts from cells infected with *vhs*1 GAPD mRNA was every bit as stable as in extracts from mock-infected cells (Fig. 2, lanes 10 through 14; Fig. 3). In vitro decay was specific for mRNAs, as evidenced by the fact that 28S rRNA was equally stable in mock-, wild type-, and *vhs*1-infected cell extracts. All told, the rank order of in vitro decay rates of GAPD mRNA was the same as that observed in mock, wild-type, and *vhs*1 infections in vivo.

The *vhs* protein is a structural component of virions and is therefore able to induce degradation of host mRNAs in the absence of prior de novo viral gene expression (17, 22, 55, 66, 69). Thus, virion host shutoff is induced by UV-inactivated virus as well as after infection of cells in the presence of dactinomycin to block viral transcription (17, 22, 55, 66, 69). To determine whether the accelerated degradation of GAPD mRNA observed in extracts from cells infected with wild-type virus was induced by a virion component or required de novo viral gene expression, in vitro degradation extracts were prepared from cells 5 h after a productive wild-type virus infection or 5 h after infection with 50 PFU of wild-type virus or *vhs*1 per cell in the presence of 5 μg of dactinomycin per ml. Degradation of the GAPD mRNA was equally rapid in extracts from cells infected with wild-type virus in the presence and absence of dactinomycin (Fig. 4). In contrast, GAPD mRNA was stable for at least 5 h in extracts from cells infected with *vhs*1. Thus, the accelerated degradation of host mRNAs that was observed in extracts from cells infected with wild-type HSV-1 was not dependent upon de novo viral gene expression and was therefore induced by a component of the infecting virions.

In vitro degradation of viral mRNAs. Although *vhs* mutants were originally isolated on the basis of their defects in the degradation of cellular mRNAs and the shutoff of host protein synthesis, recent studies indicate that the *vhs* protein plays a central role in determining the half-lives of both viral and cellular mRNAs within the infected cell (32, 44, 45).

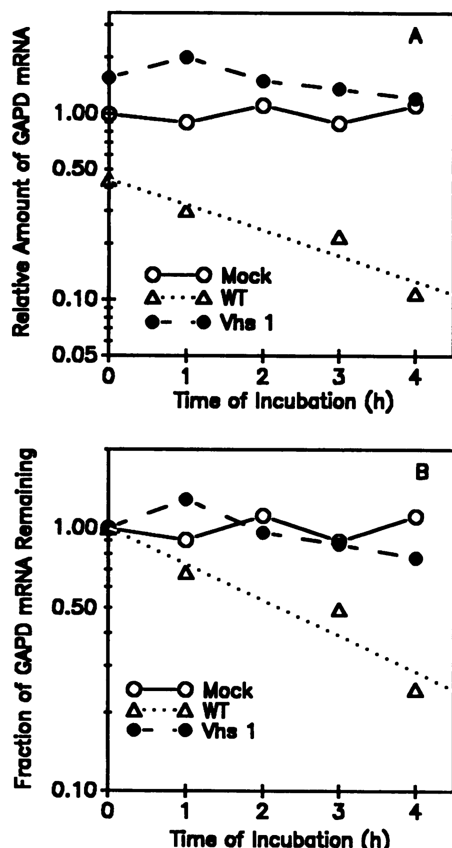

FIG. 3. Quantitation of in vitro decay of host mRNAs. The autoradiogram shown in Fig. 2 was scanned, and the amount of GAPD mRNA in each lane was normalized to the amount of 28S rRNA. In panel A the relative amounts of GAPD mRNA in mock-infected (○), wild-type virus-infected (△), and *vhs*1-infected (●) cell extracts are plotted in arbitrary units. In panel B the amount of GAPD mRNA present in a sample from any of the three types of extract is expressed as a fraction of the amount present at 0 h in that kind of extract.

