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Biochemistry

Degradation of cellular mRNA during infection by herpes simplex virus

(Friend leukemia cells/globin mRNA degradation/cDNA/protein synthesis)

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ABSTRACT **The fate of preexisting mRNA sequences was examined after infection by herpes simplex virus. Murine erythroid cells transformed by Friend leukemia virus were used as the host. Such cells, when exposed to 2% dimethyl sulfoxide, produce large amounts of globin and globin mRNA. The protein and its mRNA are easily recognized at 4 days by electrophoresis in high percentage acrylamide gels and by hybridization to cDNA, respectively. Herpes simplex virus replicates in these cells. By 2 hr after infection the rate of protein synthesis decreases to 30% of the level in mock-infected cells and only 49 ± 8% (SEM) of the globin mRNA sequences present prior to infection could be detected by hybridization to cDNA. At 4 hr after infection, when the rate of protein synthesis in infected cells is at a maximum, only about 15% of the globin mRNA sequences remained. Control experiments support the hypothesis that globin mRNA sequences are degraded after infection by herpes simplex virus.**

One of the consequences of infection by many animal viruses is the inhibition of synthesis of host macromolecules. This is seen as a decrease in functioning polyribosomes concomitant with an abrupt drop in the rate of amino acid incorporation. This decay is generally followed by recruitment of the nonfunctioning ribosomal subunits for the synthesis of virus-specified polypeptides. The mechanisms responsible for these changes are obscure. Some viruses, e.g., adenovirus (1) and poxvirus (2, 3), possess virion-associated inhibitors that shut down cellular protein synthesis.

Alternatively, picornaviruses require the expression of viral genomic information to effect shutdown (4, 5). Cellular mRNA is then released from the decaying polyribosomes and appears to be incapable of re-entering the ribosomes (6). This cellular mRNA appears to be as stable in infected cells as it is in uninfected cells, as judged by its ability to be translated in a cell-free protein-synthesizing system (7). In addition, qualitative analysis of the 5′-methylated cap structures in host mRNAs present after picornavirus infection reveals no significant changes in the cap as a consequence of virus infection (8). Therefore, it appears likely that inhibition of host protein synthesis is not a result of nucleolytic degradation of host mRNAs. Indeed, viral mRNAs can suppress the translation of nonviral mRNAs by outcompeting them in cell-free translation systems (7, 9). The translational control mechanism allowing for the specific translation of viral mRNAs appears to act at the level of initiation of protein synthesis (10).

Infection by herpes simplex virus (HSV) also results in a rapid decline in the rate of amino acid incorporation, a decrease in the number of polyribosomes, and selective translation of viral mRNAs (11, 12). We have examined the effect of HSV infection on the metabolism of a preexisting cellular mRNA sequence and its translation product. Friend erythroleukemia cells (FL cells), grown in the presence of 2% dimethyl sulfoxide to induce the synthesis of globin mRNA, were infected with HSV. The amount of globin mRNA was monitored by hybridization to globin cDNA and synthesis of globin by polyacrylamide gel electrophoresis. Our results demonstrate that infection of induced FL cells with HSV results in a rapid shutoff of globin synthesis followed by degradation of globin mRNA.

Abbreviations: FL cells, Friend erythroleukemia cells; HSV, herpes simplex virus; MOI, multiplicity of infection; C_rt, initial concentration of RNA (mol/liter) × time (sec).

MATERIALS AND METHODS

Cells and Virus. FL cells clone 19 (13) were obtained from P. A. Marks and grown in Dulbecco's modified Eagle's medium supplemented with 15% horse serum (Flow Laboratories). Vero cells were maintained in modified Eagle's medium supplemented with 10% calf serum (Flow Laboratories). The F strain of HSV type 1 was provided by B. Roizman. The virus was propagated at low multiplicity of infection (MOI) in Vero cells, and its titer was determined by plaque assay on Vero cell monolayers.

