

Evidence for Translational Regulation by the Herpes Simplex Virus Virion Host Shutoff Protein[▽]

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The herpes simplex virus (HSV) virion host shutoff protein (vhs) encoded by gene UL41 is an mRNA-specific RNase that triggers accelerated degradation of host and viral mRNAs in infected cells. We report here that vhs is also able to modulate reporter gene expression without greatly altering the levels of the target mRNA in transient-transfection assays conducted in HeLa cells. We monitored the effects of vhs on a panel of bicistronic reporter constructs bearing a variety of internal ribosome entry sites (IRESs) located between two test cistrons. As expected, vhs inhibited the expression of the 5' cistrons of all of these constructs; however, the response of the 3' cistron varied with the IRES: expression driven from the wild-type EMCV IRES was strongly suppressed, while expression controlled by a mutant EMCV IRES and the cellular ApaF1, BiP, and DAP5 IRES elements was strongly activated. In addition, several HSV type 1 (HSV-1) 5' untranslated region (5' UTR) sequences also served as positive vhs response elements in this assay. IRES activation was also observed in 293 and HepG2 cells, but no such response was observed in Vero cells. Mutational analysis has yet to uncouple the ability of vhs to activate 3' cistron expression from its shutoff activity. Remarkably, repression of 5' cistron expression could be observed under conditions where the levels of the reporter RNA were not correspondingly reduced. These data provide strong evidence that vhs can modulate gene expression at the level of translation and that it is able to activate cap-independent translation through specific *cis*-acting elements.

The virion host shutoff protein (vhs) encoded by herpes simplex virus (HSV) gene UL41 is an endoribonuclease that is packaged into the tegument of mature HSV virions. Once delivered into the cytoplasm of newly infected cells, vhs triggers shutoff of host protein synthesis, disruption of preexisting polysomes, and degradation of host mRNAs (reviewed in reference 62). The vhs-dependent shutoff system destabilizes many cellular and viral mRNAs (36, 46, 67). The rapid decline in host mRNA levels presumably helps viral mRNAs gain access to the cellular translational apparatus. In addition, the relatively short half-lives of viral mRNAs contribute to the sharp transitions between the successive phases of viral protein synthesis by tightly coupling changes in the rates of synthesis of viral mRNAs to altered mRNA levels (46). These effects enhance virus replication and may account for the modest reduction in virus yield displayed by vhs mutants in cultured Vero cells (55, 61).

vhs also plays a critical role in HSV pathogenesis: vhs mutants are severely impaired for replication in the corneas and central nervous systems of mice and cannot efficiently establish or reactivate from latency (63, 65, 66). Mounting evidence indicates that this attenuation stems at least in part from an impaired ability to disarm elements of the innate and adaptive host immune responses (reviewed in reference 62). For example, vhs suppresses certain innate cellular antiviral responses, including production of proinflammatory cytokines and che-

mokines (68); dampens the type I interferon system (11, 45, 49, 78); and blocks activation of dendritic cells (58). Moreover, vhs mutants display enhanced virulence in knockout mice lacking type I interferon (IFN) receptors (37, 45) or Stat1 (48) and are hypersensitive to the antiviral effects of IFN in some cells in tissue culture (11, 49, 68). Thus, vhs is arguably a *bona fide* virulence factor.

vhs present in extracts of HSV virions or purified from bacteria has nonspecific RNase activity capable of degrading all RNA substrates (15, 70, 71, 79). However, vhs is highly selective *in vivo*, targeting mRNAs and sparing other cytoplasmic RNAs (36, 46). *In vivo* and in mammalian whole-cell extracts, vhs-induced decay of at least some mRNAs initiates near regions of translation initiation and proceeds in an overall 5'-to-3' direction (12, 13, 29, 52). Moreover, vhs binds to the translation initiation factors eIF4H, eIF4B, and eIF4A II, all components of the cap recognition factor eIF4F (10, 16, 17). Thus, it has been proposed that vhs selectively targets actively translated mRNAs through interactions with eIF4F components (17). Consistent with this hypothesis, recent data document that eIF4H is required for vhs activity *in vivo* (59).

A previous report from this laboratory documented that the internal ribosome entry sites (IRESs) of the picornaviruses poliovirus and encephalomyocarditis virus (EMCV) strongly target vhs-induced RNA cleavage events to sequences immediately 3' to the IRES in an *in vitro* translation system derived from rabbit reticulocyte lysates (RRL) (13). IRES elements are highly structured RNA sequences that are able to direct cap-independent translational initiation (reviewed in references 21, 25, 30, and 64). In the case of the poliovirus and EMCV elements, this is achieved by directly recruiting the eIF4F scaffolding protein eIF4G, thus bypassing the require-

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ment for the cap-binding eIF4F subunit, eIF4E (reviewed in reference 30). Based on these data, we suggested that vhs is strongly targeted to the picornavirus IRES elements via interactions with eIF4 factors.

A growing number of cellular mRNAs have been proposed to bear IRES elements in their 5' untranslated regions (5' UTRs). These include many that are involved in cellular stress responses, apoptosis, and cell cycle progression (24, 64, 74). Given the striking ability of picornavirus IRES elements to target vhs RNase activity *in vitro*, we asked whether viral and cellular IRES elements are able to modify the susceptibility of mRNAs to vhs *in vivo*. During the course of preliminary experiments designed to test this hypothesis, we unexpectedly discovered that vhs is able to strongly activate gene expression controlled by some cellular IRES elements and HSV 5' UTR sequences in *in vivo* bicistronic reporter assays. These observations are the subject of the present report.