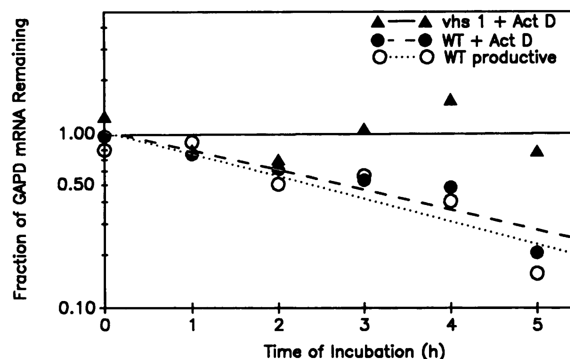

FIG. 4. In vitro degradation in extracts from cells infected in the presence of dactinomycin. Standard in vitro degradation extracts were prepared from cells 5 h after infection with 50 PFU of wild-type virus (●) or *vhs*1 (▲) per cell in the presence of 5 μg of dactinomycin per ml or 5 h after infection with 50 PFU of wild-type virus per cell in the absence of any drugs (○). The extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for GAPD mRNA and 28S rRNA by Northern blotting as described in the legend to Fig. 2. Autoradiograms were scanned with a Hoeffer model GS300 scanning densitometer, and the amount of GAPD mRNA in each sample was normalized to the amount of 28S rRNA. The amount of GAPD mRNA remaining at various times was plotted as a fraction of the amount present at 0 h.

Measurements of the half-lives of 10 different viral mRNAs in cells infected with wild-type virus or the mutant *vhs*1 revealed the following: (i) in wild-type infections the half-lives of all 10 messages, representing all kinetic classes of viral mRNA, were very similar; (ii) the *vhs*1 mutation resulted in dramatic increases in the stabilities of all 10 messages (44, 45). Thus, the *vhs* protein induces the largely nonselective degradation of both viral and cellular mRNAs.

To determine whether degradation of viral mRNAs was also accelerated in our in vitro message degradation system, in vitro degradation extracts were prepared from cells 5 h after infection with wild-type virus or *vhs*1 and analyzed for the degradation of the mRNA encoding the viral thymidine kinase (TK). TK mRNA was degraded rapidly in in vitro extracts from cells infected with wild-type HSV-1 but was relatively stable for at least 5 h in extracts from cells infected with *vhs*1 (Fig. 5). Therefore, once again the in vitro results paralleled those observed in vivo.

Characterization of the in vitro mRNA degradation system. In all respects examined to this point, in vitro degradation of host and viral mRNAs observed in HSV-infected HeLa cell extracts paralleled *vhs*-induced mRNA degradation observed in vivo. We therefore undertook a series of experiments to characterize some of the biochemical requirements of the in vitro mRNA degradation system.

A preliminary experiment was undertaken to determine the effect of RNasin upon *vhs*-induced mRNA decay. Parallel degradation extracts were prepared from HeLa cells 5 h after infection with 20 PFU of either wild-type HSV-1 or *vhs*1 per cell. RNasin (100 U/ml) was included in half of the reaction mixtures and omitted from the other half. Regardless of whether the inhibitor was present, mRNAs were very

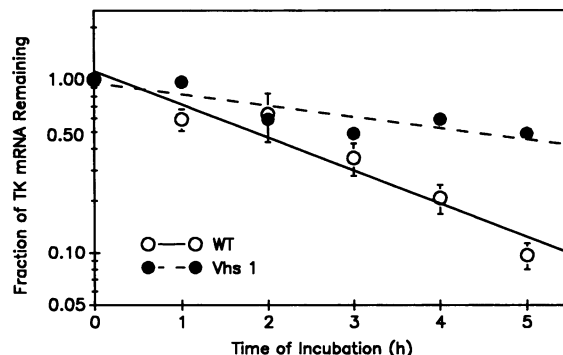

FIG. 5. In vitro degradation of viral mRNAs. Standard in vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 PFU of wild-type HSV-1 (○) or *vhs*1 (●) per cell. The extracts were incubated at 30°C for the indicated times, at which point samples were withdrawn and total RNAs were extracted and analyzed for TK mRNA and 28S rRNA by Northern blotting as described in the legend to Fig. 2. Autoradiograms were scanned with a Hoeffer model GS300 scanning densitometer, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA. The amount of TK mRNA remaining at various times was plotted as a fraction of the amount present at 0 h. Error bars indicate the standard errors of the means determined from replicate experiments.