Growth and Assay of HSV-1 in FL Cells. Exponentially growing FL cells were adjusted to 3×10^5 cells per ml and grown for 4 days in the presence or absence of 2% dimethyl sulfoxide. Twelve hours prior to infection, the cell density was adjusted to 4×10^5 cells per ml. The cells were concentrated by centrifugation to 1×10^7 cells per ml in phosphate-buffered saline supplemented with 1% glucose, 1% inactivated horse serum, 0.1 mM calcium, and 0.5 mM magnesium and infected at an MOI of 10 by gently shaking for 1 hr at 37°. Unadsorbed virus was removed by centrifugation and the infected cells were resuspended in fresh growth medium to 5×10^5 cells per ml. The cultures were incubated for 2 days and samples were removed at intervals for titering. The infected cells were frozen, thawed, and sonicated to release virus; virus titers were determined by plaque assay on Vero cell monolayers.

Rate of Protein Synthesis. FL cells were concentrated to 6×10^7 cells per ml and infected at an MOI of 10 for 30 min at 37°. Unadsorbed virus was removed by centrifugation and the cells were resuspended to 2×10^6/ml. At intervals, 1-ml aliquots were removed, washed once with phosphate-buffered saline, and resuspended in modified Eagle's medium containing one-tenth the usual concentration of leucine, 1% dialyzed horse serum, and 2 μCi of [^{3}H]leucine per ml. After 30 min, the cells were washed with phosphate-buffered saline and the incorporation of [^{3}H]leucine was determined by trichloroacetic acid precipitation onto Millipore filters. The radioactivity of dried filters was measured in a Packard Tricarb Scintillation spectrometer. The incorporation of [^{3}H]leucine at 0° was taken as background. Protein concentration was determined by the method of Lowry *et al.* (14).

Pattern of Proteins Synthesized. At intervals, infected cells were pulse labeled by resuspending 5×10^6 cells in 1 ml of modified Eagle's medium containing one-tenth the usual

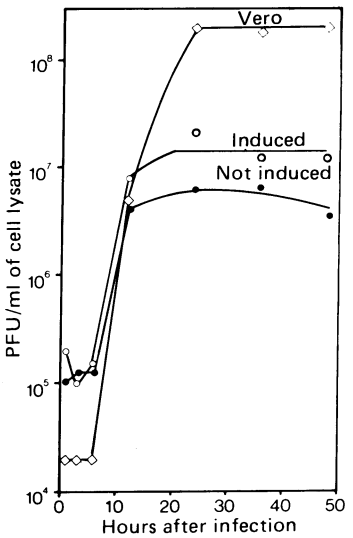

FIG. 1. Growth of HSV. Twelve hours prior to infection FL cells were diluted with fresh medium to 4×10^5/ml. Cells were infected at an MOI of 10 for 30 min. At intervals a portion of the infected culture was removed and assayed for production of infectious HSV-1 by plaquing on Vero cell monolayers. PFU, plaque-forming units.

concentration of leucine, valine, and threonine; 0.5 μCi each of [^{14}C]leucine, [^{14}C]valine, and [^{14}C]threonine was added and the cells were incubated at 37° for 30 min. Whole cell lysates were prepared by washing the cells in phosphate-buffered saline followed by resuspension in 50 mM Tris, pH 7.0/2% sodium dodecyl sulfate/5% 2-mercaptoethanol/5% sucrose/0.005% bromophenol blue (wt/vol). Cytoplasmic extracts were prepared from cells washed in phosphate-buffered saline by resuspension in phosphate-buffered saline containing 0.5% Nonidet P-40 for 5 min at 0° followed by centrifugation at 1000 $\times$ g. Then sodium dodecyl sulfate, mercaptoethanol, sucrose, and bromophenol blue were added to 2%, 5%, 5%, and 0.005% (wt/vol), respectively, and the samples were stored at −20° prior to analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. For analysis, samples were heated to 100° for 2 min and then layered onto slab gels of varying acrylamide concentration. Gels were prepared in a Bio-Rad model 220 apparatus using the discontinuous Tris-glycine system described by Laemmli (15). Electrophoresis was at 50 V until the marker dye entered the main gel and then it was increased to 100 V. When the marker dye reached the end of the gel, the electrophoresis was terminated. The gels were stained and then dried under reduced pressure and exposed to Cronex x-ray films. When necessary, gels were processed for fluorography as described by Bonner and Laskey (16).