MATERIALS AND METHODS

Plasmids. The pβgal/CAT (23), pβgal/EMCV/CAT, pβgal/Apaf1/CAT, pβgal/Dap5/CAT, and pβgal/BiP/CAT constructs were obtained from Martin Holcik, University of Ottawa, and the rhinovirus 2A protease vectors p2A and p2Amut (53) were from Joachim Seipelt, Medical University of Vienna.

Plasmids containing the GW3 and GW8 mutant IRESs were originally obtained as *in vitro* expression constructs from Eckard Wimmer, State University of New York (77). These mutated EMCV IRESs were excised as EcoRI/BssHII fragments, blunt ends were created using Klenow polymerase, and the gel-purified products were ligated into pβgal/CAT that had been digested with XhoI and similarly blunt ended.

pβgal/TK/CAT, pβgal/UL12/CAT, and pβgal/gC/CAT were each created by PCR amplification of the appropriate sections of the HSV type 1 (HSV-1) bacmid KOS37 (18) extending from the transcriptional start site to the last nucleotide upstream of the translation start codons of thymidine kinase (TK), UL12, and glycoprotein C (gC) mRNA (nucleotides [nt] 109, 159, and 143, respectively), using primer pairs that added terminal XhoI sites to facilitate subsequent cloning into pβgal/CAT. The primers used were as follows: TK forward, 5' GGCTCGAGATACCGAGCGACCCTGCAG; TK reverse, 5' GGCTCGAGACGCGCTCTCTACAAGGCGC; UL12 forward, 5' GGCTCGAGTATCTGTCGTCGGTGGCGCTG; UL12 reverse, 5' GGCTCGAGTCCGAGACGACGTGGGGG; gC forward, 5' GGCTCGAGACCCCTCACTATCGAGGGG; and gC reverse, 5' GGCTCGAGCCCCGACGCTCCC. All oligonucleotides used in this study were synthesized by Integrated DNA Technologies, Inc.

A plasmid bearing a stable stem-loop in the 5' UTR of pβgal/BiP/CAT (pSL/βgal/BiP/CAT) was fashioned using the stem-loop sequence described by Attal et al. (4). Two oligonucleotides that contain the stem-loop sequence (5' AGCTGGGCGGGCGCGCGCCGCGCCGAGGTAC and 5' CTGGCCGGGCGCGCGCGCGCCGCGCA) were annealed, phosphorylated, and ligated into βgal/BiP/CAT that had previously been digested with HindIII and KpnI.

Transfections. HeLa cells in 24-well plates were transfected with bicistronic reporter plasmids along with specified effector plasmids using Lipofectamine 2000 (Invitrogen). The medium was changed the next morning, and the following day lysates were prepared using 1× reporter lysis buffer (Promega). β-Galactosidase and chloramphenicol acetyltransferase (CAT) assays were then performed on portions of the lysates by standard procedures. To perform RNA analyses, the transfections were scaled up to 60-mm dishes containing 12-mm coverslips. To harvest, the coverslips were transferred to wells of a 24-well plate, and lysates were prepared for activity analyses; RNA from the remainder of the dish was harvested using TRIzol (Invitrogen). Purification of poly(A)⁺ RNA was accomplished using an Oligotex mRNA mini kit (Qiagen) according to the manufacturer's instructions.

Northern blot analysis. RNA samples were electrophoresed through a 1.2% agarose-formaldehyde gel and transferred to a GeneScreen membrane (NEN). All hybridizations were done using ExpressHyb (Clontech) according to the user's manual. The CAT probe was derived from PCR amplification of the CAT gene using the primers 5' ATCACTGGATATACCACCGTTG and 5' CTGGTGAACTCACCAGG. The probe was radiolabeled with ³²P by random priming.



FIG. 1. Schematic representation of the bicistronic reporter constructs used in this study (not drawn to scale). The test IRES elements were inserted between the 5' β-galactosidase (βgal) and 3' CAT cistrons. The locations of the CMV IE promoter and two alternative polyadenylation signals are indicated.

Primer extension. Primer extension was performed essentially as previously described (7). The ³²P-labeled primer used was 5'-GCGGCCGTACTAGTGATCCGAG. Annealing reaction mixtures were incubated at 65°C for 1 h, followed by slow cooling to room temperature. For the extension reactions, the enzyme used was SuperScript II (Invitrogen).

***In vitro* vhs decay assays.** The *in vitro* vhs decay assays have been described elsewhere (12, 13). To summarize, vhs RNA was translated in rabbit reticulocyte lysate (Promega) and incubated with reporter RNA substrates that had been generated by *in vitro* transcription (Ambion) in the presence of [³²P]CTP. Aliquots of the reaction mixture were removed at various times and immediately added to TRIzol (Invitrogen) containing carrier RNA (Ambion). The samples were extracted, and RNA was recovered by precipitation. The RNA pellet was then dried and resuspended in RNase-free water. RNA samples were analyzed by electrophoresis through agarose-formaldehyde gels.

RESULTS AND DISCUSSION

vhs activates gene expression driven from a mutant version of the EMCV IRES. The ability of the poliovirus and EMCV IRES elements to strongly target vhs-induced RNA cleavage events in the RRL-based *in vitro* assay system (13) raised the possibility that vhs might selectively target mRNAs bearing certain IRES elements *in vivo*. As one approach to testing this possibility, we surveyed the effects of vhs on the activities of a variety of IRES elements using a standard bicistronic reporter assay system (51). In this assay, IRES activity is monitored by testing the ability of the element to activate expression of a 3' cistron when inserted between two reporter open reading frames (ORFs) (in this case β-galactosidase and chloramphenicol acetyltransferase) driven from a common upstream human cytomegalovirus (HCMV) immediate-early (IE) promoter (pβgal/CAT) (Fig. 1) (23). As detailed below, these experiments unexpectedly revealed that vhs strongly activates expression driven by some IRES elements.

