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Degradation of Cellular mRNAs Induced by a Virion-Associated Factor During Herpes Simplex Virus Infection of Vero Cells

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We have used Northern blot hybridization to study the accumulation of specific cellular mRNAs in Vero cells infected with herpes simplex virus (HSV) type 1 or type 2. HSV-1 infection decreased the cytoplasmic levels of β- and γ-actin, β-tubulin, and histone H3 and H4 mRNAs, though not all at the same rate. HSV-2 infection resulted in a more rapid decrease in actin and histone mRNA levels compared with HSV-1 infection. The turnover rate of each type of mRNA studied was accelerated in HSV-infected cells compared with the rate in uninfected cells. Cellular mRNA degradation was induced by HSV infection under conditions of (i) inhibition of de novo protein synthesis, (ii) inhibition of de novo RNA synthesis, (iii) infection with HSV-1(17) *ts*K, which fails to produce early and late viral gene products at the nonpermissive temperature, and (iv) infection with purified virions in the presence of actinomycin D. We have concluded that, in Vero cells, cellular mRNA degradation is induced by a factor associated with the infecting HSV virion and thus does not require de novo RNA or protein synthesis. Despite the overall inhibition of cellular mRNA accumulation, a novel 2.2-kilobase cytoplasmic actin transcript was produced in HSV-infected cells when viral gene expression was allowed. The level of accumulation of cytoplasmic host mRNAs was compared with the rate of cellular protein synthesis under different conditions of infection. This analysis suggests that both HSV-1 and HSV-2 require an additional function(s) to completely inhibit cellular protein synthesis.

The infection of permissive cells with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) results in a rapid decline in the synthesis of host polypeptides, allowing the selective translation of viral mRNAs (17, 45). Infection with HSV-2 typically results in a more rapid inhibition of host protein synthesis compared with HSV-1 (7, 16, 32, 34). These viruses appear to shut off host polypeptide synthesis via a multistep process (18, 29, 38), which is not completely understood. One immediate consequence of infection is the disaggregation of cellular polyribosomes to monoribosomes (11, 29, 45, 46), an event which coincides with host shutoff and which is mediated by a heat-labile structural component of the HSV virion (29).

Another event which contributes to the inhibition of host protein synthesis is the virus-induced degradation of host cytoplasmic mRNAs. Nishioka and Silverstein have shown that, in HSV-1-infected Friend erythroleukemia cells, 85% of globin mRNA sequences are degraded by 4 h post-infection (28, 29). They also found that degradation was a stochastic process and that it required de novo infected-cell protein synthesis (29). In polyoma virus-transformed cells infected with HSV-1, virus-induced mRNA degradation has been suggested to account for the pronounced reduction in cytoplasmic levels of moderately abundant host mRNAs, including polyoma mRNA (26, 33). Stenberg and Pizer (41) have studied the metabolism of adenovirus RNAs in HSV-1-infected, adenovirus type 5-transformed human kidney cells (cell line 293-31). Transcription of adenovirus RNA was inhibited by 75% after a few hours of infection, but accumulation of cytoplasmic adenovirus RNA was inhibited by 95%. The authors suggested that a degradation function could account for this effect and that it required viral polypeptide synthesis, since treatment with cycloheximide appeared to block the HSV-induced inhibition of mRNA accumulation. The above studies were performed by hybridizing RNAs from infected cells to filter-bound DNAs or to DNA in solution. β-Actin and glyceraldehyde-3-phosphate dehydrogenase mRNAs of BHK cells were analyzed by Northern blot hybridization and shown to turn over much more rapidly as a result of HSV-1 infection (19).

In this report, we describe the effects of HSV-1 and HSV-2 infection on the accumulation of cytoplasmic mRNAs coding for actin, β-tubulin, and histone proteins in Vero cells. These are mRNAs which are common to all eucaryotic cells, but differ in their structure and regulation. Blot hybridization of size-fractionated RNA was used to analyze the steady-state levels of mRNA, allowing us to also detect degradation products and other alterations in size. We have found that (i) the cytoplasmic levels of six different mRNAs decline during HSV infection, though not all at the same rate; (ii) infection with HSV-2 causes a more rapid decrease in the accumulation of some mRNAs than infection with HSV-1; (iii) HSV-1- and HSV-2-induced mRNA degradation is instrumental in inhibiting the levels of every type of mRNA studied; and (iv) the accelerated mRNA degradation is induced by a factor associated with the infecting HSV virion and thus does not require de novo RNA or protein synthesis. In addition, we describe a novel 2.2-kilobase (kb) cytoplasmic actin transcript which is produced as a result of HSV gene expression in Vero cells.

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MATERIALS AND METHODS

Cells and virus. Vero cells were maintained and virus stocks were prepared as previously described (14). The virus strains used were HSV-1(F), HSV-2(G), HSV-1(17) and its temperature-sensitive (*ts*) mutant *ts*K (from Saul Silverstein; 35–37), and HSV-1(HFEM) and its *ts* mutant *ts*B7 (from David Knipe; 1, 20).

Viral infection and treatment with inhibitors. Replicate

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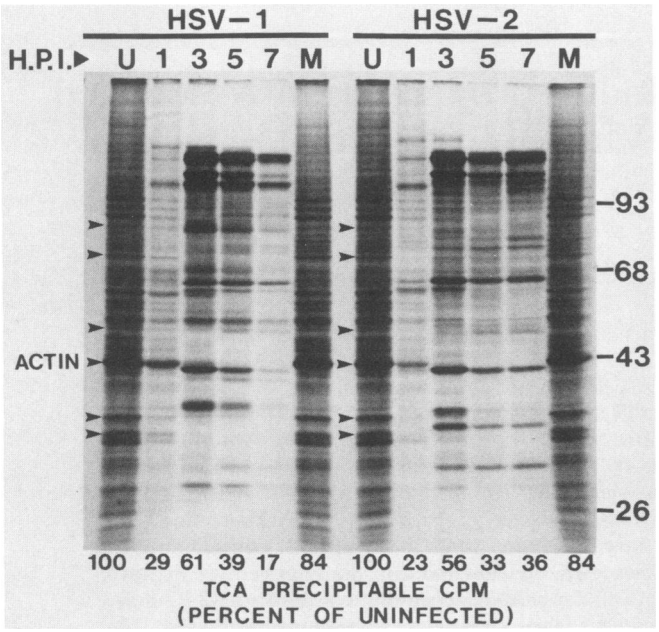

