JOURNAL OF VIROLOGY, Apr. 2005, p. 4090–4098 Vol. 79, No. 7
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.7.4090–4098.2005

Herpes Simplex Virus Infection Stabilizes Cellular IEX-1 mRNA

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Received 20 September 2004/Accepted 21 October 2004

Herpes simplex virus (HSV) virion host shutoff protein (vhs) destabilizes cellular and viral mRNAs. Previous work from several laboratories has indicated that vhs accelerates the turnover of most host mRNAs and provided evidence that at least some of these are degraded via endonucleolytic cleavage near regions of translational initiation followed by 5′→3′ decay. In contrast, several recent reports have argued that vhs is selective, preferentially targeting a subset of mRNAs including some that bear AU-rich instability elements (such as the stress-inducible IEX-1 mRNA). These reports concluded that vhs triggers deadenylation, 3′ cleavage, and 3′→5′ decay of IEX-1 mRNA. However, we report here that HSV infection does not increase the rate of degradation of IEX-1 mRNA; rather, actinomycin D chase assays indicate that the transcript is stabilized relative to that in uninfected cells in both the presence and absence of functional vhs. Moreover, deadenylated but otherwise intact IEX-1 mRNA was readily detected in uninfected cells cultured under our experimental conditions, and its relative abundance did not increase following HSV type 1 (HSV-1) infection. We confirm that HSV infection increases the relative abundance of a discrete 0.75-kb 3′-truncated IEX-1 RNA species in a vhs-dependent manner. This truncated transcript was also detected (albeit at lower levels) in cells infected with vhs mutants and in uninfected cells, where it increased in abundance in response to tumor necrosis factor alpha, cycloheximide, and puromycin. We conclude that IEX-1 mRNA is not preferentially degraded during HSV-1 infection and that HSV-1 instead inhibits the normal turnover of this mRNA.

Herpes simplex virus (HSV) rapidly shuts off expression of most cellular genes during lytic infection in tissue culture (34). Shutoff is a multitiered process that involves inhibition of host mRNA biogenesis (19, 39), accelerated degradation of cytoplasmic mRNAs (23, 44), and selective translational repression (18, 25). The virion host shutoff (vhs) protein encoded by the gene UL41 plays a key role in the shutoff process (24, 33) by triggering inhibition of host protein synthesis and accelerated decay of host and viral mRNAs (23, 30, 44; reviewed in reference 38). vhs displays amino acid sequence similarity to a family of cellular nucleases (8, 14, 15), and G. S. Read and colleagues have assembled strong genetic and biochemical evidence that vhs has inherent RNase activity (15). It therefore seems plausible that many or all of the regulatory properties of vhs stem from its actions as a nuclease. vhs is dispensable for virus replication in tissue culture (33, 37). However, vhs mutants are severely attenuated in animal models of HSV infection (26, 41-43). Mounting evidence indicates that this attenuation stems from the inability of vhs mutants to effectively quench certain host responses to infection, including the type I interferon system (17, 28, 35, 45; reviewed in reference 38).

vhs is selective in that it degrades mRNA and spares other cytoplasmic RNA species (22, 23, 30, 51). It binds host translation initiation factors eIF4B and eIF4H (7, 16), and these interactions have been suggested to deliver vhs to mRNAs (16). Consistent with this hypothesis, vhs appears to degrade the 5′ end of HSV thymidine kinase mRNA before the 3′ end in infected cells (20). In addition, vhs initiates RNA decay via endonucleolytic cleavage near regions of translation initiation in an in vitro assay system derived from rabbit reticulocyte lysates (10, 11). Recent studies have shown that subsequent RNA decay in this system occurs in an overall 5′→3′ direction (31).

The vhs-dependent shutoff system is able to inhibit the synthesis of the majority of the host proteins that can be detected by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis in assays conducted in the absence of ongoing cellular or viral transcription (33), and vhs has been shown to destabilize many host and viral mRNAs (23, 30). These observations have been taken to indicate that vhs displays little if any selectivity, globally destabilizing most mRNAs. However, several recent reports from the Roizman laboratory have argued that mRNA decay triggered by vhs is highly selective, preferentially targeting a subset of mRNAs including some that bear AU-rich instability elements (AREs) (12, 13, 47). This hypothesis emerged from studies of the effects of HSV type 1 (HSV-1) infection on the cellular stress-inducible IEX-1 mRNA. IEX-1 mRNA was strongly induced following HSV-1 infection (46). However, two RNA bands corresponding to IEX-1 degradation intermediates were also observed in the infected cells: deadenylated but otherwise intact mRNA (band B) and a 3′-truncated species lacking a portion of the 3′ untranslated region (UTR) (band C) (12, 47). These degradation intermediates appeared to increase in relative abundance at the expense of fully intact IEX-1 mRNA as HSV-1 infection proceeded. The authors reported that bands B and C were not present in uninfected cells or in cells infected with an HSV-1 vhs mutant. It was therefore concluded that vhs provokes degradation of IEX-1 mRNA through deadenylation, endonucleolytic cleavage in the 3′ UTR, and 3′→5′ decay of IEX-1 mRNA (12). Similar vhs-dependent processes were proposed to target the 3′ regions of the ARE-bearing mRNAs encoding IκBα and c-*fos*. In contrast, no evidence for such vhs-dependent decay

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was obtained with several virus-induced cellular mRNAs that lack AREs (12). Based on these findings, the authors suggested that the vhs system selectively targets ARE-bearing transcripts, thereby blocking a subset of the host responses to infection (12). Consistent with this hypothesis, HSV infection was shown to enhance the synthesis of tristetraprolin (13), a cellular protein that plays a key role in the turnover of some ARE-bearing mRNAs (1). In addition, evidence was presented to suggest that vhs binds tristetraprolin (13).

AREs are present in the 3′UTRs of a large number of inherently unstable cellular mRNAs, where they serve to regulate mRNA function at the levels of RNA degradation and translation (5, 49, 52). Examples of ARE-bearing mRNAs include those encoding cytokines (e.g., tumor necrosis factor alpha [TNF-α] and type I interferons), immediate-early response transcription factors (e.g., c-*fos* and c-*myc*), and regulators of apoptosis (eg., Bcl-2). Although AREs are functionally diverse and appear to vary in the mechanisms used to engage the cellular mRNA turnover machinery, intensive studies have shown that degradation of many ARE-bearing mRNAs proceeds through deadenylation followed by 3′→5′ decay (49). AREs serve to control mRNA stability in response to external signals, and several proinflammatory cytokines are able to stabilize select ARE-bearing transcripts by activating the p38 mitogen-activated protein kinase signaling pathway and the downstream MK2 kinase (21, 50). Recent data indicate that stabilization is achieved at least in part via MK2-mediated phosphorylation of tristetraprolin (40).

