

# mRNA Degradation by the Virion Host Shutoff (Vhs) Protein of Herpes Simplex Virus: Genetic and Biochemical Evidence that Vhs Is a Nuclease

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**During lytic infections, the virion host shutoff (Vhs) protein (UL41) of herpes simplex virus destabilizes both host and viral mRNAs. By accelerating the decay of all mRNAs, it helps redirect the cell from host to viral gene expression and facilitates the sequential expression of different classes of viral genes. While it is clear that Vhs induces mRNA degradation, it is uncertain whether it is itself an RNase or somehow activates a cellular enzyme. This question was addressed by using a combination of genetic and biochemical approaches. The Vhs homologues of alphaherpesviruses share sequence similarities with a family of mammalian, yeast, bacterial, and phage nucleases. To test the functional significance of these similarities, Vhs was mutated to alter residues corresponding to amino acids known to be critical to the nuclease activity of cellular homologues. In every instance, mutations that inactivated the nuclease activity of cellular homologues also abolished Vhs activity. Recent experiments showed that Vhs interacts with the cellular translation initiation factor eIF4H. In this study, the coexpression of Vhs and a glutathione *S*-transferase (GST)–eIF4H fusion protein in bacteria resulted in the formation of a complex of the proteins. The wild-type Vhs/GST–eIF4H complex was isolated and shown to have RNase activity. In contrast, Vhs mutations that altered key residues in the nuclease motif abolished the nuclease activity of the recombinant Vhs/GST–eIF4H complex. The results provide genetic and biochemical evidence that Vhs is an RNase, either alone or as a complex with eIF4H.**

During lytic herpes simplex virus (HSV) infections, viral and cellular gene expression is regulated through a complex set of transcriptional and posttranscriptional controls (57). Of the posttranscriptional mechanisms, one of the best characterized is the destabilization of host and viral mRNAs by the HSV virion host shutoff (Vhs) protein (UL41) (49). Soon after infection, copies of the Vhs polypeptide, which enter cells as components of infecting virions, accelerate the degradation of host mRNAs (20, 58, 66). This activity, together with the inhibition of pre-mRNA splicing by the immediate-early polypeptide ICP27 (24, 25), helps redirect the cell from the synthesis of host proteins to that of viral proteins. In addition, following the onset of viral transcription, the Vhs protein accelerates the turnover of viral mRNAs belonging to all kinetic classes (36, 45, 46, 66). In this role, it helps determine viral mRNA levels and facilitates the sequential expression of different classes of viral genes (46).

While not lethal, mutations that inactivate Vhs result in a severalfold reduction of virus growth in cell cultures (50, 51), and wild-type virus rapidly outgrows Vhs mutants in mixed infections (37). A number of studies suggest that Vhs plays a significant role in HSV pathogenesis (4, 38, 61, 63–65). Thus, the replication of Vhs mutants is markedly reduced in mouse cornea, trigeminal ganglion, and brain (38, 64), and Vhs is required for the efficient establishment of latency (63), although this latter phenotype probably reflects a requirement

for Vhs for efficient virus replication in peripheral tissues (63). The mechanisms by which Vhs affects pathogenesis are unclear but may involve a role in reducing the expression of major histocompatibility complex class I (69) or suppressing cytokine production by infected cells (67).

Although it is clear that Vhs induces mRNA turnover, a central unanswered question is whether Vhs is itself an RNase or, instead, activates a cellular enzyme. Several studies demonstrated Vhs-dependent cleavage of target mRNAs in cytoplasmic extracts of infected cells (34, 62). Similarly, rabbit reticulocyte lysates containing *in vitro*-translated Vhs induced Vhs-dependent endonuclease cleavage of target mRNAs (15, 16, 75). In these *in vitro* systems, Vhs degraded mRNAs, while rRNAs were unaffected, an observation that parallels the specificity of Vhs for mRNAs that is seen *in vivo* (45, 46, 58, 66). In addition, recent studies suggested that, *in vivo* and in rabbit reticulocyte lysates, Vhs does not cleave mRNAs at random sites but rather appears to initiate degradation near regions of translation initiation. Thus, the degradation of mRNAs, which are translated by cap-dependent scanning, appears to be initiated near the 5' end (15, 32), and *in vitro*-translated Vhs preferentially induces cleavage at sites downstream from a picornavirus internal ribosome entry site (16, 41). Extracts of partially purified virions contain an RNase activity that is blocked by Vhs-specific antisera and is absent from the virions of Vhs mutants (75). However, in several respects, this RNase activity lacks the selectivity of the Vhs activity *in vivo*. First, it is not restricted to mRNAs. Second, it cleaves target RNAs at multiple locations throughout the molecule without showing an apparent preference for sites near the 5' end. These observations suggest that targeting of the Vhs activity may require

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one or more cellular factors that are present in reticulocyte lysates but absent from virion preparations. While all of these studies are consistent with Vhs being a nuclease, in each case the preparations of Vhs protein probably contained cellular polypeptides, making it impossible to exclude the possibility that Vhs activates a cellular RNase.

To investigate this question more fully, we used a combination of genetic and biochemical approaches. Vhs has been reported to share sequence similarities with a number of cellular nucleases (12, 17). These studies were extended by using hidden Markov modeling, with the result that the Vhs homologues of alphaherpesviruses were found to share more extensive similarities with a larger family of human, yeast, bacterial, and phage nucleases. In particular, nine charged or hydrophilic residues are highly conserved between Vhs and the cellular nucleases. For several of the nucleases, the conserved amino acids are located in the active site and are critical to nuclease activity. Alteration of the corresponding residues of Vhs by site-directed mutagenesis was found to abolish the ability of Vhs to degrade mRNAs. In other experiments, Vhs was expressed in bacteria along with a fusion protein of glutathione *S*-transferase (GST) and the mammalian translation initiation factor eIF4H. Vhs and eIF4H recently were shown to interact in mammalian cells (19), and the two proteins formed a complex when coexpressed in bacteria. A complex of GST-eIF4H and wild-type Vhs was isolated and shown to have RNase activity, while complexes containing either of two mutant forms of Vhs did not have such activity. These results provide genetic and biochemical evidence that Vhs is an RNase, either alone or as part of a complex with eIF4H.

#### MATERIALS AND METHODS

**Cells.** Vero cells were purchased from the American Type Culture Collection and maintained in Eagle's minimum essential medium (GIBCO) supplemented with 10% (vol/vol) calf serum and antibiotics as described previously (17, 46, 51).

**Plasmids.** The plasmid pKOSamp contains the Vhs (UL41) open reading frame from HSV type 1 (HSV-1) strain KOS cloned into the vector pcDNA1.lamp (Invitrogen) downstream from the cytomegalovirus immediate-early promoter as well as a promoter for T7 RNA polymerase (18). It was the parent plasmid for constructing all site-directed mutations in Vhs.

**Homology searches and alignments.** To search for Vhs homologues, the UL41 open reading frame from HSV-1 (KOS) was compared to other known protein-encoding sequences by using the BLAST search program (2), and the results were refined by hidden Markov modeling (3, 13, 21, 35). The initial identification of cellular and phage nucleases with similarities to Vhs was achieved through a BLAST search with just the central region of UL41, including amino acids 165 through 265. Subsequent comparison of the sequences of the nucleases with the entire UL41 sequence revealed similarities outside of this central region of Vhs.

Vhs homologues from the alphaherpesviruses were aligned in a multiple alignment by using the ClustalW alignment algorithm of MacVector, version 6.0 (Oxford Molecular, Campbell, Calif.). Alignment of the Vhs proteins with the cellular and phage nucleases was done in several steps. First, the nucleases were divided into four groups (RAD2 DNA repair nucleases, xeroderma pigmentosum [XPG] proteins, flap endonucleases [FEN-1], and DNA polymerases) based on their relative homologies to each other. The ClustalW algorithm was then used to align the proteins in each group, after which the different groups were aligned with each other by performing a multiple alignment to achieve the best alignment of all of the proteins. Finally, the alignment of some proteins was adjusted by visual inspection, since the computer algorithm did not recognize some conserved motifs that were readily apparent by eye.