We first examined the effects of vhs on expression driven by the canonical EMCV IRES. As controls, we also tested two inactive mutant derivatives of the IRES, GW3 and GW8 (77). The 36-nt GW8 deletion disrupts the J/K structural domains that are critical for binding of eIF4G, eIF4A, and eIF4B to the IRES (32, 33), while the 3-nt GW3 deletion alters the apex of the distal I domain, which does not interact with eIF4G. Fixed amounts of the EMCV IRES reporter plasmids were transfected into HeLa cells along with increasing quantities of a vhs expression vector, and reporter gene expression was assessed ~43 h later (Fig. 2). The wild-type EMCV IRES supported high levels of 3' cistron (CAT) expression in the absence of vhs, while the GW3 and GW8 mutants were inactive, as expected. We have previously shown that CAT expression mediated by the EMCV IRES in the pβgal/CAT vector system occurs via translation of the intended full-length bicistronic reporter mRNA and hence represents *bona fide* internal initiation of translation (57). As expected, vhs strongly inhibited

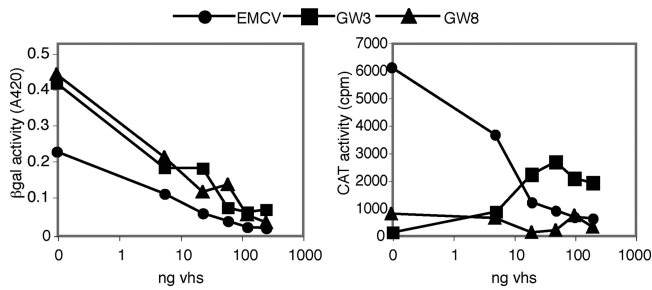


FIG. 2. Effects of vhs on 5' and 3' cistron expression from reporter constructs bearing wild-type and mutant EMCV IRES elements. HeLa cells were transfected with the indicated reporter plasmids and increasing amounts of a vhs expression vector (pCMVvhs). Cell extracts were then assayed for β -galactosidase (left panel) and CAT (right panel) enzymatic activities.

expression of the 5' (cap-dependent) β -galactosidase cistrons of all three constructs, similar to its effects on monocistronic reporters in similar transient-transfection assays (28, 47). The differences in β -galactosidase expression between the plasmids in the absence of vhs were not consistently observed. Also as expected, vhs strongly inhibited expression of the 3' CAT cistron driven from the wild-type EMCV IRES. Remarkably, CAT expression from the GW3 construct was consistently activated by intermediate doses of the vhs expression vector before declining at the highest doses used; in contrast, vhs had no such effect on the GW8 element. Thus, the 3-nt deletion in the GW3 IRES radically altered the response of the 3' cistron to vhs, without altering the ability of vhs to inhibit expression of the 5' cistron encoded by the same mRNA.

It seemed possible that the GW3 lesion abolishes the ability of the GW3 IRES to target vhs-induced endoribonucleolytic cleavage events and thus unmask a positive regulatory activity of vhs that not is evident on the wild-type element. We therefore compared the abilities of wild-type and mutant IRES elements to target vhs-dependent RNA cleavage events in the *in vitro* RRL assay for vhs-induced RNA decay. To this end, uniformly labeled EMCV transcripts bearing the wild-type, GW3, and GW8 IRES elements at their 5' ends were added to RRL containing pretranslated vhs, and the fate of the RNA was followed over time (Fig. 3). As shown previously (13), the wild-type EMCV IRES targets vhs-induced endonucleolytic cleavage events to a cluster of closely spaced sites located immediately 3' to the IRES, giving rise to a ca. 600-nt 5' fragment bearing the IRES and an 1,800-nt 3' fragment cor-

responding to the 3' portion of the transcript. The 5' fragment is quite stable over the course of the reaction, while the 3' fragment undergoes rapid 5'-to-3' decay (52). We found that the GW3 IRES generated the same initial cleavage products as RNA bearing the wild-type IRES, although the 600-nt IRES-bearing fragment appeared to be less stable than its wild-type counterpart. Thus, the GW3 mutation does not abolish the ability of the EMCV IRES to target vhs-dependent RNA cleavage events in the RRL system. In contrast, RNA bearing the GW8 mutation gave rise to a heterogeneous set of decay products similar to that previously observed for RNAs lacking an IRES (12). Whether the lack of targeting activity of the GW8 element is due to an inability of the GW8 IRES to bind eIF4G remains to be determined. Overall, these data indicate that the very different *in vivo* responses of the wild-type and GW3 EMCV IRES elements to vhs *in vivo* do not correlate with ability of these elements to target RNA cleavage events *in vitro*. Evidence arguing that 3' cistron activation does not involve generation of monocistronic CAT RNA via cleavage of the reporter RNA is presented below.

vhs activates 3' cistron expression driven by some cellular IRES elements and HSV-1 5' UTR sequences. The preceding data indicate that at least one RNA sequence is capable of serving as a positive vhs response element *in vivo*. To determine if the activation effect is limited to a mutant form of the EMCV IRES, we monitored the responses of the IRES elements derived from the cellular mRNAs encoding BiP (41), Dap5 (22), and ApaF1 (8) in the same bicistronic reporter assay (Fig. 4). Once again, vhs strongly inhibited expression of the 5' β -galactosidase cistrons of all of the constructs and blocked expression of the 3' CAT cistron driven from the wild-type EMCV IRES. As previously emphasized (35), the cellular IRES elements were much less active than the EMCV IRES in the absence of vhs, although activity was significantly above that of the no-IRES control. In all three cases expression of the 3' cistron was strongly induced by vhs, with CAT levels increasing to well above basal levels before declining at the highest doses of the vhs expression vector. Activation of the BiP construct was especially robust, ranging from 10- to >50-fold over the course of repeated experiments and giving rise to substantially higher levels of expression than the EMCV IRES in the absence of vhs. 3' cistron activation appeared to require specific sequences or RNA structural elements in the intercistronic region, as CAT expression from the parental bicistronic construct lacking any IRES was not enhanced, and a construct bearing a 655-nt "stuffer" fragment of HSV DNA (lacking