FIG. 1. Shutoff of cellular protein synthesis by HSV-1 and HSV-2. After infection by either HSV-1(F) or HSV-2(G) at an MOI of 20 PFU per cell, Vero cells were labeled at various intervals for 20 min with [^{35}S]methionine. Uninfected cells (U) were labeled at 0 h and mock-infected cells (M) were labeled at 7 h postinfection. Cell equivalent amounts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular mass markers are shown to the right in kilodaltons. Arrows to the left indicate cellular proteins whose synthesis is inhibited more rapidly by infection with HSV-2 than with HSV-1. Below the fluorogram are the total trichloroacetic acid-precipitable counts per minute incorporated at each time point compared with that incorporated by the uninfected cell control. H.P.I., Hours postinfection.

cultures of Vero cells were infected with 10 or 20 PFU of HSV per cell at 37°C with the zero time point defined as the time of addition of virus. After a 1-h adsorption period, the inoculum was removed and replaced with fresh maintenance medium. For experiments involving *ts* mutants, the inoculum and overlay media were preincubated at 32.5 or 39.5°C, and the cells were maintained at the appropriate temperature throughout infection. For experiments involving *ts*B7, flasks were submerged in a 39.5°C water bath after infection or mock infection.

For experiments with metabolic inhibitors, 25 μM anisomycin or 4 to 5 μg of actinomycin D per ml was added to cells before inoculation with virus and was present throughout the infection.

Analysis of polypeptides in infected and mock-infected cells. Cells were pulse-labeled for 20 min with 20 μCi of [^{35}S]methionine per ml in methionine-free medium supplemented with 2% dialyzed fetal calf serum. At the end of the labeling period, the cells were harvested, lysed with sodium dodecyl sulfate, and prepared for electrophoresis as previously described (14). Labeled polypeptides were analyzed by electrophoresis through 9% polyacrylamide gels cross-linked with bisacrylamide (22). Gels were processed as described before (14).

Cloned DNA probes. Human fibroblast γ-actin and β-tubulin clones were obtained from P. Gunning, P. Ponte, and L. Kedes. These clones were isolated from a cDNA library constructed by Okayama and Berg (31). The γ-actin cDNA clone pHF-1 includes sequences from amino acid 151 to the polyadenylated tail of the mRNA; it hybridizes to both β- and γ-actin mRNAs (12; P. Gunning, personal communication). The β-tubulin cDNA clone pHFβT-1 is a virtually full-length clone which hybridizes specifically to β-tubulin mRNAs. This clone has the same sequence as the β-tubulin cDNA clone (Dβ-1) described by Hall et al. (13; Gunning, personal communication). The cDNA inserts were isolated from these plasmids by restriction enzyme digestion and separation on agarose gels and were routinely used to prepare nick-translated probes.

Plasmids pHu4A and pHh5B, given to us by N. Heintz, carry human genomic DNA fragments containing the histone H4 and H3 genes, respectively (15). Because pHh5B contains a repetitive element in addition to the H3 gene (N. Heintz, personal communication), a 1-kb *Hin*dIII fragment containing only the H3 gene was subcloned into pBR322 in our laboratory. This subclone (pH3) and pHu4A were nick translated as entire plasmids for use in hybridization.

RNA purification. Total cytoplasmic RNA was harvested by cellular lysis with 0.5% Nonidet P-40 and isolated by phenol extraction and ethanol precipitation essentially as described previously (14).

Gel electrophoresis and blot analysis. Electrophoresis of RNA through 1.2% formaldehyde-agarose gels, Northern transfer of RNA to nitrocellulose, preparation of ^{32}P-labeled nick-translated probes, and hybridization conditions were all as described before (14), except that yeast tRNA or total RNA was used as a carrier in hybridization mixes. Autoradiographs of probed RNA blots were scanned with an LKB Ultrascan (model 2200) laser beam densitometer for quantitation of individual bands (14).

Virus neutralization. An HSV-1 general antiserum was prepared by repeated immunization of rabbits with Nonidet P-40 extracts of HSV-1-infected RK13 cells. This antiserum reacts specifically in immunoprecipitation with a number of viral polypeptides (Schek and Bachenheimer, unpublished data). Virus neutralization was carried out by incubating 3×10^8 PFU of HSV-1 or HSV-2 in phosphate-buffered saline with or without 10% antiserum for 40 min at 37°C. The mixture was diluted in medium and added to Vero cells; a portion was removed for assay of infectivity. Incubation with antiserum decreased the infectivity of HSV-1 and HSV-2 by 4 and 2 logs, respectively.

Virion purification. Purified virions were isolated from HSV-1(F)-infected Vero cells by dextran gradient sedimentation, essentially as described by Cassai et al. (4).

RESULTS

Inhibition of host protein synthesis by HSV-1 and HSV-2. To determine whether HSV-2 would inhibit host protein synthesis more rapidly and completely than HSV-1 under our conditions of infection, we compared their effects on cellular protein synthesis in Vero cells (Fig. 1). Replicate cultures of cells were infected with HSV-1(F) or HSV-2(G) and then labeled with [^{35}S]methionine at different intervals postinfection. The [^{35}S]methionine-labeled polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and also quantitated by trichloroacetic acid precipitation.

The kinetics of incorporation of [^{35}S]methionine were similar when HSV-1 and HSV-2 infection were compared (Fig. 1). At the earliest time of labeling, 1 to 1.5 h postinfection, HSV-2 infection caused a slightly greater decrease in the overall amount of polypeptide synthesis compared with the uninfected control. However, [^{35}S]methionine incorporation at this time included both cellular and viral polypep-

