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The Virion Host Shutoff Protein of Herpes Simplex Virus Type 1: Messenger Ribonucleolytic Activity In Vitro

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Shortly after tissue culture cells are infected with herpes simplex virus (HSV) type 1 or 2, the rate of host protein synthesis decreases 5- to 10-fold and most host mRNAs are degraded. mRNA destabilization is triggered by the virion host shutoff (vhs) protein, a virus-encoded, 58-kDa protein located in the virion tegument. To determine whether it can function as a messenger RNase (mRNase), the capacity of vhs protein to degrade RNA in vitro in the absence of host cell components was assessed. Two sources of vhs protein were used in these assays: crude extract from virions or protein translated in a reticulocyte-free system. In each case, wild-type but not mutant vhs protein degraded various RNA substrates. Preincubation with anti-vhs antibody blocked RNase activity. These studies do not prove that vhs protein on its own is an mRNase but do demonstrate that the protein, either on its own or in conjunction with another factor(s), has the biochemical property of an mRNase, consistent with its role in infected cells.

Herpes simplex virus (HSV) and other lytic viruses facilitate their replication by the preferential or exclusive production of viral proteins, at the expense of host cell gene expression. HSV does so by inhibiting host gene transcription and RNA processing and by destabilizing host (and viral) mRNAs (2, 25, 34, 39, 40, 46, 51). In tissue culture cells, host protein synthesis and mRNA levels decline by approximately 90% within 3 h postinfection with HSV type 1 or HSV type 2 and virtually all proteins produced thereafter are viral (16, 21, 34, 40). Three observations demonstrate that mRNA destabilization is triggered by the virion-induced host shutoff (vhs) protein, which is located in the virion tegument and is imported into the cell at the onset of infection. (i) Destabilization occurs in the absence of viral gene expression; i.e., it is not blocked by inactivating virions with UV light or by treating infected cells with actinomycin D or cycloheximide to inhibit viral gene expression (13, 15, 16, 40, 49). (ii) It does not occur in cells infected with virus containing mutations in the UL41 gene, which encodes the vhs protein (14, 22, 32, 34, 35, 44). (iii) The vhs protein is the only viral gene product required to inhibit reporter gene expression in transfected Vero cells (22, 32).

A remarkable feature of the shutoff process is its specificity for mRNA. Host rRNA and tRNA are unaffected, while viral and host mRNAs are rapidly degraded (25, 31). These observations raise two important questions. (i) Is the vhs protein itself a messenger RNase (mRNase), or does it induce a latent cellular RNase? (ii) What accounts for the specific degradation of mRNA? Little in the primary sequence of the protein provides a clue as to its function or specificity. It is a 58-kDa phosphoprotein (35, 44), has limited homology to a small segment of poly(A) binding protein (47), and binds to VP16, the viral transcription factor required for immediate-early gene expression (45), but it lacks any primary sequence similarity to known RNases.

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To address the mechanism of vhs protein-induced mRNA degradation, our laboratory and the Read laboratory have reproduced the early destabilization phenomenon in cell extracts (24, 47). When postpolysomal supernatant (S130) from HSV-infected cells is incubated with polysomes from uninfected cells, mRNAs like globin, which are normally quite stable, are rapidly degraded. mRNA destabilization is not observed with S130 from uninfected cells or from cells infected with a virus encoding defective vhs protein. These and other observations confirm that host shutoff is vhs protein dependent (22, 32). However, all of the previously described in vitro assays contained host cell components so it was not possible to assess whether the vhs protein possesses intrinsic RNase activity. To address this issue, we used two approaches. The first was to incubate herpes virion extracts with various RNA and ribonucleoprotein (RNP) substrates. The second was to translate vhs mRNA in vitro and to incubate the translation product with RNA substrates. Taken together, the data reveal that the vhs protein has ribonucleolytic activity in vitro.

MATERIALS AND METHODS

Cells and virus. Vero cells were maintained in Dulbecco's modified Eagle medium plus 10% donor calf serum (GIBCO BRL, Gaithersburg, Md.) plus penicillin-streptomycin (GIBCO BRL). K562 and murine erythroleukemia (MEL) cells were maintained in suspension in RPMI 1640 plus 10% donor calf serum plus penicillin-streptomycin (47). HSV strains KOS, vhs-1, and ΔSma were the kind gifts of G. Sullivan Read. KOS is the wild-type (wt) strain with a UL41 gene encoding active vhs protein; the UL41 gene of vhs-1 has a point mutation (Thr to Ile at amino acid 214) that inactivates early host shutoff (34); ΔSma has a 588-bp deletion in its UL41 gene, resulting in an inactive vhs protein lacking amino acids 137 to 344 of a total of 489 (35). Cytoplasm from infected MEL cells was prepared as described previously (47). To prepare virus stocks, approximately 5×10^8 Vero cells were infected at a multiplicity of infection of 0.01 in Dulbecco's modified Eagle medium plus 2% donor calf serum with antibiotics. Medium was harvested after a cytopathic effect was evident (typically 2 days for KOS, 3 days for mutant strains), and virions were purified at room temperature by the method of Visalli and Brandt (50). Briefly, medium was centrifuged at 500 $\times g$ for 10 min and then at 25,000 $\times g$ for 1 h to pellet virions, which were purified by centrifugation twice through 36% sucrose cushions at 30,000 $\times g$ for 80 min. Virion pellets were resuspended in 200 μl of reticulocyte swelling buffer [10 mM KOAc, 1.5 mM $Mg(OAc)_2$, 10 mM Tris-HCl (pH 7.6)] plus 2 mM dithiothreitol (RSB-DTT) and stored at −80°C.

Virion extracts. All steps were performed at 4°C, unless otherwise noted. Aliquots (20 μl) of purified virions were mixed with 30 μl of ice-cold 1.67× lysis buffer, so that the final composition of the buffer was 400 mM KOAc, 0.9 mM

$Mg(OAc)_2$, 0.03% (vol/vol) Nonidet P-40 (NP-40), 2 mM DTT, 0.1 mM EDTA, 100 μg each of leupeptin and pepstatin A per ml, 0.5 mM phenylmethylsulfonyl fluoride, and 25 mM Tris-HCl (pH 7.5). Lysis buffer was prepared fresh for each extraction, and phenylmethylsulfonyl fluoride was added immediately before use. The mixture was left on ice for 1 h with occasional gentle mixing. Insoluble material was removed by centrifugation at 16,000 × *g* for 20 min, and the supernatants were transferred to fresh tubes and stored at −80°C, where they retained activity for 2 to 3 months. For some experiments, a slight modification was used. One volume of virions was mixed with 1 volume of modified lysis buffer (2% NP-40, 5 mM DTT, and 50 mM Tris-HCl [pH 7.5]), and the mixture was incubated on ice for 15 min to remove envelope proteins; no vhs was solubilized by this procedure, although ~50% of VP16 was solubilized (data not shown). The capsid-tegument structures, which include the vhs protein, were pelleted for 10 min at 16,000 × *g*, the supernatant was removed, and the pellet was resuspended in 50 μl of 1× lysis buffer per 20 μl of input virions and processed as described above. Both methods yielded equally active vhs protein.

In vitro translation. Micrococcal nuclease-treated rabbit reticulocyte lysate was purchased from Promega (Madison, Wis.). Translations (100 μl) contained 50% (vol/vol) lysate, 0.5 μCi of [^{35}S]Trans-Label (ICN Biomedicals, Costa Mesa, Calif.) per ml, and 50 ng of template mRNA per μl and were incubated at 30°C for 2 h.

