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Cellular Protein Synthesis Shutoff by Mengovirus: Translation of Nonviral and Viral mRNA's in Extracts from Uninfected and Infected Ehrlich Ascites Tumor Cells

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The mechanism whereby picornaviruses inhibit host protein synthesis while their own synthetic processes proceed unabated has remained elusive. One of our approaches to this problem was to study the ability of cell-free extracts derived from uninfected and mengovirus-infected Ehrlich ascites tumor cells to translate viral and nonviral mRNA's under various conditions of incubation. Our results indicate that viral messengers (from mengovirus and encephalomyocarditis virus) and cellular messengers [L cell and Ehrlich ascites tumor poly(A)-containing mRNA's, rabbit globin mRNA, and chicken embryo lens crystallin mRNA] are translated equally well in both extracts. We also examined the simultaneous translation of viral and nonviral mRNA's in extracts from uninfected Ehrlich ascites tumor cells. Our results indicate that under certain conditions mengovirus RNA can suppress completely the translation of globin mRNA. The significance of these results in terms of the shutoff of host protein synthesis is discussed.

Mengovirus infection results in a rapid inhibition of host RNA and protein synthesis (4). Viral protein synthesis, however, remains unaffected throughout infection (3, 4, 11). The mechanism by which this differential inhibition is effected is not known, although several ideas have been considered throughout the years. During the first 3 h after infection, there is a fall in the rate of amino acid incorporation into protein and a decrease in the number of cellular polysomes (9).

From our studies on the fate of L cell poly(A)-containing mRNA's after mengovirus infection, we know that, at 3 h after infection, a considerable number of host mRNA's still exists in polysomes. By 5 h after infection, most of these mRNA's are replaced by viral messages and nearly all of the proteins synthesized are virus specific. We have suggested (9) that late in infection, when a large amount of viral RNA is present and is being translated, the displacement of host mRNA from polysomes by viral message could result from competition at the initiation step of protein synthesis between host and viral mRNA's. To test this possibility, we studied the translation of nonviral (globin and lens crystallin) mRNA's and viral mRNA's from mengovirus and encephalomyocarditis (EMC) virus added simultaneously to extracts from uninfected Ehrlich ascites tumor (EAT) cells. We found that viral RNA can suppress the translation of nonviral mRNA's under certain conditions. These results are in agreement with those of Lawrence and Thach (13) obtained with EMC virus and a mouse plasmacytoma cell-free system.

Although competition of viral and host mRNA's for factors needed for translation may explain the inhibition of host protein synthesis late in infection, it does not account for the decrease in host protein synthesis early in infection. To explain the latter, one must invoke some other mechanism. It is possible, for example, that soon after infection a virus-specific protein is synthesized that inhibits host, but not viral, mRNA translation. In this case one might expect that extracts from infected cells would translate host mRNA's less well than extracts from uninfected cells, but viral RNA would be translated equally well in both extracts. It is also possible that a virus-specific protein is made that stimulates viral protein synthesis. In this case viral mRNA would be expected to be translated better in extracts from infected cells than in extracts from uninfected cells. To test these possibilities, we compared the ability of extracts from uninfected and mengovirus-infected EAT cells to translate nonviral mRNA's such as globin, lens crystallin, L cell, and EAT cell mRNA's and viral mRNA's from mengovirus and EMC virus. Our extracts from infected cells were prepared at 3 h

after infection, a time when host protein synthesis is decreased by at least 50% and before significant viral protein synthesis is detectable. In agreement with the results of others (13), we found that there is no difference in translation efficiency in the two extracts, using either viral or nonviral mRNA's as templates for protein synthesis.

MATERIALS AND METHODS

Buffers. The buffers used were: HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma); Tris-hydrochloride (Schwarz/Mann); phosphate-buffered saline, pH 7.5; HE (10 mM HEPES, pH 7.6, 2 mM dithiothreitol, 1 mM magnesium acetate, 10 mM KCl); HKMT (30 mM HEPES, pH 7.6, 120 mM KCl, 5 mM magnesium acetate, 2 mM dithiothreitol); TTE (10 mM Tris-hydrochloride, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA); TM (10 mM Tris-hydrochloride, pH 7.5, 2 mM magnesium acetate).

Cell culture. EAT cells, kindly provided by Milton Taylor, were grown in the peritoneal cavity of Swiss albino mice. Each animal was infected with 2×10^7 freshly obtained cells or with 1×10^8 preserved cells (8). The ascites fluid was harvested 7 to 8 days later.

Mouse L-929 cells were maintained as previously described (9).

Growth and purification of mengovirus. EAT cells washed several times with phosphate-buffered saline were suspended in minimal essential medium at a concentration of 4×10^7 cells/ml and were infected with mengovirus at a multiplicity of infection of 10. After 45 min of adsorption at room temperature, the virus-cell suspension was diluted to 4×10^6 cells/ml with minimal essential medium and made 10% in fetal bovine serum. Infection was allowed to continue for 24 h at 37 C. The 24-h postinfection virus-cell suspension was frozen and thawed once, the cellular debris was pelleted at $10,000 \times g$ for 10 min at 4 C, and the supernatant was made 6% (vol/vol) in polyethylene glycol (carbowax 6000, pH adjusted to 7.5 with 1 M Tris-hydrochloride) with a 30% (wt/vol) stock solution. This solution was slowly stirred for 24 h, and the precipitate formed was collected by centrifugation at $15,000 \times g$ for 15 min at 4 C. The pellets were resuspended in a small volume of phosphate-buffered saline and stored at −70 C. This material constitutes our crude virus stock. Further purification of the virus proceeded with the addition of 25 μg of electrophoretically purified DNase per ml and incubation at 37 C for 20 min, followed by incubation with 50 μg of trypsin per ml at 37 C for 30 min. This suspension was then centrifuged at $10,000 \times g$ for 5 min at 4 C, and the supernatant was layered on 2 ml of 15% sucrose in TM and centrifuged for 2 h at $150,000 \times g$ at 4 C. The pellet was resuspended in TTE, layered on a Cs_2SO_4 solution, 1.36 g/cm^3, pH 8.0, and centrifuged in a Beckman SW27 rotor at 25,000 rpm at 10 C for 20 h. The viral band (density, 1.33 to 1.34 g/cm^3) was collected, diluted threefold with 10 mM Tris-hydrochloride (pH 7.5), layered over a 2-ml cushion of 30% sucrose in TM, and centrifuged for 3 h at $150,000 \times g$. The virus pellet was resuspended in 100 mM Tris-hydrochloride (pH 8.0) at a concentration of 5×10^{13} particles/ml, assuming a particle to PFU ratio of 100 (20).