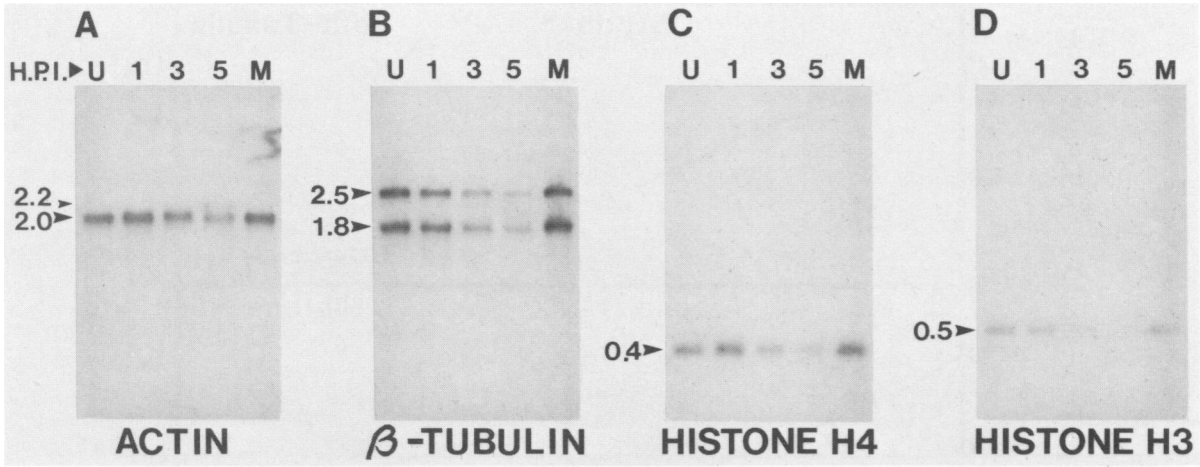

FIG. 2. Accumulation of cellular mRNAs after HSV-1 infection. Cytoplasmic RNA was harvested at various intervals after infection of Vero cells with 20 PFU of HSV-1(F) per cell. Uninfected cells (U) were harvested at 0 h and mock-infected cells were harvested at 7 h postinfection. Equal quantities of RNA were fractionated, analyzed by Northern blot hybridization, and probed for specific cellular mRNAs as indicated. To the left of each blot is shown the length of the detected RNAs in kilobases. The 2.2-kb RNA detected by the actin probe is produced in response to HSV infection. H.P.I., Hours postinfection.

tide synthesis (Fig. 1). When individual bands corresponding to host polypeptides were compared (see arrows, Fig. 1), more label was incorporated into these bands in HSV-1-infected cells than in HSV-2-infected cells at 1 to 1.5 h postinfection. As an example of this, we quantitated the amount of radioactive material in the actin band, which in this gel system is well resolved from the band representing the viral thymidine kinase. In HSV-1-infected cells, the rate of actin synthesis declined by 68%; in HSV-2-infected cells, it declined by 91%. When cells were labeled from 3 to 3.5 h postinfection, actin synthesis had ceased in HSV-2-infected cells, whereas a small amount of synthesis continued in HSV-1-infected cells. We noted a parallel difference in the magnitude of inhibition of synthesis of certain other cellular polypeptides when HSV-1- and HSV-2-infected cells were compared at early times (Fig. 1). However, when cells were labeled from 5 to 5.5 and 7 to 7.5 h postinfection, no cellular protein synthesis was apparent in either HSV-1- or HSV-2-infected cells. Therefore, whereas both HSV-1 and HSV-2 infection completely inhibited protein synthesis in Vero cells by 5 h postinfection, HSV-2 infection caused a more rapid shutoff at earlier times.

Effect of infection with HSV-1 or HSV-2 on host mRNA accumulation. To assess the effect of HSV infection on the accumulation of specific cellular mRNAs, we used several recombinant plasmids carrying message-specific sequences (see Materials and Methods). Cytoplasmic RNA was harvested at different times after HSV-1(F) infection of Vero cells or from mock-infected cells after 5 h. Equal quantities of RNA from each time point were analyzed by preparing replicate Northern blots and hybridizing with the specific probes (Fig. 2). In uninfected cells, the probe containing γ-actin DNA sequences (Fig. 2A) hybridized to comigrating 2.0-kb RNAs coding for β-actin and γ-actin. The β-tubulin cDNA probe (Fig. 2B) hybridized to 2.5- and 1.8-kb RNAs, which both translate in vitro to yield β-tubulin polypeptides (24). The histone H4 and H3 probes containing genomic DNA sequences hybridized to 0.4- and 0.5-kb RNAs, respectively (Fig. 2C and D). We have examined the cytoplasmic distribution of these transcripts by sedimentation of uninfected cell extracts through sucrose gradients and found them to be associated with polyribosomes (data not shown). Since growing cells were used in this and the following experiments, we refer to the material in these bands as mRNA.

Figure 2 shows that, as infection with HSV-1 progressed, the cytoplasmic concentration of each mRNA species decreased compared with the 0-h or mock-infected control. As mentioned above, the actin cDNA probe hybridizes to two isotypes of actin mRNA which migrate close to one another. Using isotype-specific probes, we have determined that the β- and γ-actin mRNAs are present in comparable concentrations in Vero cells and that the amount of each isotype decreased to the same extent as a result of HSV-1 infection (data not shown).

Using the actin cDNA probe, we detected a novel 2.2-kb transcript which was produced after 3 h of HSV-1 infection (Fig. 2A). It hybridized detectably with the γ-actin- but not the β-actin-specific probe (data not shown).

The amount of individual transcripts present at different times postinfection was quantitated and expressed as a percentage of the levels found in uninfected cells (Fig. 3). The levels of actin and histone H4 and H3 mRNAs decreased at a similar rate; at 5 h postinfection the amounts remaining were 45, 38, and 34%, respectively (Fig. 3A, C, and D). By comparison, the β-tubulin mRNAs (Fig. 3B) decreased much more rapidly; by only 1 h postinfection just 35% of each of these transcripts remained.

To compare the effects of HSV-1 and HSV-2 infection on the accumulation of cellular mRNAs, we extracted cytoplasmic RNA after infection with HSV-2(G) and determined the concentration of specific mRNAs (Fig. 3). The effect of HSV-2 infection on the levels of β-tubulin mRNA was similar to that of HSV-1 infection (Fig. 3B). However, there was a substantial difference in their effects on the accumulation of actin and histone H4 mRNAs. Histone H4 mRNA levels decreased to 31% of the control by only 1 h postinfection and continued to decline more gradually thereafter (Fig. 3C). Actin mRNA levels decreased to 3% of the control by only 3 h postinfection (Fig. 3A). The novel 2.2-kb actin transcript was also detected in HSV-2-infected cells by 3 h postinfection and in larger amounts than in HSV-1-infected cells (data not shown).