**Site-directed mutagenesis.** The Vhs-expressing plasmid pKOSamp was the parent plasmid used for all site-directed mutagenesis procedures. The wild-type Vhs allele was mutagenized by using a Chameleon double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions but with the modifications described previously (18). Mutagenic primers were de-

signed to create not only the desired change in the Vhs coding sequence but also a new restriction enzyme site to be used in screening the mutant alleles. Mutagenized plasmids were screened by restriction enzyme analysis, sequenced to confirm the nucleotide changes, and analyzed by *in vitro* transcription and translation with a TnT T7 quick coupled transcription-translation system (Promega Corp., Madison, Wis.) to confirm that they encoded proteins of the expected molecular masses (18). Mutants were named for the number of the residue that is altered, preceded by the wild-type amino acid and followed by the amino acid to which it is changed. For example, in D34N, aspartic acid at residue 34 is changed to asparagine.

**DNA isolation and sequencing.** Plasmids for transfection and sequencing were prepared from bacterial lysates by using MidiPrep and MaxiPrep systems as recommended by the manufacturer (Qiagen Inc., Chatsworth, Calif.). Sequencing of the Vhs alleles was performed with an Applied Biosystems model 377 DNA sequencer at the Molecular Biology Core Facility of the University of Missouri—Kansas City (18).

**Transient expression assay of Vhs activity.** Vhs activity was measured by determining the ability of a transfected UL41 allele to inhibit the expression of a cotransfected reporter plasmid containing the *Escherichia coli lacZ* gene under the control of the simian virus 40 early promoter and enhancer (17, 18, 47). Transfection was performed by using a Profection mammalian transfection system (Promega) according to the manufacturer's instructions. Vero cells were plated on the day before transfection in 60-mm-diameter petri dishes at a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. Cultures were transfected with 0.6-ml aliquots containing calcium phosphate coprecipitates of 3  $\mu$ g of the reporter plasmid pSV- $\beta$ -galactosidase (Promega) and 0.73 pmol of either a UL41-containing effector plasmid or the expression vector pcDNA1.lamp. The precipitates also contained enough salmon sperm carrier DNA to bring the total amount of DNA to 12  $\mu$ g.

Cell extracts were prepared 40 to 48 h after transfection and assayed for reporter gene expression by using a  $\beta$ -galactosidase enzyme assay system (Promega) and a Thermo Max microplate reader (Molecular Devices, Sunnyvale, Calif.) as described previously (17, 18, 47). For each transfection involving a UL41-containing effector plasmid, the amount of  $\beta$ -galactosidase activity was expressed as a fraction of that observed in a transfection involving 0.73 pmol of the empty expression vector pcDNA1.lamp.

**Western blotting.** The expression of the Vhs polypeptide in extracts of transfected cells was confirmed by Western blotting with a polyclonal rabbit antiserum raised against a Vhs-LacZ fusion protein as described previously (51).

**Expression of recombinant Vhs and GST-eIF4H.** Recently, the Vhs protein was shown to interact with the eukaryotic translation initiation factor eIF4H *in vitro* in yeast and mammalian cells (19). In the present study, efforts were undertaken to coexpress Vhs and a fusion protein of eIF4H and GST in *E. coli*. To this end, the Vhs gene was cloned into the *NcoI* site of pET19b (Novagen) to yield a plasmid encoding an untagged Vhs protein. pGST-4H was described previously (19) and encodes a fusion protein of eIF4H and GST. For the coexpression studies, an *EcoRV-SaI* fragment was excised from pGST-4H and inserted between the same sites of pLysS (Novagen) to yield a plasmid encoding GST-eIF4H under the control of an isopropyl- $\beta$ -D-galactopyranoside (IPTG)-inducible *tac* promoter.

*E. coli* BL21(DE3) was transformed with both the Vhs- and the GST-eIF4H-expressing plasmids and selected for resistance to both carbenicillin and chloramphenicol. The cells were grown to mid-log phase and induced for 3 to 4 h with 1 mM IPTG. Induced cells were harvested and lysed, and protein complexes that bound glutathione-Sepharose 4B were isolated by using a bulk GST purification module from Amersham-Pharmacia Biotech. Proteins were eluted from glutathione-Sepharose, dialyzed overnight against 50 mM sodium phosphate (pH 7.0), and applied to a 1-ml HiTrap SP Sepharose HP column (Amersham-Pharmacia Biotech) by using a Waters 650E advanced protein purification system. Proteins were eluted with a nonlinear 0 to 1 M NaCl gradient. Fractions were concentrated by using Centricon YM-10 filters (Amicon) and analyzed for protein content and RNase activity.

**RNase activity.** Target RNAs encoding HSV thymidine kinase were synthesized by *in vitro* transcription of linearized pBK2 with SP6 RNA polymerase in the presence of a 5' cap analogue (Stratagene) as described previously (32). The resulting 1,340-nucleotide transcripts contained a 35-nucleotide poly(A) tail encoded by the plasmid.

RNase activity was measured by using the reaction conditions of Zelus and coworkers (75). Reactions were initiated by mixing 5  $\mu$ l of concentrated gradient fractions with 45  $\mu$ l of degradation buffer (80 mM potassium acetate, 1.5 mM magnesium diacetate, 2 mM dithiothreitol, 0.1 mM EDTA, 10 U of RNasin [Promega], 25 mM Tris-HCl [pH 7.5]) containing 0.4  $\mu$ g of target RNA. After incubation at 37°C for various intervals, the RNAs were extracted with phenol-

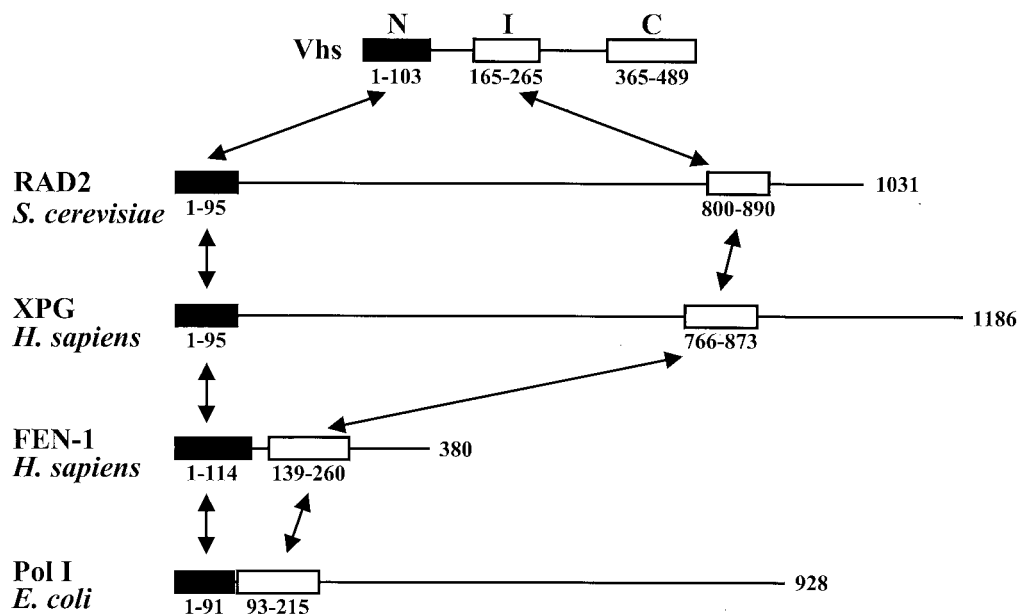


FIG. 1. Primary structures of Vhs and selected human, yeast, and bacterial nucleases. The primary structure of the Vhs polypeptides is depicted in the first line, with the three homology regions shared by the UL41 homologues from various alphaherpesviruses depicted by the black, white, and grey rectangles labeled N, I (internal), and C. The coordinates of the residues encompassing each of the homology regions differ slightly among the UL41 homologues, as do the distances separating them in the primary sequence. The coordinates shown below the first line refer to the Vhs polypeptide of HSV-1. The primary structures of the RAD2 protein from *S. cerevisiae*, the human XPG and FEN-1 polypeptides, and DNA polymerase I from *E. coli* are diagrammed in the second through fifth lines (26, 68). Each of these polypeptides has an amino-terminal domain (depicted by a black rectangle) that is similar to the amino-terminal domain of Vhs and an internal domain (depicted by a white rectangle) that is similar to the internal domain of Vhs. The amino acids that comprise the domains of each of the proteins are shown below the corresponding rectangles, and the overall length of each polypeptide is shown at the right. The distances separating the amino-terminal and internal domains of each polypeptide differ considerably from nuclease to nuclease. Arrows connect homologous domains in the different proteins.

chloroform, precipitated from ethanol, and analyzed by electrophoresis through 1.2% agarose gels (34).