Isolation of mengovirus RNA. To Cs_2SO_4 purified virus, resuspended as mentioned above, sodium dodecyl sulfate (SDS) and EDTA, pH 6.0, were added to a final concentration of 0.5% and 10 mM, respectively, and the solution was incubated at 37 C for 10 min. The viral RNA was extracted twice with an equal volume of a 1:1 mixture of hot (60 C) phenol and chloroform saturated with 0.1 M NaCl and 1 mM EDTA. The aqueous layer was adjusted to 0.3 M KCl, and the RNA was precipitated by the addition of 2 volumes of 95% ethanol. It was stored at −20 C overnight. The precipitate was collected by centrifugation at $15,000 \times g$ for 20 min, rinsed once with 95% ethanol, and resuspended in sterile, double-distilled water. All glassware used was baked at 200 C for 12 h.

Isolation of cellular mRNA's. EAT or L-929 cells (5×10^9 to 10×10^9 total) were resuspended in 2.5 volumes of HE buffer minus the KCl and made 0.5% (vol/vol) in NP-40 (Particle Data Laboratories, Ltd.). After standing in ice for 10 min, the suspension was Vortex-stirred for 30 s, placed in ice for another 5 min, and centrifuged at 4 C for 15 min at $10,000 \times g$. The supernatant was made 0.1 M in NaCl, 20 mM in EDTA, and 0.5% in SDS (vol/vol) from a 10% (wt/vol) stock solution and was extracted three times with an equal volume of hot phenol-chloroform (1:1) saturated with 0.1 M NaCl and 1 mM EDTA. Alternatively, 10 U of RNase inhibitor per ml (Searle, England) was added to the supernatant, which was then layered over 2 ml of 30% sucrose in TM and centrifuged for 4 h at $150,000 \times g$ at 4 C. The ribosomal-polysomal pellet was suspended in TTE and extracted as above with phenol and chloroform. The RNA was precipitated from the final aqueous layer by the addition of 0.2 volumes of 1.0 M NaCl and 2 volumes of 95% ethanol and was stored at −20 C overnight. The poly(A)-containing mRNA fraction was obtained by oligo(dT)-cellulose (Collaborative Research, Inc.) chromatography, as described by Aviv and Leder (1).

Lens crystallin mRNA was prepared from 12- to 15-day-old chicken embryo lenses by the methods of Zelenka and Piatigorsky (22).

Preparation of uninfected and mengovirus-infected cell-free extracts. Approximately 2×10^9 to 3×10^9 thoroughly washed EAT cells were resuspended in phosphate-buffered saline at a concentration of 4×10^7 cells/ml and aliquoted into equal halves. One-half was then mock-infected with phosphate-buffered saline, and the other half was infected with mengovirus at a multiplicity of infection of 50 to 100. After an adsorption period of 1 h at room temperature, sufficient minimal essential medium plus 10% fetal bovine serum was added to both cultures to give a final cell concentration of 4×10^6/ml. Incubation at 37 C was ended 3 h later by pouring the medium into chilled centrifuge bottles. The cells were collected by centrifugation at $2,000 \times g$ for 5 min at 2 C. Both infected and unin-

fected cells were resuspended in 2 volumes of HE buffer, allowed to swell for 10 min, and lysed with 15 to 20 strokes of a teflon-glass homogenizer. The lysate was adjusted to the concentration of HKMT buffer with 10 times concentrated HKMT buffer and centrifuged for 10 min at 30,000 × *g* at 4 C. Both supernatants (S30's) were made 1.0 mM in ATP, 0.2 mM in GTP, 5.0 mM in phosphoenolpyruvate, 50 μg/ml in pyruvate kinase, and 10^{-6} M in amino acids and then incubated for 1 h at 37 C. This was followed by centrifugation at 4 C for 10 min at 20,000 × *g*. The supernatants were dialyzed for 4 to 5 h (the dialysis buffer was changed once during this period) against 100 volumes of HKMT. The preincubated and dialyzed S30's were then aliquoted and stored in liquid nitrogen. Over 20 extracts were analyzed in the course of these studies.

Conditions for cell-free protein synthesis. Reactions were carried out in a volume of 50 μl or some multiple of this amount. They contained 20 mM HEPES (pH 7.6); 80 mM KCl (or as indicated); 3.0 mM $MgAc_2$ (or as indicated); 1.0 mM ATP; 0.20 mM GTP; 5.0 mM phosphoenolpyruvate; 50 μg of pyruvate kinase per ml; 0.25 μCi of either a ^{14}C-labeled amino acid mix (NEC-445, New England Nuclear Corp.) or of a ^{3}H-labeled amino acid mix (NET-250, New England Nuclear Corp.) supplemented with the complementary unlabeled amino acids Asn, Cys, Gln, His, Met, and Try at a concentration of 1×10^{-6} M or 0.25 μCi of [^{3}H]leucine (5 Ci/mmol) supplemented with the other nineteen amino acids at a concentration of 10^{-6} M each; S30 fraction containing 100 to 200 μg of protein (optimized for each preparation); and mRNA at a concentration of 25 μg/ml (or as indicated). Incubation took place at 37 C for 1.5 or 2 h as indicated.

To the cell-free system translating crystallin mRNA, 20 μg of ascites tRNA per ml, prepared by the method of Boime and Aviv (5), was added. The addition of this tRNA was not, however, essential for crystallin mRNA translation.