Effects of metabolic inhibitors on HSV-induced inhibition of

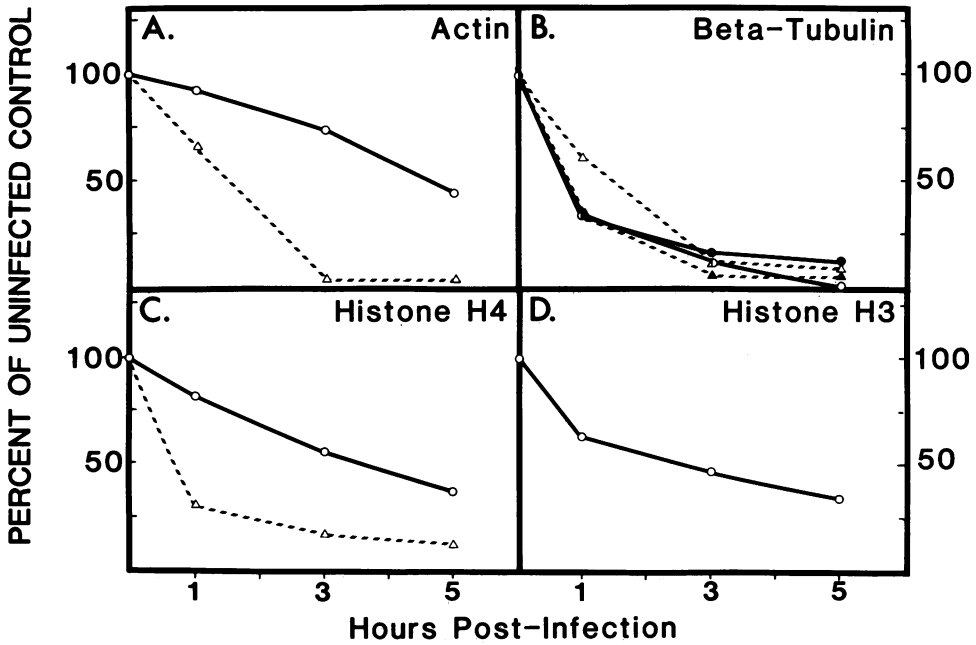

FIG. 3. Kinetics of accumulation of cytoplasmic mRNAs during HSV-1 and HSV-2 infection. The concentrations of specific mRNAs at different times postinfection were quantitated by scanning densitometry and expressed as a percentage of the concentrations found in uninfected cells. Cells were infected with HSV-1(F) (○) or HSV-2(G) (△). For the curves representing β-tubulin mRNA accumulation, closed symbols represent the 1.8-kb mRNA and open symbols represent the 2.5-kb mRNA.

accumulation of host mRNAs. The kinetics of HSV-induced inhibition of host mRNA accumulation (Fig. 3) suggested that host mRNAs were actively degraded after infection. We performed the following experiment (i) to determine whether de novo RNA or protein synthesis was required for the decrease in accumulation of cellular transcripts and, using actinomycin D, (ii) to determine whether a virus-induced RNA degradation activity accelerated the rate of turnover of these cellular mRNAs, as previously reported for other systems (19, 28, 29, 41). Cells were infected with HSV-1(F) or HSV-2(G) or were mock infected in the presence or absence of anisomycin or actinomycin D. The levels of specific transcripts at 6 h postinfection were analyzed by Northern blot hybridization.

(i) Actin mRNA. Treatment of mock-infected cells with actinomycin D for 6 h resulted in a decrease in the amount of actin mRNAs to 62% of that in untreated cells (Fig. 4; Table 1, experiment 1). However, the amount of RNA remaining in this band was approximately 1% after 6 h of HSV-1 or -2 infection in the absence of drug. This result confirms that a virus-induced activity which degrades cytoplasmic cellular mRNAs is important in inhibiting their accumulation. When cells were treated with actinomycin D from the time of HSV-1 or HSV-2 infection, the levels of actin mRNA were <0.5% by 6 h postinfection. This result indicates that virus-induced degradation of actin mRNAs can occur in the absence of viral gene expression. Our conclusion is strengthened by the results of treatment with anisomycin. When anisomycin was used to block protein synthesis in mock-infected cells, the levels of actin mRNA increased over twofold (Fig. 4; Table 1, experiment 1). Various other eucaryotic mRNAs have been shown to be stabilized in the presence of translational elongation inhibitors (see, for example, references 42, 47). Despite this effect of anisomysin, only 8 and 1% of actin mRNA remained in treated HSV-1- or HSV-2-infected cells, respectively. Along with a decrease in the amount of intact RNA, we observed a smear of lower-molecular-weight material in these lanes (Fig. 4). This presumably represented products of actin mRNA degradation which are transiently stabilized in the presence of an-

TABLE 1. Effect of treatment with actinomycin D (ActD) or anisomycin (Anis) on host cytoplasmic mRNA accumulation in HSV-infected cells

Conditions of incubation	% mRNA remaining[a]				
	Actin (β + γ)	β-Tubulin		Histone	
		1.8 kb	2.5 kb	H3	H4
Expt 1 (6 h postinfection)					
Mock	100	100	100		
Mock + Anis	231	11	11		
Mock + ActD	62	24	33		
HSV-1(F)	1	<0.5	<0.5		
HSV-1 + Anis	8	<0.5	<0.5		
HSV-1 + ActD	<0.5	<0.5	<0.5		
HSV-2(G)	1	<0.5	<0.5		
HSV-2 + Anis	1	<0.5	<0.5		
HSV-2 + ActD	<0.5	<0.5	<0.5		
Expt 2 (2 h postinfection)					
Mock		100	100	100	100
Mock + Anis		104	107	242	213
Mock + ActD		144	162	24	19
HSV-1(F)		1	<0.5	21	18
HSV-1 + Anis		5	8	12	87
HSV-1 + ActD		1	<0.5	<0.5	<0.5
HSV-2(G)		<0.5	<0.5	8	8
HSV-2 + Anis		3	3	1	5
HSV-2 + ActD		<0.5	<0.5	<0.5	<0.5

[a] The percent mRNA remaining was calculated by dividing the concentration of mRNA in each sample by its concentration in mock-infected cells incubated in the absence of inhibitors.

isomycin. Thus, in HSV-1-or HSV-2-infected cells, accelerated degradation of actin mRNA occurs in the absence of de novo viral RNA or polypeptide synthesis.

A long exposure of the Northern blot probed for actin sequences is shown in Fig. 4 to demonstrate the effect of treatment with inhibitors on the production of the HSV-induced 2.2-kb actin transcript. The 2.2-kb band was not detected in infected cells treated with actinomycin D or anisomycin. This result indicates that de novo viral or cellular gene expression or both are required for HSV-infected cells to produce the 2.2-kb actin transcript.

(ii) β-Tubulin mRNA. The accumulation of β-tubulin mRNAs was also examined in cells treated with inhibitors (Table 1, experiment 1). Treatment of mock-infected cells with actinomycin D for 6 h resulted in a decline in the levels of 1.8- and 2.5-kb β-tubulin mRNAs to 24 and 33%, respectively. Anisomycin treatment lowered the levels of both β-tubulin mRNAs in mock-infected cells to 11%. Tubulin mRNA accumulation is regulated by the pool size of free tubulin monomers (3, 6); this result therefore suggests that anisomycin treatment causes an increase in cellular levels of unpolymerized tubulin.