Isolation of rabbit reticulocyte polysomes and K562 cell messenger RNP (mRNP). Rabbit reticulocytes were purchased from Green Hectares Farm (Oregon, Wis.). Polysomes and postpolysomal supernatants (S130) from reticulocytes and K562 cells were prepared as described previously (37). Ribosome-free mRNP from K562 cells was prepared at 4°C by lysing 1.5×10^9 cells in 15 ml of RSB-DTT in a Dounce homogenizer. After centrifugation at 20,000 × *g* for 10 min, the supernatant was removed and adjusted to 2 M NaCl by slowly adding a two-thirds volume of 5 M NaCl. Heparin was added to 0.5 mg/ml, and salt-washed polysomes were prepared by centrifugation through a 30% sucrose pad at 130,000 × *g* for 4 h at 2°C. The polysome pellet was resuspended in 1.5 ml of RSB-DTT and stored at −80°C. A 250-μl aliquot of polysomes was treated with 30 mM EDTA at 37°C for 2 min to dissociate mRNP from ribosomal subunits, and mRNP was separated from the ribosomes by centrifugation through a 5 to 20% sucrose gradient at 4°C for 20 h at 100,000 × *g*. Fractions were collected, and those containing gamma globin mRNP were identified by Northern (RNA) blotting (see below). An aliquot of each fraction was also electrophoresed in an agarose mini-gel, which was stained with ethidium bromide, to detect rRNA. Only fractions containing gamma globin mRNP and lacking rRNA were used.

Plasmids. To construct plasmids containing the full-length UL41 (vhs)-coding region, plasmid pHS-ΔSma (35) was used as a template for PCR with the following primers: 5′-CGGAATCCCATGGGTTTGTTCGGGA-3′ (the start codon is in boldface type, the *Nco*I site is underlined) and 3′-GGGAATTCTGCAGTTTGGGGCTACTC-5′ (the stop codon is in boldface type, the *Pst*I site is underlined). After cleavage with *Nco*I and *Pst*I, the 891-nucleotide (nt) PCR product was ligated into *Nco*-*Pst*-cut pEMCLucBgA$_2$TA$_{33}$, a gift of Jon Wolff, to create pEMC-ΔSma. This plasmid contains the encephalomyocarditis virus internal ribosomal entry sequence (11), the human beta globin mRNA 3′ untranslated region (UTR), and a poly(A) tract. The 588-nt *Sma*I fragment of pHS (containing full-length vhs) was ligated into the *Sma*I site of pEMC-ΔSma to create pEMC-vhs. The vhs-1 mutation was recreated in pEMC-vhs by site-directed mutagenesis with the Altered Sites mutagenesis kit (Promega) and the following oligonucleotide: 5′-ACAGGAGGTCAATGTCCGTGGT-3′. pEMC plasmids were linearized with *Nsi*I or *Eco*RI for in vitro transcription.

The bacterial expression vector pKK-6xHis-vhs was used to produce vhs protein with six amino-terminal histidine residues. Oligonucleotides encoding an initiator methionine codon and six histidine residues (5′-CATGTCCCATCACCATCACCATCACCCATG-3′ and 5′-CATGGAGTGATGGTGATGGTGATGGGAC-3′) were annealed and ligated into the *Nco*I site of pKK233-2 (Pharmacia, Piscataway, N.J.), destroying the 5′ *Nco*I site and recreating the 3′ *Nco*I site, to construct pKK-6xHis. The *Nco*I-*Pst*I restriction fragment of pEMC-vhs was ligated into *Nco*I-*Pst*I-digested pKK-6xHis to create pKK-6xHis-vhs.

The plasmid pSP6βc (26), a full-length cDNA clone encoding human beta globin, was used to make mRNA substrates. The plasmid was digested with *Hin*dIII and transcribed in vitro to synthesize capped, full-length, globin ^{32}P-mRNA. It was digested with *Eco*RI and transcribed to produce a 438-nt 5′ beta globin transcript lacking the 3′ UTR and poly(A) tract. To produce the 267-nt 3′ beta globin transcript, an *Eco*RI-*Hin*dIII fragment of pSP6βc was cloned into *Eco*RI-*Hin*dIII-digested pGEM4 (Promega). DNA from this plasmid was then linearized with *Hin*dIII prior to transcription. The 370-nt beta globin transcript used in the experiments shown in Fig. 4 and 5 was created by cloning a *Bam*HI-*Hin*dIII fragment of pSP6βc into *Bam*HI-*Hin*dIII-digested pGEM4 and then linearizing with *Hin*dIII. To produce the 135-nt beta globin 3′ UTR transcript used in the experiment shown in Fig. 7, plasmid pG4-3′βg (−50) was created. A PCR fragment produced by amplifying pSP6βc with the primers 5′-GGGAATTCTAAACTGGGGGATAT-3′ and 5′-TAATAACGACTCACTATAGGG-3′ (T7 sequencing primer) was digested with *Eco*RI and *Hin*dIII and cloned into *Eco*RI-*Hin*dIII-digested pGEM4 as described above.

In vitro transcription and RNA labeling. Large-scale synthesis of unlabeled mRNA for in vitro translation was performed as per Krieg and Melton (23) with T7 or SP6 RNA polymerase (New England Biolabs, Beverly, Mass.). Small-scale synthesis of human beta globin ^{32}P-mRNA was performed by the same method in a final volume of 20 μl, except that 2 μl of [α-^{32}P]GTP (Amersham, Arlington Heights, Ill.) and 1 mM cap analog (New England Biolabs) were included. ^{32}P-RNA substrates were also prepared with the Megascript kit (Ambion, Austin, Tex.). Radiolabeled RNAs were poly(A) selected by standard methods (20). Unlabeled homopolymeric RNAs (Sigma) were reduced in size by alkaline hydrolysis, dephosphorylated with alkaline phosphatase, and electrophoresed in a 6% polyacrylamide–7 M urea gel. RNA (~160 nt) was eluted overnight in 0.5 M ammonium acetate–0.2% (wt/vol) sodium dodecyl sulfate (SDS)–2 mM EDTA at room temperature, phenol-chloroform extracted, and ethanol precipitated. 5′-end labeling with T4 polynucletide kinase (New England Biolabs) was performed by standard methods (20). For some experiments, unlabeled poly(A) was extended with [α-^{32}P]ATP and *Saccharomyces cerevisiae* poly(A) polymerase (United States Biochemical, Cleveland, Ohio), as per the manufacturer's instructions. RNase T1 and *Bacillus cereus* RNase were purchased from United States Biochemical.