Cell-free reaction product analyses. For gel electrophoresis analysis, an equal volume of a 2% (wt/vol) SDS solution was added to each reaction mixture at the end of the incubation time (2 h), followed by the addition of 4 ml of ice-cold acetone. This mixture was allowed to stand in ice for 1 h and was then centrifuged for 10 min at 5,000 × *g*. The pellet was dissolved in 50 μl of 0.1 M Tris-hydrochloride, pH 7.5. SDS and 2-mercaptoethanol were each added to 1% concentration, and the sample was heated for 3 min at 95 C. The samples were made 15% (wt/vol) in sucrose and 0.01% (wt/vol) in bromophenol blue and were analyzed on 7.5% polyacrylamide-SDS gels prepared as previously described (9).

Viral protein markers (25-min pulse, 60-min chase) were prepared as described by Butterworth et al. (7). Lens crystallin markers were obtained as previously described (17); high specific activity, 86 Ci/mmol, [^{3}H]leucine was used as label. Rabbit globin, >99% pure, was purchased from ICN Pharmaceuticals, Inc.

To simply determine stimulation of amino acid incorporation by added mRNA, reactions were stopped by the addition of 1.5 ml of cold 5% (wt/vol) trichloroacetic acid. Samples were then heated at 95 C for 15 min and cooled in ice. The precipitates were collected on 0.45-μm membrane filters (Millipore Corp.), washed three times with 5% trichloroacetic acid, and dissolved in a triton scintillation cocktail. Samples were counted in an Isocap 300 scintillation counter (Searle Analytic, Inc.).

Competition data analysis. To analyze for competition, the total counts per minute in the globin or crystallin peak on the gel was determined for each mRNA concentration. In the case of mengovirus mRNA-directed products, the total counts per minute in only the "A" protein peak was determined. For instance, in Fig. 8G counts per minute of gel slices 9 through 20 were added. The total number of counts in the globin, crystallin, or mengovirus capsid precursor protein "A" peaks was, thus, established for each experimental situation.

RESULTS

Characteristics of mRNA's tested. Several mRNA's were used as templates in determining the protein biosynthetic activity of extracts from uninfected and infected cells. Mengovirus RNA was obtained from purified virus, as described above. It migrated in 2.5% polyacrylamide-SDS gels (15) as a single peak, a little slower than 28S RNA (data not shown).

Rabbit globin mRNA was obtained commercially (Searle, England). It had a sedimentation coefficient of 9S and directed the synthesis of only one polypeptide, which comigrated with a rabbit globin marker (see Fig. 3).

Poly(A)-containing mRNA's from EAT and L-929 cells were isolated from oligo(dT)-cellulose columns. These mRNA's represent a heterogeneous mixture of molecules consisting of many species. In 2.5% SDS-polyacrylamide gels, they migrated with mobilities of molecules in the molecular weight range of 0.3×10^{6} to 3.8×10^{6} (data not shown).

Lens crystallin mRNA was isolated from 12- to 15-day-old chicken embryo lenses. This mRNA is involved in the synthesis of lens crystallins (α, β, and δ), and 70 to 80% of this RNA codes for δ-crystallin (17, 22). It directed the synthesis, in vitro, of primarily one polypeptide, which comigrated with the crystallin marker (molecular weight, ~50,000; see Fig. 4).

EMC virus RNA, obtained from Cs_2SO_4 purified virus, was the kind gift of Robert Thach.

Translation of viral mRNA in extracts from infected and uninfected cells. Cell-free extracts were prepared (see above) from uninfected EAT cells and cells that had been infected with mengovirus for 3 h. This latter time point was chosen because host protein synthesis is maximally inhibited (9) and viral protein synthesis is not yet very pronounced. Infection was verified by letting aliquots of cells grow

overnight and then staining them with trypan blue the next day. More than 85% of the infected cells were dead after 24 h. In contrast, less than 20% of the mock-infected cells were dead after the same time period.

The S30 fractions were preincubated to run off host ribosomes from endogenous mRNA and dialyzed, as indicated above. Figure 1a shows that the rate of translation of mengovirus RNA in extracts from uninfected and infected cells was the same. Incorporation of amino acids was dependent on the addition of exogenous mengovirus RNA, and the reaction was linear for about 80 min.

Conditions that play important roles in translation in vitro were also examined. Figure 2b demonstrates that, at different magnesium ion concentrations, mengovirus RNA was translated equally well in extracts from infected and uninfected cells. The optimum concentration, approximately 3 mM, was the same in each case. Also, irrespective of whether the extract was prepared from infected or uninfected cells, the amount of mengovirus RNA required to saturate the cell-free system was the same, about 30 μg/ml (Fig. 1c). Similarly, the optimum amount of S30 fraction required to translate mengovirus RNA was the same, regardless of whether the extract was derived from uninfected or infected cells (Fig. 1d). This optimum amount was approximately 100 μg of S30 protein per 50 μl of reaction mixture. Finally, Fig. 1e demonstrates that the translation of mengovirus RNA was the same in extracts from uninfected and infected cells under a large range of KCl concentrations.

Translation of nonviral mRNA's in extracts from infected and uninfected cells. The lack of