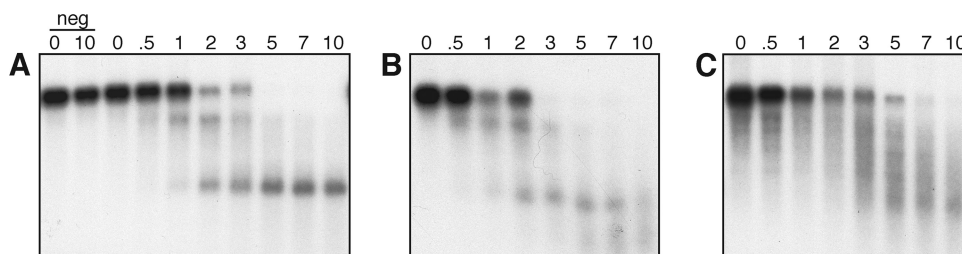


FIG. 3. The EMCV GW3 mutant IRES retains vhs targeting activity *in vitro*. Uniformly labeled EMCV transcripts containing wild-type (A), GW3 (B), and GW8 (C) IRES elements at their 5' ends were added to RRL containing pretranslated vhs, and aliquots were removed after the indicated times (minutes) and analyzed by gel electrophoresis. neg, RRL lacking pretranslated vhs.

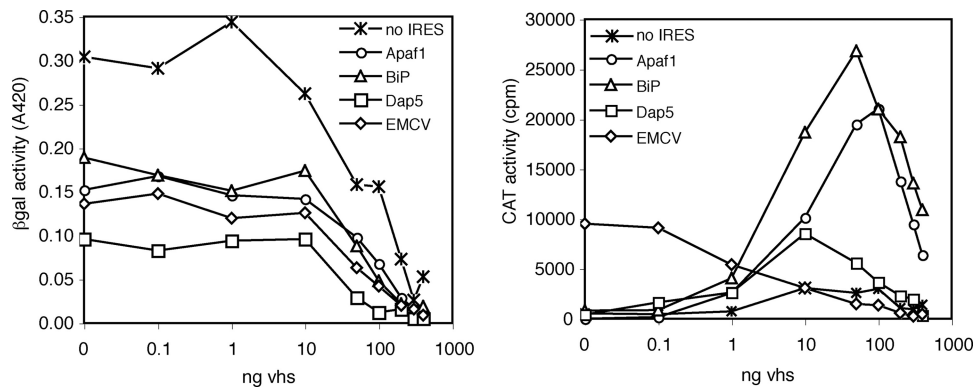


FIG. 4. Effects of vhs on 5' and 3' cistron expression from reporter constructs bearing various IRES elements. HeLa cells were transfected with the indicated reporter plasmids and increasing amounts of pCMVvhs. Cell extracts were then assayed for β -galactosidase (left panel) and CAT (right panel) enzymatic activities.

IRES activity and devoid of ATG triplets) inserted into the intercistronic region was not induced (data not shown). vhs-induced activation of the BiP IRES was also observed in HepG2 and 293 cells, documenting that the effect is not restricted to HeLa cells (not shown). However, vhs did not activate expression driven from the BiP IRES in Vero cells, although 5' cistron expression was strongly suppressed (Fig. 5). This finding is discussed further below.

The ability of certain picornavirus and cellular sequences to serve as positive vhs response elements in the bicistronic assay in HeLa cells prompted us to ask if HSV-1 5' UTRs display similar activity. To this end, we inserted the DNA sequences encoding the 5' UTRs of the HSV-1 UL12, thymidine kinase (TK), and glycoprotein C (gC) mRNAs into the p β Gal/CAT vector and tested their ability to promote 3' CAT cistron expression in the presence and absence of vhs (Fig. 6). None of the HSV-1 UTRs detectably enhanced 3' cistron activity in the absence of vhs; hence, these sequences do not serve as constitutive IRES elements. However, all three elements produced a positive response in the presence of vhs. These data demonstrate that some HSV-1 5' UTRs can serve as positive vhs response elements in the bicistronic reporter assay.

Activation correlates with the shutoff activity of vhs. The ability of vhs to strongly activate the expression of the 3' cistrons of certain bicistronic constructs was surprising and raised the possibility that vhs may have regulatory functions distinct from its well-studied host shutoff activity. As one ap-

proach to addressing this possibility, we asked whether the ability of vhs to activate 3' cistron expression displays the same mutational sensitivity profile as its host shutoff function. To this end, we examined a set of 12 single-amino-acid substitution mutants that have been previously characterized for shutoff activity and their ability to bind eIF4H and eIF4AII (15, 16). Ten of the mutants are devoid of shutoff activity, while the T211S and D261N constructs are partially active (15). Strikingly, T211S and D261N were also the only mutants capable of significantly activating 3' cistron expression from the construct bearing the BiP IRES (Table 1). A similar correlation between shutoff and 3' cistron activation was observed using a set of 10 in-frame linker insertion mutations distributed across the vhs open reading frame (reference 28 and data not shown). These data raise the possibility that activation of 3' cistron expression relies on the host shutoff function of vhs, which is thought to stem from its RNase activity. Exhaustive random mutagenesis, such as has been applied to the Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) host shutoff proteins SOX and BGLF5 (19, 56), will be required to further test this hypothesis.