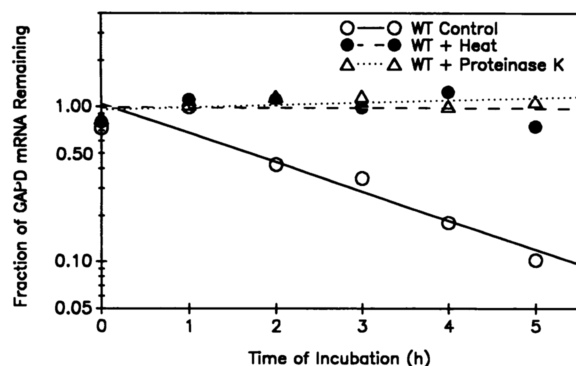

FIG. 6. Effect of heat and proteinase K pretreatment upon *vhs*-induced in vitro degradation. Standard in vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. Extracts were pretreated either by the addition of proteinase K and digestion for 30 min or by heating to 90°C for 10 min (●), after which the extracts were returned to 4°C. Control unpretreated extracts were left at 4°C until the start of incubation (○). The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and analyzed for GAPD mRNA and 28S rRNA by Northern blotting, and the amount of GAPD mRNA in each sample was normalized to the amount of 28S rRNA.

stable in extracts from cells infected with *vhs*1, whereas they decayed rapidly in extracts from cells infected with wild-type virus (data not shown). In *vhs*1-infected cells extracts, there was slightly more mRNA decay in the absence of RNasin than when it was included in the reaction mix. We attribute this to nonspecific RNases that are inhibited by RNasin. Because it did not inhibit *vhs*-induced degradation and its inclusion yielded slightly cleaner results, RNasin was included as a component in the standard in vitro reaction buffer and was used in all of the other experiments described in this report.

The next set of experiments was undertaken to determine whether the in vitro *vhs* activity could be inactivated by pretreating the extracts with heat or proteinase K. Three parallel in vitro degradation extracts were prepared from HeLa cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. One extract was heated to 90°C for 10 min and then chilled on ice. A second extract was supplemented with proteinase K and digested at 30°C for 30 min, and the third was left untreated. All three extracts were then analyzed for in vitro *vhs* activity. Pretreatment of the extracts by either heating or proteinase K digestion completely abolished *vhs*-mediated in vitro degradation (Fig. 6). These results are consistent with the involvement of one or more heat-labile proteins in *vhs*-mediated message turnover.

Recently Brewer and Ross showed that a factor that is present in the postpolysomal supernatant fraction from the cytoplasm of K562 cells and that specifically accelerates the decay of c-*myc* and c-*myb* mRNAs is inactivated by brief digestion with micrococcal nuclease (7). To determine whether similar micrococcal nuclease pretreatment of extracts from HSV-1-infected cells would inactivate the in vitro *vhs* activity, standard in vitro degradation extracts were prepared from HeLa cells 5 h after mock infection or infection with 20 PFU of wild-type HSV-1 per cell. The extracts were supplemented with micrococcal nuclease and $CaCl_2$ and then preincubated at 30°C for 10 min. EGTA was then added to chelate the Ca^{2+} and inactivate the micrococcal nuclease, and the extracts were chilled briefly on ice.

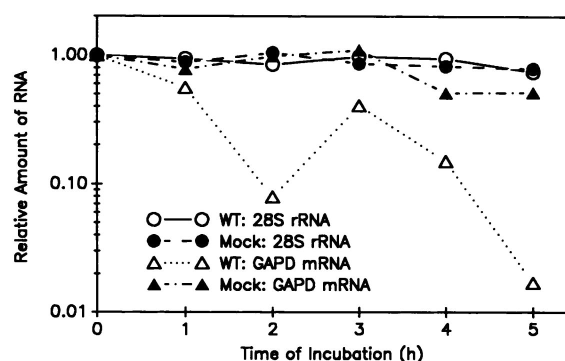

FIG. 7. Decay of exogenous GAPD mRNA and 28S rRNA in micrococcal nuclease-treated in vitro degradation extracts. Standard in vitro mRNA degradation extracts were prepared from cells 5 h after mock infection or infection with 20 PFU of wild-type HSV-1 per cell. The extracts were pretreated as described in the text with micrococcal nuclease in the presence of added Ca^{2+}. EGTA was added to chelate the Ca^{2+}, and deproteinized total cytoplasmic RNA from an equivalent number of cells was added to each extract. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and analyzed for GAPD mRNA and 28S rRNA by Northern blotting. The relative amount of 28S rRNA in mock-infected (●) and wild-type virus-infected (○) cell extracts is plotted as a fraction of the amount present at 0 h. In contrast to other figures in this paper, the relative amount of GAPD mRNA detected in mock-infected (▲) and wild-type virus-infected (△) cell extracts is plotted without being normalized to the amount of 28S rRNA present in the sample.