Stimac et al. (43) have recently shown β-tubulin mRNA levels in S49 mouse lymphoma cells to be unaffected by treatment with another elongation inhibitor, cycloheximide, over a 3-h period. For this reason, and because of their relatively short half-lives, we also measured β-tubulin mRNA accumulation after 2 h in the presence of inhibitors (Table 1, experiment 2). Over this time period, the levels of β-tubulin mRNAs were unchanged in anisomycin-treated, mock-infected cells. However, we were surprised to find that 2 h of actinomycin D treatment resulted in higher levels of β-tubulin mRNAs compared with untreated mock-infected cells. The target of regulation of β-tubulin mRNA levels appears to be nontranscriptional (5); thus, the inhibition of RNA synthesis may result in the stabilization of cytoplasmic β-tubulin mRNA.

Either 2 or 6 h of infection with HSV-1 or HSV-2 resulted in essentially complete disappearance of β-tubulin mRNA (Table 1). This occurred either in the presence or absence of anisomycin or actinomycin D, indicating that viral gene expression is not required to decrease β-tubulin mRNA levels and that at least part of the effect results from virus-induced mRNA degradation. RNAs smaller than 2.5 kb accumulated in cells which were both infected and treated with anisomycin for 2 h. These degradation products were decreased in amount by 6 h postinfection (data not shown).

(iii) Histone mRNAs. The accumulation of histone H3 and histone H4 mRNAs was examined in cells treated with or without inhibitors for 2 h (Table 1, experiment 2). Actinomycin D treatment reduced the levels of H3 and H4 mRNAs to 24 and 19%, respectively, probably due to the normally rapid turnover of these mRNAs in mock-infected cells. However, in cells infected with HSV-1 or HSV-2 in the presence of actinomycin D, <0.5% of H3 and H4 mRNAs remained by 2 h postinfection.

The levels of H3 and H4 mRNAs in mock-infected cells were increased over twofold by treatment with anisomycin (Table 1, experiment 2). Stimac et al. (43) have described a similar stabilizing effect on histone mRNAs in S49 cells after only a short period of protein synthesis inhibition. Despite this effect, we found that the amount of histone mRNA was substantially lower in anisomycin-treated infected cells than in anisomycin-treated mock-infected cells. HSV-2 infection was more effective than HSV-1 infection in reducing the accumulation of histone mRNAs in the presence of anisomycin. Degradation products of H3 and H4 mRNAs were observed in anisomycin-treated HSV-2-infected but not HSV-1-infected cells (not shown). In summary, these results show that actin, β-tubulin, and histone mRNAs are rapidly degraded as a result of HSV infection and that this does not require a product of de novo viral gene expression.

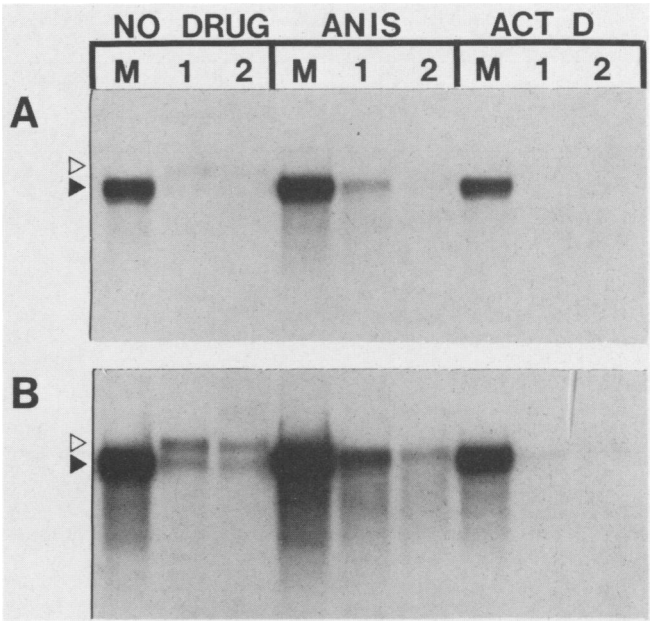

FIG. 4. Effects of drug treatment on accumulation of actin cytoplasmic transcripts. Cells were infected with 10 PFU of HSV-1 (1) or HSV-2 (2) per cell or were mock infected (M). Infections were done in the absence of drug or in the presence of 25 μM anisomycin (ANIS) or 4 to 5 μg of actinomycin D (ACT D) per ml. Cytoplasmic RNA was isolated at 6 h postinfection and actin transcripts were detected as in the legend to Fig. 2. (A) shows a 2-day exposure and (B) shows a 2-week exposure of the same autoradiogram. The closed arrow indicates the 2.0-kb β- and γ-actin mRNAs. The open arrow indicates the 2.2-kb novel actin transcript produced in HSV-infected cells only in the absence of anisomycin and actinomycin D.

Accumulation of host mRNAs in HSV-1(17) *ts*K-infected cells. To further investigate the requirements for an HSV-induced decrease in host cytoplasmic mRNA levels, we studied the effect of infection with conditional lethal viral mutants blocked at two different levels of gene expression. Since these were *ts* mutants, we first examined the effect of temperature on the decrease in actin mRNA accumulation induced by wild-type HSV strains. When cells are infected with HSV-1(F), HSV-1(KOS), or HSV-2(G) for 6 h at either 32.5 or 39.5°C, the level of cytoplasmic actin mRNAs was reduced to ≤6% that in uninfected cells (Table 2, experiment 1). Thus, the temperature of incubation did not have an effect on the ability of these wild-type strains to induce actin mRNA degradation. At 39.5°C, all three strains caused the production of the novel 2.2-kb actin transcript which appeared as a discrete band. Infection at 32.5°C resulted in the production of actin RNAs migrating slightly more slowly than the 2.0-kb mRNAs found in uninfected cells, but a discrete 2.2-kb transcript was not observed (data not shown).