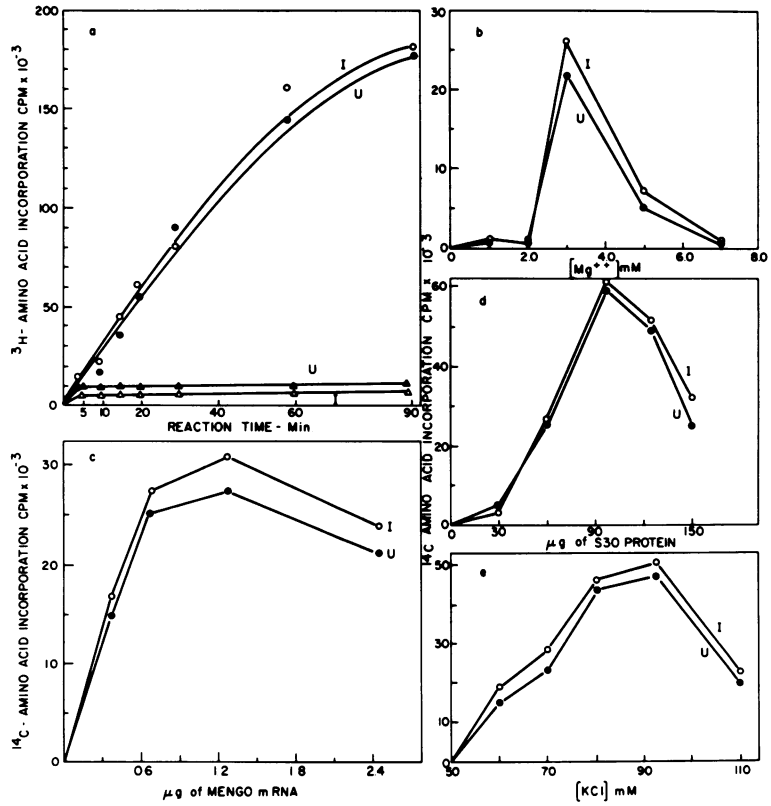

FIG. 1. *Optimization of conditions for the translaiion of mengovirus mRNA in extracts from uninfected and infected cells. (a) Reactions were scaled up to 300 μl; 3.0 μCi of ^{3}H-labeled amino acid mixture was added per reaction mixture, and 6 μg of mengovirus RNA was added where indicated. Duplicate aliquots of 20 μl were taken at the indicated times and precipitated with trichloroacetic acid, as described in the text. (b, d, e) Each point is the average of duplicate 50-μl reactions; 1.2 μg of mengovirus RNA was added to each. (c) Mengovirus RNA was added, as indicated, to duplicate 50-μl reaction mixtures. Symbols: ○, extracts from infected cells; ●, extracts from uninfected cells; △, minus mengovirus RNA in extracts from infected cells; ▲, minus mengo RNA in extracts from uninfected cells.*

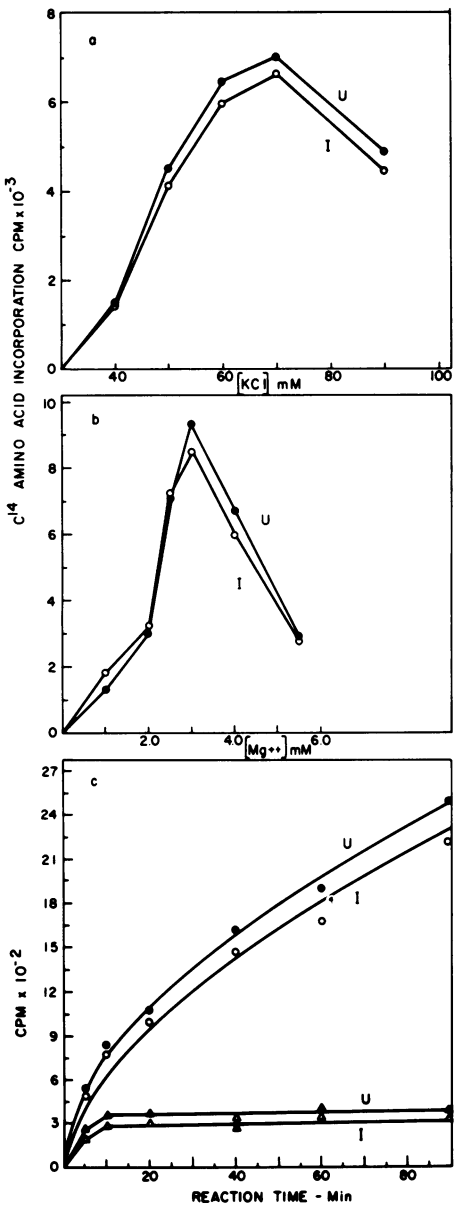

FIG. 2. *Optimization of conditions for the translation of globin mRNA in extracts from uninfected and infected cells. (a, b) Each point is the average of duplicate 50-μl reactions; 1 μg of globin mRNA was added to each. (c) Reaction was scaled up to 200 μl; 4 μg of globin mRNA was added. Duplicate aliquots of 15 μl were withdrawn at the time points indicated, and trichloroacetic acid was added, as described in the text. Symbols: ○, extracts from infected cells; ●, extracts from uninfected cells; △, minus globin mRNA in extracts from infected cells; ▲, minus globin mRNA in extracts from uninfected cells.*

difference in the ability of extracts from uninfected and infected cells to translate mengovirus RNA suggested that viral RNA translation is not significantly better in extracts from infected cells. The crucial test as to whether the in vivo shutoff could be duplicated in vitro was to determine whether extracts from infected cells could discriminate against nonviral mRNA's. Globin and lens crystallin mRNA's were used as nonviral mRNA's. As shown in Fig. 2a and b, globin mRNA was translated equally well in extracts from uninfected and infected cells, even under different magnesium ion and KCl concentrations. The magnesium and KCl optima were identical in each case. Furthermore, as with the translation of mengovirus RNA, the rate of amino acid incorporation directed by globin RNA was the same in extracts derived from infected and uninfected cells (Fig. 2c).

To verify that the product synthesized in the extracts primed by globin mRNA was indeed globin, the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. Figures 3a and b demonstrate the presence of only one major peak of radioactivity. This peak migrated to the same location as marker globin shown in the top panel. The total radioactivity in the peak made by extracts from infected cells (Fig. 3b) was about the same as that from uninfected cells (Fig. 3a).

Similar results were obtained with lens crystallin mRNA (Fig. 4b and c). Using this RNA as message, only one major peak of radioactivity was observed, presumably δ-crystallin (17). This peak migrated as authentic lens crystallin (Fig. 4a) isolated from lens tissue. No difference in translation was observed in extracts from uninfected (Fig. 4b) and infected (Fig. 4c) cells.

Because both globin and crystallin mRNA's were obtained from terminally differentiated cells and may, thus, not be representative of true host mRNA's, similar experiments were carried out with total poly(A)-containing mRNA from L cells and EAT cells. As shown in Table 1, Experiment 3, both L cell and EAT cell mRNA's were translated equally well in extracts from uninfected and infected cells, in agreement with our results using globin and crystallin mRNA's.