Micrococcal nuclease treatment is routinely used to deplete in vitro translation extracts of endogenous mRNAs and to render translation dependent upon exogenously added messages (49). Therefore, to provide a target for the *vhs*-induced degradative activity, after micrococcal nuclease pretreatment the extracts were supplemented with deproteinized total cytoplasmic RNA from an equivalent number of uninfected HeLa cells. The extracts were then incubated at 30°C and analyzed for in vitro decay of exogenous GAPD mRNA and 28S rRNA.

Figure 7 shows the decay of exogenous GAPD mRNA and total 28S rRNA. Three conclusions can be drawn from the results of this experiment. First, pretreatment of the extract from wild-type virus-infected cells with micrococcal nuclease did not inhibit the rapid degradation of exogenous GAPD mRNA. That this degradation was due to the *vhs* activity and was not the results of residual micrococcal nuclease activity is indicated by the fact GAPD mRNA was relatively stable in the micrococcal nuclease-treated extracts from mock-infected cells. Second, the fact that exogenous GAPD mRNA was degraded in the wild type-infected cell extracts suggests that the in vitro mRNA degradation system will be useful for studying the decay of both exogenous and endogenous mRNAs. Third, 28S rRNA was stable in extracts from both mock-infected and wild-type virus-infected cells. Since initially the total amount of 28S rRNA was a 50:50 mixture of endogenous and deproteinized exogenous 28S rRNA, the results indicate that in wild type-infected cell extracts deproteinized exogenous GAPD mRNA was degraded much more rapidly than deproteinized exogenous 28S rRNA. This result is an additional indication that the RNase activity seen in wild type-infected cell extracts was specific for mRNAs and was not the result of a nonspecific RNase.

The next set of experiments were undertaken to examine

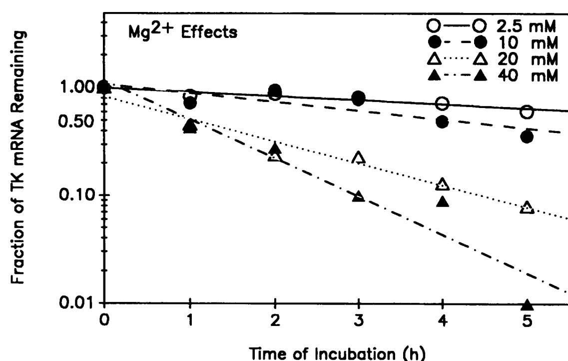

FIG. 8. Mg^{2+} dependence of *vhs*-induced mRNA degradation. In vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. The extracts were prepared in standard reaction buffer modified to contain 2.5 mM (○), 10 mM (●), 20 mM (△), or 40 mM (▲) $Mg(OAc)_2$. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and analyzed for viral TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.

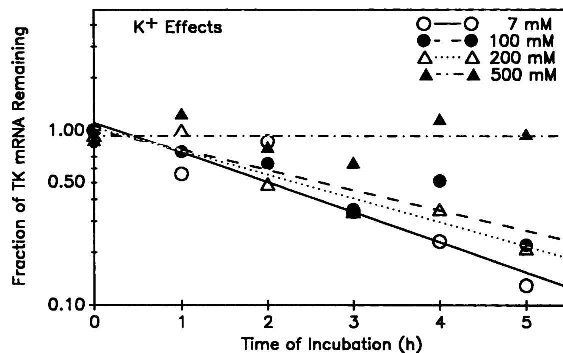

FIG. 9. K^+ dependence of *vhs*-induced mRNA degradation. In vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. The extracts were prepared in standard reaction buffer modified to contain 7 mM (○), 100 mM (●), 200 mM (△), or 500 mM (▲) KCl. The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and analyzed for viral TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.