The hypothesis that HSV infection and vhs selectively target ARE-bearing mRNAs for destruction is very intriguing, because as noted above, most cytokine mRNAs (including beta interferon [IFN-β]) bear AREs. The hypothesis might therefore provide an elegant explanation for the role of vhs in blunting the type I IFN response and other aspects of innate immunity summarized above. However, the hypothesis appears to conflict with an earlier report indicating that HSV infection actively stabilizes certain ARE-bearing mRNAs including IFN-β, a process accompanied by binding of the viral immediate-early protein ICP27 to the 3′ ARE (2). Moreover, we noted that the evidence for vhs-induced destabilization of IEX-1 mRNA was based mostly on detecting increased quantities of presumed mRNA degradation intermediates in situations where the overall levels of the transcript were enhanced rather than on more-direct measures of the rate of mRNA degradation. We therefore reevaluated the effects of HSV infection and vhs on IEX-1 mRNA. We found that HSV infection stabilizes IEX-1 mRNA relative to results with uninfected cells and that vhs has little effect on this stabilization process. We confirmed the previous reports that the presence of functional vhs increases the relative abundance of a specific 3′-truncated IEX-1 RNA species (band C) and conclude that HSV-1 infection and possibly vhs interfere with the normal turnover of IEX-1 mRNA.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HEL cells (obtained from the American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 mM sodium pyruvate. The HSV-1 strains used in this study were KOS, F (originally from B. Roizman), and the KOS-derived vhs mutant virus ΔSma, which contains a 588-nucleotide deletion in the UL41 gene (32). Virus absorption, infections, and mock infections were carried out at 37°C in order to minimize stress-mediated induction of IEX-1 mRNA (47). Care was taken to limit the time that cultures were handled at room temperature to less than 2 min. Control experiments demonstrated that the mock-infection protocol did not increase IEX-1 mRNA levels in HeLa cells. UV inactivation of HSV-1 was performed by using a UV-Stratalinker 2400 (Stratagene) for 1 min. This method of preparing UV-inactivated viruses has previously been reported to reduce titers of virus by a factor of $\sim 10^4$ (27).

IEX-1 plasmid and probes. An IMAGE cDNA clone containing nucleotides 523 to 1236 of IEX-1 mRNA (5481250; Invitrogen) was used to generate probes for Northern blot analysis of IEX-1 RNA. The authenticity of the IMAGE clone was confirmed by sequencing the entire insert region. IEX-1 probe 1 was a ~450-base-pair EcoRI-Aat II fragment isolated from clone 5481250. IEX-1 probe 2 was a 260-base-pair Aat II-XhoI fragment from the same plasmid. These plasmid-derived probes were radiolabeled with ^{32}P by random priming. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected by using an oligonucleotide (5′ TTGACTCCGACCTTCACCTTCCCCAT 3′) 5′ end labeled using polynucleotide kinase and [γ-^{32}P]ATP.

RNA preparation. Total RNA was isolated from cells in 60-mm-culture dishes using TRIzol reagent (Invitrogen) according to standard protocols. Poly(A)$^+$ RNA was selected by using an Oligotex mRNA minikit (QIAGEN) according to the manufacurer's instructions. RNA was deadenylated by treating total RNA with oligo(dT) and RNase H. Ten micrograms of RNA was incubated with 500 ng of oligo(dT) 12-18 (Invitrogen)/μl and 0.5 U of RNase H (Invitrogen) in digestion buffer (20 mM Tris [pH 7.5], 10 mM MgCl2, 0.5 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 30-ng/μl bovine serum albumin) for 1 h at 30°C. RNA was ethanol precipitated before electrophoresis.

Northern blot analysis. RNA samples (10 μg) were electrophoresed through a 1.3% agarose-formaldehyde gel and transferred to a Genescreen membrane (NEN). Blots were hybridized to radiolabeled probes specific for IEX-1 or GAPDH mRNA. Hybridization to IEX-1 probes 1 and 2 was done at 68°C in ExpressHyb (Clontech) according to the user's manual. The GAPDH oligonucleotide probe was hybridized at 55°C in a modified Westneat buffer (6.6% sodium dodecyl sulfate, 250 mM morpholinepropanesulfonic acid [pH 7.0], 5× Denhardt' solution, 1 mM EDTA). All quantification was performed with a STORM 860 phosphorimager (Molecular Dynamics).

Cytokines and inhibitors. Interleukin 1 beta (IL-1β) was purchased from Research Diagnostics, Inc., and TNF-α was a gift from Hoffman-La Roche to L. Guilbert. Actinomycin D, cycloheximide, and puromycin were from Sigma.

RESULTS AND DISCUSSION

Uninfected cells contain IEX-1 transcript B and trace amounts of IEX-1 transcript C. Previous studies have suggested that transcripts of the stress-inducible cellular IEX-1 gene increase in abundance following HSV-1 infection and then are degraded in a vhs-dependent fashion via deadenylation and cleavage in the 3′ UTR (12, 47). Three classes of IEX-1-related transcripts were detected by Northern blot analysis of RNA extracted from cells infected with wild-type HSV-1 (diagrammed in Fig. 1E): a broad band of ca. 1.3 kb representing intact IEX-1 mRNA bearing poly(A) tails of varied length (band A), a discrete 1.1-kb band corresponding to nonadenylated but otherwise full-length RNA (band B), and a 3′-truncated species of ca 0.75 kb reported to arise via vhs-dependent endonucleolytic cleavage in the 3′ UTR (band C). Band A was present in both infected and uninfected cells, but bands B and C were reported to arise only after infection with HSV-1 expressing functional vhs. Band B appeared to be generated through deadenylation of the intact mRNA, since it increased in abundance while band A either declined or remained relatively constant during infection (12, 47).