During the course of these comparative studies, we observed an occasional (2 out of 20) extract in which nonviral, but not viral, mRNA's were translated less well in the extract from infected cells than in the extract from uninfected cells (Experiments 1 and 2, Table 1). Some extracts from infected cells were also found to translate both viral and nonviral

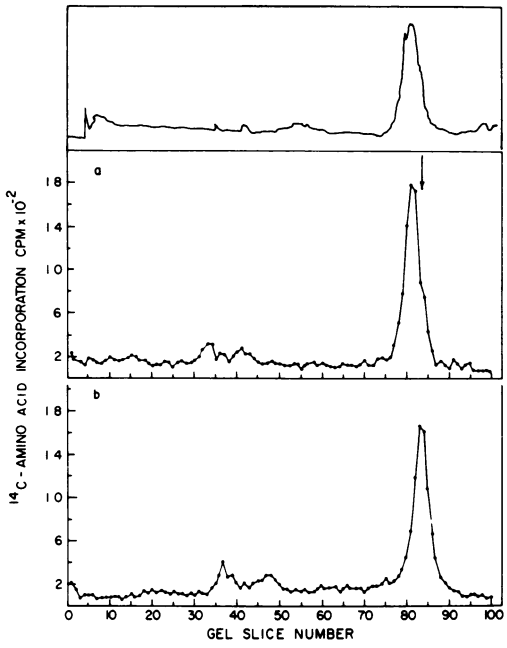

FIG. 3. *Polyacrylamide-SDS gel electrophoresis patterns of products synthesized in vitro, using globin mRNA as template in preincubated extracts from uninfected and infected cells. (a) A 1-μg amount of globin mRNA was added to the extract from uninfected cells. (b) A 1-μg amount of globin mRNA was added to the extract from infected cells. The curve on the top of panel (a) is a densitometer (Joyce, Loebl) tracing, at 620 nm, of Coomassie blue-stained globin marker (20 μg) run in a parallel gel. The arrow indicates the middle of the tracking dye. The dimensions of all the gels used in this study were 0.6 by 10 cm.*

mRNA's less well then those from uninfected cells (Table 1, Experiment 4). We do not have an explanation for these variations in the properties of the extracts from time to time. Since the large majority of our extracts showed no difference in translation (such as Experiment 3, Table 1), we have chosen to take this stand. However, we have not ruled out the possibility that there is present a labile inhibitor of host translation in extracts from infected cells which, depending on uncontrollable factors in the preparation of the extract, is partially or totally inactivated, thus giving different results from preparation to preparation.

The possibility that double-stranded RNA may be involved in the inhibition of nonviral and viral RNA translation in some of the extracts from infected cells was considered. Figure 5 shows that poly(rI):poly(rC) inhibited both viral and nonviral mRNA translation. Thus, it is possible that, in those extracts from infected cells in which both viral and nonviral protein synthesis were inhibited (Table 1, Experiment 4), double-stranded RNA might have been involved. These results suggest that, if any differential inhibition of nonviral message were found in the extract from infected cells, it would not result from double-stranded RNA, since both viral and nonviral mRNA's are inhibited. These findings agree with those reported for the Krebs ascites cell-free extract (19).

Translation of nonviral mRNA's in non-

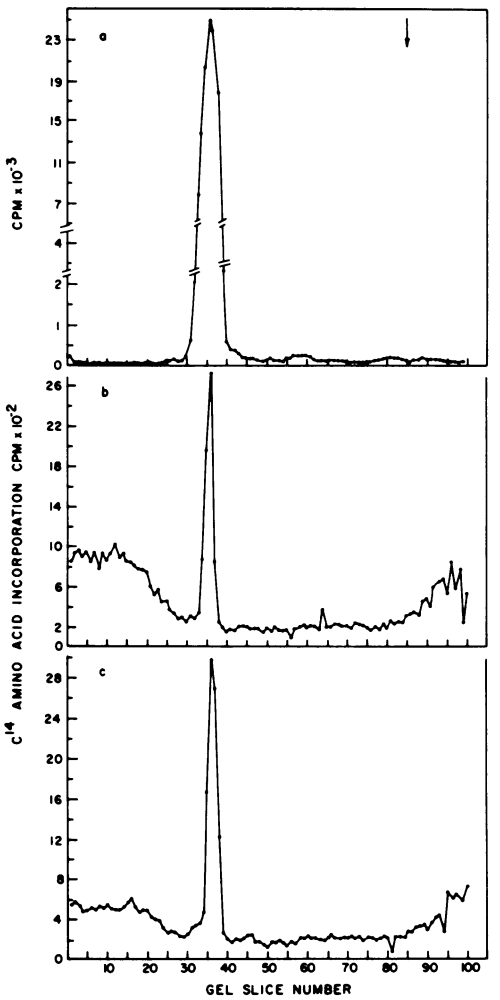

FIG. 4. *Polyacrylamide-SDS gel electrophoresis patterns of products synthesized in vitro, using crystallin mRNA as template in preincubated extracts from uninfected and infected cells. (a) Crystallin marker was prepared as described in the text. (b) Cell-free products were directed by 2.5 μg of crystallin mRNA in the extract from uninfected cells. (c) Same as (b), but in the extract from infected cells. The arrow indicates the middle of the dye band.*

TABLE 1. *Messenger RNA translation in uninfected and mengovirus-infected cell-free extracts*

Exp. no.	Additions	Counts/min of ^{14}C-labeled amino acid incorporation	
		S30 U	S30 I
1	-mRNA	4,112	3,250
	+mengo RNA (1.2 μg)	42,402	39,680
	+L cell mRNA (0.8 μg)	17,045	8,216
	-poly U[a]	1,061	951
	+poly U[a]	34,604	36,000
2	-mRNA	4,248	3,068
	+EAT mRNA (1.0 μg)	8,894	2,783
	+mengo RNA (1.2 μg)	41,531	45,009
	-poly U[a]	1,203	1,114
	+poly U[a]	67,395	69,075
3	-mRNA	2,486	2,136
	+EAT mRNA (1.0 μg)	8,336	8,290
	+L cell mRNA (1.0 μg)	7,652	6,593
	+Mengo RNA (1.2 μg)	22,650	23,186
	+EMC RNA (1.2 μg)	25,010	22,564
4	-mRNA	3,681	2,009
	+mengo RNA (1.0 μg)	22,125	15,997
	+globin mRNA (1.0 μg)	10,406	5,166
	-poly U[b]	735	690
	+poly U[b]	16,724	15,538

[a] Poly(U) was added to a concentration of 80 μg/ml or 4 μg/50 μl of reaction mixture. A total of approximately 100,000 counts/min of [^{14}C]phenylalanyl tRNA was added; about 50,000 of these were trichloroacetic acid precipitable.