## RESULTS

**Similarities between Vhs and cellular nucleases.** A comparison of the Vhs (UL41) homologues of HSV-1 and the other alphaherpesviruses reveals that they contain three regions of high sequence homology (5, 17, 31). These consist of amino-terminal and carboxyl-terminal regions of approximately 100 amino acids and a conserved internal region (Fig. 1, first line). Two less conserved regions that vary in length depending upon the Vhs homologue separate the conserved regions. Mutations that affect Vhs activity generally fall within the conserved regions, while those in the less conserved regions often do not affect activity (17, 18, 31, 47).

Previous studies revealed sequence similarities between regions of Vhs and several cellular nucleic acid binding proteins and nucleases (12, 17). To extend these studies, the Vhs sequence of HSV-1 was used to perform a BLAST search (2), and the results were refined by hidden Markov modeling (3, 13, 14, 35). The results revealed similarities between the amino-terminal and internal regions of Vhs proteins and a larger family of mammalian, yeast, bacterial, and phage nucleases, including members of the RAD2 DNA repair nucleases, XPG proteins, flap endonucleases (FEN-1), and DNA polymerases (Table 1). The primary structures of the Vhs proteins and representative members of the cellular nucleases are compared in Fig. 1. The nucleases contain two regions of homology: a

conserved amino-terminal region and an internal region (10, 26, 39, 68). These, in turn, are similar to the conserved amino-terminal and internal regions of the Vhs proteins. The nucleases differ in overall size and in the length of the primary sequence separating the conserved amino-terminal and internal regions. No obvious homology was observed between the cellular nucleases and the conserved carboxyl-terminal region of the Vhs proteins.

Figure 2 shows a sequence comparison of the amino-terminal and internal regions of the Vhs proteins and representative members of the cellular nucleases. The Vhs polypeptides of the alphaherpesviruses are highly homologous, with a large number of identical or conserved residues (Fig. 2, alleles below the double line). A smaller, but significant, amount of similarity is observed in both the amino-terminal and the internal regions of the Vhs proteins and the cellular nucleases. The similarity is most striking for eight charged residues and one hydrophilic residue (highlighted in black in Fig. 2) that are conserved across all of the aligned proteins. These include three residues (D34, D82, and K96) in the amino-terminal region and six amino acids (E192, D194, T211, D213, D215, and D261) in the internal region.

Within the amino-terminal region, an invariant aspartate is present at position 34 of the HSV-1 polypeptide and the corresponding residues of the other Vhs proteins as well as all of the cellular nucleases listed in Table 1 and Fig. 2. Aspartate is found at position 82 of the HSV-1 polypeptide and the corresponding residues of all but two of the other viral and cellular

TABLE 1. Nuclease family

Category	Protein <sup>a</sup>	Organism <sup>b</sup>	Abbreviation	Entrez accession no.
DNA polymerase associated proteins	DNA polymerase I	<i>B. burgdorferi</i>	Bbu POLI	2688462
	DNA polymerase I	<i>B. caldotenax</i>	Bca POLI	416913
	DNA polymerase I	<i>Bacillus stearothermophilus</i>	Bst POLI	3041672
	DNA polymerase I	<i>Escherichia coli</i>	Eco POLI	118825
	DNA polymerase I	<i>Helicobacter pylori</i>	Hpy POLI	2494177
	DNA polymerase I	<i>Streptococcus pneumoniae</i>	Spn POLI	118827
	DNA polymerase	<i>Thermus aquaticus</i>	Taq POLI	1942938
	Exonuclease	<i>E. coli</i>	Eco EXO	2507020
	Exonuclease	<i>Mycoplasma pneumoniae</i>	Mpn EX53	2494184
	Exonuclease	<i>Bacillus subtilis</i>	Bsu YPCP	1730895
	YY30	<i>Mycobacterium tuberculosis</i>	Mtu YY30	1731347
	Exodeoxyribonuclease	Bacteriophage T5	T5 EXO5	119684
	RNase H	Coliphage T4	T4 RNH	133162
	Exodeoxyribonuclease	Bacteriophage T3	T3 EXRN	119705
	IXPG proteins	XPG	<i>C. elegans</i>	Cel XPG
XPG		<i>Xenopus laevis</i>	Xle XPG	267421
XPG		<i>Homo sapiens</i>	Hsa XPG	267420
XPG		<i>Mus musculus</i>	Mmu XPG	549454
Flap endonucleases	Flap endonuclease 1	<i>X. laevis</i>	Xle FEN1	2674207
	Flap endonuclease 1	<i>M. musculus</i>	Mmu FEN1	729476
	Flap endonuclease 1	<i>H. sapiens</i>	Hsa FEN1	729475
RAD2 DNA repair proteins	YEN1	<i>Saccharomyces cerevisiae</i>	Scy YEN1	731457
	RAD2	<i>C. elegans</i>	Cel RAD2	529362
	Exonuclease I	<i>Schizosaccharomyces pombe</i>	Spo EXOI	1706728
	DIN7	<i>S. cerevisiae</i>	Scy DIN7	2501673
	Exonuclease I	<i>S. cerevisiae</i>	Scy EXOI	1706421
	TOSCA	<i>Drosophila melanogaster</i>	Dme TOSCA	1419489
	RAD2	<i>S. cerevisiae</i>	Scy RAD2	131811
	RAD13	<i>S. pombe</i>	Spo RAD13	131777
	RA27	<i>S. cerevisiae</i>	Scy RA27	140964
	RAD2	<i>S. pombe</i>	Spo RAD2	730469
	YA31	<i>S. pombe</i>	Spo YA31	1175380
	RAD2	<i>A. fulgidus</i>	Afu RAD2	2650376
	RAD2	<i>Methanobacterium thermoautotrophicum</i>	Mth RAD2	2622760
	RAD2	<i>M. jannaschii</i>	Mja RAD2	2127857

<sup>a</sup> Nucleases with which Vhs shares homology.

<sup>b</sup> Organisms from which the Vhs homologues are obtained.

proteins, in which it is glutamate. A third conserved residue is a basic amino acid at position 96 of the HSV-1 sequence. For all of the viral and cellular homologues, this residue is either lysine or arginine.

Within the internal region, acidic amino acids are conserved at positions 192, 194, 213, 215, and 262 of the HSV-1 polypeptide. Aspartate is found at the residue corresponding to position 213 in every one of the proteins and at positions 215 and 262 of all but one of the other viral and cellular homologues. Similarly, glutamate is found at the residue corresponding to position 192 in all but one of the proteins, in which it is aspartate; the residue corresponding to position 194 is uniformly either aspartate or glutamate. The other highly conserved site within the internal region is a serine or threonine at the residue corresponding to position 211 of the HSV-1 polypeptide.