In vitro mRNA decay reactions. Assays containing cell polysomes were performed as previously described (47). As a control for experiments with virion extracts, some (background) reaction mixtures contained equivalent volumes of virion extraction buffer but no virion material. For reactions with ^{32}P-RNA substrates (no polysomes), 1 μl of virion extract (~1 μg of total protein) was incubated with ~50,000 cpm of substrate in 25-μl reaction mixtures containing 80 mM KOAc, 1.5 mM $Mg(OAc)_2$, 2 mM DTT, 0.1 mM EDTA, 10 U of RNasin, and 25 mM Tris-HCl (pH 7.5). For Fig. 4, 1 μl of virion extract and the same concentration of the other components were incubated in a 10-μl final volume. To assay in vitro-translated vhs protein, reaction mixtures (final volume, 20 μl) contained 10 μl of the reticulocyte translation reaction mixture, 6 μl of RSB, 2 μl of 400 mM KOAc, and 2 μl of ^{32}P-RNA (approximately 50,000 cpm) or rabbit reticulocyte polysomes (200 ng of total RNA). The final concentrations of potassium and magnesium in these assays were 70 and 0.5 mM, respectively. Aliquots were removed at the times indicated in each figure, added to 100 μl of urea lysis buffer (8 M urea, 0.2% [wt/vol] SDS, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.5]), extracted with phenol-chloroform, and precipitated with 2.5 volumes of 100% ethanol overnight at −20°C. The RNA was recovered by centrifugation, resuspended in 10 μl of RNA loading dye (72% formamide, 12.5 mM EDTA, 1 mg each of bromophenol blue and xylene cyanol, 20 mM sodium phosphate [pH 7.0]), and electrophoresed in 6% polyacrylamide–7 M urea gels. Gels were fixed, dried, exposed to X-Omat film (Kodak, Rochester, N.Y.) for various times without screens, and quantitated by PhosphorImager analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Northern analysis. Agarose gel Northern blots were performed as previously described (19). The 4% polyacrylamide–7 M urea gel shown in Fig. 2C was transferred to nitrocellulose by semi-dry electroblot (Hoefer Scientific Instruments, San Francisco, Calif.) in 1× TBE (90 mM Tris-borate [pH 8.0], 1 mM EDTA) at 5 V for 30 min and UV cross-linked. Hybridizations were performed as previously described (19). Random-primed probes were generated as per Feinberg and Vogelstein (12). The rabbit beta globin cDNA probe was a *Bgl*II-*Hin*dIII restriction fragment of pRSV-Globin (American Type Culture Collection, Rockville, Md. [17]). To assess gel loading, membranes were stripped and reprobed with a ^{32}P-labeled oligonucleotide complementary to human 28S rRNA (5′-ACTTTCCCTTACGGTACTTGTTGACTATGG-3′), as previously described (27).

Bacterial expression of histidine-tagged vhs protein and generation of polyclonal antiserum. *Escherichia coli* JM109 cells transformed with pKK-6xHis-vhs were grown in Luria broth and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C, resulting in the expression of insoluble, histidine-tagged vhs protein, which was purified from inclusion bodies by Ni^{2+}-nitrilotriacetic acid agarose affinity chromatography according to the manufacturer's instructions (Qiagen, Chatsworth, Calif.). The purified vhs protein was then dialyzed against 0.1% trifluoroacetic acid (Sigma) and lyophilized. To prepare standards for the semiquantitative immunoblot of Fig. 1B, 6xHis-vhs protein was further purified by electrophoresis in an SDS–10% polyacrylamide gel. Full-length 6xHis-vhs protein was eluted in 0.1% SDS–150 mM NaCl–0.1 mM EDTA–50 mM sodium phosphate (pH 8.0), and its concentration was estimated by densitometry in comparison with known amounts of bovine serum albumin (Pierce Biochemical, Rockford, Ill.) in an SDS–10% polyacrylamide gel stained with Coomassie blue.

To produce polyclonal antiserum against vhs protein, 500 μg of lyophilized 6xHis-vhs protein was resuspended in 0.5 ml of sterile phosphate-buffered saline, emulsified with 0.5 ml of Freund's complete adjuvant, and injected subcutaneously into a New Zealand White rabbit. Subsequent antigen boosts (every 4 weeks) contained 500 μg of 6xHis-vhs protein and Freund's incomplete adjuvant. Blood and serum were obtained 2 weeks after each booster injection by standard methods (18). Immunoglobulin G (IgG) was purified and desalted into 80 mM KOAc–10 mM Tris-HCl (pH 7.5) with protein A-Sepharose and exocellulose columns (Pierce) according to the manufacturer's instructions and concentrated by ultrafiltration (Amicon, Beverly, Mass.). Its purity was assessed by Coomassie blue staining of an SDS gel, and all preparations used in these experiments were free of RNase activity, as judged by incubating the material with ^{32}P-RNA and electrophoresing the RNA in a gel.

Immunoblots. Aliquots of in vitro translation reaction mixtures or virion extracts were denatured by boiling in 2% SDS plus 10% β-mercaptoethanol and electrophoresed in 10% polyacrylamide–SDS gels. The proteins were transferred

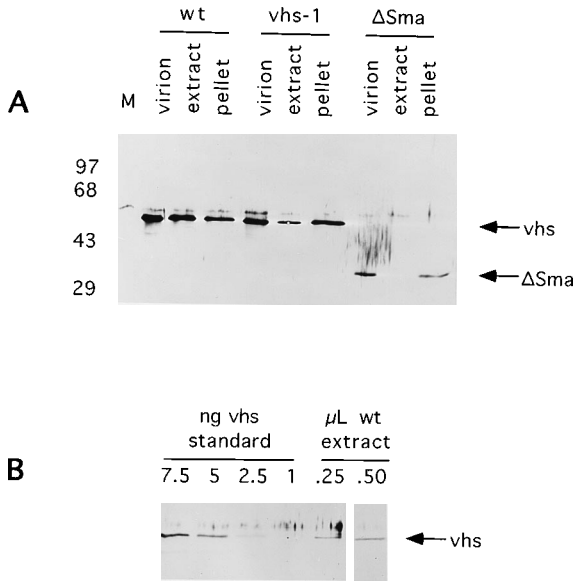

FIG. 1. Solubilization of vhs protein from herpes virions. (A) Comparison of wt and mutant vhs proteins by Western blotting. The tegument portion of purified HSV virions was extracted with a potassium acetate–NP-40 buffer (Materials and Methods). Aliquots corresponding to equivalent numbers of whole virions (lanes virion; 5 μg), soluble extract (lanes extract; 1.6 μg), and insoluble pellet (lanes pellet; 3.4 μg) were boiled in 2% (wt/vol) SDS–10% (vol/vol) β-mercaptoethanol, electrophoresed in an SDS–10% polyacrylamide gel, and transferred to nitrocellulose by electroblotting. The blot was probed with rabbit polyclonal anti-vhs antiserum and then with peroxidase-conjugated goat anti-rabbit IgG antiserum, exposed to enhanced chemiluminescence reagents, and autoradiographed. Lane M, prestained molecular mass markers (not visible); the molecular masses (in kilodaltons) are noted on the left. Lanes are also marked to indicate the presence of wt (strain KOS) virus, vhs-1 virus, or vhs ΔSma virus. Bands corresponding to 58 kDa (wt and vhs) and 31-kDa (ΔSma) vhs proteins are noted by arrows on the right. Immunoreactive material detected just above the wt vhs band is an artifact of the electrophoresis procedure (compare lane M with other lanes). An identical gel was silver stained to confirm equal loadings of virion protein (data not shown). (B) Quantitation of vhs protein in virion extracts. wt virion extract was electrophoresed in the same gel with bacterium-derived, SDS-PAGE-purified, histidine-tagged vhs protein standards of known concentrations. The gel was then analyzed by blotting and processed as described above. The intensities of vhs protein bands were measured by laser densitometry with multiple exposures. A standard curve was constructed, and the concentration of vhs protein was estimated to be 5 to 10 ng/μl of virion extract.

to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) by a mini-blot cell system (Bio-Rad) by standard methods (18). Following a brief rinse in wash buffer (0.05% [vol/vol] NP-40, 150 mM NaCl, 0.5% dry-milk solids, 25 mM Tris-HCl [pH 8.0]), membranes were incubated for 1 h with 5 μl of anti-vhs serum in 10 ml of wash buffer, washed three times for 5 min each, incubated with 5 μl of goat anti-rabbit IgG-peroxidase conjugate (Sigma) in 15 ml of wash buffer for 30 min, washed as described above, exposed to enhanced chemiluminescence reagents (Amersham), and autoradiographed. To estimate the amount of vhs protein in each viral extract preparation (Fig. 1B), samples were run side-by-side with histidine-tagged vhs protein that had been purified by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted as above. Autoradiographs of several exposures were analyzed by densitometry (Pharmacia LKB, Uppsala, Sweden).