The *ts* mutant HSV-1(17) *ts*K synthesizes an aberrant immediate early regulatory protein, infected cell polypeptide (ICP) 4, during infection at 39.5°C, resulting in the overproduction of immediate early proteins and the failure to produce early and late viral gene products (35–37). We infected

TABLE 2. Accumulation of cytoplasmic actin mRNAs in HSV-infected cells

Virus	% mRNA remaining[a] in cells infected at:	
	32.5°C	39.5°C
Expt 1		
HSV-1(F)	6	2
HSV-1(KOS)	3	4
HSV-2(G)	3	1
Expt 2		
HSV-1(17)	15	6
HSV-1(17) *ts*K	10	<0.5
Expt 3		
HSV-1(HFEM)	37	7
HSV-1(HFEM) *ts*B7	19	45

[a] HSV-infected and mock-infected cells were incubated at 32.5 or 39.5°C from the time of virus addition until 6 h postinfection. The percent actin mRNA remaining was calculated by dividing the concentration of actin mRNA in infected cells by its concentration in a parallel plate of mock-infected cells incubated at the same temperature.

cells with *ts*K or its parent strain at the permissive (32.5°C) or nonpermissive (39.5°C) temperature and quantitated the level of cytoplasmic actin mRNA. These two viruses inhibited the accumulation of actin mRNA to a similar extent at both 32.5 and 39.5°C (Table 2, experiment 2). In fact, the inhibition was greatest when *ts*K infection took place at 39.5°C. The accumulation of β-tubulin mRNAs was also severely inhibited by infection with either strain 17 or *ts*K at either temperature (data not shown). These results show that HSV-induced inhibition of cellular mRNA accumulation occurs in the absence of early and late viral gene products and thus agree with the results of experiments that use metabolic inhibitors. Since the levels of actin and β-tubulin mRNA were much lower after 6 h of *ts*K infection than after 6 h of treatment of uninfected cells with actinomycin D (Table 1), this experiment also shows that accelerated mRNA degradation can occur under these conditions of limited viral gene expression.

A long exposure of the Northern blot probed for actin mRNA from this experiment is shown in Fig. 5. At 32.5°C, both HSV-1(17) and *ts*K produced actin transcripts slightly altered in migration, similar to the wild-type strains previously examined. At 39.5°C, HSV-1(17) induced the synthesis of a discrete 2.2-kb band, which at 6 h postinfection was more prominent than the 2.0-kb actin mRNA band. However, *ts*K infection did not result in production of the 2.2-kb transcript at 39.5°C. Therefore, either a functional ICP-4 protein or an early or late viral gene product must be at least partially responsible for the appearance of the 2.2-kb actin transcript in HSV-infected cells.

Accumulation of host mRNAs in HSV-1(HFEM) *ts*B7-infected cells. The HSV-1(HFEM) mutant *ts*B7 is defective in the release of viral DNA from capsids (1). When cells are infected at 39°C with *ts*B7, viral capsids accumulate at the nuclear membrane and no viral polypeptide production is detectable (1, 20). We infected cells with *ts*B7 or its parent strain at the permissive (32.5°C) and nonpermissive (39.5°C) temperatures and quantitated the levels of cytoplasmic actin mRNA. Infection with either virus inhibited actin mRNA accumulation at 32.5°C (Table 2, experiment 3), although not as effectively as other viral strains (Table 2, experiment 1). Upon incubation at 39.5°C, infection with HSV-1(HFEM) was more effective at decreasing actin mRNA levels than at 32.5°C. Conversely, infection with *ts*B7 was less effective at 39.5°C in decreasing actin mRNA levels. The effects of HFEM or *ts*B7 infection on β-tubulin mRNA levels paralleled their effect on actin mRNA accumulation: at 39.5°C, HFEM reduced the levels of both β-tubulin mRNAs to 8%, whereas 38 and 96%, respectively, of the 1.8- and 2.5-kb transcripts remained in *ts*B7-infected cells. We have found, in repeated experiments, that the ability of wild-type HFEM to inhibit cellular mRNA accumulation at 39.5°C was somewhat variable; however, *ts*B7 infection was always substantially less effective in this function at 39.5°C than at 32.5°C. Thus, the defect in *ts*B7 partially prevented the decreased accumulation of cellular mRNAs at the nonpermissive temperature (see below).

Consistent with our previous results, the 2.2-kb transcript was observed in cells infected with strain HFEM, but never in cells infected with *ts*B7 at the nonpermissive temperature (data not shown).

Effects of antibody neutralization, multiplicity of infection (MOI), and virion purification on ability of inocula to induce cellular mRNA degradation. The experiments with metabolic inhibitors and *ts*K show that HSV-induced inhibition of cellular mRNA accumulation does not require the expression of viral genes. In particular, the accelerated degradation of mRNA appears to be the result of an activity present in the viral inoculum. This activity is present both in inocula consisting of crude lysates from infected cells and in those consisting of resuspended virus pellets from clarified infected cell culture medium (unpublished data). We performed a series of experiments to determine whether the factor which induces cellular mRNA degradation could be distinguished from infectious virus particles.

In the first experiment, a general anti-HSV-1 antiserum (see Materials and Methods) was used to neutralize virus before infection. The accumulation of actin and β-tubulin mRNAs was measured after infection with neutralized or unneutralized HSV-1(F) or HSV-2(G). All infections were

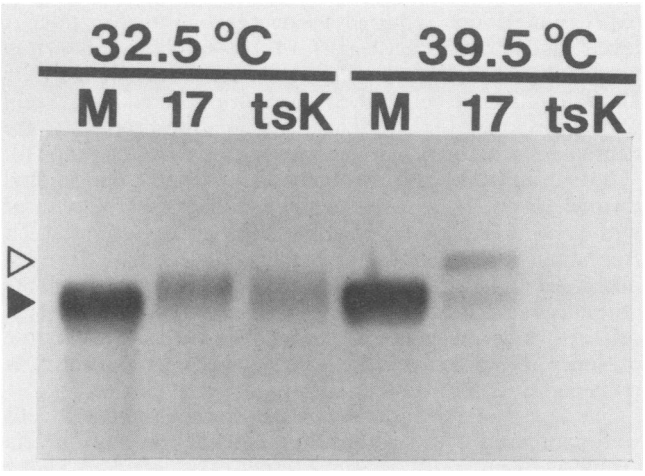

FIG. 5. Accumulation of actin transcripts in cells infected with *ts*K or parental strain 17. Cells were mock infected (M) or infected with HSV-1(17) or *ts*K at 32.5 or 39.5°C. Cytoplasmic RNA was isolated at 6 h postinfection and actin transcripts were detected as in the legend to Fig. 2. The closed arrow indicates the 2.0-kb β- and γ-actin mRNAs. The open arrow indicates the 2.2-kb actin transcript detected only in cells infected with the wild-type strain 17 at 39.5°C.

performed in the presence of actinomycin D to (i) allow us to assay for mRNA degradation as the only factor in lowering mRNA levels and (ii) eliminate any mRNA degradation activity which might arise as a result of viral gene expression.

Incubation of mock-infected cells with the antiserum had a small negative effect on the level of β-tubulin mRNAs, but not of actin mRNAs (Table 3, experiment 1). Incubation of HSV-1(F) and HSV-2(G) with the antiserum prevented virus-induced degradation of 56 to 75% of actin and 2.5-kb β-tubulin mRNAs and 90 to 100% of 1.8-kb β-tubulin mRNA. Since the level of neutralization of HSV-1 and HSV-2 reduced the infectivity of the inocula 10^4- and 10^2-fold, respectively, it was surprising that HSV-induced mRNA degradation was not completely prevented. These results suggested that (i) some neutralized virus particles enter cells without resulting in an infection but still induce degradation through an associated activity, or (ii) an activity which induces degradation is free in the inoculum and is not completely neutralized by anti-HSV-1 antibody, or both.