**Functional significance of the conserved amino acids.** For a number of the nucleases, structural and genetic studies indicate that many of the conserved residues are located in the active site and are essential for catalytic activity (Tables 2 and 3). Crystal structures have been determined for several of the FEN-1 nucleases (28, 29) as well as T4 RNase H (44), T5 5' exonuclease (11), and the 5' exonuclease domain of *TaqI* DNA

polymerase (33). Each of these nucleases binds two or more divalent metal ions, and the conserved residues play key roles in metal binding (10). For the FEN-1 nucleases, one Mg<sup>2+</sup> binding site is formed by amino acids corresponding to residues D34, D82, E192, and D194 of Vhs, while a second Mg<sup>2+</sup> site is formed by amino acids corresponding to Vhs residues D213, D215, and D261 (28) (Table 2). Alteration of any of these residues to alanine by site-directed mutagenesis abolishes the nuclease activity of human FEN-1, a finding further supporting the conclusion that these amino acids are important for catalysis (59, 60). Similar results were obtained for T4 RNase H. For this nuclease, aspartates homologous to Vhs residues D34, D82, D194, and D213 are important for the binding of one metal ion, while amino acids corresponding to Vhs residues D194, D215, and D261 form hydrogen bonds with the water molecules that are coordinated by a second metal ion (44) (Table 2). In addition, a serine homologous to threonine 211 of Vhs forms a hydrogen bond with the carboxylate of the aspartic acid corresponding to residue D34 of Vhs. Site-directed mutagenesis that alters the residue homologous to D213 of Vhs or any of the aspartates forming the first metal binding site abolishes or greatly reduces the nuclease activity of T4 RNase H, as does altering the serine homologous to threonine



## Amino Terminal Region

Eco POLI	~ PQNPLTLDGSSYLRYAYHAFPPLTN	SAG	EPTGAMYGYLNM~~ KPTHAAVVFD	DAKGT	FRDEL	FEHYKSH
Taq POLI	~ PKGRVLLVDGHHLAIRTFHAKGLTTS	RG	EPVQAVYGFASK~~	DGDVAIVVFD	AKAPS	FRHEA YGGYKAG
Eco EXO	~ VAYHLLIYDALNLRIRIHAVQ		GSPCVETCOHA~~	QPTHAVAVFD	DENRSS	SGWRHOR LPDYKAG
Mtu YY30	~ LRSPLVLLDGSAMWFRSEFGVPS SITAP	DG	RPVNAVYGFIDS~~	RPNRLAVCLD	LDWRP	QFRVDL IPSYKAH
T5 EXO5	~ SRRNLMIVDGTNLGFRFKHNSKPPFASYSVSTIQSLAKSY		~~	SARTTIVLGD	KGKSV	FRLH LPEYKGN
T4 RNH	~ YKEGICLIDFS	QIALSTALVNF	DKEKINLSMVRHL	ILNSIRFN	~~	GYTKIVLCIDNAKSG YWRRDF AYYYKDN
T3 EXRN	~ DDKGILVMDGDWLVFQAMSAAEFDASWEEI	IWHRCCD	HAKARQIEDS	~~	VGAPIVLAFTDS	VN WRKLVDPNYKAN
Hsa XPG	~ G KILAVDISIWLNOALKGVDRRHGNSIE		NPHLLTLFHRLC	~~	FFRIRPIFVFD	GDAPLLKKQTLVKRQR
Hsa FEN1	~ G RKVATDASMSIQFLIAVR	QGGDVLQ	NEEGETTSHLMGMFYRTI	~~	ENGIKPYVYVFD	GKPPQLKSGELAKRSE
Cel RAD2	~ D KTLSIDG HIWLY	ESLKGCEAH	QQQTP NSYLVTFFRIQ	~~	ELKIPIVVD	N INASSAHE SKDQ
Sec DIN7	~ Y QTLAIDG YAWLH	RASCACAF	ELVMNKPINKYLQFFIKRL	~~	RLKIKPYIVFD	GDSLFFVKNHTETRRK
Sec EXO1	~ G EVLAIDG YAWLH	RAACSCAYEL	AMGKPTDKYLQFFIKRF	~~	TFKVEPYLVFD	GDAIPYKSTESKRRD
Dme TOSCA	~ G STVAVDT YCWLH	KGVFGCAE	LARGEDTDVYIQYGLKYV	~~	SYDIKPIVFD	GOHLPAKALTEKRRD
Sec RAD2	~ D KRMAVDASIWYQFLKAVRDQEGNAVKN		SHITGFERRIC	~~	YFGRIPVEVFD	GGVPLVKRETIRQRKE
Sec RA27	~ G RKVAIDASMSLYQFLIAVRQDGGQLTNEAGET	TSHLMGMFYRTL	~~	DNGIKPCYVFD		GKPPDLKSHELTKRSS

VZV Dumas	~ GVLTPVAVD VWNVMYTLERLY	PVGKREN	LHGSPVTHCLGVLRLL	~~	YYPIFVLE	RC TDG PLSRGAKAI
PRV Kaplan	~ GVLTPVAVD DLWVMYTLMEKHY	QET	TEDNATTTARCLRLRML	~~	YEPIFVSD	RG IFNGRIARGAKAI
BHV Cooper	~ GVMTPVAVD LWNVMYTLERFC	GDAF	GGVGDAAATARCFSLRML	~~	YYPIFVAD	RG IHGDRRATRGAIAI
Gallid HV-2	~ GAGTPVAVD LWNVMYTLERFY	PEKI	DGVDSITLRLCYLRLLL	~~	YYPVFSVSD	RG MYGNVKALYGAIAI
EHV AB4P	~ GIMTPVAVD DLWVMYTLMEKFD	QERN	FPLDGAATARCFSLRLL	~~	YYPIFVSD	RG IYDGRVKQGAIAI
HSV-2 333	~ GYFTPVAVD LWNVMYTLVVKYQ	RRYP	SYDREAITLHCLCMLRVT	~~	LFPIFVID	RG VECTEPVVFGAIAI
HSV-1 KOS	~ GYFTPVAVD LWNVMYTLVVKYQ	RRYP	SYDREAITLHCLCRLKVF	~~	LFPIFVID	RG VNCMEPVVFGAIAI

KOS #      26      30      35      40      45      50      55      60      65      70      75      80      85      90      95

## Internal Region

Eco POLI	~HAMVKAMGLPLLAVSG	VEADDVIGTL	LAREAEEKAG	RPVISTG	DKDMAQIVT	PNTLINTM~~	ELIIDFLALMGDSNDNI	
Taq POLI	~KELVDLLGLARLEVP	YEADDVLSL	LAKKAEKEG	YEVILTADK	DEYQLSDR	THVLFPE~~	DQWADYRALTGDESNDL	
Eco EXO	~RAAFEQRGVPCWSTSG	NEADDLAAT	LAVKVTQAG	HQAVS	DKGYCOLL	SPTLRIRDYF~~	QQLPDYWGLAGISSSKV	
Mtu YY30	~MELLDLAFCLAMAGAP	FEADDVLGT	LATRERR	DPVVYSG	DRDLQYVAD	PPVPRVL~~	AAAYEALALRGDPSDGL	
T5 EXO5	~AFELCKTTFPTFTIRG	VEADDMAAYI	VKLIGHLY	DHVLIS	DGDWDTL	TDKVSRFST~~	EQFISEKAIMGLGDNI	
T4 RNH	~DELKAYMPTIVMDIK	YEADHIAVL	VKFSLEG	HKIIS	SSDQFTQ	LHKYP NKKOWS~~	EIDCMTKILGDKKDNV	
T3 EXRN	~EALFEREEFYCIPEPM	EGDDVMGVI	ASNPSAFGARKAI	ISCDK	DKFTIPNCD	FLWCTT~~	DWWHLFQTIKGDMDGY	
Hsa XPG	~QELLRLFCIPYIQAPM	EAEAQCAI	LDLTDQTS	TITDDSDI	WLFGARH	VYRNF~~	DRNKLIAYLLGSDYT	
Hsa FEN1	~KHLLSLMGIPYLDAPS	EAEASCAAL	YKAGVYA	AATEDMD	CLTFGSP	VLMRHLT~~	NQEQLVLCILLGSDYC	
Cel RAD2	~NALLTELCIKVITAPG	DGEAQCAR	LEDLGVTSG	CITDFDYF	LFGGK	LYRDFD~~	SRPHLITAILLGDYF	
Sec DIN7	~IDYCKLHSIPYIVAPF	EADPQM	VYLEKMG	LIGQ	IISEDSD	LELVFGG	KTITKLN~~	SEQQRNLVCLAGDYT
Sec EXO1	~ICYCKLNGIRYIVAPF	EADSQM	VYLEKQKNI	VQG	IISEDSD	LELVVFGC	RRLITKLN~~	TNEEITMVCLSGDYT
Dme TOSCA	~IRECRSRNVDCIVAPY	EADAQMA	WNRADVAQY	IITEDSD	ETLFGAK	NIIEKLD~~	HFDKRRMCILSGDYT	
Sec RAD2	~QELLSRFGIPYITAPM	EAEAQCAE	LQLNLVDG	IITDDSD	VFLFGG	TKIYKNMF~~	DRKNMIELAQLLSDYT	
Sec RA27	~QKLLGLMGIPIYIAPT	EAEAQCAE	LAKKGVYA	AASEMD	ITL	CYRTP	FLLRHLT~~	TIEQLVLCIMLGDYC