[b] Poly(U) concentrations as above. A total of about 55,000 counts/min of [^{14}C]phenylalanyl tRNA was added; of these, about 15,000 were acid precipitable.

preincubated, uninfected, and infected extracts. As seen above, the selective translation of viral mRNA in infected cells was not exhibited in vitro. The lack of difference could result from the loss of a virus or virus-induced host inhibitor. During the preparation of an extract, the preincubation step is one of the most likely to cause inactivation of labile translational components. Thus, we investigated nonviral mRNA translation in non-preincubated extracts from infected and uninfected cells. Figure 6 shows that in the non-preincubated extract, globin mRNA was translated equally well in extracts from uninfected (Fig. 6b) and infected (Fig. 6d) cells. Similar results were observed using lens crystallin mRNA as message (data not shown). Thus, the lack of selectivity of the extract from infected cells does not result from the decay of a labile inhibitor during preincubation. The greater activity of the non-preincubated extract in globin mRNA translation (cf. Fig. 3 and 6) suggests that some component necessary for translation does lose activity during the preincubation step. However, it is not a component that confers the property of differential translation upon the extract from infected cells.

Competition between nonviral and viral mRNA. As mentioned in the Introduction, it is possible that late in infection the displacement of host messages from polysomes could result from competition between viral and host messages for components necessary for translation. To test for competition, extracts from uninfected cells were used because we wished to know the amount of viral mRNA present in the system. Extracts from infected cells contain endogenous viral RNA, which would have been difficult to quantitate. Nonviral and viral mRNA's were used in quantities that saturated the extract. Figure 7 shows titration curves for globin, crystallin, and mengovirus mRNA's. The experiments reported in Fig. 7, 8, and 9 were carried out with preincubated extracts.

Globin versus viral mRNA translation. Globin and mengovirus mRNA were added simultaneously to the reaction mixture, which was then activated by the addition of the cell-free extract. In the experiment in Fig. 8, the mengovirus mRNA concentration was kept constant at 15 μg/ml, and the concentration of globin mRNA was varied from 1.3 to 30 μg/ml. As can be seen, both globin mRNA and viral mRNA were translated at all of the concentrations tested. However, the amount of cell-free product directed by each message in the presence of the other was decreased. When the extract was saturated with respect to both globin and mengovirus RNAs, viral RNA translation was reduced by approximately 25%, and globin translation was reduced by 36%, in comparison with control values.

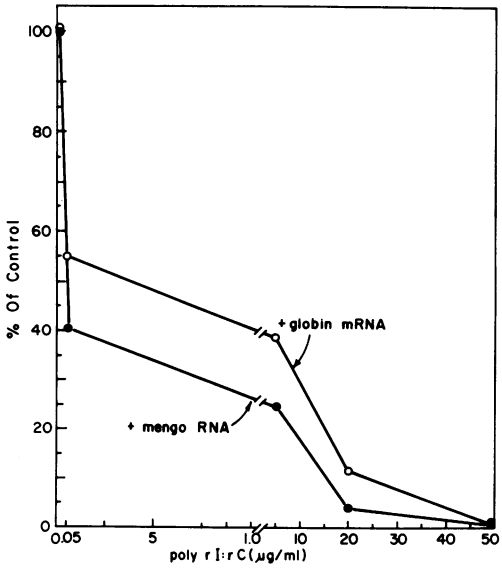

FIG. 5. *Effect of poly(rI):poly(rC) on the translation of mengovirus RNA and globin mRNA in uninfected EAT extracts. Symbols: ○, globin mRNA translation; ●, mengo RNA translation.*