the Mg^{2+} dependence of the in vitro degradation system. Parallel in vitro degradation extracts were prepared from HeLa cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. Individual reaction mixtures were supplemented with concentrated $Mg(OAc)_2$ to bring the Mg^{2+} concentration to the desired value. The extracts were incubated for 5 h and analyzed for the decay of the viral mRNA encoding TK. In vitro degradation of TK mRNA showed a strong dependence upon the concentration of Mg^{2+} ion (Fig. 8). Although a significant amount of mRNA degradation could be observed at an Mg^{2+} concentration of 10 mM (Fig. 8) or 5 mM (data not shown), increasing the Mg^{2+} concentration to 20 mM or higher significantly increased the degradation rate in wild-type extracts (Fig. 8). That the effect of raising the Mg^{2+} concentration was not simply the result of inducing nonspecific changes in mRNP structure or the activation of nonspecific nucleases is indicated by the fact that a significant difference in the mRNA decay rates in wild type- and *vhs*1-infected cell extracts was observed at an Mg^{2+} concentration of 20 mM (see Fig. 10); 20 mM was chosen as the optimal Mg^{2+} concentration for subsequent experiments.

A similar experiment was undertaken to determine the effect of varying the K^+ concentration upon the rate of *vhs*-induced decay of TK mRNA (Fig. 9). Parallel in vitro degradation reactions were prepared from HeLa cells 5 h after infection with 20 PFU of wild-type HSV-1 per cell. In these reactions the Mg^{2+} concentration was held constant at 20 mM, whereas the concentration of K^+ ion was varied from 7 to 500 mM. Efficient degradation of TK mRNA was observed at K^+ concentrations from 7 to 200 mM, whereas increasing the K^+ concentration to 500 mM severely inhibited the degradation reaction (Fig. 9). We chose 7 mM K^+ as optimal.

The final experiment was designed to determine whether *vhs*-induced in vitro mRNA degradation was dependent upon the components of an energy-generating system. Parallel in vitro degradation extracts were prepared from HeLa cells at 5 h after infection with 20 PFU of either wild-type HSV-1 or *vhs*1. Half of the reactions contained all of the components of the standard reaction, whereas ATP, GTP, creatine phosphate, and creatine phosphokinase were omitted from the other half. Efficient *vhs*-induced degradation of TK mRNA occurred in the presence (Fig. 10A) and absence (Fig. 10B) of the components of an energy-generating system.

DISCUSSION

In this report we describe an in vitro mRNA degradation system, consisting of cytoplasmic extracts from HSV-1-infected HeLa cells, that appears to accurately reproduce the degradation of host and viral mRNAs induced by the wild-type *vhs* protein in vivo. This conclusion is supported by several important parallels between the in vitro data and in vivo observations. First, host messages were degraded rapidly in extracts prepared from cells productively infected with wild-type HSV-1 but not in extracts from mock-infected cells or cells infected with the mutant *vhs*1. Second, the accelerated turnover of host mRNAs occurred in extracts from cells infected with wild-type virus in the presence of dactinomycin, indicating that it was induced by a component of the infecting virions and was not dependent upon de novo viral gene expression. Third, accelerated turnover of viral mRNAs was observed in extracts from cells productively infected with wild-type HSV-1 but not in extracts from *vhs*1-infected cells. In each of the above cases, the most important observation supporting the fidelity of the in vitro system was the striking difference between the mRNA decay rates in extracts from cells infected with wild-type virus and in extracts from *vhs*1-infected cells. This observation indicates that the accelerated in vitro degradation of mRNAs was dependent upon infection of the cells with virions containing a functional *vhs* polypeptide and was not simply the consequence of a nonspecific RNase liberated during cell fractionation or induced as a nonspecific consequence of viral infection. Finally, although the wild-type *vhs* function induced accelerated in vitro turnover of both viral and cellular mRNAs, endogenous 28S rRNA was equally stable in extracts from mock-infected cells and in extracts from cells infected with either wild-type virus or the *vhs*1 mutant. This lends further support to the conclusion that the degradative activity seen in wild type-infected cell extracts was specific for mRNAs and was not due to a contaminating