In a second experiment, cells were infected with a preparation of purified HSV-1(F) virions (see Materials and Methods). The levels of actin and β-tubulin mRNAs were compared with those in cells that were mock infected or infected with an equal MOI (8 PFU per cell) of a crude preparation of HSV-1(F). All infections were performed in the presence of actinomycin D. Infection with either purified or unpurified virus induced virtually complete degradation of actin and β-tubulin mRNAs (Table 3, experiment 2). Thus, a factor which is associated with the infectious particle itself can induce accelerated degradation of cellular mRNAs.

The last experiment in this series was performed to further address the possibility that the "degradation factor" might be associated with another structure in addition to infectious virus in our routine inocula. The MOI with HSV-1 or HSV-2 was progressively reduced, and the level of actin mRNAs was quantitated at 6 h postinfection in the presence of actinomycin D. Infection with HSV-2(G) was more effective in the induction of actin mRNA degradation compared with HSV-1(F) at each corresponding MOI (Table 4). Both viruses became progressively less effective in inducing degradation as their MOI was decreased. However, at an MOI of 1, 0.5, or 0.2 PFU per cell, more actin mRNA was degraded by HSV-1 or HSV-2 than would be predicted based on the proportion of cells escaping infection. The results indicate that the degradation factor, in addition to its association with infectious HSV, also exists free in the inoculum or associated with some other structure, most likely noninfectious viral particles.

TABLE 3. Accumulation of host cytoplasmic mRNAs in cells infected with neutralized or purified HSV

Conditions of infection[a]	% mRNA remaining[b]		
	Actin (β + γ)	β-Tubulin	
		1.8 kb	2.5 kb
Expt 1			
Mock	100	100	100
Mock + Ab	114	78	73
HSV-1(F)	5	5	<0.5
HSV-1(F) + Ab	72	110	75
HSV-2(G)	<0.5	<0.5	<0.5
HSV-2(G) + Ab	63	95	56
Expt 2			
Mock	100	100	100
HSV-1(F), crude	<0.5	<0.5	<0.5
HSV-1(F), purified	<0.5	<0.5	<0.5

[a] All infections were performed in the presence of 4 to 5 μg of actinomycin D per ml. For experiment 1, viral inocula were incubated in the presence or absence of anti-HSV-1 antiserum for 40 min at 37°C immediately before exposure to Vero cells.

[b] Cells were harvested at 6 h postinfection. The percent mRNA remaining was calculated by dividing the concentration of each mRNA in each sample by its concentration in a parallel plate of mock-infected cells.

DISCUSSION

The inhibition of cellular protein synthesis by HSV infection is a multistep process (18, 29, 38) and the exact mechanisms involved are different for HSV-1 and HSV-2 (16). In agreement with previous studies (26, 28, 29, 33, 41), we have found that the cytoplasmic accumulation of six different Vero cell mRNAs is progressively inhibited during HSV-1 or -2 infection. The levels at which HSV could affect the accumulation of cellular mRNAs include transcription, nuclear processing, transport, and stability. Stenberg and Pizer (41) found that HSV-1 infection of 293 cells greatly reduced the rate of adenovirus E1a and E1b RNA transcription by 3 h postinfection. However, in Vero cells, the transcription of actin, β-tubulin, and histone mRNAs continues at unaltered or even higher rates after HSV-1 infection (D. R. Yager and S. L. Bachenheimer, manuscript in preparation).

It is not known whether HSV infection inhibits the normal processing of cellular mRNAs or their transport to the cytoplasm. However, in this report we have described a novel actin transcript in HSV-infected cells having altered migration on formaldehyde-agarose gels. This transcript is complementary to a γ-actin cDNA probe, but appears to be larger than β- or γ-actin mRNA. We have ruled out the possibility that our probe is homologous with a virus-encoded RNA by showing that it does not hybridize to HSV-1 DNA (data not shown). Production of the 2.2-kb actin transcript requires functional ICP-4 or early or late viral polypeptide synthesis. It is tempting to speculate that a specific change in the infected-cell transcriptional apparatus results in either (i) the use of a new initiation or termination site or (ii) the induction of transcription of a previously silent actin gene. However, it is also possible that the infected-cell nucleus fails to correctly process the normal γ-actin mRNA precursor which, like the β-actin mRNA precursor, probably contains several small introns (23). At least 20% of the 2.2-kb actin transcripts are polyadenylated (data not shown). Further structural studies of this RNA may yield insight into the specificity of HSV-induced changes in the infected-cell transcriptional or post-transcriptional apparatus.

The results of experiments with metabolic inhibitors showed that infection with either HSV-1 or HSV-2 causes accelerated degradation of actin, β-tubulin, and histone mRNAs. This effect is instrumental in the ability of HSV to inhibit cellular mRNA accumulation; at 6 h postinfection, at least 61% of the reduction in actin mRNA levels is due to virus-induced degradation. Recently, Fenwick and McMenamin (10) have demonstrated by in vitro translation that Vero cell mRNA becomes functionally inactivated after HSV infection in the presence of actinomycin D or cycloheximide, or after infection with UV-irradiated virus. In addition, they found that the effect of HSV-2 infection on host cell mRNA was more marked than that of HSV-1 infection. Our data strongly suggest that the functional inactivation of host cell mRNAs results from their physical

degradation in HSV-infected cells. Using metabolic inhibitors, we have shown that HSV-induced degradation of cellular mRNAs also occurs in the absence of host and viral gene expression. The ability of the HSV-1 mutant *ts*K to induce cellular mRNA degradation confirms that this function requires neither a fully functional ICP-4 nor early or late viral gene expression. The HSV-1(HFEM) *ts* mutant *ts*B7 was able to decrease the level of actin mRNAs to about 45% of the uninfected cell level during infection at 39.5°C. At this temperature, cells infected with *ts*B7 accumulate viral capsids at the nuclear membrane and viral polypeptides are not expressed (1, 20). Given the phenotype of this mutant, it is most likely that the virion-associated factor which induces mRNA degradation is only partially released from *ts*B7 virions. Alternatively, the release of this factor is completely blocked at the nonpermissive temperature, but some enters the cell in a non-virion-associated form. We have recently analyzed the accumulation of cellular mRNAs in cells infected with the HSV-1 host shutoff mutant *vhs*-1 (38). In contrast to the parental wild-type strain KOS, this mutant fails to induce the degradation of actin and β-tubulin mRNAs in the absence of viral gene expression (our unpublished data). Similar results have been obtained by T. Strom and N. Frenkel (personal communication). This finding supports our conclusion that a virion-associated factor induces cellular mRNA degradation, since the *vhs*-1 mutant was previously shown to be defective in a virion-associated function acting to rapidly shut off host polypeptide synthesis (38).