We sought to confirm these findings as a prelude to more-detailed studies of the mechanism of vhs action in this system. In the first experiment, HeLa cells were mock infected or infected with 5 PFU of wild-type HSV-1 strain KOS or the

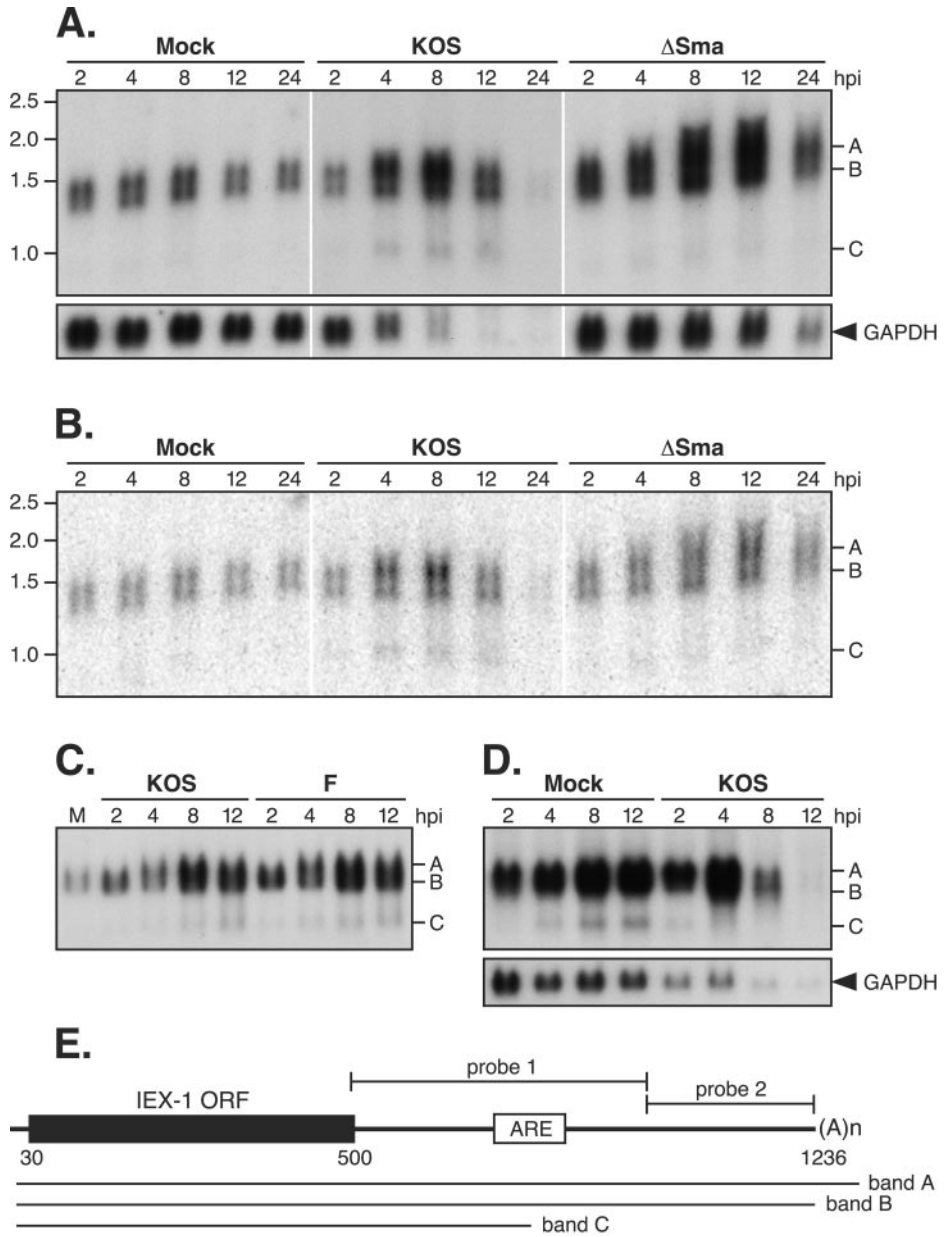

FIG. 1. Effects of HSV infection on IEX-1 RNA in HeLa and HEL cells. (A-D) HeLa (A, B, C) or HEL (D) cells were mock infected or infected with wild-type HSV-1 (strain KOS or F) or a vhs deletion mutant of KOS (ΔSma) at a multiplicity of infection of 5, and total RNA harvested at the indicated times (h) postinfection (hpi) was analyzed for IEX-1 transcripts by Northern blot hybridization, using probe 1, diagrammed in panel E. The mobilities of IEX-1 bands A to C are indicated. Panels A, C, and D display film exposures. Panel B displays a lighter phosphorimager exposure of the blot shown in panel A. The blots shown in panels A and D were also probed for GAPDH mRNA, indicated with an arrowhead. (E) Structures of IEX-1 transcripts. The diagram illustrates the structures of the three classes of IEX-1 transcripts detected in this report and indicates the locations of the IEX-1 open reading frame (IEX-1 ORF), ARE, and 3′ poly(A) tail [(A)n]. Bands A, B, and C correspond to full-length, deadenylated, and 3′-truncated transcripts, respectively. The relative positions of probes used in Northern blot analyses are also shown.

vhs-deficient mutant ΔSma (32)/cell, and total RNA harvested at various times postinfection was analyzed by Northern blot hybridization, using a radiolabeled IEX-1 probe corresponding to the 5′ portion of the 3′ UTR (Fig. 1E, probe 1). The RNA samples were also scored for GAPDH mRNA, using an oligonucleotide probe. The results (Fig. 1A and B) confirmed that IEX-1 mRNA levels increase following infection with wild-type HSV-1 and that three classes of transcripts can be resolved by gel electrophoresis. Similar results were obtained after infection with HSV-1 strain F (Fig. 1C) and infection with either strain at a multiplicity of 10 PFU/cell (data not shown). The electrophoretic mobilities of the three classes of IEX-1 transcripts were consistent with those previously reported for bands A to C (12, 47), and the RNA species are therefore labeled as such in Fig. 1. IEX-1 transcript levels peaked at about 8 to 12 h postinfection with KOS and declined signifi-