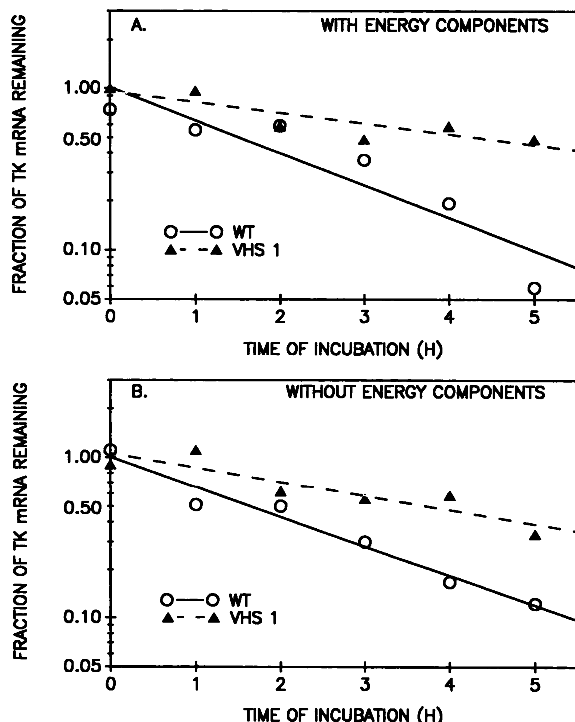

FIG. 10. Dependence of *vhs*-induced mRNA degradation upon the components of an energy-generating system. In vitro mRNA degradation extracts were prepared from cells 5 h after infection with 20 PFU of wild-type HSV-1 (O) or *vhs*1 (▲) per cell. The extracts were prepared either in standard reaction buffer (A) or in standard reaction buffer from which ATP, GTP, creatine phosphate, and creatine phosphokinase had been omitted (B). The extracts were incubated at 30°C for the indicated times. Samples were withdrawn and analyzed for viral TK mRNA and 28S rRNA by Northern blotting, and the amount of TK mRNA in each sample was normalized to the amount of 28S rRNA.

nonspecific RNase that should have been present in all three kinds of extract.

Analysis of the crude in vitro system showed that one or more factors necessary for in vitro *vhs* activity was inactivated by heating the extracts to 90°C or by brief proteinase K digestion. These data are consistent with the involvement of one or more heat-labile proteins in *vhs*-induced degradation. In contrast, pretreatment of wild-type extracts with micrococcal nuclease did not inhibit the subsequent degradation of added exogenous mRNA, indicating that the factors required for in vitro *vhs* activity are apparently insensitive to micrococcal nuclease treatments known to inactivate a number of small RNAs and ribonucleoproteins (30) as well as a factor that accelerates in vitro decay of c-*myc* and c-*myb* mRNAs (7). Furthermore, the finding that exogenous mRNAs were rapidly degraded in extracts from cells infected with wild-type virus but were relatively stable in extracts from mock-infected cells suggests that the in vitro degradation system will be suitable for studying the *vhs*-induced decay of both exogenous and endogenous mRNAs. It is also worth noting that in these experiments the source of exogenous mRNA was total deproteinized cytoplasmic RNA containing a mixture of mRNA and rRNAs. Thus, the results shown in Fig. 7 indicate that added deproteinized mRNA was degraded more rapidly than added deproteinized rRNAs in wild-type extracts. This adds further support to the conclusion that the in vitro *vhs* activity observed in wild type-infected cell extracts was specific for mRNAs and did not simply result from a contaminating nonspecific RNase.

Preliminary biochemical characterization of the in vitro mRNA degradation system from HSV-infected cells indicates that it is similar in a number of respects to in vitro mRNA degradation systems from uninfected cells described previously by Ross et al. (3, 4, 6, 7, 51, 52, 60–63) and others (48, 70). In particular, the *vhs*-induced mRNA degradation activity was not inhibited by the placental RNase inhibitor RNasin and was dependent upon added divalent cation. Efficient *vhs*-induced degradation occurred at K^+ ion concentrations of up to 200 mM but was inhibited by 500 mM K^+, and mRNA degradative activity was not dependent upon the addition of ATP, GTP, creatine phosphate, or creatine phosphokinase. In each of these respects, the *vhs*-induced RNase activity was similar to that of the exonuclease shown by Ross and co-workers to induce degradation of histone mRNAs in extracts from K562 erythroleukemia cells (62).