RESULTS

Virion extracts containing wild-type vhs protein accelerate the decay of polysomal mRNA and degrade protein-free mRNA substrates. Most host mRNAs are destabilized, some by 10-fold or more, in HSV-infected cells, and this phenomenon has been reproduced in two in vitro mRNA decay systems using cytosol or cytosolic subfractions from infected cells (24, 47). Both host and viral mRNAs were at least 10-fold less stable when incubated with infected-cell cytosol than when incubated with uninfected-cell cytosol. The mRNA-destabilizing activity was localized to the soluble or S130 fraction, not to the polyribosomes, and ribosomal and transfer RNAs remained stable. mRNAs were not destabilized in cytosol from cells infected with vhs-defective mutant strains. Therefore, the cell-free reactions mirrored virion host shutoff in infected cells.

These and other observations raised two central questions. (i) How does vhs protein trigger host mRNA degradation? Is it an mRNase or does it act indirectly, for example, by activating a host RNase or by blocking a host RNase inhibitor? (ii) How does the RNase, whatever it is, target mRNA while sparing rRNA and tRNA? The work described here focused on the

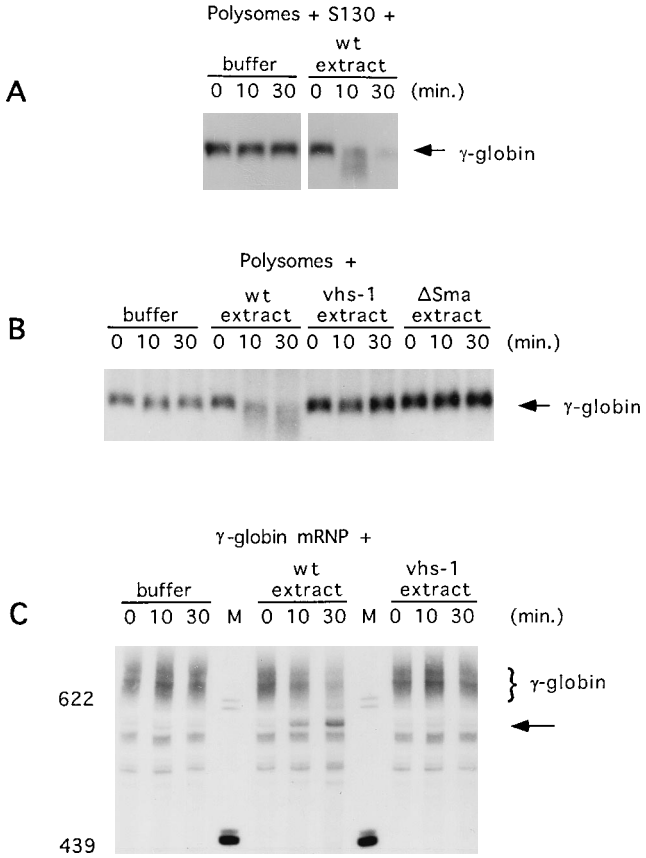

FIG. 2. Accelerated decay of gamma globin mRNA present in polysomes and mRNP by extracts from wt virus. (A) Polysomes from K562 erythroleukemia cells (10^6 cell equivalents per reaction) were incubated at 37°C with postpolysomal supernatant (S130) from mock-infected MEL cells (10^6 cell equivalents per reaction) and either virion extraction buffer (left panel) or extract from wt virions (right panel). Aliquots were removed at the times indicated, and total RNA was extracted with phenol-chloroform, precipitated with ethanol, and analyzed by Northern blot analysis. The probe was a human gamma globin cDNA labeled by random priming (Materials and Methods). Arrow, full-length gamma globin mRNA. (B) In vitro mRNA decay reactions were performed with K562 polysomes alone (no S130) plus virion extraction buffer or extract from either of three viral strains as indicated. Samples were processed and analyzed as in panel A. (C) Free (non-polysome-bound) mRNP was prepared by centrifuging EDTA-dissociated K562 cell polysomes in a sucrose gradient. The mRNPs (1.5×10^6 cell equivalents per reaction) were incubated with buffer or virion extract, as for panel A. Total RNA was processed and hybridized as described above, except it was electrophoresed in a 4% polyacrylamide–7 M urea gel and transferred to nitrocellulose by electroblotting. The position of a gamma globin mRNA decay intermediate produced with wt virion extract is shown by the arrow at right. DNA size markers (pBR322 DNA cleaved with *Hae*II and ^{32}P-labeled with polynucleotide kinase) were coelectrophoresed and blotted; the positions of the 622- and 439-bp fragments are shown at the left. Full-length gamma globin mRNA appears more diffuse in this gel than in panels A and B because of increased resolution in the polyacrylamide gel, compared with agarose gels.