Isolation of RNA. Whole cell RNA was extracted at 55° using phenol saturated with water. Cytoplasmic RNA was prepared from Dounce homogenized cells by phenol extraction. These RNAs were stored under 2 volumes of ethanol at −20° prior to use.

DNA·RNA Hybridization. Globin mRNA was isolated from reticulocytes of phenylhydrazine-treated DBA mice by phenol extraction, oligo(dT)-cellulose chromatography, and velocity sedimentation through 15–30% sucrose gradients in 10 mM Tris·HCl (pH 7.0) at 26,000 rpm in the SW27 rotor for 48 hr. The material in the 9S peak was collected, precipitated with ethanol, and after denaturation in formamide analyzed for homogeneity by electrophoresis in 3% acrylamide gels. Only one band migrating at 9 S could be detected on the gels. As a second criterion of purity the RNA was translated in a wheat

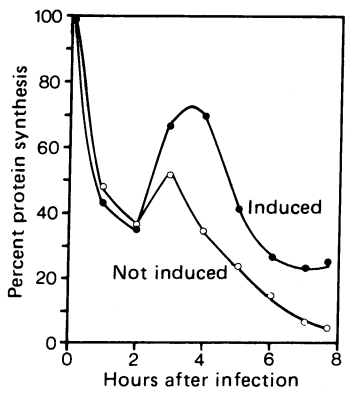

FIG. 2. Incorporation of amino acids by infected, induced, or noninduced FL cells. Exponentially growing FL cells, either induced by exposure and maintained in exponential growth for 4 days or left to grow exponentially, were infected or mock-infected with HSV-1 at an MOI of 10. At the indicated intervals a 1-ml aliquot of cells was removed, washed with phosphate-buffered saline, and pulse-labeled for 30 min with 2 μCi of [^{3}H]leucine. The rate of protein synthesis was measured in terms of cpm/μg of protein and is expressed as the percentage of the mock-infected control.

germ cell-free protein-synthesizing system. Globin was the only polypeptide detected. Complementary DNA (globin cDNA) was prepared from the purified globin mRNA using the RNA-dependent DNA polymerase from avian myeloblastosis virus as described by Kacian and Myers (17). This cDNA back-hybridized to globin mRNA with a $C_rt_{1/2}$ of 4×10^{-3} mol·sec·liter^{-1}, and was full length as judged by acrylamide gel electrophoresis. [C_rt is the initial concentration of RNA (mol/liter) $\times$ time (sec).] The hybridization conditions were as described by Ramirez *et al.* (18). The reactions were terminated by introducing the sample into 0.45 ml of S1 digestion buffer (30 mM NaOAc, pH 4.5/1.8 mM $ZnCl_2$/0.3 M NaCl/10 μg of heat-denatured calf thymus DNA per ml). The samples were divided in half and either treated with 50 units of S1 nuclease for 30 min at 45° or incubated at 45° without enzyme as a control for recovery of cDNA. All hybridizations were corrected for efficiency of hybridization of the cDNA probe back to purified 9S mouse globin RNA. This value varied from 85 to 100%, depending on the cDNA preparation.

RESULTS

Growth of HSV in FL Cells. To examine the fate of globin mRNA after productive infection, it was necessary to demonstrate that FL cells could support the replication of HSV. The ability of FL and induced, terminally differentiated FL cells to support HSV replication was compared with that of Vero cells. Fig. 1 shows that the time course of HSV accumulation is the same as in Vero cells, although the final yield of infectious particles is 1% of that in Vero cells.

Effect of Infection on Protein Synthesis. The effect of infection on the rate of protein synthesis was studied by examining the incorporation of radiolabeled amino acids into acid-insoluble material at intervals after infection. The results were expressed as percent of radiolabeled amino acid incorporation by mock-infected cells (Fig. 2). The rate of protein synthesis declined rapidly, reaching a minimum at 2 hr after infection. Thereafter, the rate recovered from 2 to 4 hr after infection and then continuously declined until, by 15 hr after infection, the rate of incorporation by infected cells was 1% of the rate of control mock-infected cultures.