vhs does not generate detectable levels of monocistronic CAT RNA. vhs has opposing effects on the expression of the 5' and 3' cistrons of reporter RNAs bearing positive vhs response elements. Given the correlation between 3' cistron activation and the shutoff activity of vhs, as well as the previous evidence that vhs-induced decay of some mRNAs proceeds 5' to 3', we considered the possibility that vhs activates 3' cistron expression by removing the 5' portion of the bicistronic transcript, generating monocistronic RNA encompassing the 3' CAT open reading frame. This hypothesis stems from a critique of the bicistronic assay for IRES activity published by Kozak (35). Kozak suggested that at least some IRES elements function poorly if at all from intercistronic positions; rather, their apparent ability to drive second-cistron expression in the bicistronic assay was proposed to stem from unanticipated processes that give rise to monocistronic mRNA bearing the test cistron in the 5' position. Proposed mechanisms include cryptic promoters or splice acceptors in the "IRES" insert or RNA breakage mediated by nucleases. Indeed, the activities of several putative cellular IRESs have since been shown to arise

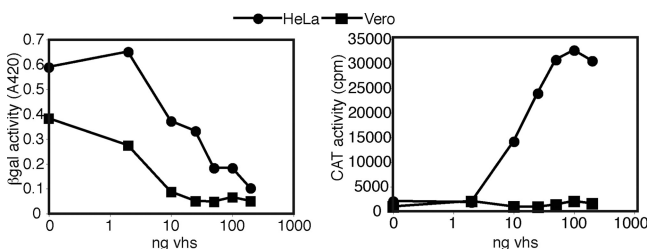


FIG. 5. vhs does not activate the BiP IRES in Vero cells. HeLa and Vero cells were transfected with the p β Gal/BiP/CAT reporter plasmid and increasing amounts of pCMVvhs. Cell extracts were then assayed for β -galactosidase (left panel) and CAT (right panel) enzymatic activities.

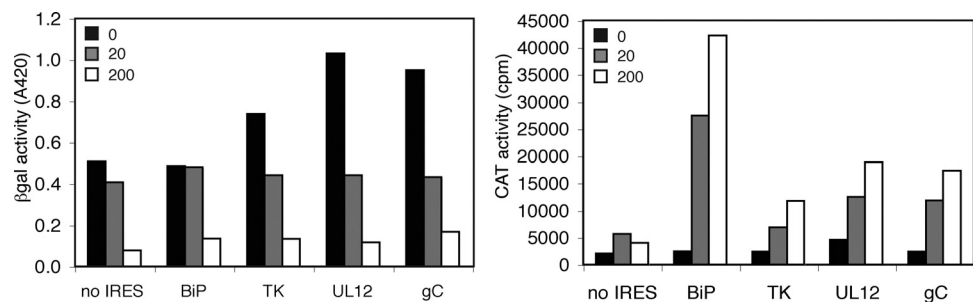


FIG. 6. Several HSV-1 5' UTR sequences serve as vhs positive response elements. HeLa cells were transfected with the reporter plasmids bearing the indicated elements inserted into the intercistronic regions, along with 0, 20, or 200 ng of pCMVvhs. Cell extracts were then assayed for β-galactosidase and CAT enzymatic activities.

primarily through cryptic splicing or promoter sequences within the “IRES” (5, 6, 21, 39, 57, 75, 76). However, to the best of our knowledge, 3' cistron activation via nucleolytic cleavage has not yet been documented.

To address this possibility, we examined the effects of vhs on the levels and structure of the reporter RNA arising from the pβGal/BiP/CAT plasmid by Northern blot analysis (Fig. 7). Samples of the transfected cultures were analyzed in parallel for β-galactosidase and CAT enzymatic activity to assess the degree of 5' cistron repression and 3' cistron activation. In control cultures lacking vhs, the pβGal/BiP/CAT plasmid gave rise to a single prominent band of the length predicted for full-length bicistronic mRNA (ca. 5.5 kb), with no evidence of smaller RNAs of the size predicted for monocistronic CAT transcripts (ca. 1.5 to 2.0 kb). Surprisingly, in five independent experiments the negative effects of vhs on 5' cistron expression were substantially greater than the effects on reporter mRNA levels, with reductions in protein expression and RNA levels averaging 75% and 15%, respectively. For example, in the experiment depicted in Fig. 7, the highest dose of vhs reduced β-galactosidase levels 4-fold and enhanced CAT expression 13-fold, but reporter RNA levels declined by only 30%. Of note, vhs did not provoke the appearance of discrete novel RNA species of the size predicted for monocistronic CAT transcripts (ca. 1.5 to 2.0 kb) in this or any other experiment, even in heavily overexposed images. Similar results have been

obtained with the construct bearing the gC 5' UTR (data not shown).

vhs-induced decay of at least some mRNAs initiates at or close to the 5' end in HSV-infected cells and in the RRL-based *in vitro* assay system (12, 29). Thus, it seemed possible that the reporter mRNA was “decapitated” by vhs-induced endonucleolytic cleavage very close to the 5' end. However, primer extension analysis using a radiolabeled primer that anneals to sequences extending from ca. 70 to 93 nt downstream of the