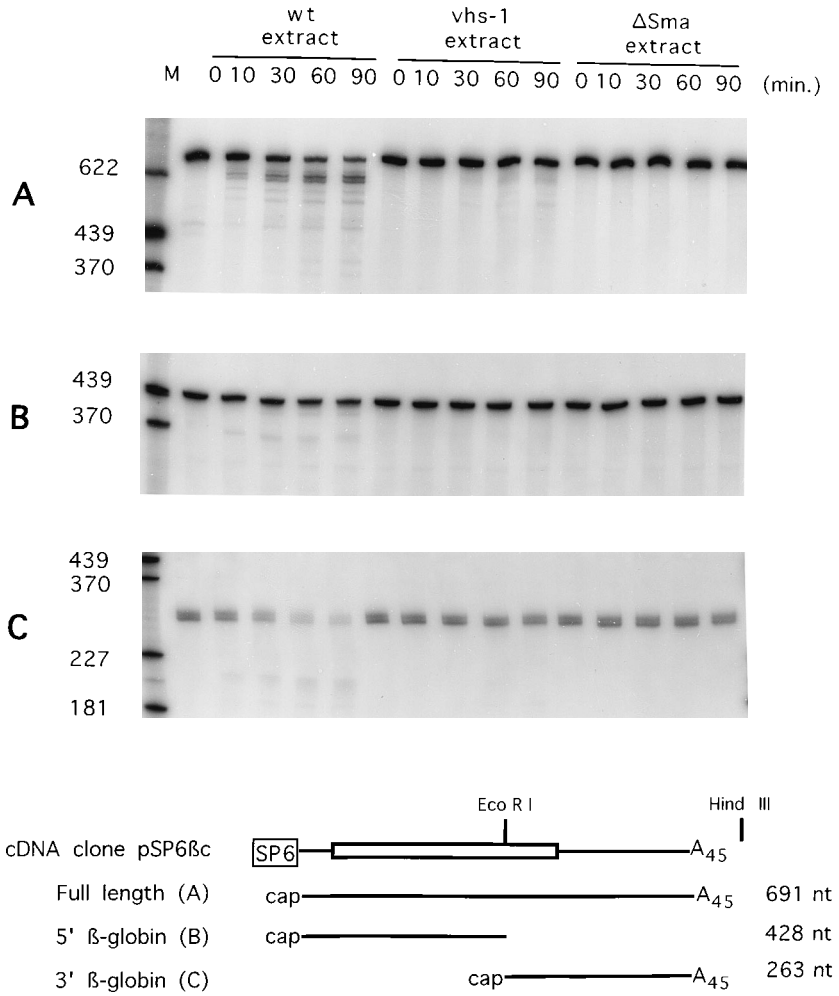

FIG. 3. Accelerated decay of deproteinized, polyadenylated mRNA substrates by wt virion extracts. (A to C) Virion extracts were incubated with equimolar amounts of in vitro-transcribed, human beta globin ^{32}P-mRNA substrates (Materials and Methods). Aliquots were removed at the indicated times, added to 80% formamide–10 mM EDTA loading dye, and electrophoresed in polyacrylamide–7 M urea gels (4% for panels A and B, 6% for panel C). Lanes M, DNA size markers, as per the legend to Fig. 2C. Beneath panels A to C are diagrams of the RNA substrates. (Top diagram) Full-length human beta globin cDNA clone SP6βc, including the SP6 promoter site. The *Hin*dIII site is located at the 3′ end of the poly(A). Full-length RNA pSP6βc (diagram A) was digested with *Hin*dIII and transcribed with SP6 RNA polymerase to produce an ~690-nt, capped, polyadenylated mRNA. 5′-beta globin-RNA pSP6βc (diagram B) was digested with *Eco*RI and transcribed to generate a 428-nt, capped, nonpolyadenylated RNA. For the 3′ beta globin RNA represented in diagram C, an *Eco*RI-*Hin*dIII fragment of pSP6βc was cloned into *Eco*RI-*Hin*dIII-digested pGEM4. This clone was digested with *Hin*dIII and transcribed to generate a 263-nt, capped, polyadenylated RNA.

first question and took two approaches. One was to prepare vhs protein-containing extracts from purified virions, incubate the extracts with various mRNA substrates, and ask whether the mRNA was destabilized. The second, complementary approach was to synthesize vhs protein in vitro in a reticulocyte-free system and to incubate the protein product with RNA substrates.

Preliminary experiments were performed to verify that vhs protein could be efficiently solubilized from virions and to quantitate the amounts solubilized. Purified virions were incubated in a high-salt buffer plus nonionic detergent, capsids were pelleted, and the solubilized material (extract) was analyzed by Western blotting (immunoblotting) with anti-vhs antibody made with purified immunogen from *E. coli*. Proteins from the wt and point mutant (vhs-1) strains (see Materials and Methods) were easily detectable and migrated at the expected molecular mass of ~58 kDa (Fig. 1A). The contaminating band above the vhs protein was present in all lanes and migrated at the position of keratin. Approximately 30 to 40% of the vhs protein was solubilized by this method, and the assay was linear over a range of protein concentrations (Fig. 1B). Comparing band intensities in the extracts and in the bacterium-derived standard revealed that each microliter of extract contained 5 to 10 ng of vhs protein (Fig. 1B).

Extract from vhs ΔSma virus contained little or no vhs protein of the expected size (31 kDa) (Fig. 1A). However, some ΔSma vhs protein was detected in the virion preparation. This result seemed inconsistent with a previous report that ΔSma protein is not packaged efficiently into virions (35). However, there is no inconsistency because ΔSma protein is only absent from virions harvested before extensive cytopathic effect; it is present in a form difficult to extract in virions harvested late in infection, as was done in our experiments (reference 33a and data not shown).

To determine whether crude viral extract could trigger mRNA destabilization, wt extract was incubated with polysomes plus S130, polysomes alone, or mRNP from a human erythroleukemia cell line, K562. Total RNA was then purified

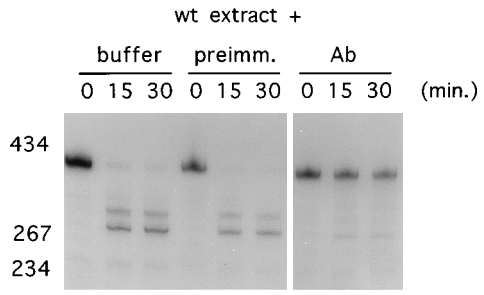

FIG. 4. Inhibition of vhs-induced mRNA degradation by anti-vhs antibody. In vitro mRNA decay reactions with wt viral extract were performed and analyzed as described for Fig. 3. The substrate was a 370-nt, capped, poly(A)$^+$ ^{32}P-RNA representing the 3′ half of human beta globin mRNA. The DNA from which it was derived was constructed by cloning a *Bam*HI-*Hin*dIII fragment of pSP6βc into pGEM4. Where noted, IgG was added immediately before the RNA. Buffer, control without antibody; Preimm., 50 μg of purified IgG from preimmune serum; Ab, 50 μg of IgG from the serum of an immunized rabbit. DNA size markers (in base pairs) are noted on the left.

and analyzed by Northern blotting with a probe to gamma globin mRNA. This mRNA is very stable when incubated with extracts from uninfected cells (47) and was also stable in extraction buffer (Fig. 2, first three lanes of panels A to C). It was destabilized at least 12-fold with extract from purified wt virions, as compared with buffer. Another host mRNA, encoding glyceraldehyde 6-phosphate dehydrogenase (GAPDH), was destabilized to approximately the same extent (data not shown). rRNA was not degraded in the reactions shown in Fig. 2A and B (data not shown). Destabilization required functional vhs protein and was not observed with extracts from the point mutant (vhs-1) or the deletion mutant (ΔSma). Therefore, crude, wt virion extract triggers mRNA degradation in the absence of host postribosomal supernatant (S130) (Fig. 2B) or ribosomes (Fig. 2C). A discrete ~580-nt decay intermediate was observed in a polyacrylamide Northern blot with mRNP but has not been further mapped (Fig. 2C). With polysomes plus S130 (cytosol), the mRNA half-lives with buffer and with wt virion extract were >3 h and 14 min, respectively. With mRNP, mRNA half-lives were >3 h and 40 min. Therefore, vhs-induced mRNA degradation occurred approximately threefold faster when cytosol was present than with mRNP. Perhaps this rate difference resulted from a two-step decay process. In step 1, vhs protein cleaves each mRNA molecule only once or a few times, presumably in some critical region (see also Fig. 3 and 7 and Discussion). In step 2, loss-of-mass degradation of the cleavage products is effected rapidly by cytosolic host RNases, which are abundant and attack a variety of RNA substrates (1, 5, 37, 38).

The experiment shown in Fig. 2 did not assess whether any host cell factors were required for mRNA destabilization. To address this question, virion extracts were incubated with ^{32}P-labeled, ^{7}mG-capped, poly(A)$^+$, full-length, protein-free human beta globin mRNA in reactions lacking host cell components. wt virion extract but not ΔSma extract cleaved the mRNA (half-life = 40 min for wt virion extract versus >3 h for ΔSma) (Fig. 3A). The vhs-1 extract exhibited weak degradative activity (half-life = 150 min versus 40 min for wt virion extract), indicating that the point mutation does not fully inactivate the RNase. wt extract also degraded a polyadenylated substrate corresponding to the 3′ one-half of the mRNA (Fig. 3C). A nonpolyadenylated substrate corresponding to the 5′ one-half of the mRNA was degraded more slowly (for wt extract, half-life = 90 min versus 40 min for substrates B and A) (Fig. 3B). These data demonstrate that virion extract contains an RNase activity dependent on functional vhs protein but independent of host cell components. The data do not address whether any differences in decay rates result from poly(A), the 3′ UTR, or other segments of these substrates (but see also Fig. 8).