VZV Dumas	~ASLIRWVGAYAVEAVD	I	EAEACANL	FHTRTVAL	VYTTID	DDEL	FMGCDI	LDAI~~	TYTEFLVAFVRCQDLH
PRV Kaplan	~VNLIRHMGYAYVDVSD	MEADDVCANL	YHTNTVAQ	VHTID	DMIT	LIGCDM	ILDIAA	~~	DYSEFLAAFVRCHTDLH
BHV Cooper	~VSLIRFLGYAVDAAE	MEADDVCANL	FHTNTVAH	IYTTID	DMIT	LMGCDL	ILDAA	~~	TYGFLATFVRCHTDLH
Gallid HV-2	~MRMIRFMGYPYVDAGT	MEADDICANL	YHTNTVAY	VLSSD	DELI	LMGCDI	IIDL	LT	~DYSTFLNFLRCHTDLH
EHV AB4P	~VNLIRHLGYPYVNACN	MEADDVCANL	YHTNTVAQ	IYTTID	DDELI	LMGCDI	ILDIM	~~	TYDFLTFVRCHTDLH
HSV-2 333	~IRVLRALGYAYINSGQ	MEADDACANL	YHTNTVAY	VYTTID	DDELI	LMGCDI	VLDIS	~~	SYQFLALFVRCHTDLH
HSV-1 KOS	~IRVLRALGYAYINSGQ	MEADDACANL	YHTNTVAY	VYTTID	DDELI	LMGCDI	VLDIS	~~	SYQFLALFVRCHTDLH

KOS #      175      180      185      190      195      200      205      208      210      215      220      225      228      247      250      255      260

FIG. 2. Sequences of the amino-terminal and internal domains of Vhs and selected cellular nucleases. The amino acid sequences of the amino-terminal and internal domains of the Vhs polypeptides from seven alphaherpesviruses are shown below the double line; the sequences of the corresponding domains of selected cellular and phage nucleases (listed in Table 1) are shown above the double line. The amino acid coordinates of the Vhs polypeptide from HSV-1 strain KOS are shown at the bottom of each panel. Amino acids that are identical to the residues in HSV-1 strain KOS are shown in white lettering on a dark grey background. Amino acids that represent a conservative change from the residues in HSV-1 strain KOS are shown in black lettering on a light grey background. The names of cellular and phage nucleases for which structural and/or genetic data have identified key residues that are in the active site are highlighted by a light grey background in the leftmost column. The active-site residues from these nucleases that are conserved in other cellular and phage nucleases and in the Vhs polypeptides are shown in white lettering on a black background. These residues were the focus of site-directed mutagenesis of the Vhs polypeptides (see Fig. 3 and 4). VZV, varicella-zoster virus; PRV, pseudorabies virus; BHV, bovine herpesvirus; Galid HV-2, Gallid herpesvirus 2; EHV, equine herpesvirus.

211 of Vhs (6) (Table 3). Interestingly, a point mutation that changes threonine 214 of Vhs to isoleucine abolishes detectable Vhs activity. These and similar results for other nucleases (Tables 2 and 3) indicate that these conserved residues

are components of a motif shared by a large group of nucleases.

**Site-directed mutagenesis of Vhs.** If one believes that the sequence similarities shared by Vhs and the cellular and phage

TABLE 2. Residues implicated in activity and metal binding by structural studies of nucleases<sup>a</sup>

Vhs residue	Corresponding residue in:			
	T5 5' exonuclease (10, 11)	Taq I DNA polymerase (10, 33)	T4 RNase H (10, 44)	Human FEN-1 (10, 28, 29)
D34	D26 (Mn-1)	D18 (M-1)	D19 (Mg-1)	
D82			D71 (Mg-1)	
K96				
E192	E128 (Mn-1)	E117 (M-3)		
D194		D119 (M-1, M-3)	D132 (Mg-1, M-2)	
D195	E131 (Mn-1)	D120 (M-3)		
T211			S153↔D19	
D213	D153 (Mn-1, Mn-2)	D142 (M-1, M-2)	D155 (Mg-1)	
D215	D155 (Mn-2)	D144 (M-2)	D157 (Mg-2)	D181 (Mg-2)
D261	D204 (Mn-2)		D200 (Mg-2)	

<sup>a</sup> Key conserved residues of the Vhs polypeptide are shown in column 1. The corresponding residues of cellular and phage nucleases are shown in columns 2 through 5. T5 5' exonuclease and T4 RNase H have been shown to bind two manganese (Mn-1 and Mn-2) and two magnesium (Mg-1 and Mg-2) ions, respectively. Human FEN-1 has been shown to bind two magnesium ions, while Taq I DNA polymerase binds three metal ions (M-1, M-2, and M-3). Residues that have been shown in structural studies to play key roles in metal binding are indicated for the different nucleases. S153 and D19 of T4 RNase H have been shown to interact by hydrogen bonding. Data are from the references listed at the top of each column.

nucleases indicate that Vhs is a nuclease, then mutations that alter key conserved residues of the nuclease motif should abolish Vhs activity, just as they do for the nucleases discussed above. To test this prediction, Vhs was altered by site-directed mutagenesis to change eight acidic residues (D34, D82, E192, D194, D195, D213, D215, and D261) as well as threonine 211. The acidic amino acids were changed to their basic counterparts, aspartate to asparagine and glutamate to glutamine. Threonine 211 was changed to serine in one mutant and alanine in another. The activities of the mutant Vhs alleles were assessed by using a transient expression assay of Vhs activity in which Vero cells were transfected with plasmids encoding a *lacZ* reporter gene and mutant or wild-type Vhs alleles. Vhs activity was determined by the ability of a transfected Vhs allele to inhibit *lacZ* activity. This assay was previously used to compare the mRNA degradation activities of various mutant

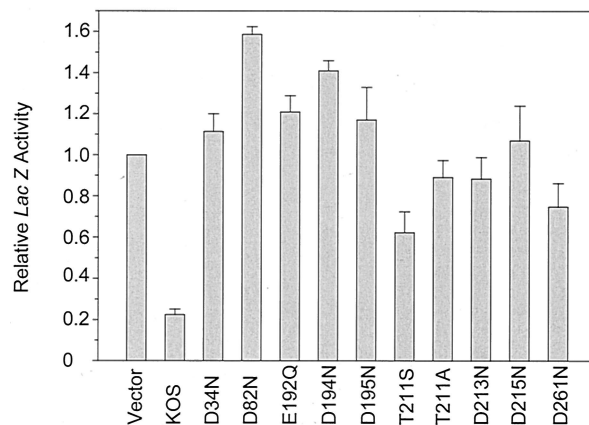


FIG. 3. Ability of wild-type Vhs and site-directed Vhs mutants to inhibit the expression of a cotransfected reporter gene. Triplicate cultures of Vero cells were transfected with 3 µg of the reporter plasmid pSV-β-galactosidase plus 0.73 pmol of pcDNA1.1*lamp* (Vector) or UL41-containing effector plasmids encoding wild-type Vhs or the indicated mutant forms of Vhs. Transfection mixtures also contained enough salmon sperm carrier DNA to bring the total amount of DNA to 12 µg. Cell extracts were prepared 40 to 48 h after transfection and assayed for β-galactosidase activity as described in the text. For each transfection, the amount of β-galactosidase activity was expressed as a fraction of that observed in the transfection involving the vector pcDNA1.1*lamp*. Errors bars indicate the standard error of the mean.

Vhs alleles as well as the Vhs polypeptides expressed by HSV-1 and HSV-2 strains (17, 18, 23, 47).