Selective Synthesis of Viral Proteins. To determine if the synthesis of virus-specific polypeptides could be demonstrated

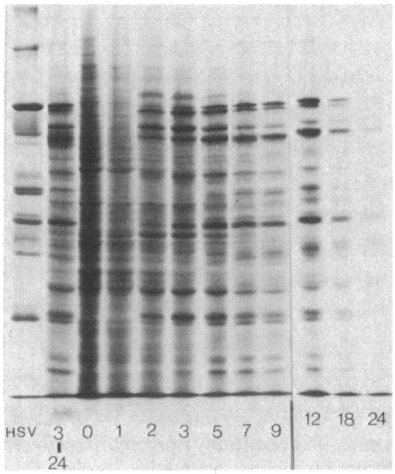

FIG. 3. Pattern of protein synthesis in infected cells. Autoradiogram of 8.5% polyacrylamide gel slab containing electrophoretically separated polypeptides from HSV-1-infected cells labeled with ^{14}C-labeled amino acids for 30 min at the times indicated after infection. HSV, polypeptides from purified virion; 3–24, cells labeled from 3 to 24 hr after infection.

in the infected, induced cells and if the pattern of protein synthesis changed during the course of infection, infected cells were labeled with ^{14}C-labeled amino acids for 30 min at various times after infection. Radiolabeled proteins from whole infected cell lysates were separated by electrophoresis through 8.5% acrylamide gels. The results showed that host protein synthesis was rapidly inhibited and that a new class of proteins was synthesized (Fig. 3). Many of these polypeptides could be identified as viral structural proteins by their comigration with polypeptides from highly purified virus. These results are in good agreement with the selective switch in synthesis from host to viral specific polypeptides seen in cells infected with HSV-1 and HSV-2 (12, 19). We demonstrated that *de novo* globin

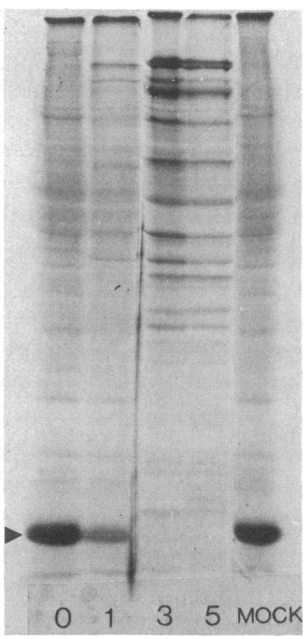

FIG. 4. Globin synthesis is shut off in infected cells. Autoradiogram of 12.5% polyacrylamide gel slab containing electrophoretically separated polypeptides from the cytoplasm of cells infected with 10 plaque-forming units of HSV-1 per cell, pulse-labeled for 30 min at the indicated intervals (hr) after infection.

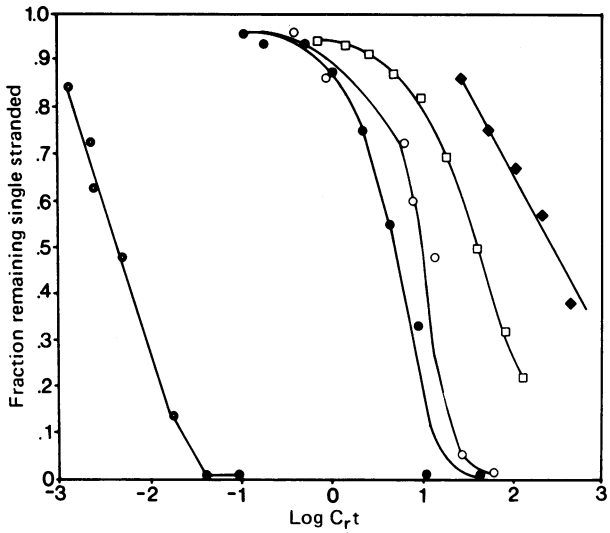

FIG. 5. Hybridization of globin mRNA extracted from FL after infection with HSV. Varying amounts of whole cell RNA were hybridized to a constant amount of [^3H]globin cDNA. The data are presented as a plot of the fraction of cDNA remaining single stranded as a function of the input concentration of RNA (C_r) in moles of nucleotides per liter times the time of hybridization (t). (O) Mouse globin mRNA; (●) RNA from induced cells; (○) RNA from induced cells infected for 2 hr at an MOI = 10; (□) RNA from induced cells infected for 4 hr; (■) RNA from noninduced cells.

synthesis was inhibited after infection by analyzing the newly synthesized polypeptides present in the cytoplasm by electrophoresis on 12.5% sodium dodecyl sulfate/acrylamide gels. Globin synthesis was rapidly suppressed by 1 hr after infection and was undetectable at 3 hr after infection (Fig. 4).