Since virion extracts contain many proteins (reference 28 and data not shown), the results shown in Fig. 3 did not conclusively demonstrate that vhs protein was responsible for degrading the RNA. To answer this question, we exploited a rabbit anti-vhs antibody. The immunogen was highly purified vhs protein expressed in *E. coli* and thus contained no other HSV or host cell epitopes. Virion extract was preincubated with antibody or preimmune serum and then incubated with

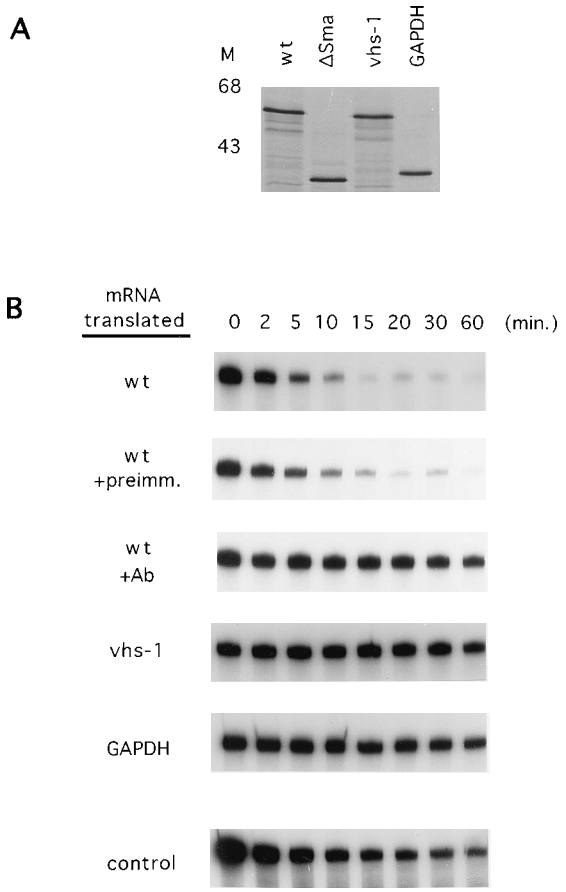

FIG. 5. Destabilization of ^{32}P-mRNA by in vitro-translated vhs protein in a reticulocyte lysate-based mRNA decay assay. (A) In vitro translation. mRNAs encoding wt vhs protein (strain KOS), the vhs-1 and vhs ΔSma mutant proteins, and rat GAPDH were transcribed in vitro and used to program a micrococcal nuclease-treated rabbit reticulocyte lysate containing [^{35}S]methionine. Aliquots were electrophoresed in a 10% polyacrylamide–SDS gel and autoradiographed. Lane M, protein molecular mass markers; molecular masses are noted in kilodaltons on the left. On the basis of these and other markers, each protein migrated at its expected molecular mass (58 kDa for wt and vhs-1; 31 kDa for ΔSma; 37 kDa for GAPDH). (B) mRNA decay. Reticulocyte lysates containing equivalent amounts (in nanomoles) of the indicated translation products were mixed with the same human beta globin polyadenylated ^{32}P-RNA used in the experiment shown in Fig. 4 and incubated at 37°C. Aliquots were removed at the indicated times, and RNA was purified and electrophoresed in 6% polyacrylamide–7 M urea gels, which were analyzed with a PhosphorImager and X-ray film without screens. wt + preimm. and wt + Ab indicate that preimmune or anti-vhs IgG (25 μg per reaction), respectively, was added immediately before the substrate. The control consisted of extract from wt virions and was mixed with the substrate and added to a reticulocyte lysate in which no translation had occurred (blank lysate). The amounts (in nanomoles) of translated protein were identical in each decay reaction, as determined by PhosphorImager analysis.

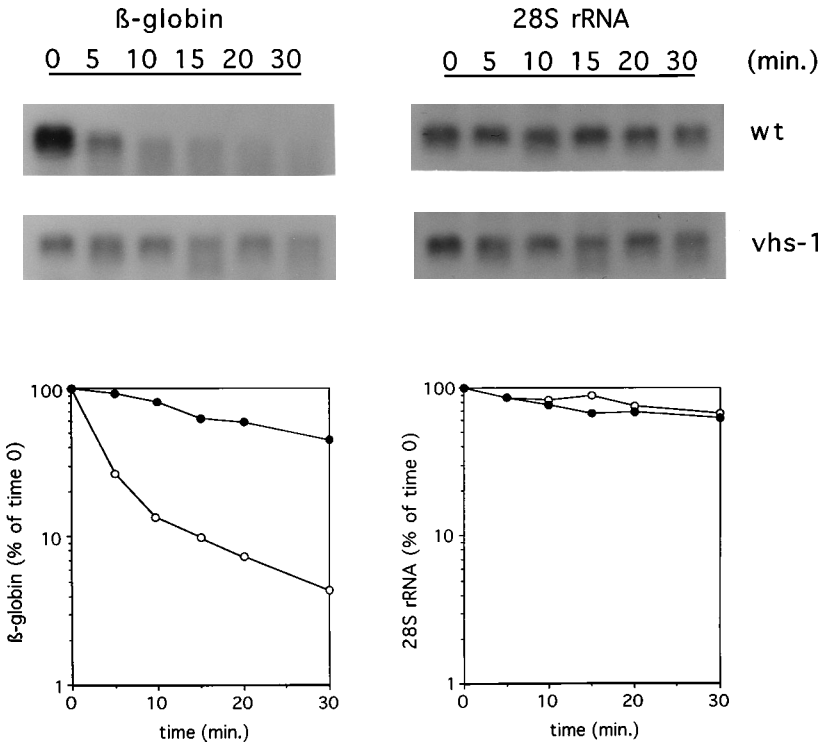

FIG. 6. Specificity of vhs protein for mRNA in a polysome-containing in vitro mRNA decay assay. Reticulocyte lysate-based in vitro decay reactions were performed as described for Fig. 5, except that rabbit reticulocyte polysomes were used as the substrate instead of ^{32}P-RNA. Aliquots were removed at the times indicated, and total RNA was isolated and analyzed by Northern blotting. The blot was first hybridized with a rabbit beta globin random primer probe (left blot). It was then stripped and reprobed with an oligonucleotide complementary to 28S rRNA (right blot). The fraction of beta globin or 28S rRNA remaining is shown in the graphs below. Unfilled circles, wt vhs; filled circles, vhs-1.