In these experiments, the wild-type Vhs allele reduced *lacZ* expression to a level 15 to 20% that seen in cells transfected with the reporter gene plus the empty expression vector lacking a Vhs allele (Fig. 3). In contrast, seven of the mutants containing alterations in acidic residues (D34N, D82N, E192Q, D194N, D195N, D213N, and D215N) exhibited reporter gene expression that was at least as great as that seen with the empty expression vector, indicating that they had no detectable Vhs activity in this assay. The same was true for one of the mutants containing an alteration in amino acid 211, T211A. Two of the mutants, T211S and D261N, showed slight inhibition of reporter gene activity, indicating that they had some activity. However, the more active of the two, T211S, reduced reporter

TABLE 3. Site-directed mutagenesis of key nuclease residues<sup>a</sup>

Vhs residue	Mutated residue in:				
	T4 RNase H (6)	Human FEN-1 (59, 60, 73)	Human XPG (73)	<i>M. tuberculosis</i> YY30 (43)	<i>E. coli</i> DNA polymerase I (74)
D34	D19N (nuclease -, sub. binding -)	D34A (nuclease -, sub. binding <<)		D21N (nuclease -)	D13N (nuclease -)
D82	D71N (nuclease -, sub. binding -)	D86A (nuclease -, sub. binding <)	D77A (reduced)	D73N (nuclease -)	D63A (nuclease -)
K96	K87A (nuclease -, sub. binding -)	R103A (nuclease +)			
E192		E158A (nuclease -, sub. binding -)		E123Q (nuclease -)	E113A (nuclease -)
D194	D132N (nuclease -, sub. binding -)			D125N (nuclease -)	D115A (nuclease -)
D195				D126N (nuclease -)	D116A (nuclease -)
T211	S153A (nuclease <, sub. binding <)				
D213	D155N (nuclease -, sub. binding -)	D179A (nuclease +)		D148N (nuclease -)	D138N (nuclease -)
D215	D157N (nuclease <<, sub. binding <<)	D181A (nuclease -, sub. binding +)	D812A (nuclease -)	D150N (nuclease -)	D140N (nuclease -)
D261	D200N (nuclease +, sub. binding +)	D233A (nuclease -, sub. binding -)		D202N (nuclease -)	D188A (nuclease -)

<sup>a</sup> Key conserved residues of the Vhs polypeptide are shown in column 1. Mutations that have been constructed in the corresponding residues of cellular and phage nucleases are shown in columns 2 through 6, along with their effects upon nuclease activity and substrate (sub.) binding. -, no activity; <<, greatly decreased activity; <, decreased activity; +, active. Data are from the references listed at the top of each column.

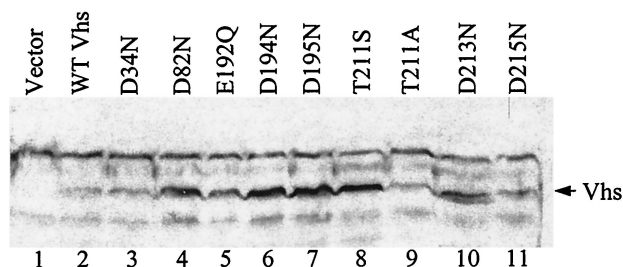


FIG. 4. Expression of mutant and wild-type Vhs polypeptides in transfected cells. Vero cells were transfected with 0.73 pmol of pcDNA1.1amp (Vector) or plasmids encoding wild-type Vhs (WT Vhs) or the indicated mutant forms of Vhs. Whole-cell lysates were prepared 48 h after transfection and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with polyclonal rabbit antiserum raised against a Vhs-LacZ fusion protein. The arrow to the right of lane 11 indicates the position of the Vhs (UL41) polypeptide.

gene expression to only 60% that seen in transfections with the empty expression vector (Fig. 3).

To ensure that the lack of activity of the Vhs mutants was not simply due to reduced levels of the Vhs polypeptide, lysates were prepared from cells transfected with wild-type or mutant Vhs alleles and were analyzed by Western blotting for the Vhs polypeptide. The Vhs protein was detected in all of the lysates from cells transfected with a Vhs allele but not in those from control cells transfected with the empty expression vector (Fig. 4). Significantly, cells transfected with each of the mutant alleles contained at least as much Vhs polypeptide as did cells transfected with the wild-type allele (Fig. 4, compare lane 2 with lanes 3 to 11). Interestingly, cells transfected with some of the mutant alleles contained significantly more Vhs protein than did cells transfected with the wild-type allele (for example; D82N, D194N, D195N, and T211S). This finding was reported previously for other mutants lacking Vhs activity and is presumably due to the fact that an active Vhs protein degrades its own mRNA, thereby reducing the amount of the protein that is produced (47). In any event, the lack of activity that was observed for the mutant alleles clearly was not due to a lower level of expression of the Vhs polypeptide.

**Preparations containing recombinant Vhs and GST-eIF4H have RNase activity.** The above experiments demonstrated that the conserved residues of the nuclease motif are important for Vhs activity and provided strong genetic data that Vhs is itself a nuclease. Nevertheless, to date, the Vhs protein has not been purified and shown to have nuclease activity. To this end, we attempted to purify recombinant Vhs expressed in *E. coli*. Initial efforts to express His-tagged or GST-tagged Vhs resulted in a protein that was insoluble, except in buffers containing high concentrations of guanidine hydrochloride, urea, or Sarkosyl (data not shown). Attempts to refold the Vhs protein by dialysis or rapid dilution into buffers that lacked a denaturant proved unsuccessful.

As an alternative approach, we took advantage of our recent observation that, in mammalian cells, Vhs interacts with the cellular translation initiation factor eIF4H. We tried coexpressing untagged Vhs and a GST-eIF4H fusion protein, with the hope that a complex of the two proteins would be more soluble than Vhs alone. *E. coli* was transformed with plasmids

encoding GST-eIF4H and one of three Vhs alleles: wild type, D194N, or D215N. As shown above, the latter two mutants express proteins which have alterations of key residues in the nuclease motif and which do not inhibit reporter gene expression in the transient expression assay. Nevertheless, both mutant proteins have been shown to still bind eIF4H (19). Control bacteria were transformed with the plasmid encoding GST-eIF4H and the vector lacking Vhs. Following induction with IPTG, the bacteria were lysed and the soluble fraction was applied to a column of glutathione-Sepharose to isolate complexes containing GST-eIF4H. GST-eIF4H was the most prominent protein in the bound fraction from each of the four types of bacteria (Fig. 5A). In addition, a 58-kDa polypeptide was observed in bacteria expressing wild-type Vhs or either of the two mutant forms of Vhs (Fig. 5A, lanes b to d) but not in cells expressing just GST-eIF4H (Fig. 5A, lane e). This polypeptide comigrated with Vhs from infected Vero cells and was verified as Vhs by Western blotting (data not shown).

Material that eluted from glutathione-Sepharose was applied to a HiTrap SP Sepharose HP cation-exchange column and eluted with a gradient of 0 to 1 M salt. Vhs-containing fractions were identified by Western blotting. Peak amounts of wild-type Vhs and mutant Vhs eluted in fraction 12 of the gradient (Fig. 5B). As expected, Western blotting detected no Vhs protein in fractions from bacteria expressing just GST-eIF4H. Figure 5C shows a Coomassie blue-stained gel of the material in fractions 10 through 13 from bacteria expressing wild-type Vhs. A prominent band corresponding to GST-eIF4H was observed in all four fractions, while the 58-kDa Vhs polypeptide was observed predominantly in fraction 12 and to a lesser extent in fraction 13. Several other proteins were observed in the peak fractions. Whether they were breakdown products of Vhs or of GST-eIF4H or bacterial proteins that copurified with them is unknown.

To test for RNase activity, peak fractions containing GST-eIF4H and each of the three forms of Vhs were incubated for 3 or 16 h with a capped, polyadenylated target RNA produced by *in vitro* transcription. Three hours of incubation with partially purified GST-eIF4H and wild-type Vhs caused degradation of most of the target RNA (Fig. 6B, lanes d and e), while incubation for 16 h resulted in complete degradation of the target RNA to products that were no longer visible on the gel (Fig. 6C, lanes c and d). In contrast, GST-eIF4H alone or preparations containing GST-eIF4H and either D194N or D215N failed to induce any detectable degradation (Fig. 6B, lanes a to c and f to k, and Fig. 6C, lanes a and b), even after 16 h of incubation, indicating that the mutant polypeptides lacked nuclease activity and that no nonspecific bacterial nuclease copurified with Vhs and GST-eIF4H. Although the peak fractions did not contain a purified complex of Vhs and GST-eIF4H, they were highly enriched for the two proteins. Significantly, Vhs and GST-eIF4H were the only eukaryotic or viral proteins present. Taken together, the results indicate that partially purified preparations of recombinant Vhs and GST-eIF4H have RNase activity.