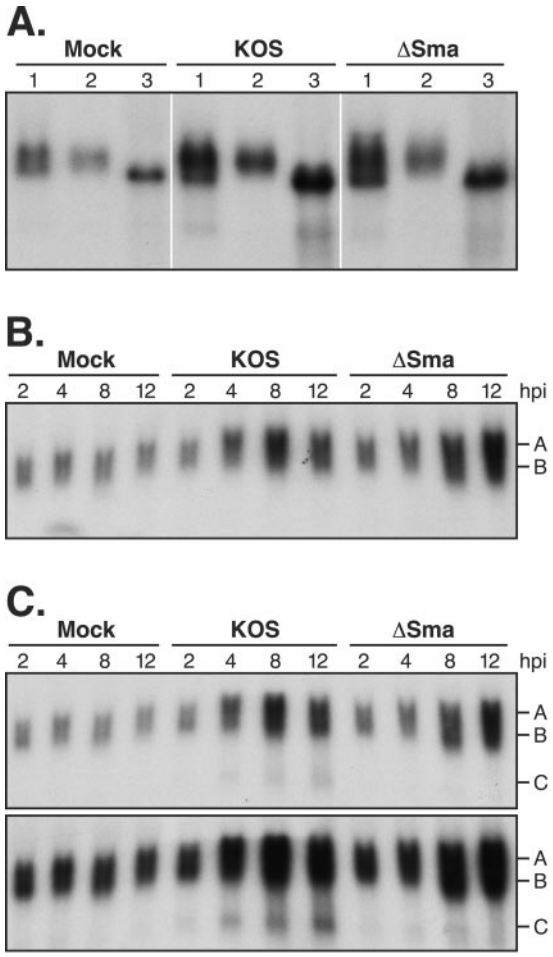

FIG. 2. Structures of IEX-1 RNAs detected in HeLa cells. HeLa cells were mock infected or infected with HSV-1 KOS or ΔSma at a multiplicity of infection of 5 PFU/cell. (A) Status of the poly(A) tail. RNA extracted 8 h postinfection was analyzed by Northern blot hybridization by using probe 1 (see Fig. 1E). Lanes 1, total RNA; lanes 2, poly(A)$^+$ RNA; lanes 3, deadenylated total RNA. (B and C) Transcripts detected by probes 1 (B) and 2 (C). Total RNA extracted at the indicated times (h) postinfection (hpi) was analyzed by Northern blot hybridization, using the IEX-1 probes 1 and 2, diagrammed in Fig. 1D). The mobilities of IEX-1 transcripts A to C are indicated. The bottom portion of panel displays a longer exposure to facilitate visualization of band C.

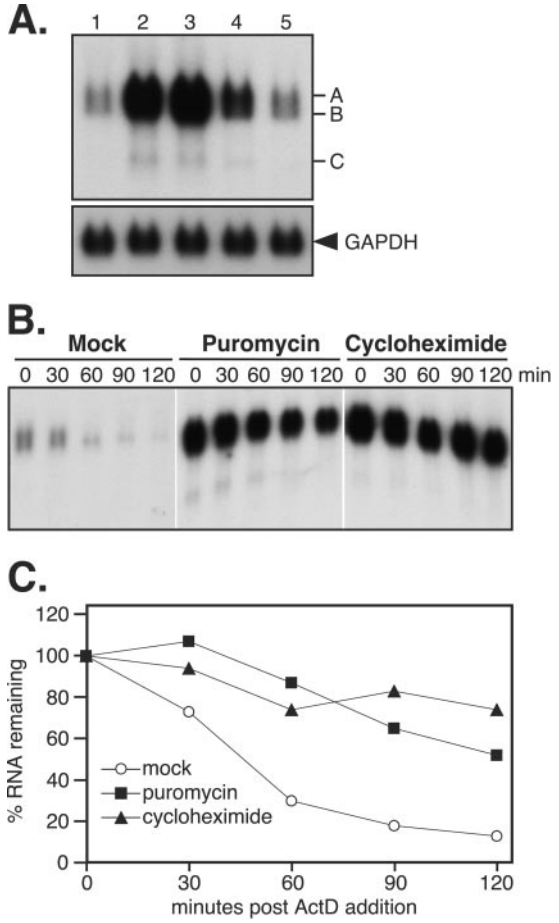

FIG. 3. Induction of IEX-1 transcripts A to C in uninfected cells by treatments that stabilize IEX-1 mRNA. (A) Effects of translational inhibitors and cytokines on IEX-1 transcript levels. HeLa cells were mock treated (lane 1) or treated with 230 μM puromycin (lane 2), 100 μg of cycloheximide/ml (lane 3), 10 μg of TNF-α/ml (lane 4), or 200 ng of IL-β (lane 5)/ml. Total RNA extracted 2.5 h posttreatment was analyzed by Northern blotting for IEX-1 (upper panel) and GAPDH (lower panel) RNAs. IEX-1 probe 1 was used. (B) Effects of translational inhibitors on IEX-1 mRNA stability. HeLa cells were treated as described above, and then actinomycin D (10 μg/ml) was added 2.5 h later. Total RNA was then harvested at 30-min intervals and analyzed for IEX-1 transcripts by Northern blotting, using probe 1 (see Fig. 1D). (C) The intensities of bands A plus B obtained in the experiment depicted in part B were quantified by phosphorimage analysis and plotted.

cantly at later time points (Fig. 1A, B, and C). This late decline was delayed during infection with ΔSma, a result that mirrors the delayed decline of viral immediate-early and early mRNAs observed during infection with vhs mutants (30). Previous reports have concluded that IEX-1 bands B and C are detected only during infection in the presence of functional vhs (12, 47). However, in our experiments RNA migrating at the position of band B was also detected after infection with ΔSma and in uninfected HeLa cells (Fig. 1A, B, and C and Fig. 2). Moreover, its abundance relative to band A did not change greatly over the course of infection with wild-type HSV-1 KOS or F (Fig. 1A, B, and C) and was not obviously influenced by the status of vhs. In addition, band C was also detected in HeLa cells infected with ΔSma and in uninfected cells, although its abundance relative to bands A and B was lower in these cases (Fig. 1A, 2, and 3), and clear demonstrations of band C in uninfected cells usually required very heavy exposures. Bands B and C were also observed with RNA extracted from HeLa cells infected with an independently isolated vhs null derivative of KOS and with the 333vhsB vhs null mutant of HSV-2 strain 333 (48) (data not shown). In addition, bands B and C were readily detected with RNA from uninfected human embryonic lung (HEL) fibroblasts (Fig. 1D). Interestingly, in the experiment depicted in Fig. 1D, the IEX-1 transcripts (including band C) increased significantly in abundance after mock infection of HEL cells, possibly as a consequence of stresses associated with the mock infection protocol. This increase, which was not observed in all experiments conducted with HEL cells,

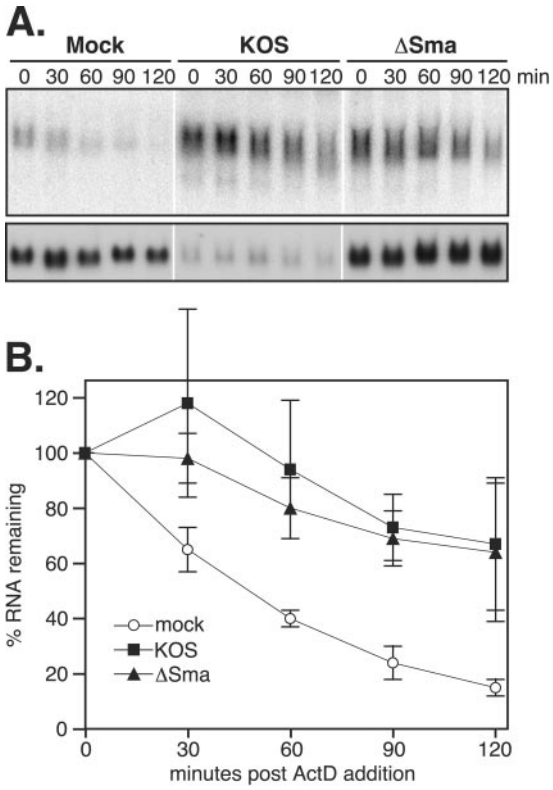

FIG. 4. HSV-1 infection stabilizes IEX-1 RNA. (A). HeLa cells were mock infected (mock) or infected with the wild type (KOS) or a vhs mutant (ΔSma) at 5 PFU per cell. Actinomycin D was added 6 h later, and total RNA extracted at the indicated times (min) thereafter was scored for IEX-1 transcripts (upper panel) and GAPDH mRNA (lower panel) by Northern blotting. (B) The sums of the intensities of bands A plus B obtained in (A) were quantified by phosphorimager analysis, and the results obtained in three independent experiments are plotted.

illustrates that the levels of all three IEX-1 RNA species can vary between samples prepared from uninfected cells (discussed further below). IEX-1 RNA levels increased in KOS-infected HEL cells but declined more rapidly than in HeLa cells.