^{32}P-RNA, and degradation was monitored by gel electrophoresis. The anti-vhs antibody inhibited vhs RNase activity; buffer and preimmune serum had no effect (Fig. 4). RNA decay was also blocked when vhs protein was immunodepleted from the extract (data not shown). These results demonstrate an absolute requirement for vhs protein to degrade RNA.

mRNA degradation by in vitro-translated vhs protein. To confirm the results of Fig. 1 to 4 by an independent assay, we asked whether vhs protein synthesized in a cell-free translation system triggers mRNA decay without affecting rRNA. A positive result would support the notion that vhs protein is the only viral component required (22, 32), establish further the specificity for mRNA, and provide a rapid, convenient in vitro assay for generating potentially interesting vhs mutants. RNAs encoding wt, vhs-1, or vhs ΔSma protein and a control host protein, GAPDH, were prepared by in vitro transcription. Each mRNA was then translated in a rabbit reticulocyte extract and generated a protein of the expected size (Fig. 5A). Crude reticulocyte extracts containing equivalent amounts (in nanomoles) of translated protein were mixed with polyadenylated beta globin ^{32}P-RNA and incubated for various times, and total RNA was purified and analyzed. The ^{32}P-RNA was degraded slowly (half-life = 60 min) with lysate that had translated GAPDH mRNA (Fig. 5B). In reactions with wt vhs translation product, degradation was accelerated approximately 30-fold (Fig. 5B, top). RNA destabilization was dependent on vhs protein, because adding anti-vhs antibody abrogated the effect, whereas preimmune serum did not. Neither vhs-1 (Fig. 5B) nor ΔSma (data not shown) mutant proteins were active.

To confirm these results, and to assess the specificity of the in vitro translation product for mRNA but not rRNA or tRNA, reticulocyte translation extracts containing wt or vhs-1 proteins were incubated with unlabeled rabbit reticulocyte polyribosomes and the decay of rabbit beta globin mRNA and 28S rRNA was analyzed by Northern blotting. Some mRNA and rRNA was degraded in reactions with all of the lysates, even those in which no mRNA was translated (Fig. 6 and data not shown). While we have not investigated this background degradation further, the apparent lack of specificity of the nuclease activity suggests it might be RNase L (52). More importantly, wt vhs protein destabilized rabbit globin mRNA ~10-fold more than vhs-1 protein, confirming the result of Fig. 5. Neither wt nor vhs-1 translation product accelerated rRNA degradation (Fig. 6, right side). Therefore, although the vhs translation product has ribonuclease activity, it is highly specific for mRNA. If it were nonspecific, rRNA should have been degraded at least as rapidly as mRNA, since ribosomes are abundant in the reticulocyte lysate.

Cleavage sites in the polyadenylated beta globin RNA substrate. It is important to determine how vhs protein targets mRNAs and where it cleaves. Does it recognize mRNA-specific sequences and structures like poly(A) and if so, how? To begin to answer these questions, wt virion extract was incubated with a short beta globin RNA substrate [66 nt of the 3′ UTR plus a 45-nt poly(A) tract; the sequence is shown at the bottom of Fig. 7]. The RNA was uncapped and was ^{32}P-labeled at its 5′ terminus. (No difference was observed between capped and uncapped substrates in any of our experiments [data not shown].) Following incubation, total RNA was extracted and analyzed by electrophoresis in a sequencing gel with appropriate ladders and standards. Two prominent and two faint decay

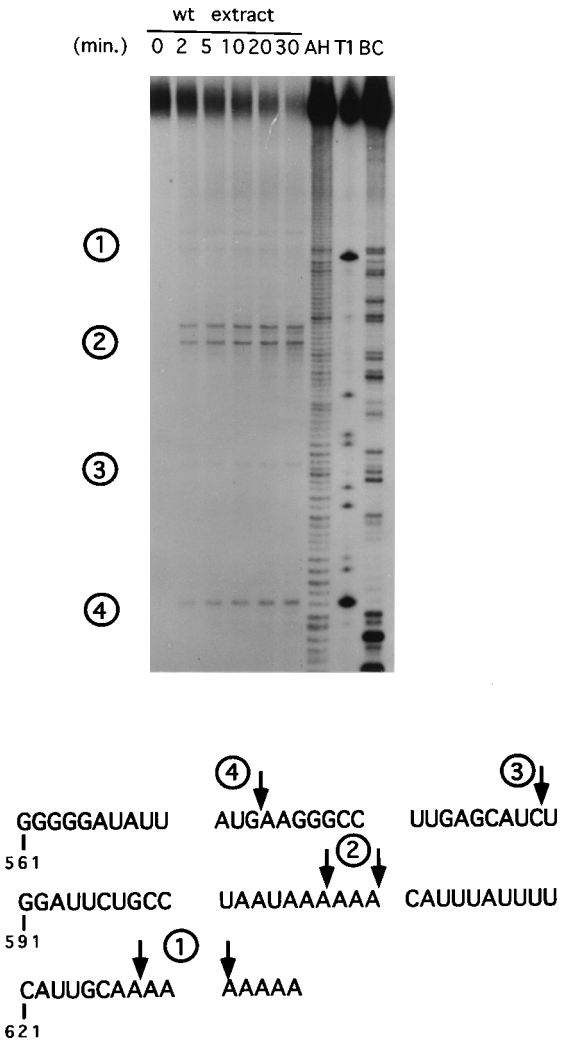

FIG. 7. mRNA decay intermediates in reactions with wt virion extract. A 135-nt, polyadenylated RNA derived from the 3′ UTR of human beta globin mRNA and including the last 66 nt of the 3′ UTR was transcribed in vitro and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (diagram at bottom). The RNA was incubated with wt virion extract for the times noted and under the same conditions as described for the experiment shown in Fig. 3, except that the temperature was lowered to 15°C to slow the reaction rate. Aliquots were electrophoresed in an 8% polyacrylamide–7 M urea sequencing gel and autoradiographed. Lane AH, alkaline hydrolysis ladder of the substrate RNA; lane BC, limited digestion with *B. cereus* RNase; lane T1, limited digestion with RNase T1. Circled numbers at the left indicate cleavage sites shown in the diagram below. The sequence shows only 9 nt of the 45-nt poly(A) tract.

products were detected (Fig. 7, bands 2 and 4 and 1 and 3, respectively). No intermediate-sized bands were observed, suggesting (but not proving) that the products were generated endonucleolytically. Bands 2 and 4 in Fig. 7 correspond to RNAs cleaved in purine-rich regions, while band 1 indicates cleavage within or at the 5′ end of the poly(A) tract. There appear to be no precursor-product relationships among these products, because the bands do not appear sequentially (band 1, then 2, then 3, etc.). Therefore, each band was presumably generated by independent cleavages.

To determine whether the vhs RNase might have some preference for poly(A) or A-rich regions of protein-free substrates, wt and mutant viral extracts were incubated with ^{32}P-poly(A). Only the wt extract completely degraded poly(A) (Fig. 8A). The vhs-1 extract degraded it 10- to 20-fold more slowly than wt vhs, while the ΔSma extract was inactive. wt extract was then incubated with ^{32}P-poly(A), -(C), or -(U), and the half-lives were 8, ~60, and 20 min, respectively (Fig. 8B). We conclude that the vhs RNase has some specificity with protein-free RNAs, preferring A- or U-rich regions to C-rich regions, but also that its high specificity for mRNA observed in infected cells and in cell-free reaction mixtures containing polysomes (Fig. 1 and 6) is not observed with protein-free RNAs.

DISCUSSION

Four observations presented here indicate that the vhs protein functions as an RNase in cell-free reactions. (i) Only host-shutoff-competent virion extracts exhibit vhs-dependent RNase activity. (ii) Only reticulocyte lysates containing wt vhs protein display enhanced RNase activity. (iii) In reaction mixtures containing polysomes, the RNase activity of virion extracts is mRNA specific. rRNA is spared, which accurately reflects vhs protein specificity in infected cells. (iv) Antibodies against *E. coli*-derived vhs protein inhibit the RNase activity in both assay systems. Although these data do not unequivocally prove that the in vitro RNase activity of the protein reflects its primary intracellular function, there is a strong correlation between in vitro function and biological activity. Thus, both mutants that were tested (vhs-1 and ΔSma) are RNase deficient in vitro and destabilization deficient in infected cells (34, 35). These data also tend to exclude other possible mechanisms for vhs-induced host shutoff. For example, the vhs protein does not induce a host cell mRNase, because no host components are required to degrade ^{32}P-RNA in the presence of viral extract (Fig. 3).