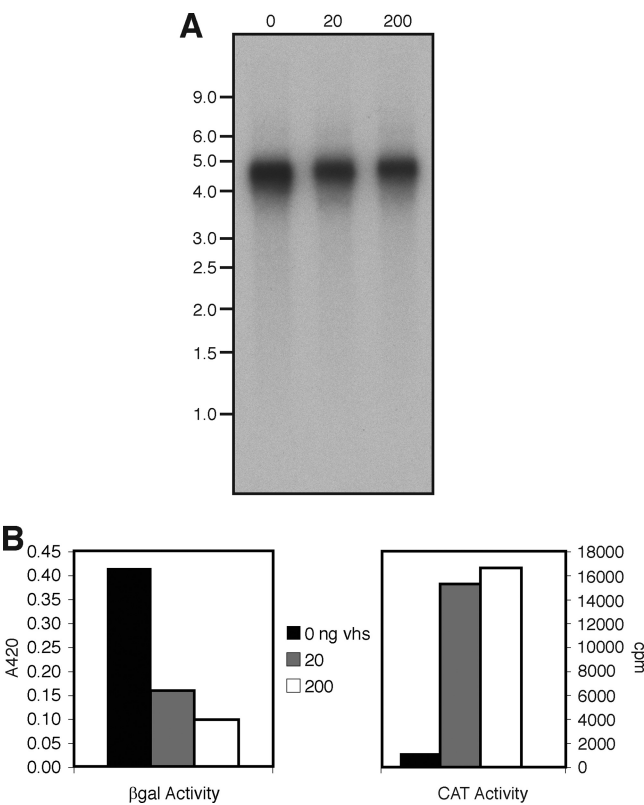


FIG. 7. vhs modulates 5' and 3' cistron expression without greatly altering reporter mRNA levels or structure. (A) Northern blot analysis of poly(A)⁺ RNA extracted from HeLa cells transfected with pβgal/BiP/CAT and 0, 20, or 200 ng of pCMVvhs, probed for CAT sequences. The electrophoretic mobilities of marker RNAs of the indicated sizes (kb) are displayed on the left. (B) β-Galactosidase and CAT activities in extracts of the transfected cells.

TABLE 1. 3' cistron activation correlates with host shutoff				
vhs allele	Shutoff activity	Binding to:		3' cistron activation (%) ^a
		eIF4H	EIF4A II	
Wild type	+++	+	+	100
D34N	—	+	+	0
D82N	—	+	+	2
E192Q	—	+	+	4
D194N	—	+	+	2
D195N	—	+	+	0
T211S	++	+	+	96
T211A	—	—	+	1
D213N	—	+	+	4
T214I	—	—	+	5
D215N	—	+	+	11
D261N	+	+	+	41
R435H	—	—	+	4

^a Percentage of wild-type vhs level.

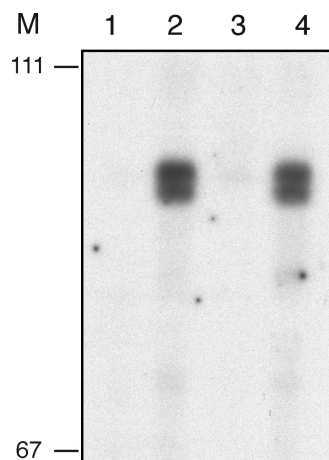


FIG. 8. vhs does not alter the 5' end of the reporter mRNA. Total RNA extracted from HeLa cells transfected with pβgal/BiP/CAT in the presence or absence of pCMVvhs was analyzed by primer extension as described in Materials and Methods, using a ^{32}P -labeled oligonucleotide primer complementary to residues 70 to 93 of the βgal/BiP/CAT reporter RNA. Products were resolved on 8% polyacrylamide sequencing gels. HeLa cells were transfected with pUC19 (lane 1), βgal/BiP/CAT and pUC19 (lane 2), pCMVvhs and pUC19 (lane 3), or βgal/BiP/CAT, pCMVvhs, and pUC19 (lane 4). Lane M, sizes of marker pUC19 MspI cleavage fragments in nt.

transcription initiation site revealed that reporter mRNA extracted from cells expressing vhs gave rise to the same ca. 93-nt extension products as RNA extracted from cells lacking vhs (Fig. 8). These data document that the mRNA retained an intact 5' region. The Superscript II reverse transcriptase used in this experiment efficiently copies the 5' cap into the cDNA product (60), and the gel system used readily resolves single-nucleotide size differences. Therefore, the data also suggest that the reporter mRNA retains the 5' cap. Taken in combination with the Northern blot data, these results indicate that vhs can repress 5' cistron expression without inducing a corresponding loss or 5' alteration of the reporter mRNA. In addition, the data provide no support for the hypothesis that 3' cistron activation is achieved via degradation of the reporter mRNA to generate monocistronic CAT mRNA. The simplest interpretation is that both effects occur the translational level.

The finding that vhs can extinguish reporter gene expression in the absence of significant mRNA loss contrasts with the results reported by Pak et al. (47), who found that vhs greatly reduces the levels of reporter mRNA in similar transient-co-transfection assays. The experiments of Pak et al. differed from those presented in Fig. 7 in two potentially significant ways. First, Pak et al. conducted their assays in Vero cells rather than HeLa cells. Second, they used a monocistronic CAT reporter plasmid (pSV3CAT) which lacks an IRES. We found that vhs strongly represses CAT activity specified by pSV3CAT in HeLa cells without triggering a corresponding loss of reporter mRNA (Fig. 9), a result that parallels our findings with the pβGal/BiP/CAT reporter. In contrast, we confirmed that vhs induces extensive loss of pSV3CAT reporter mRNA in Vero cells (data not shown). Thus, cell line-specific differences in the effects of vhs on reporter mRNA stability appear to account for the distinct results obtained in the two studies. Although the