Analysis of Globin mRNA Sequences. To determine the fate of globin mRNA sequences during the course of productive infection, we assayed the relative amount of globin sequences by hybridization to labeled cDNA. Whole cell or cytoplasmic RNA extracted from induced FL cells and from infected cells at 2 and 4 hr after infection was hybridized to a complementary copy of globin mRNA from DBA mouse reticulocytes. The results demonstrated that, as infection proceeded, the amount of globin mRNA present and capable of hybridizing to globin cDNA decreased (Fig. 5). Because the intensity of induction (amount of globin mRNA produced) varied, the results of two experiments are summarized in Table 1. In one experiment, whole cell RNA from induced control cells drove 50% of our cDNA probe into a nuclease-resistant form at a C_rt of 5.0×10^0.

Table 1. Quantitation of globin mRNA sequences remaining after infection

Time after infection	$C_rt_{1/2}$	% remaining
	Experiment 1*	
Control	5×10^0	100
2 hr	1.2×10^1	41
4 hr	4.0×10^1	13
	Experiment 2†	
Control	4×10^0	100
2 hr	7×10^0	57
4 hr	2.2×10^1	18

* Total cellular RNA.
† Cytoplasmic RNA.

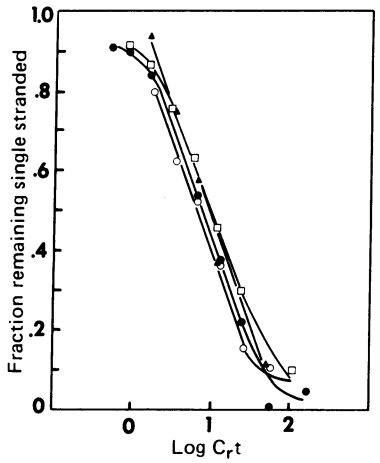

FIG. 6. Stability of globin mRNA in the absence of protein or RNA synthesis. Varying amounts of cytoplasmic RNA from FL cells treated with inhibitors of protein or RNA synthesis were hybridized to [^{3}H]globin cDNA. The data are presented as described in the legend to Fig. 5. (●) RNA from cytoplasm of exponentially growing induced FL cells; (□) cytoplasmic RNA from induced cells treated with 10 μg of cycloheximide per ml for 4 hr; (▲) cytoplasmic RNA from induced FL cells treated with 10 μg of actinomycin per ml for 4 hr; (○) cytoplasmic RNA from the same population of cells 4 hr later.

At 2 hr after infection a C_rt of 1.2×10^1 was required to drive 50% of our probe to DNA·RNA hybrid; by 4 hr after infection a C_rt of 4.0×10^1 was necessary to drive 50% of the probe into DNA·RNA hybrid. In another experiment (Table 1) we examined the capacity of RNA extracted from the cytoplasm of induced and infected cells to form a stable hybrid with our cDNA probe. The results of these two experiments (Table 1) demonstrate that when either whole cell or cytoplasmic RNAs were examined for globin sequences by hybridization to cDNA the amount of globin sequences declined as the infection proceeded. By 2 hr after infection, 41 and 57% of the globin sequences present at the time of infection were detectable; by 4 hr, only 13 and 18% of the globin sequences initially present could be detected. These results indicate that globin mRNA is degraded after infection.

Because one of the results of infection by HSV is the depression of cellular RNA synthesis (20) and because the steady-state level of a particular mRNA reflects the balance between its synthesis and degradation, it was necessary to demonstrate that the decrease in the amount of globin mRNA was not a reflection of its turnover in the face of decreasing *de novo* mRNA synthesis. Induced FL cells were treated with either actinomycin D or cycloheximide. After 4 hr RNA was isolated and the amount of globin sequences was quantitated by hybridization of cDNA. As shown in Fig. 6, no significant change in the amount of globin sequences could be detected during the 4-hr period of treatment with drugs. These results demonstrate that neither inhibiton of RNA synthesis nor inhibition of protein synthesis alone can account for the degradation of globin mRNA (Fig. 5) seen after HSV infection.