## DISCUSSION

The Vhs protein is known to accelerate the decay of host and viral mRNAs (49). However, despite considerable study, it has



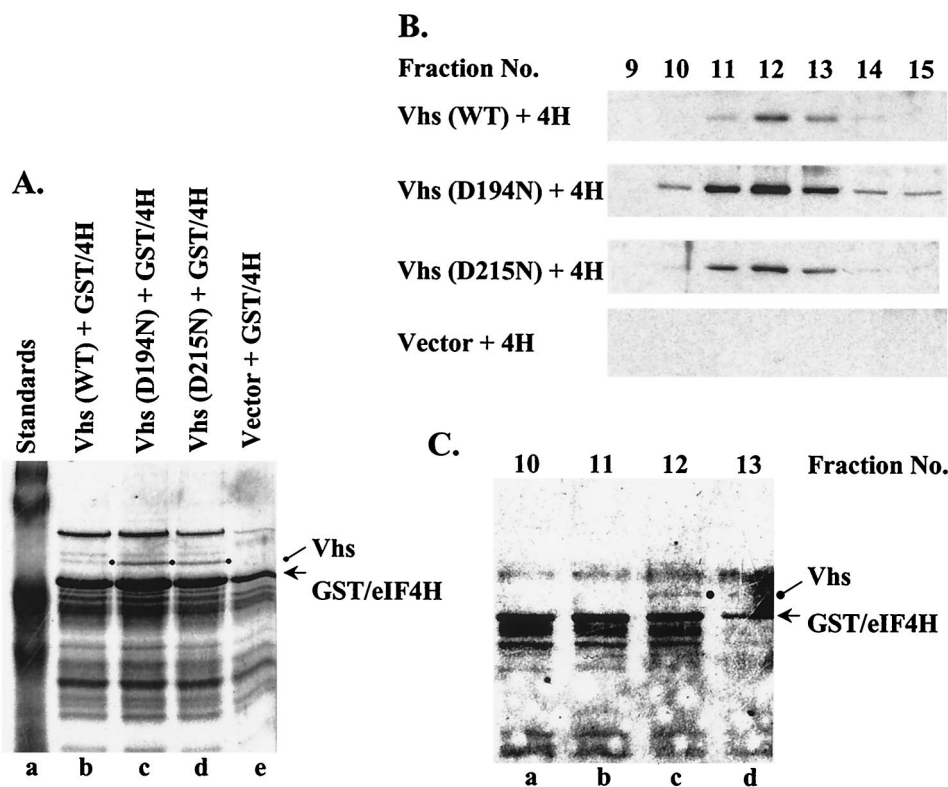


FIG. 5. Expression of recombinant Vhs and GST-eIF4H. (A) Coomassie blue-stained gel of recombinant proteins bound to glutathione-Sepharose and eluted with 10 mM glutathione. Proteins are from *E. coli* expressing GST-eIF4H (GST/4H) (lanes b to e) and wild-type (WT) Vhs (lane b), D194N (lane c), or D215N (lane d). Material in lane e is from bacteria expressing GST-eIF4H and no Vhs. Vhs is indicated by a closed circle to the right of lanes b to d, and GST-eIF4H is indicated by an arrow. (B) Material that eluted from glutathione-Sepharose was applied to a column of HiTrap SP Sepharose and eluted with a gradient of 0 to 1 M salt. Fractions were analyzed for Vhs by Western blotting. 4H, eIF4H. (C) Coomassie blue-stained gel of HiTrap SP Sepharose fractions 10 to 13 from cells expressing wild-type Vhs and GST-eIF4H. Vhs is indicated by a closed circle, and GST-eIF4H is indicated by an arrow.

remained unclear whether Vhs is itself an RNase or somehow activates a cellular enzyme. The present study demonstrates that bacterial fractions which are highly enriched for recombinant Vhs and GST-eIF4H have RNase activity. In addition, Vhs shares significant sequence similarities with a number of cellular and phage nucleases, and mutations that alter key conserved residues in Vhs and the nucleases abolish the activities of both. Taken together, the results provide strong evidence that Vhs is an mRNA RNase (mRNase), either alone or as part of a complex with eIF4H.

At present, it is unclear whether recombinant Vhs/GST-eIF4H is an endonuclease, an exonuclease, or both. Data suggesting that it is an endonuclease come from the observation of Elgadi and coworkers that rabbit reticulocyte lysates containing *in vitro*-translated Vhs contain a Vhs-dependent endonuclease (15). In the present study, recombinant Vhs/GST-eIF4H did not produce discrete degradation intermediates but instead degraded target mRNAs to products that were not observed on the gel. If Vhs is an endonuclease, then such a result could be explained if it cleaved each RNA molecule many times or cleaved each molecule at one or a few sites but at sites that differed from molecule to molecule. In either scenario, the isolated Vhs nuclease would lack the specificity that it demonstrates *in vivo*, where it shows a strong preference for mRNAs and appears to initiate mRNA degradation at sites of transla-

tion initiation (16, 32, 46). This information suggests that one or more additional factors may be required for the targeting of Vhs that is observed *in vivo*.

In this regard, our observation that Vhs binds the translation factor eIF4H suggests a mechanism for targeting the Vhs nuclease (19). Evidence that this interaction is biologically important is provided by the observation that several Vhs point mutations which abolish its ability to bind eIF4H also abolish its ability to degrade mRNAs *in vivo* (19). eIF4H shares sequence homologies with eIF4B and appears to be functionally similar in that both stimulate the RNA helicase activity of eIF4A (52–56). eIF4A, in turn, is a component, along with eIF4E and eIF4G, of the tripartite cap binding complex eIF4F (22). Thus, the available data suggest that eIF4H acts at an early stage of translation initiation to help unwind mRNA secondary structures and facilitate ribosome scanning (27).

Our data are consistent with two alternative models of Vhs activity (Fig. 7). In model 1, Vhs is itself a nuclease. It requires no other factors to have basal, relatively nonselective endonuclease activity. Binding to eIF4H targets Vhs to mRNAs, as opposed to non-mRNAs, and to regions of translation initiation. In model 2, Vhs is an essential component of an mRNase, perhaps the component that contains the active site. However, by itself the Vhs protein lacks activity. Binding of Vhs to eIF4H serves two purposes. First, it activates the nuclease activity of