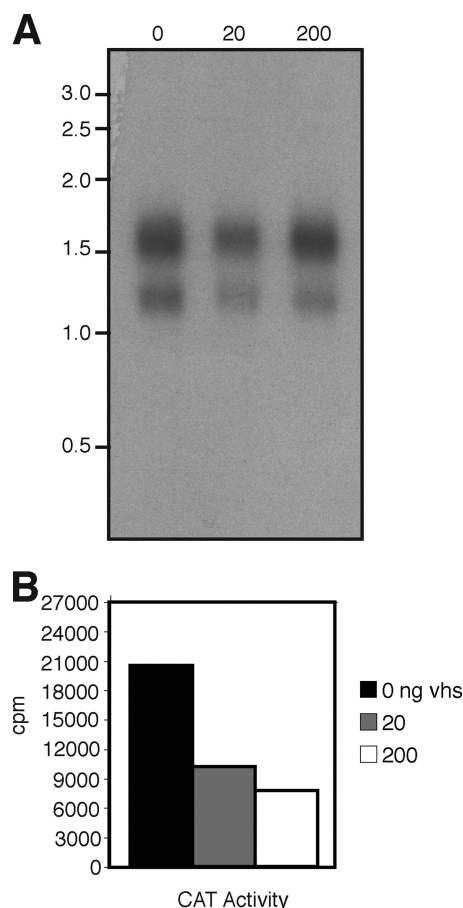


FIG. 9. vhs suppresses expression from pSV3CAT without triggering a corresponding loss of reporter mRNA in HeLa cells. (A) Northern blot analysis of poly(A)⁺ RNA extracted from HeLa cells transfected with pSV3CAT and 0, 20, or 200 ng of pCMVvhs, probed for CAT sequences. The electrophoretic mobilities of marker RNAs of the indicated sizes (kb) are displayed on the left. (B) CAT activity in extracts of the transfected cells.

molecular basis for this difference remains to be determined, it is important to note that HeLa cells are competent to support vhs-induced mRNA decay during HSV infection (59).

Cellular IRES elements (including the BiP sequence) are normally located in the 5' UTRs of monocistronic mRNAs. Such mRNAs can therefore potentially be translated by both cap-dependent and cap-independent mechanisms. Indeed, IRES activity is routinely assayed in bicistronic reporter assays to eliminate the contribution of cap-dependent initiation to reporter cistron expression. It was therefore of interest to determine the effect of vhs on a monocistronic CAT reporter bearing the BiP IRES in the 5' UTR. We found that vhs efficiently suppressed CAT expression from this construct in HeLa cells (data not shown). The simplest interpretation of this finding is that cap-dependent translation of this reporter mRNA is substantially more efficient than the cap-independent activity of the IRES under the conditions of our assays.

IRES activation does not require translation of the 5' cistron. Given the opposing effects of vhs on expression of the 5' and 3' cistrons of bicistronic constructs bearing positive vhs response elements, it seemed possible that activation of the 3'

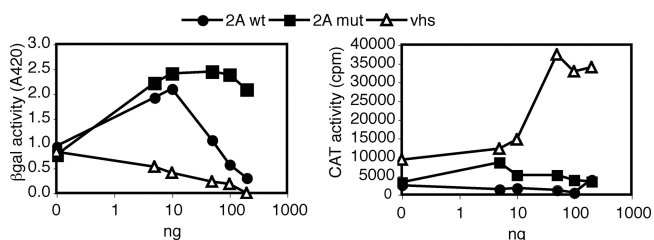


FIG. 10. Rhinovirus 2A protease does not activate 3' cistron expression. HeLa cells were transfected with the β gal/BiP/CAT bicistronic reporter plasmid and increasing amounts of expression vectors encoding vhs or wild-type (wt) or mutant (mut) HRV2 2A protease. Cell extracts were then analyzed for β -galactosidase (left panel) and CAT (right panel) enzymatic activities.

cistron stems from inhibition of cap-dependent translation of the 5' cistron. As one test of this idea, we asked if the BiP IRES is activated when cap-dependent translation of the 5' cistron is inhibited via expression of the human rhinovirus 2A protease (20). The 2A proteases of members of the *Rhinovirus* and *Enterovirus* genera of the *Picornaviridae* cleave eIF4G into two fragments, separating the N-terminal domain, which binds the cap-binding protein eIF4E and poly(A)-binding protein, from the C-terminal domain, which binds eIF4A and eIF3 (reviewed in reference 40). Previous studies have documented that the BiP IRES remains functional *in vitro* and in poliovirus-infected cells following cleavage of eIF4G (26, 27, 42, 73). As shown in Fig. 10, expression of β -galactosidase was effectively inhibited by high doses of a vector expressing wild-type 2A protease, while a vector encoding a catalytically inactive form of the protease (53) had no such inhibitory effect (although low doses of both vectors consistently enhanced 5' cistron expression, an effect that we have previously noted [57]). Despite the inhibition of 5' cistron expression, 3' cistron activity was not significantly enhanced. Thus, inhibition of cap-dependent translation via cleavage of eIF4G does not activate expression driven from the BiP element in the absence of vhs.

Conversely, we asked whether translation of the 5' cistron is required in order for vhs to activate 3' cistron expression. We inserted a stable stem-loop into the 5' UTR of the β Gal/BiP/CAT reporter, just upstream of the β -galactosidase-coding sequences, to block cap-dependent scanning and translation of the 5' ORF (50). As shown in Fig. 11, the stem-loop severely inhibited expression of β -galactosidase but had no effect on the ability of vhs to activate CAT expression. Taken in combination, these data argue against a role of cap-dependent translation of the 5' ORF in modulating the BiP IRES.