One caveat is that we have not succeeded in demonstrating

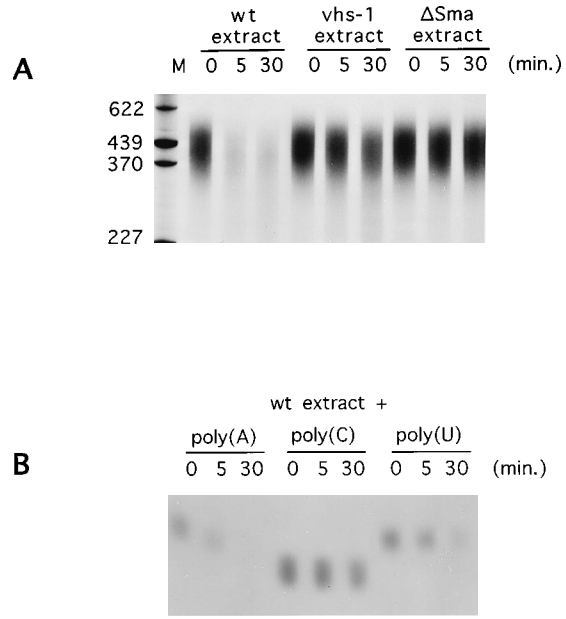

FIG. 8. Degradation of homopolymeric RNAs by wt virion extract. (A) Poly(A) (~160 nt) was incubated with yeast poly(A) polymerase plus [α-^{32}P]ATP to prepare the radiolabeled substrate. ^{32}P-poly(A) (size range, 350 to 500 nt) was incubated with wt or mutant virion extract, electrophoresed in a 6% polyacrylamide–7 M urea gel, and autoradiographed, as described in the legend to Fig. 3. Lane M, DNA size markers, as described in the legend to Fig. 2. (B) Poly(A), poly(C), and poly(U) RNAs were prepared as described in Materials and Methods, labeled with [γ-^{32}P]ATP and polynucleotide kinase, and used as substrates with the wt virion extract, as described above for panel A.

the RNase activity of vhs protein purified to homogeneity, having been unable to renature it from bacterial extracts or SDS-polyacrylamide gels (data not shown). Since both sources of vhs protein used in our assays are crude (virion extract and reticulocyte lysate), we also cannot absolutely exclude the possibility that a cofactor is required for RNase activity. However, three observations argue against this possibility. (i) The inactivity of proteins from mutant genes and the inhibition of wt vhs protein by antibody strongly support the vhs-as-mRNase model. (ii) Reaction mixtures containing viral extract and ^{32}P-RNA contain no added host components, and reaction mixtures containing reticulocyte lysate and ^{32}P-RNA contain no other viral proteins. In both cases, however, mRNase activity is observed. It seems unlikely that viral extracts and rabbit reticulocyte lysates have a common cofactor required to activate vhs protein. (iii) The vhs protein is the only viral gene product required to induce host mRNA destabilization in transfected cells (22, 32). It is known that VP16, the HSV-encoded immediate-early transcription factor, forms a complex with vhs protein (35, 45), is approximately 5- to 10-fold more abundant than vhs protein in virions (35), and could theoretically block the RNase activity. Immunoblot analysis confirms that our virion extracts contain VP16 (reference 33 and data not shown), but this material clearly does not abolish vhs function in vitro (Fig. 3) and VP16 is not required for mRNA destabilization in transfected cells (22, 32). Perhaps the vhs-VP16 interaction facilitates packaging of one or both proteins.

The remarkable specificity of vhs protein for mRNA seems to require that the mRNA be packaged as an RNP, because stringent specificity is lost with deproteinized substrates (e.g., compare Fig. 6 and 8). This observation is neither unexpected nor unusual, because other RNases that are thought to degrade mRNA or to process rRNA behave similarly. They exhibit specificity with their natural RNP substrates in intact cells and in cell-free reaction mixtures but have little or no specificity when incubated with protein-free RNAs in vitro (5, 6, 8–10, 36, 37, 41, 42). Therefore, it will be essential to identify which feature(s) of the mRNP structure is recognized by vhs protein. Since polyadenylated substrates are degraded somewhat faster than nonpolyadenylated substrates (Fig. 3), perhaps the poly(A)-poly(A)-binding protein complex is the recognition signal, and poly(A) cleavage is the vhs-dependent decay step. This model is consistent with the accumulation of deadenylated mRNA in HSV-infected cells (30, 43).

There are two major mechanisms whereby vhs protein could effect the wholesale destruction of mRNAs. (i) It could, on its own, totally destroy each mRNA molecule. Although total destruction is observed in vitro with deproteinized substrates incubated for extended periods, we consider this mechanism unlikely in cells because it is inconsistent with the striking specificity of the protein. Cells contain thousands of different mRNAs, which have no common sequence or structural motif, aside from their caps and poly(A) tracts. Therefore, once an mRNA molecule was partially degraded, it (the degradation product) would lose any distinguishing feature(s) that marked the mRNA for attack in the first place. Once such features were lost, how could the vhs mRNase continue to degrade it unless it was highly processive? (ii) A more likely possibility is that vhs protein, unlike fungal, bacterial, and pancreatic RNases, has evolved as a low-activity, high-specificity enzyme whose major function is to cleave mRNA molecules at one or a few critical sites that normally serve to protect each mRNA from destruction. In this model, mRNA undergoes limited cleavage by vhs protein, after which potent host RNases finish the job. This two-step decay pathway is consistent with the observed generation of discrete decay intermediates when mRNP or deproteinized mRNA is cleaved by vhs (Fig. 2C, 3, 4, and 7). Polysomes contain abundant, highly active nucleases capable of degrading a variety of RNA substrates, particularly those lacking a poly(A) or cap (1, 3, 38). The model is also consistent with general mRNA decay pathways in mammalian and yeast cells. Poly(A) appears to act as a shield, protecting mammalian cell mRNAs from rapid destruction, and its removal is often the first step in the decay process (4, 36). In yeast cells, many mRNAs are degraded by a multi-step pathway in which decapping is an essential early event (29). Once decapping occurs, the mRNA 5′ terminus becomes exposed to potent 5′ to 3′ exoribonucleases (48).

Studies of the vhs protein have provided important insights about how HSV appropriates the host cytoplasm for its own use, and further work on its specificity and mechanism could also provide crucial information on how host mRNases and rRNA processing enzymes recognize and cleave their RNP substrates (7, 36). Its roles in viral replication, pathogenesis, and latency (if any) are presumably linked to its biochemical activity but are also incompletely understood. UL41 loss-of-function mutations affect HSV replication minimally in tissue culture infections (14, 34) but reduce viral pathogenicity by at least 2 orders of magnitude in newborn mice (2). Thus, it should be instructive to analyze UL41 mutants in order to correlate mRNase activity with biological function. Having devised in vitro assays for vhs RNase activity, it should also be possible to identify and select gain-of-function mutants, whose phenotypes in productive infections and in the early stages of latency could be particularly interesting.

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