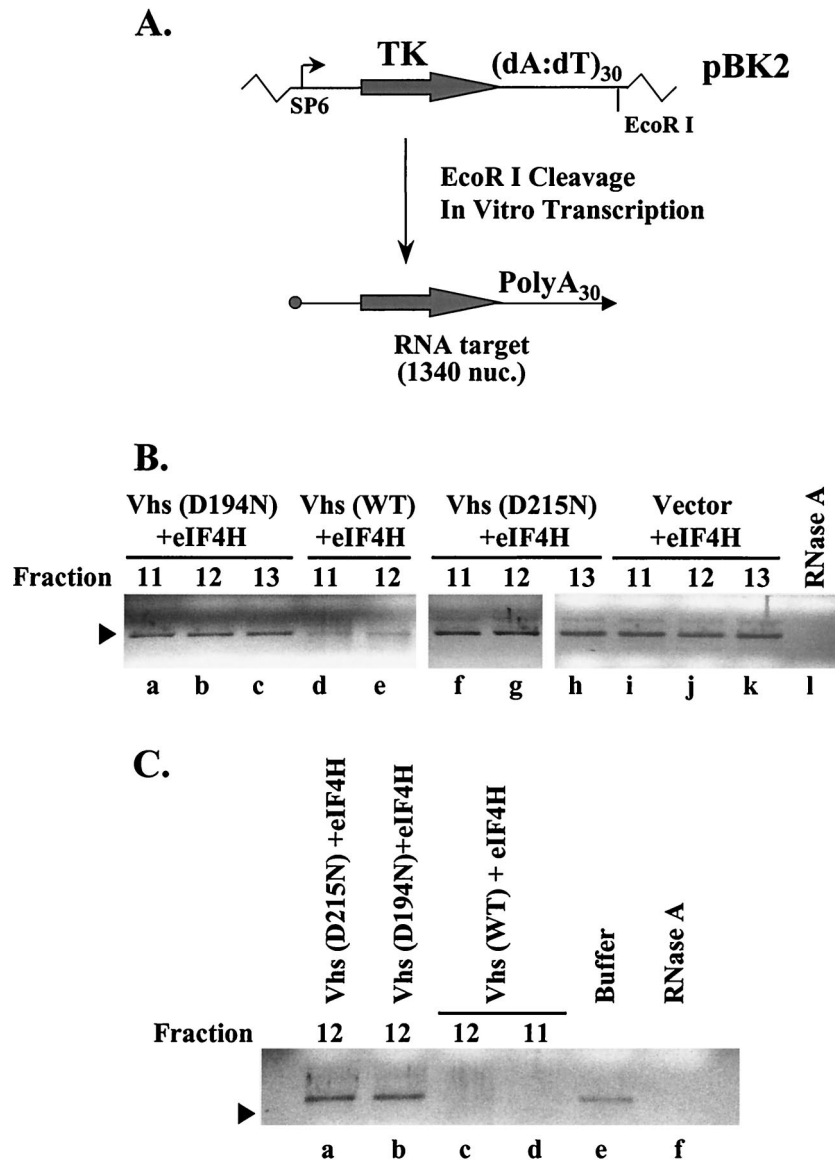


FIG. 6. RNase activity of peak fractions containing Vhs and GST-eIF4H. (A) *EcoRI* cleavage of pBK2 followed by in vitro transcription with SP6 RNA polymerase in the presence of a 5' cap analogue resulted in the production of a capped, 1,340 nucleotide (nuc.) target RNA with a 35-nucleotide poly(A) tail encoded by the plasmid. TK, thymidine kinase. (B and C) Target RNAs were incubated for 3 h (B) or 16 h (C) with degradation buffer (C, lane e), with RNase A (B, lane l, and C, lane f), or with the indicated HiTrap SP Sepharose fractions from bacteria expressing just GST-eIF4H (B, lanes i to k), GST-eIF4H plus Vhs D194N (B, lanes a to c, and C, lane b), GST-eIF4H plus Vhs D215N (B, lanes f to h, and C, lane A), or GST-eIF4H plus wild-type (WT) Vhs (B, lanes d and e, and C, lanes c and d). RNAs were extracted with phenol-chloroform, precipitated from ethanol, electrophoresed through 1.2% agarose gels, and visualized by staining with ethidium bromide.

Vhs, and second, it targets Vhs to mRNAs and regions of translation initiation. Recently, Lu and coworkers published data that would appear to favor model 2 (40). Unfractionated lysates from yeast cells that expressed the Vhs polypeptide lacked RNase activity until after they were supplemented with unfractionated rabbit reticulocyte lysates. This observation is consistent with a model in which one or more mammalian factors are required to activate the nuclease activity of Vhs. However, alternative explanations are possible. An important test of these models will be to purify Vhs to homogeneity and determine whether it has nuclease activity in the absence of eIF4H.

The Vhs homologues of the alphaherpesviruses share three blocks of homology (Fig. 1), only two of which, the amino-terminal and internal domains, are related to regions of the cellular and phage nucleases. This result raises the question as to the function of the carboxyl-terminal domain of Vhs. One possibility is that the carboxyl-terminal domain contains sequences required for the interaction of Vhs with eIF4H. In apparent support of this model, a point mutation that changes arginine 435 to histidine abolishes the interaction, as does shortening Vhs from the carboxyl terminus to 454 amino acids (19). However, the interaction is also abolished by several point mutations within the internal domain, as well as by the

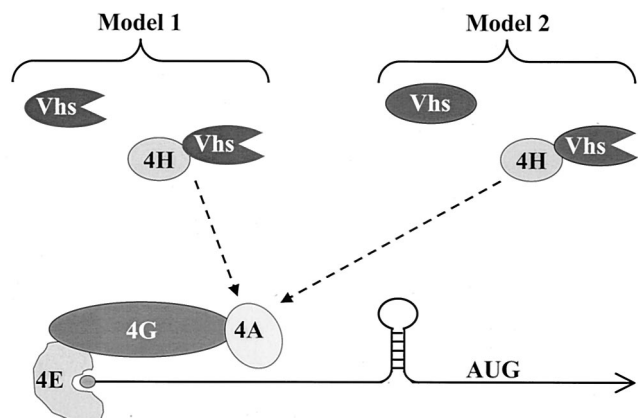


FIG. 7. Models for Vhs targeting. In model 1, Vhs is itself an RNase. It is targeted to mRNAs and to regions of translation initiation through its interaction with eIF4H. The functional, and perhaps physical, interaction of eIF4H with eIF4A is depicted by the broken arrow. In model 2, Vhs is an essential component of an RNase. However, it does not become active until after binding to eIF4H. The active nuclease is targeted by virtue of its eIF4H component.

removal of the first 88 residues from the amino terminus of the protein (19). This finding may indicate that the interaction domain of Vhs is formed by the folding together of residues from different parts of the protein. Alternatively, it is possible that the interaction domain consists of contiguous residues in the primary sequence, but mutations in other parts of the molecule inhibit the interaction with eIF4H by disrupting the folding of the protein. Clearly, additional experiments are required to define the domain of Vhs required for its interaction with eIF4H.

In view of the role of Vhs in mRNA degradation, it is interesting that many of the eukaryotic, bacterial, and phage nucleases with which it shares similarities are involved in DNA repair and replication. One possible explanation is that the conserved residues in Vhs and the cellular nucleases are part of a motif shared by a number of DNases and RNases and that residues responsible for making Vhs an RNase lie elsewhere.

The observation that Vhs binds a helicase accessory factor is particularly interesting in view of studies showing that, in bacteria and yeasts, nucleases involved in mRNA decay also interact with RNA helicases. In bacteria, the degradation of many mRNAs is catalyzed by the degradosome, a multiprotein complex containing endo- and exonucleases (RNase E and polynucleotide phosphorylase), as well as enolase and RhlB, a member of the DEAD-box family of ATP-dependent RNA helicases (8, 9, 48, 72). The purified degradosome has an ATP-dependent activity that facilitates its ability to degrade structured RNAs, and antibody against RhlB inhibits this activity (48). These results suggest that the unwinding of RNA secondary structures by RhlB plays an important role in the degradosome-mediated degradation of mRNAs. Similarly, the exosome is a large complex of proteins that mediates a number of RNA-processing reactions in yeast cells and perhaps mammalian cells (1, 42, 70, 71). Among these, at least in yeast cells, is the 3'-to-5' degradation of mRNAs (30, 71). The yeast exosome contains at least 10 core proteins, several of them exonucleases, as well as a number of associated factors, including

the Ski2p RNA helicase (1, 7, 71). Genetic studies indicate that Ski2p facilitates the 3'-to-5' degradation of mRNAs, suggesting that the helicase may be important for mRNA decay via disruption of RNA secondary structures or RNA-protein interactions or, perhaps, by targeting of RNA to the exosome (30, 71). Whether the eIF4H-stimulated helicase activity of eIF4A facilitates or in some way modulates the nuclease activity of Vhs remains an important unanswered question.

The above models of Vhs activity hypothesize that the interaction with eIF4H is important for targeting Vhs to mRNAs and regions of translation initiation. However, in this study, preparations containing recombinant Vhs and GST-eIF4H lacked the specificity that is observed for Vhs in vivo. This result suggests that the interaction with eIF4H may be necessary for the targeting of Vhs but not sufficient for it. The interaction between Vhs and eIF4H may be just one in a chain of protein-protein or protein-RNA interactions that are important for normal Vhs activity. Other important interactions may include those between eIF4H and eIF4A, between eIF4A and eIF4G, between eIF4G and eIF4E, or between eIF4G and internal ribosome entry site elements. These and other possibilities are under investigation.

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