As expected, GAPDH mRNA levels declined to almost undetectable levels following infection of HeLa and HEL cells with KOS, and this decline was severely impaired (but not eliminated) by the ΔSma vhs mutation (Fig. 1A and D). These data confirm that HSV-1 KOS is fully competent to provoke vhs-dependent mRNA loss of a known target of vhs action under the conditions used in our experiments. In contrast to the clear vhs-dependent loss of GAPDH mRNA, vhs had relatively little effect on the levels of IEX-1 RNA at early and intermediate times postinfection (for example, 8 h postinfection, a time by which loss of GAPDH mRNA was virtually complete [Fig. 1A and 4A]). These observations were difficult to reconcile with the notion that IEX-1 mRNA is selectively targeted by vhs.

Confirmation of IEX-1 transcript structure. The foregoing results differed in two key respects from those reported previously (12, 47). First, IEX-1 band A was not progressively replaced with band B in a vhs-dependent fashion as infection proceeded; rather, the ratio between bands A and B remained relatively constant and was not greatly influenced by the presence or absence of vhs. Moreover, band B was readily detected in uninfected cells, where its abundance relative to band A was not obviously different from that observed during HSV-1 infection. Second, band C was detected during infection in both the presence and absence of functional vhs and also in uninfected HeLa and HEL cells (albeit with more difficulty in uninfected HeLa cells). We did, however, confirm that the relative abundance of band C is greatest during infection with vhs-competent virus. Given these differences, we examined whether the three classes of IEX-1 transcripts detected in our experiments display the same structural characteristics as those described in previous reports.

We first examined the polyadenylation status of the RNAs. To this end, we compared the electrophoretic mobility of poly(A)$^+$ RNA obtained by oligo(dT) cellulose chromatography to that of total cellular RNA and total RNA after deadenylation via treatment with oligo(dT) and RNase H (Fig. 2A). The poly(A)$^+$ RNA (lanes 2) migrated as a relatively broad band that comigrated with band A of total RNA (lanes 1), confirming that band A corresponds to intact poly(A)$^+$ mRNA. As previously reported (12), neither band B nor band C was recovered in the poly(A)$^+$ fraction, demonstrating that these transcripts lack a 3′ poly(A) tail. In addition, band A was converted to band B when total RNA was deadenylated (lanes 3), confirming that band B represents deadenylated but otherwise intact mRNA. The data obtained in this experiment reinforce our conclusion that deadenylated band B is present in total RNA samples prepared from uninfected cells and cells infected with ΔSma as well as cells infected with KOS and that its abundance relative to band A is not obviously influenced by HSV infection or the presence or absence of functional vhs (Fig. 2A).

We next sought to confirm that band C is a 3′-truncated product that lacks a portion of the 3′ UTR present on intact IEX-1 mRNA (12) (diagrammed in Fig. 1A). Consistent with this suggestion, band C did not react with a probe derived from the 3′–most portion of the 3′ UTR (probe 2 diagrammed in Fig. 1E; Fig. 2B), while bands A and B were readily detected using this probe. In contrast, band C was readily detected in the KOS samples when the same membrane was reprobed with probe 1 and exposed to a comparable degree (Fig. 2C, upper exposure). A heavier exposure, presented at the bottom of Fig. 2C, confirms the presence of band C in the mock and ΔSma samples.

Taken in combination, these data document that bands A to C detected in our experiments display the same structural characteristics as those described in previous studies (12, 47).

Bands B and C can be detected after treatments that stabilize IEX-1 mRNA. The foregoing data established that IEX-1 band B can be readily detected in uninfected HeLa cells and HEL cells under the culture conditions used in our laboratory and that band C is also present, albeit at substantially lower levels. Thus, the host machinery can generate these RNA species in the absence of HSV gene products. Intensive analysis of the mode of decay of inherently unstable mRNAs, including those bearing AREs, has indicated that at least some are degraded via deadenylation followed by 3′→5′ exonucleolytic decay (5, 49). It therefore seems plausible that bands B and C

correspond to intermediates generated by this normal cellular decay pathway: band B likely represents the initial deadenylation product, and band C may correspond to an intermediate produced during the subsequent 3′→5′ decay of the mRNA body. It is interesting that the 3′ end of band C has been mapped to within the ARE (12), raising the possibility that band C arises due to a kinetic barrier to the progress of the 3′→5′ exonuclease imposed by a protein complex bound to the ARE. In this context we note that Esclatine et al. (12) inferred that band C is produced by vhs-dependent endonucleolytic cleavage; however, the evidence provided in that study did not distinguish between an endo- and exonucleolytic origin for this RNA. The pattern of discrete IEX-1 mRNA degradation intermediates that we observe in uninfected cells is somewhat unusual, since the decay intermediates of many ARE transcripts are extremely unstable and therefore do not accumulate to readily detectable levels. However, cases similar to that of IEX-1 have been previously noted. For example, the deadenylated but otherwise intact decay product of granulocyte-macrophage colony-stimulating factor mRNA (analogous to IEX-1 band B) is readily detected by Northern blot analysis in several cell types (reviewed in reference 1).