Possible mechanisms and potential significance. The data presented in this report demonstrate that vhs can regulate reporter gene expression both positively and negatively without causing major changes in the levels of the reporter RNA when it is expressed in the absence of other HSV proteins in transiently transfected HeLa cells. The ability of vhs to inhibit reporter gene expression without triggering a corresponding loss or detectable alteration of the reporter mRNA is very intriguing and has not been reported previously, presumably because the effect is masked by vhs-induced RNA degradation in Vero cells. The simplest explanation of our findings is that vhs is able to inhibit cap-dependent translation initiation in

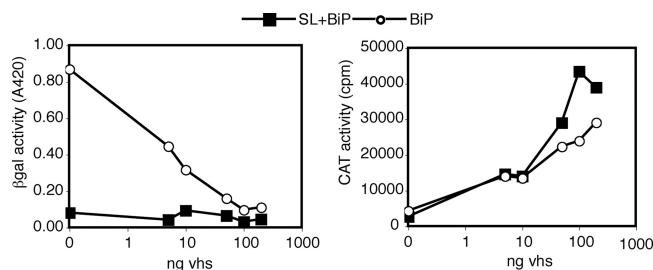


FIG. 11. Translation of the 5' cistron is not required for 3' cistron activation. HeLa cells were transfected with the β gal/BiP/CAT bicistronic reporter plasmid (BiP) or a derivative bearing a stem-loop inserted into the 5' UTR (SL+BiP) along with the indicated amounts of pCMVvhs. Cell extracts were then analyzed for β -galactosidase (left panel) and CAT (right panel) enzymatic activities.

addition to destabilizing mRNA, perhaps via induction of a cellular stress response or through its documented interactions with one or more eIF4 factors such as eIF4A, eIF4B, and eIF4H. It will be important to determine why vhs-induced RNA decay is less robust in HeLa cells than in Vero cells and to evaluate the relative contributions of mRNA decay and translational repression to vhs-induced host shutoff during HSV infection of a variety of cell types. In this context, it is interesting to note that although vhs inhibits the synthesis of most cellular proteins detectable by one-dimensional gel electrophoresis (55) and globally disrupts polysomes during HSV infection (72), transcriptional profiling experiments using microarrays suggest that only a subset of host mRNAs are strongly depleted during infection (54, 69). Because microarrays will detect some mRNA degradation fragments as well as intact mRNAs, it is possible that these studies underestimated the effect of vhs on host mRNA degradation; however, it is also possible that vhs inhibits host protein synthesis at the translational level during HSV infection, in addition to triggering loss of a subset of mRNAs.

The other major result to emerge from our study is that vhs is able to activate 3' cistron expression from certain bicistronic reporter RNA constructs in HeLa, 293, and HepG2 cells, in a process that in HeLa cells requires particular RNA sequences or structures in the intercistronic region. The effect is not observed in Vero cells, perhaps because vhs-induced RNA degradation is more pronounced in this cell line. As shown above, activation is not accompanied by detectable generation of monocistronic mRNA encompassing the 3' cistron and hence appears to represent activation of internal initiation of translation. We can envision three quite distinct models that could account for this activity. First, it is possible that 3' cistron expression is stimulated as a consequence of vhs-induced repression of cap-dependent initiation, perhaps because the IRES elements are then able to more effectively compete for translation initiation factors. Although this possibility seems inconsistent with our finding that 3' cistron expression is not induced when cap-dependent translation is inhibited via expression of the rhinovirus 2A protease, we do not think that it can be excluded.

Second, vhs expression may provoke a cellular response that activates IRES function. Consistent with this hypothesis, the cellular IRES elements used in our study are derived from

mRNAs encoding proteins with important roles in cellular stress responses. BiP is an endoplasmic reticulum (ER)-resident chaperone that is involved in the unfolded protein response (31), ApaF1 is a component of the apoptosome (81), and DAP5 is an eIF4G homolog that plays a role in cap-independent translation during stress responses and mitosis (38, 43, 44). However, vhs does detectably not trigger apoptosis or induce phosphorylation of eIF2 α , a hallmark of many stress responses (3), including the unfolded protein response (80), in our transfection assays (data not shown). Indeed, vhs antagonizes the induction of eIF2 α phosphorylation during HSV infection (49, 78). Moreover, the vhs positive response elements that we have identified include some sequences that have no detectable IRES activity in the absence of vhs (the GW3 mutant EMCV element, and several HSV 5' UTRs). Thus, we consider this stress response scenario unlikely.

A third model is that vhs either recognizes and binds particular RNA sequences or secondary structures, some of which have no inherent IRES activity on their own, or interacts with cellular factors that bind these elements, in either case recruiting cellular translation initiation factors. We consider this the most likely mechanism. Irrespective of which scenario is correct, it will be important to determine which cellular translation initiation factors and other components are required for vhs-induced activation and whether these vary between IRES elements. Of particular note, the BiP IRES appears to be unusually dependent on eIF4A (73), raising the possibility that the known interactions between vhs and eIF4A and/or its cofactors eIF4B and eIF4H play an important role.

Our finding that several HSV 5' UTRs serve as vhs positive response elements in bicistronic reporter assays raises the possibility that vhs may act to enhance the translation of certain HSV mRNAs during infection. The TK, UL12, and gC UTRs are predicted by the mfold algorithm (82) to adopt relatively stable secondary structures (ΔG s of -34.2 , -54.5 , and -63.2 kcal/mol, respectively). The 5' UTRs of efficiently translated human mRNAs are relatively unstructured (9), and stable secondary structure (ΔG of < -50 kcal/mol) within the 5' UTR presents a strong barrier to cap-dependent scanning (9, 34, 50). Thus, it is possible that at least some HSV 5' UTRs behave as vhs-dependent IRES elements in infected cells; alternatively, vhs may stimulate cap-dependent scanning of these mRNAs through its interactions with eIF4A. Consistent with a role for vhs in translation initiation, Esclatine et al. (14) have shown that certain human cell lines display prominent cytoplasmic stress granules (sites where mRNAs bearing stalled translation initiation complexes accumulate [1, 2]) following infection with vhs-null but not wild-type HSV-1. We are currently testing the role of vhs in the translation of viral mRNAs during infection.

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