We have shown that infection of Vero cells with dextran gradient-purified HSV-1 virions induces cellular mRNA degradation in the absence of viral gene expression. We envision this to occur via one of two possible mechanisms. First, HSV infection may stimulate a latent cellular RNase or alter cellular mRNA-protein complexes, making them susceptible to a cellular nuclease. Vaccinia (39) and influenza (19) virus infection also have been shown to result in degradation of specific cellular mRNAs. As discussed by Inglis (19), the ability of very different viruses to induce mRNA degradation suggests that a common cellular nuclease might be involved as a response to some types of viral infection. Second, the nuclease may be virus encoded and virion associated. We have found that HSV-infected cell cytoplasmic extracts are able to degrade cellular mRNA in vitro under conditions in which uninfected cell cytoplasmic extracts have no degradation activity. This virus-induced activity can be detected as early as 20 min postinfection. (Schek and Bachenheimer, manuscript in preparation). We are currently using this in vitro system to test for a virion-associated nuclease.

In other cell types that have been studied, HSV gene expression seemed to be required to induce cellular mRNA degradation in infected cells. Globin mRNA was not degraded in HSV-infected Friend erythroleukemia cells in the presence of cycloheximide, or during vesicular stomatitis virus coinfection (which inhibits expression of HSV genes), or if UV-irradiated virus was used in the infection (27, 29). One explanation for the difference in our results may concern the type of cells used in these experiments. Studies of endogenous RNase inhibitors have shown that the ratio of inhibitor to cellular neutral RNase activity is high in cells characterized by high rates of RNA synthesis and accumulation (2). Friend erythroleukemia cells induced to synthesize hemoglobin accumulate globin mRNA to a high concentration (30). Thus, in these cells, de novo synthesis of the nuclease or nuclease inducer may be needed after HSV infection to degrade mRNA in the presence of higher levels of RNase inhibitor. In a different cell line, the adenovirus-transformed 293–31 cells, the accumulation of newly synthesized adenovirus RNA was not inhibited during HSV-1(17) infection when cycloheximide was used to block viral protein synthesis (41). In contrast, we have recently used Northern blot hybridization to examine the steady-state levels of cellular mRNAs in HSV-1(17)-infected 293-31 cells. Our results indicate that HSV-1 infection induces degradation of actin and β-tubulin mRNAs, as well as adenovirus RNAs, in the presence or absence of actinomycin D. Degradation of actin and β-tubulin mRNAs is also induced in HeLa cells infected with HSV-1 in either the presence or absence of viral gene expression (unpublished data). Therefore, this phenomenon is not unique to HSV-infected Vero cells.

Finally, from the results presented here, it is clear that inhibition of cellular cytoplasmic mRNA accumulation is not the sole mechanism of suppression of host protein synthesis by HSV-1 or -2. For example, in HSV-1-infected cells, actin mRNA levels are reduced by only 7% at 1 h postinfection, whereas the synthesis of actin is reduced by 68%. Therefore, some additional mechanism, such as the disaggregation of host polyribosomes (11, 29, 45, 46), must be involved immediately after infection. The same conclusion can be made for infection by HSV-2 which reduces actin mRNA levels by 34% and actin synthesis by 91%. However, it is interesting to note that the difference in ability of HSV-1 and HSV-2 to inhibit actin synthesis (23%) could be accounted for by the greater reduction of actin mRNA levels during HSV-2 infection (difference of 27%). The greater effect of HSV-2 infection on actin mRNA levels is most likely a result of the ability of HSV-2 to induce more rapid actin mRNA degradation compared with HSV-1 (Table 4). HSV-2 infection is also characterized by reduced functional stability of its immediate early mRNAs compared with those of HSV-1 (9, 32, 34). The factor associated with the HSV-2 virion may be inherently more active in inducing mRNA degradation or may be present in a greater number of copies per virion. Alternatively, our HSV-2 inocula may contain more uninfectious viral particles per PFU compared with HSV-1 inocula, since uninfectious particles probably contribute to the induction of cellular mRNA degradation.

A second discrepancy between mRNA levels and cellular protein synthesis is found during *ts*K infection. The accumulation of cellular mRNA is efficiently inhibited at the nonpermissive temperature, but complete inhibition of host protein synthesis does not take place (25; our unpublished data); thus, under these conditions, the small percentage of cellular mRNAs which escape degradation must interact effectively with polysomes. Similarly, infection with *ts*B7 at

TABLE 4. Effect of MOI on accumulation of cytoplasmic actin mRNAs

MOI	% Cells escaping infection[a]	% mRNA remaining[b] in cells infected with:	
		HSV-1(F)	HSV-2(G)
4	2	6.8	<0.5
1	37	22	3
0.5	61	46	10
0.2	82	53	21

[a] All infections were performed in the presence of 4 to 5 μg of actinomycin D per ml. The percent cells remaining uninfected at each MOI was calculated by using the Poisson distribution.

[b] Cells were harvested at 6 h postinfection. The percent mRNA remaining was calculated by dividing the concentration of mRNA in each sample by its concentration in a parallel plate of mock-infected cells.

the nonpermissive temperature inhibits actin mRNA accumulation by 55% (Table 2, experiment 3) but has little effect on host protein synthesis at 39.5°C (8, 20). These results agree with those which suggest that, at least for HSV-1, an additional function requiring expression of an early or late viral protein is needed to fully inhibit host polypeptide synthesis (16, 18, 38). This function probably directly affects the selectivity of translation in the infected cell. Thus, we find in HSV-1-infected cells that, at 3 and 5 h postinfection, the rate of actin synthesis is low compared with the amount of actin mRNA remaining in the cytoplasm. Previous studies have indicated that cellular mRNAs continue to associate with the polysomes at late times after HSV-1 infection (44) and that immediate early viral transcripts associate with the polysomes at times when they are normally no longer expressed (21). Silverstein and Engelhardt have suggested that the translation of such polysome-bound mRNAs is suppressed and have presented data demonstrating inactive polysomal structures in HSV-infected cells (40). It will be interesting to determine whether residual actin, β-tubulin, and histone mRNAs are associated with polysomes of HSV-infected cells after cellular polypeptide synthesis has ceased.

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