DISCUSSION

To understand why viral mRNAs are preferentially translated in infected cells we are investigating the effect of HSV infection on the metabolism of cellular mRNA sequences. The complexity of mRNA in cultured cells is so great (21) that it is difficult to determine the fate of a population of mRNA molecules after virus infection. It is possible to examine the metabolism of a single host mRNA by hybridizing its cDNA to infected cell mRNA. We have used this technique to follow the fate of the mRNA coding for globin during productive infection of Friend erythroleukemia cells by HSV. The cells chosen for this study were shown to produce large amounts of globin mRNA that is efficiently translated (Fig. 4). They also support the multiplication of HSV (Fig. 1). Globin mRNA, the major messenger species produced by these cells, has been extensively characterized (22, 23). Thus, this system is appropriate for studying the fate of host mRNA sequences after produtive infection.

As a preliminary step in this analysis we demonstrated that HSV infection inhibits the incorporation of labeled amino acids (Fig. 2) and the synthesis of globin (Fig. 4). The rate of protein synthesis decreases immediately after infection and reaches a minimum at 2 hr after infection. It then increases again to reach a maximum at 4 hr, and continuously declines thereafter. These data agree with results obtained using HEP-2 cells productively infected with HSV-1 (11, 12) or HSV-2 (19) and Vero cells (24). The resumption of synthesis reflects the reorganization of the apparatus for synthesizing host proteins for the selective synthesis of virus-specific proteins.

The kinetics of protein synthesis after infection indicate that there is a greater relative increase in recovery in induced cells than in exponentially growing noninduced cells (Fig. 2). This agrees with the greater magnitude of recovery seen when infection of stationary Vero cells is compared with infection of actively growing Vero cells (Silverstein and Engelhardt, unpublished data). This response suggests that the virus can more efficiently recruit inactive and/or disaggregated ribosomes for synthesis of virus proteins.

Analysis of polyacrylamide gel electropherograms of infected cells pulse labeled at 1-hr intervals shows that the synthesis of globin decreases in concert with the decline in the rate of amino acid incorporation (Fig. 4). By 1 hr after infection the amount of newly synthesized globin had drastically decreased; by 3 hr, it was undetectable. Thus, the sensitivity of synthesis of this polypeptide to infection reflects the gross changes seen in protein synthesis in the infected cell.

Hybridization of labeled globin cDNA to unlabeled RNA extracted at various times after infection allowed quantitation of the amount of globin sequences, relative to total infected cell RNA, that remained after infection. Our results (Table 1) demonstrate that the amount of globin mRNA sequences decreases as infection proceeds. We attribute this decrease to degradation and not to sequestering of nontranslated sequences because it is quantitatively similar whether cytoplasmic or whole cell RNA is assayed.

The normal half-life of globin mRNA in FL cells is 17 hr (25). Inhibition of host protein or RNA synthesis is not sufficient to account for the rapid decrease in the amount of globin mRNA sequences after productive infection (Fig. 6). The addition of 5 mM NaF to cells inhibits amino acid incorporation and disaggregates polyribosomes. Under these conditions, where initiation of protein synthesis is blocked, there is no detectable degradation of globin mRNA after 4 hr (Nishioka and Silverstein, unpublished data). Therefore, removal of mRNA from the polyribosomes is not sufficient to account for the degradation that occurs after HSV infection.

Poly(A)-containing RNA isolated from infected cells can stimulate a cell-free protein-synthesizing system from wheat germ to produce globin. The capacity of this poly(A)-containing RNA from infected cells to promote globin synthesis (i.e., amount per μg of RNA) decreases coordinately with the loss of globin mRNA sequences.

Analysis of the pattern of protein synthesis at various times after infection reveals that the synthesis of some polypeptides is more sensitive than others to viral infection (Fig. 3). For ex-

ample, histone synthesis is not completely shut off until 6 hr after infection (data not shown). We do not know if degradation of other host mRNAs occurs.

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