Degradation of some unstable transcripts, such as c-*fos* mRNA, is coupled to ongoing translation (for example, see reference 36), and as noted above, RNA degradation mediated by AREs can be inhibited in response to certain proinflammatory cytokines. We therefore examined whether select cytokines or translational inhibitors enhance the levels of IEX-1 RNA in uninfected HeLa cells, and if so, whether such induction alters the quantities of bands B and/or C and influences transcript stability. As shown in Fig. 3A, IEX-1 mRNA levels were greatly enhanced 2.5 h after exposure to the translational inhibitors puromycin (lane 2) or cycloheximide (lane 3) or to TNF-α (lane 4), while IL-1β had no effect (lane 5). These treatments also increased the abundance of bands B and C, rendering band C readily detectable. In contrast, none of the treatments altered the levels of GAPDH mRNA. In order to determine if the increased IEX-1 transcript levels provoked by the translational inhibitors correlated with enhanced mRNA stability, we performed an actinomycin D chase experiment. Cells were treated with puromycin or cycloheximide for 2.5 h, and then actinomycin D was added to block new transcription. RNA samples extracted at various time points thereafter were scored for IEX-1 mRNA levels by Northern blot analysis (Fig. 3B). The RNA signal intensities were then quantified by phosphorimager analysis. The results (Fig. 3C) confirmed the expectation that IEX-1 mRNA is quite unstable in untreated cells, decaying with a half-life of ca. 40 min, and revealed that the puromycin and cycloheximide treatments significantly stabilized the RNA (the half-life increased to more than 120 min). The half-life of IEX-1 mRNA in untreated cells determined by this assay is essentially identical to that obtained for TNF-α and granulocyte-macrophage colony-stimulating factor mRNAs using the same actinomycin D chase protocol (3, 4) and is much shorter than that of GAPDH mRNA (Fig. 4). However, it is worth noting that transcription inhibitors, such as actinomycin D, can partially inhibit mRNA decay mediated by some AREs in certain cell lines (for example, see reference 6). Therefore, our experiment may somewhat overestimate the true half-life.

Taken in combination, these results demonstrate that IEX-1 mRNA decays with the rapid kinetics expected of an ARE transcript and is stabilized by treating cells with two distinct translational inhibitors. In addition, they confirm that bands B and C are present in uninfected HeLa cells and document that they increase in (absolute) abundance in at least some situations where the normal turnover of IEX-1 mRNA is inhibited.

HSV-1 infection stabilizes IEX-1 mRNA. Taddeo et al. (47) and Esclatine et al. (12) interpreted their data to indicate that HSV-1 infection increases the rate of degradation of IEX-1 mRNA in a vhs-dependent fashion. However, these conclusions were based on detecting increased quantities of presumed degradation intermediates (bands B and C) and changes in their abundance relative to band A rather than on direct measurements of the rate of mRNA degradation. It is important to stress that such changes in the levels of degradation intermediates are not necessarily diagnostic of an enhanced rate of mRNA degradation, especially when they are monitored in a situation where the rate of mRNA biogenesis (transcription and mRNA processing) also changes in a complex fashion over time, as anticipated during HSV infection. Indeed, specific mRNA degradation intermediates could increase in abundance if virus infection imposes blocks at specific points in the degradation pathway. We therefore examined the effects of HSV-1 infection and vhs on the rate of decay of IEX-1 mRNA, as measured in an actinomycin D chase assay. HeLa cells were mock infected or infected for 6 h with HSV-1 KOS or ΔSma, and then actinomycin D was added to block new transcription. RNA samples harvested at various times after the addition of actinomycin D were then analyzed for IEX-1 transcripts by Northern blot hybridization (Fig. 4A). The results were quantified by phosphorimager analysis, and the data obtained from three such experiments are plotted in Fig. 4B. The results of these experiments confirmed the short half-life of IEX-1 mRNA in uninfected cells (ca. 40 min) and documented that as expected, GAPDH is much more stable, showing no detectable loss over the 2-h course of the experiment. The experiment also revealed that IEX-1 mRNA is considerably more stable in infected cells than in uninfected cells (half life of >120 min) and that vhs had no measurable effect on this virus-induced stabilization. These data argue that HSV-1 infection interferes with the normal turnover of IEX-1 mRNA and that IEX-1 mRNA is not detectably destabilized by vhs at 6 h postinfection. Thus, under our experimental conditions, the vhs-dependent increase in band C observed following infection is not accompanied by an increased rate of degradation of IEX-1 mRNA.

Viral gene expression is required for full induction of IEX-1 transcripts during infection. We and others have previously shown that HSV virions trigger the expression of host genes involved in antiviral defense (9, 27, 29) and that this response occurs in the absence of viral gene expression. It was therefore of interest to determine if viral gene expression is required for the induction of IEX-1 mRNA. To this end, HeLa cells were infected with untreated and UV-inactivated KOS and ΔSma, and IEX-1 transcripts were analyzed by Northern blotting (Fig. 5). As before, infection with KOS and ΔSma led to a significant increase in the levels of IEX-1 mRNA, and the increased levels were sustained for at least 12 h postinfection. In contrast, cells infected with UV-inactivated KOS and ΔSma displayed only

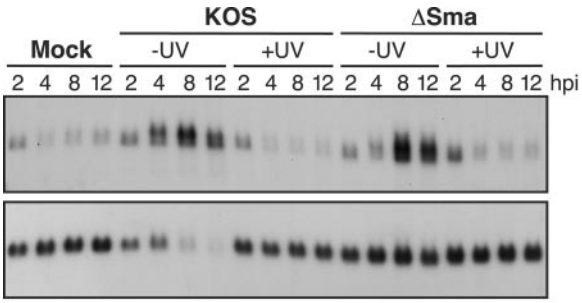

FIG. 5. Viral gene expression is required to induce IEX-1 RNA accumulation. HeLa cells were mock infected or infected with wild-type HSV-1 (KOS), UV-inactivated KOS, the vhs mutant (ΔSma), or UV-inactivated ΔSma viruses at 5 PFU per cell. Total RNA was harvested at the indicated times (hours) after infection (hpi), followed by detection of IEX-1 (upper panel) and GAPDH (lower panel) RNAs by Northern blot analysis.

transient induction, and IEX-1 mRNA returned to the levels present in uninfected cells within 4 h. Thus, viral gene expression is required for full and sustained induction of IEX-1 mRNA, and the accumulation of enhanced levels of band C. As expected, GAPDH mRNA levels were drastically reduced during infection with KOS but remained relatively constant during infection with ΔSma. This vhs-dependent loss of GAPDH mRNA was not observed with UV-inactivated KOS, presumably because cellular transcription is not inhibited by the UV-treated virus. Indeed, the vhs activity of infecting HSV-1 virions is routinely assayed in the presence of actinomycin D to block cellular mRNA synthesis (33).

Discrepancies with earlier studies. Two unexplained discrepancies exist between our data and those reported in earlier studies. First, we consistently identified band B and, with more difficulty, band C in uninfected cells and in cells infected with vhs mutants, while Taddeo et al. (47) and Esclatine et al. (12) did not. It is possible that the failure to identify band C in uninfected cells reflects the low levels of this RNA species in the absence of vhs. The failure of the previous studies to positively identify band B in uninfected cells is more problematic, since in our hands this RNA is substantially more abundant than band C. We note that transcripts with a mobility similar to that of band B are evident in the mock-infected samples displayed in Fig. 3 of the study of Escalantine et al. (12). However, the authors argued that this material represents band A molecules bearing short poly(A) tails rather than bonafide deadenylated band B RNA. In contrast, the band B detected in uninfected cells in our experiments clearly resolves from the smallest poly(A)$^+$ band A molecules and precisely comigrates with deadenylated mRNA (Fig. 2A). We have noticed that the ratio of band B to band A occasionally varies with time over the course of an experiment for unknown reasons. For example, in the experiment depicted in Fig. 5, most of the RNA detected at the 2-h time point migrated at the mobility of band B, and the relative abundance of band A increased at the later time points. This was observed in mock-infected cells and in cells infected with either KOS or ΔSma (Fig. 5). We do not yet understand the source of this variation, which presumably reflects changes in the relative rates of transcription and/or RNA decay over time. We speculate that similar variation may have hindered the identification of band B in uninfected cells in the earlier studies.