Although both required added divalent cation, the in vitro *vhs* activity reported here and the exonuclease described by Ross (62) differ somewhat in the nature of their dependence upon added Mg^{2+}. Whereas the exonuclease that degrades histone mRNAs exhibited a broad optimum ranging from 5 to 20 mM Mg^{2+} (62), the *vhs* activity observed in the extracts described here was more strongly dependent upon added Mg^{2+}. Thus, although a striking difference was observed between mRNA decay rates in extracts from wild-type virus-infected and *vhs*1-infected cells at Mg^{2+} concentrations ranging from 2.5 to 20 mM (Fig. 2 through 5, 8, and 10; unpublished data), the rate of mRNA degradation in wild-type extracts increased continuously as the Mg^{2+} concentration was raised to 20 or 40 mM. At present, the reason for this difference in the Mg^{2+} dependence of the two in vitro systems is unclear. It may reflect an increased Mg^{2+} dependence of one of the proteins involved in *vhs*-induced degradation. Alternatively, higher Mg^{2+} concentrations may favor a conformational change in mRNP structure that renders the mRNA more susceptible to *vhs*-induced degradation.

Besides providing the groundwork for future experiments, the preliminary biochemical characterization of the *vhs*-induced RNase allows it to be distinguished from several previously characterized RNases that are commonly found in cell extracts. Pancreatic RNase is resistant to boiling but is inhibited by RNasin (48). The fact that the *vhs* activity is sensitive to heating to 90°C but is not inhibited by RNasin therefore indicates that it does not involve a pancreatic-type RNase. A nucleolar exonuclease has been described that, like the *vhs*-induced RNase, is dependent upon added Mg^{2+} (35). However, unlike the *vhs*-induced enzyme, the nucleolar RNase is inactive at K^+ ion concentrations greater than 90 mM (35). A lysosomal acid RNase has been described (65). However, unlike the *vhs*-induced enzyme, it does not require added Mg^{2+}.

In several previously characterized in vitro degradation systems, the RNases responsible for mRNA turnover were found to be polysome associated. This was the case for the nucleases responsible for in vitro degradation of histone and c-*myc* mRNAs, although in both cases the decay rate was greatly accelerated by soluble factors present in a postpolysomal supernatant fraction of the cytoplasm (7, 51). In addition, Brawerman and co-workers have recently reported an RNase that is associated with polysomes as well as free mRNPs in a variety of mammalian cells (2). At present, the

subcellular localization of the proteins required for *vhs*-induced mRNA degradation is unknown.

In preliminary experiments, in vitro degradation extracts from mock-infected, wild-type HSV-1-infected, or *vhs*1-infected HeLa cells were separated by centrifugation into a polysome pellet and a postpolysomal supernatant. Polysomal mRNAs from extracts of cells infected with wild-type virus or *vhs*1 were equally stable upon suspension and incubation of the polysomes in standard reaction buffer or in the postpolysomal supernatant from mock-infected cells (31a). However, the addition of the high-speed supernatant from extracts of cells infected with wild-type virus to any of the three types of polysome resulted in rapid degradation of the polysomal mRNAs. These data indicate that one or more factors required for *vhs*-induced mRNA degradation are found in the postpolysomal supernatant fraction of extracts from cells infected with wild-type virus. Whether additional polysome- or mRNP-associated factors are also required for *vhs* activity is currently under investigation.

A central unanswered question concerning the *vhs* function is whether the *vhs* protein is itself an RNase or, instead, activates a cellular nuclease, perhaps one involved in the normal turnover of mRNAs in uninfected cells. To date, attempts to demonstrate an RNase activity in preparations of disrupted virions have been unsuccessful (31a). The reason for this may simply be that methods of virion disruption that preserve the RNase activity have yet to be found. Alternatively, one or more cellular macromolecules may be required for *vhs* activity. The similarity of the biochemical characteristics of the *vhs*-induced activity to those of in vitro degradation systems from uninfected cells (62) is consistent with this second possibility. Resolution of this question and elucidation of the detailed mechanism of *vhs*-induced message turnover will require further fractionation and characterization of the in vitro mRNA degradation system.

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