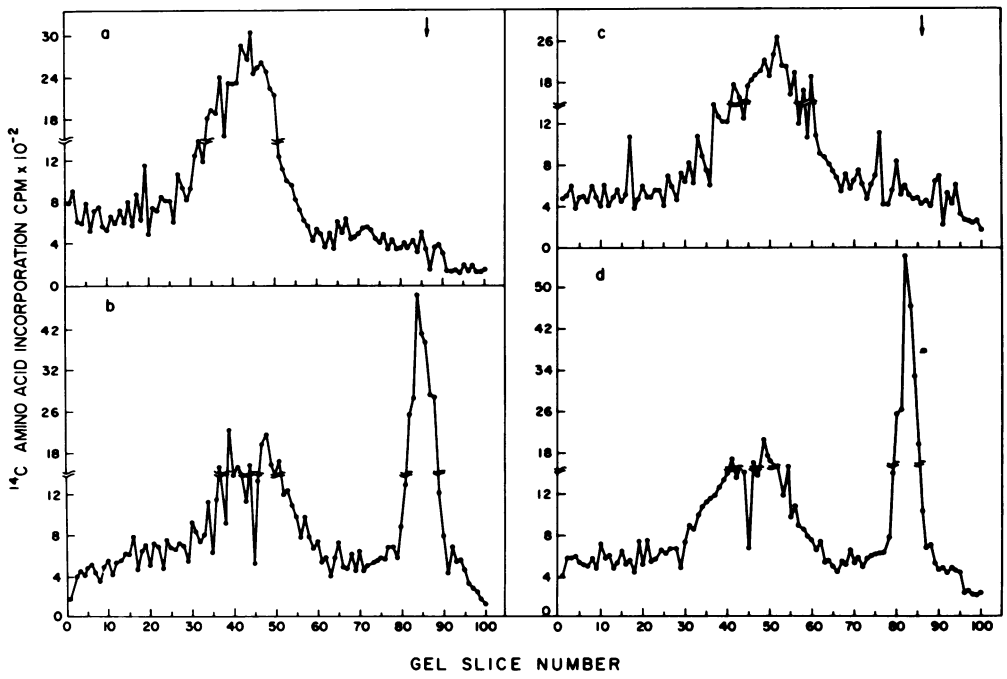

FIG. 6. *Polyacrylamide-SDS gel electrophoresis of cell-free products directed by globin mRNA in non-preincubated extracts from uninfected and infected cells. (a) Endogeneous incorporation of ^{14}C-labeled amino acids in extract from uninfected cells. (b) As in (a), but 1 μg of globin mRNA was added. (c) Endogenous incorporation in the extract from infected cells. (d) As in (c), but 1 μg of globin mRNA was added. The arrows indicate the middle of the dye band.*

This experiment was also carried out using EMC virus and globin mRNA's. The same results were obtained as with mengovirus and globin mRNA's (data not shown).

Crystallin versus mengovirus mRNA translation. As described above, both crystallin and mengovirus mRNA's were added simultaneously to the reaction mixture. Here, however, the crystallin RNA concentration was kept constant at 20 μg/ml, and the mengovirus mRNA concentration was increased from 6 to 24 μg/ml. Figure 9 illustrates that both messengers were translated at all concentrations of viral RNA. However, crystallin synthesis was decreased somewhat more than mengovirus protein "A" synthesis when the extract was saturated with respect to both crystallin and mengovirus RNA.

Globin versus mengovirus RNA translation in a non-preincubated system. From these two sets of experiments, it appeared that, regardless of whether the nonviral or viral mRNA concentration was varied (past saturating concentrations), at no point was there a complete out-competition of the nonviral mRNA by the viral mRNA. Rather, the translation of both types of messages was reduced. In the case of globin versus mengovirus RNA translation, there were 24 times more globin than mengovirus RNA molecules at the highest RNA concentrations used. To bring the number of globin and mengovirus RNA molecules closer to a ratio of 1:1, a considerably greater amount of mengovirus RNA had to be added to the extract saturated with respect to globin mRNA. However, when such high amounts of mengovirus RNA were added, there was no translation of either globin or mengovirus RNA. The viral RNA was very inhibitory at high concentrations (data not shown).

When a non-preincubated extract was used to measure competition between globin and mengovirus RNA, however, mengovirus RNA could be added to very high concentrations (greater than 50 μg/ml) without inhibiting translation. Under these conditions, as shown in Fig. 10, mengovirus RNA suppressed globin translation completely (cf. Fig. 10D and E).

DISCUSSION

In this report we have examined the possibility that mengovirus-directed inhibition of host protein synthesis results from the production of a viral protein that interferes with host, but not viral, mRNA translation. Such an interference could take place by either a positive and/or

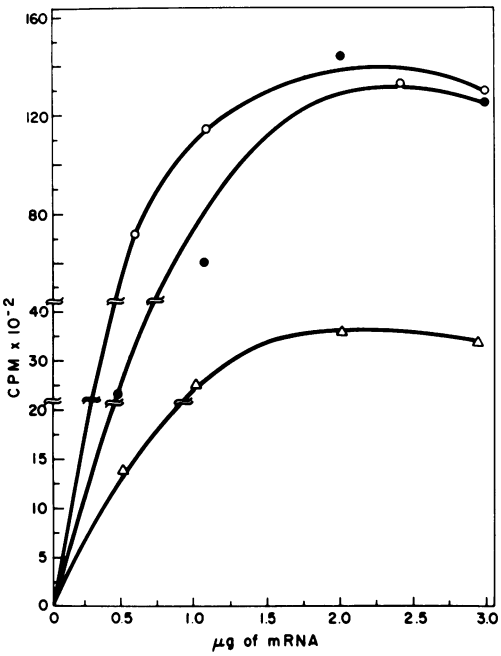

FIG. 7. *Saturation curve for globin, crystallin, and mengovirus mRNA's. mRNA's were added, as indicated, to 100-μl reaction mixtures. The cell-free products were analyzed by SDS-polyacrylamide gel electrophoresis. The total counts per minute for the peaks corresponding to crystallin, globin, and polypeptide A of mengovirus were then determined and plotted. Symbols: ●, globin; ○, polypeptide "A" of mengovirus; △, crystallin.*

negative modulation of host protein synthesis. In the first instance a viral protein could directly inhibit host, but not viral, protein synthesis. Alternatively, a viral protein could enhance viral mRNA translation, thus allowing viral mRNA to compete effectively with host mRNA. The results presented here indicate that extracts from cells infected with mengovirus for 3 h and extracts from uninfected cells do not differ in their ability to translate host and viral mRNA's. This was found to be true under a variety of conditions, including different concentrations of magnesium ions, KCl, mRNA, and S30 extract.

Clearly, these results do not lend support to either of these hypotheses. One can argue, however, that the inhibitor or stimulator is labile or short-lived and is lost in the preparation of the extract. The preincubation step does not appear to inactivate such a putative component for, as shown in Fig. 6, non-preincubated extracts gave the same results as the preincubated extracts in terms of translation of host and viral messages.

One must also consider the extent of the inhibition of host protein synthesis by mengovirus 3 h postinfection. Host protein synthesis at this time amounts to approximately 50% of that of the uninfected controls (9). Therefore, only 50% of the ribosomes (or any other translational component involved in the inhibition) is affected. It is possible that, under these conditions, the presence of the translational elements inactive for host protein synthesis was not detected. Thus, we conclude that the lack of difference in translation of nonviral and viral mRNA's in infected and uninfected extracts is not proof that such a viral modulator does not exist. Rather, we can only conclude that our crude assay system may not detect its presence.