The second discrepancy is that the earlier studies reported that band B increases in abundance relative to band A over the course of infection with wild-type HSV-1 (12, 47), while we observed no such effect in our experiments. The earlier reports argued that these changes in the A/B ratio strictly depend on vhs function. One might therefore speculate that vhs is not operational in our experimental system. However, this is clearly not the case, since GAPDH mRNA was subject to massive vhs-dependent degradation in our studies (similar controls were not included in the previous reports [12, 47]). Moreover, we detected a vhs-dependent enhancement of the relative levels of band C in cells infected wild-type HSV-1, consistent with the earlier reports. We suspect that the source of the discrepancy lies in differences in cell culture conditions or infection protocols between the two laboratories. In any case, our data establish that relatively high levels of intact IEX-1 mRNA (band A) can persist in cells after other transcripts (e.g., GAPDH) have been degraded by vhs.

Concluding remarks. Our experiments indicate that HSV-1 infection inhibits the degradation of a highly unstable cellular ARE mRNA, IEX-1 mRNA. Perhaps surprisingly, mRNA stabilization occurred in both the presence and absence of vhs. Our demonstration of enhanced stability of IEX-1 mRNA in HSV-1-infected cells conflicts with the conclusion that HSV-1 infection and vhs stimulate the degradation of this mRNA (12, 13, 47). However, we believe that our finding is compatible with most (if not all) of the data presented in those earlier studies, which measured the levels of mRNA degradation intermediates rather than rates of mRNA decay. Indeed, Esclatine et al. (12) noted in their discussion that the presence of increased levels of degradation intermediates might suggest that the virus interferes with certain aspects of the degradation pathway. Our data are in complete accord with the findings of Brown et al. (2), who showed that HSV-1 infection stabilizes certain ARE mRNAs including IFN-β, an effect that correlated with binding of the immediate-early protein ICP27 to the ARE. It will therefore be interesting to learn if stabilization of IEX-1 mRNA involves ICP27. More generally, given the stabilizing effects of translational inhibitors, it will be critical to learn if HSV-induced stabilization stems from the inhibition of host protein synthesis that occurs during infection. In this context we emphasize that robust host shutoff occurs even in the absence of vhs and depends heavily on ICP27 (reviewed in reference 38).

We have confirmed the earlier observation that vhs is required for the augmented levels of IEX-1 band C produced during infection with wild-type HSV-1 (12, 47). Inasmuch as this enhancement occurs under conditions where the rate of IEX-1 mRNA degradation is reduced, we suggest that vhs increases the abundance of band C by interfering with a rate-limiting step in the 3′→5′ decay of the mRNA body. Such interference might arise as an indirect consequence of negative effects of vhs on the expression of one or more components of the degradation machinery. Alternatively, it might reflect direct regulation of the ARE-dependent mRNA turnover apparatus, for example, via the reported interaction between vhs and tristetraprolin (13).

It may seem paradoxical that HSV-1 infection stabilizes a

category of mRNAs that includes some that are expected to be central to host antiviral responses. Perhaps HSV-1 actively interferes with the expression of the proteins encoded by some ARE transcripts through modulation at the translational level. Alternatively, it is possible that some ARE transcripts encode proteins that facilitate virus replication. Further studies are required to test these and other possibilities.

ACKNOWLEDGMENTS

We thank Rob Maranchuk and Rosalyn Doepker for expert technical assistance.

This research was supported by an operating grant from the Canadian Institutes for Health Research.

REFERENCES

1. **Blackshear, P. J.** 2002. Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. Biochem. Soc. Trans. **30:**945–952.
2. **Brown, C. R., M. S. Nakamura, J. D. Mosca, G. S. Hayward, S. E. Straus, and L. P. Perera.** 1995. Herpes simplex virus *trans*-regulatory protein ICP27 stabilizes and binds to 3′ ends of labile mRNA. J. Virol. **69:**7187–7195.
3. **Carballo, E., W. S. Lai, and P. J. Blackshear.** 2000. Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. Blood **95:**1891–1899.
4. **Carballo, E., W. S. Lai, and P. J. Blackshear.** 1998. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science **281:**1001–1005.
5. **Chen, C. Y., and A. B. Shyu.** 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20:**465–470.
6. **Chen, C. Y., N. Xu, and A. B. Shyu.** 1995. mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. Mol. Cell. Biol. **15:**5777–5788.
7. **Doepker, R. C., W. L. Hsu, H. A. Saffran, and J. R. Smiley.** 2004. Herpes simplex virus virion host shutoff protein is stimulated by translation initiation factors eIF4B and eIF4H. J. Virol. **78:**4684–4699.
8. **Doherty, A. J., L. C. Serpell, and C. P. Ponting.** 1996. The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. Nucleic Acids Res. **24:**2488–2497.
9. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J. Virol. **76:**2180–2191.
10. **Elgadi, M. M., C. E. Hayes, and J. R. Smiley.** 1999. The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. J. Virol. **73:**7153–7164.
11. **Elgadi, M. M., and J. R. Smiley.** 1999. Picornavirus IRES elements target RNA cleavage events induced by the herpes simplex virus vhs protein. J. Virol. **73:**9222–9231.
12. **Esclatine, A., B. Taddeo, L. Evans, and B. Roizman.** 2004. The herpes simplex virus 1 UL41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. Proc. Natl. Acad. Sci. USA **101:**3603–3608.
13. **Esclatine, A., B. Taddeo, and B. Roizman.** 2004. Herpes simplex virus 1 induces cytoplasmic accumulation of TIA-1/TIAR and both synthesis and cytoplasmic accumulation of tristetraprolin, two cellular proteins that bind and destabilize AU-rich RNAs. J. Virol. **78:**8582–8592.
14. **Everly, D. N., Jr., and G. S. Read.** 1997. Mutational analysis of the virion host shutoff gene (UL41) of herpes simplex virus (HSV): characterization of HSV type 1 (HSV-1)/HSV-2 chimeras. J. Virol. **71:**7157–7166.
15. **Everly, D. N. J., P. Feng, I. S. Mian, and G. S. Read.** 2002. mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease. J. Virol. **76:**8560–8571.
16. **Feng, P., D. N. J. Everly, and G. S. Read.** 2001. mRNA decay during herpesvirus infections: interaction between a putative viral nuclease and a cellular translation factor. J. Virol. **75:**10272–10280.
17. **Geiss, B. J., T. J. Smith, D. A. Leib, and L. A. Morrison.** 2000. Disruption of virion host shutoff activity improves the immunogenicity and protective capacity of a replication-incompetent herpes simplex virus type 1 vaccine strain. J. Virol. **74:**11137–11144.
18. **Greco, A., A. M. Laurent, and J. J. Madjar.** 1997. Repression of β-actin synthesis and persistence of ribosomal protein synthesis after infection of HeLa cells by herpes simplex virus type 1 are under translational control. Mol. Gen. Genet. **256:**320–327.
19. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J. Virol. **68:**7790–7799.
20. **Karr, B. M., and G. S. Read.** 1999. The virion host shutoff function of herpes simplex virus degrades the 5′ end of a target mRNA before the 3′ end. Virology **264:**195–204.
21. **Kotlyarov, A., and M. Gaestel.** 2002. Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression? Biochem. Soc. Trans. **30:**959–963.
22. **Krikorian, C. R., and G. S. Read.** 1991. In vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. J. Virol. **65:**112–122.
23. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA **84:**1926–1930.
24. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912–921.
25. **Laurent, A. M., J. J. Madjar, and A. Greco.** 1998. Translational control of viral and host protein synthesis during the course of herpes simplex virus type 1 infection: evidence that initiation of translation is the limiting step. J. Gen. Virol. **79:**2765–2775.
26. **Leib, D. A., T. E. Harrison, K. M. Laslo, M. A. Machalek, N. J. Moorman, and H. W. Virgin.** 1999. Interferons regulate the phenotypes of wild-type and mutant herpes simplex viruses in vivo. J. Exp. Med. **189:**663–672.
27. **Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J. Virol. **75:**750–758.
28. **Murphy, J. A., R. J. Duesrt, T. J. Smith, and L. A. Morrison.** 2003. Herpes simplex virus type 2 virion host shutoff protein regulates alpha/beta interferon but not adaptive immune responses during primary infection in vivo. J. Virol. **77:**9337–9345.
29. **Nicholl, M. J., L. H. Robinson, and C. M. Preston.** 2000. Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. J. Gen. Virol. **81:**2215–2218.
30. **Oroskar, A. A., and G. S. Read.** 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate early (alpha) mRNAs. J. Virol. **61:**604–606.
31. **Perez-Parada, J., H. A. Saffran, and J. R. Smiley.** 2004. RNA degradation induced by the herpes simplex virus vhs protein proceeds 5′ to 3′ in vitro. J. Virol. **78:**13391–13394.
32. **Read, G. S., B. M. Bradley, and K. Knight.** 1993. Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptides. J. Virol. **67:**7149–7160.
33. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptides synthesis and exhibiting abnormal synthesis of α (immediate-early) viral polypeptides. J. Virol. **46:**498–512.
34. **Roizman, B., and D. M. Knipe.** 2001. Herpes simplex viruses and their replication, p. 2381–2398. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Strauss (ed.), Field's virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
35. **Samady, L., E. Costigliola, L. MacCormac, Y. McGrath, S. Cleverley, C. E. Lilley, J. Smith, D. S. Latchman, B. Chain, and R. S. Coffin.** 2003. Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs− HSV vectors for dendritic cell-mediated immunotherapy. J. Virol. **77:**3768–3776.
36. **Schiavi, S. C., C. L. Wellington, A. B. Shyu, C. Y. Chen, M. E. Greenberg, and J. G. Belasco.** 1994. Multiple elements in the c-fos protein-coding region facilitate mRNA deadenylation and decay by a mechanism coupled to translation. J. Biol. Chem. **269:**3441–3448.
37. **Smibert, C. A., and J. R. Smiley.** 1990. Differential regulation of endogenous and transduced beta-globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. J. Virol. **64:**3882–3894.
38. **Smiley, J. R.** 2004. Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? J. Virol. **78:**1063–1068.
39. **Spencer, C. A., M. E. Dahmus, and S. A. Rice.** 1997. Repression of host RNA polymerase II transcription by herpes simplex virus type 1. J. Virol. **71:**2031–2040.
40. **Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W. F. Rigby, T. K. Blackwell, and P. Anderson.** 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J. **23:**1313–1324.
41. **Strelow, L., T. Smith, and D. Leib.** 1997. The virion host shutoff function of herpes simplex virus type 1 plays a role in corneal invasion and functions independently of the cell cycle. Virology **231:**28–34.
42. **Strelow, L. I., and D. A. Leib.** 1996. Analysis of conserved domains of UL41 of herpes simplex virus type 1 in virion host shutoff and pathogenesis. J. Virol. **70:**5665–5667.
43. **Strelow, L. I., and D. A. Leib.** 1995. Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. J. Virol. **69:**6779–6786.
44. **Strom, T., and N. Frenkel.** 1987. Effects of herpes simplex virus on mRNA stability. J. Virol. **61:**2198–2207.
45. **Suzutani, T., M. Nagamine, T. Shibaki, M. Ogasawara, I. Yoshida, T.**

Daikoku, Y. Nishiyama, and M. Azuma. 2000. The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. J. Gen. Virol. **81:**1763–1771.

46. **Taddeo, B., A. Esclatine, and B. Roizman.** 2002. The patterns of accumulation of cellular RNAs in cells infected with a wild-type and a mutant herpes simplex virus 1 lacking the virion host shutoff gene. Proc. Natl. Acad. Sci. USA **99:**17031–17036.
47. **Taddeo, B., A. Esclatine, W. Zhang, and B. Roizman.** 2003. The stress-inducible immediate-early responsive gene IEX-1 is activated in cells infected with herpes simplex virus 1, but several viral mechanisms, including 3′ degradation of its RNA, preclude expression of the gene. J. Virol. **77:**6178–6187.
48. **Tigges, M. A., S. Leng, D. C. Johnson, and R. L. Burke.** 1996. Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN-gamma or when virion host shutoff functions are disabled. J. Immunol. **156:**3901–3910.
49. **Tourriere, H., K. Chebli, and J. Tazi.** 2002. mRNA degradation machines in eukaryotic cells. Biochimie **84:**821–837.
50. **Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann.** 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J. **18:**4969–4980.
51. **Zelus, B. D., R. S. Stewart, and J. Ross.** 1996. The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. J. Virol. **70:**2411–2419.
52. **Zhang, T., V. Kruys, G. Huez, and C. Gueydan.** 2002. AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. Biochem. Soc. Trans. **30:**952–958.