The majority of the experiments performed in this report utilized rabbit globin and chicken lens crystallin mRNA's, two mRNA's found in cells undergoing terminal differentiation. This could mean that they do not behave as typical mRNA's. Globin mRNA, however, is "normal" in the sense that it contains poly(A) in its 3'-OH end (6) and that its 5'-terminus is "capped" (16). Moreover, experiments using total poly(A)-containing mRNA from both L cells and EAT cells yielded the same results as with the pure globin and crystallin mRNA preparations.

In this report we have also considered the possibility that late in infection the displacement of host mRNA from polysomes could result from competition by viral mRNA. Based on kinetic analyses of hemoglobin synthesis, Lodish (14) has proposed that different mRNA's have different rate constants for polypeptide chain initiation. This property of an mRNA could regulate its rate of translation. A recent study (13) has suggested that in vitro EMC virus can out-compete myeloma 10S RNA for factors necessary for translation.

In the studies described here, the ability of mengovirus RNA to compete with the nonviral mRNA's, globin, and lens crystallin was examined. When globin and viral mRNA's or crystallin and viral mRNA's were added simultaneously to a preincubated extract, both viral and nonviral RNAs were translated, though each to a lesser extent than when present alone. For example, at 15 μg of mengovirus RNA per ml and 30 μg of globin mRNA per ml, the translation of both globin and mengovirus mRNA was lowered (36 versus 25%, respectively). However, if one considers that, at these concentrations of mRNA, there were about 24 times more globin than mengovirus molecules present, then this decrease of 36 versus 25% could indicate that the viral mRNA has an advantage over globin mRNA (molecular weight of globin mRNA, 2×10^5 [12]; molecular weight of men-

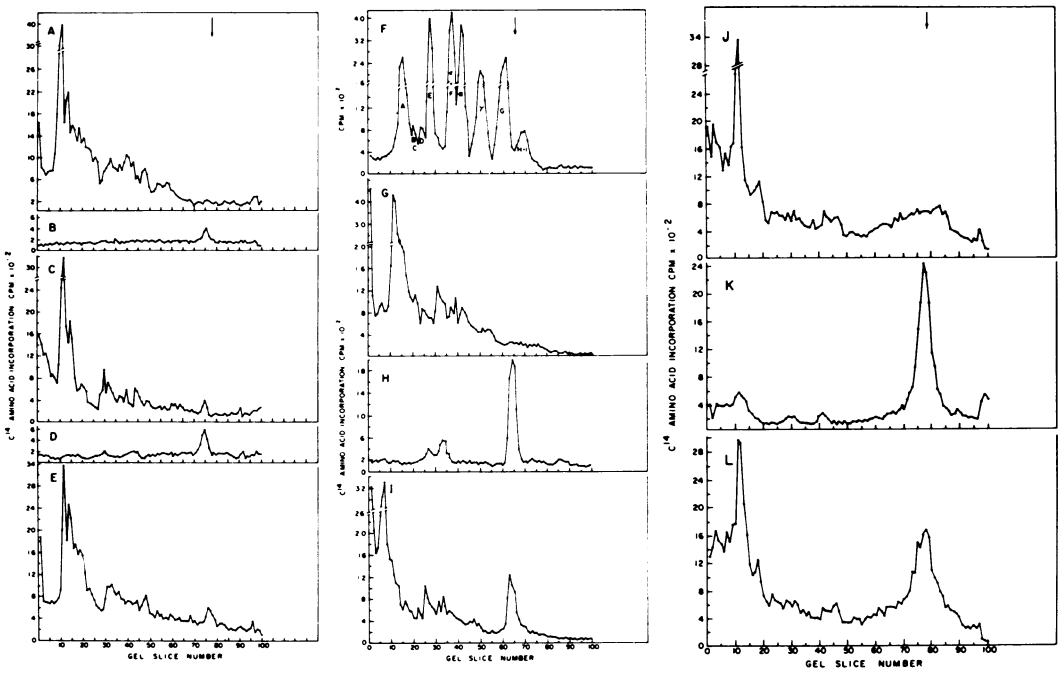

FIG. 8. *Competition experiments. Mengovirus mRNA concentration was kept constant at 15 μg/ml; globin mRNA concentration was varied. (A) Mengovirus mRNA (15 μg/ml) alone. (B) Globin mRNA (1.3 μg/ml) alone. (C) Simultaneous addition of both messengers, concentrations as in (A) and (B). (D) Globin mRNA (2.5 μg/ml) by itself. (E) Both messengers added simultaneously; globin mRNA at 2.5 μg/ml. (F) Mengovirus polypeptide marker prepared as described in the text. (G) Mengovirus mRNA (15 μg/ml) by itself. (H) Globin mRNA (10 μg/ml) alone. (I) Globin mRNA (10 μg/ml) and mengovirus (15 μg/ml) added simultaneously. (J) Mengovirus mRNA (15 μg/ml) by itself. (K) Globin mRNA alone, 30 μg/ml. (L) Simultaneous addition of both messengers; concentrations as in (J) and (K).*

govirus RNA, 2.4×10^6 [23]).

In the case of the simultaneous translation of crystallin and mengovirus mRNA's (Fig. 9), there were approximately three times more crystallin mRNA molecules present (molecular weight, 6×10^5 to 7×10^5 [17]) than mengovirus mRNA molecules at concentrations of 20 μg of crystallin per ml and 24 μg of mengovirus RNA per ml. In this case, also, the translation of both crystallin and mengovirus RNAs was reduced, though crystallin RNA was reduced to a greater extent than was mengovirus RNA.

To bring the molar ratio of globin and mengovirus mRNA's closer to 1, much more mengovirus RNA had to be added to the incubation mixture than was used in the experiments depicted in Fig. 8. However, in the preincubated extract, large amounts of mengovirus RNA was inhibitory to the translation of both mengovirus and globin RNA. It is possible that our viral RNA preparations contain minute amounts of double-stranded RNA, which, as shown in Fig. 5, is inhibitory to our cell-free extract. High concentrations of viral RNA did not inhibit translation in the non-preincubated extracts. This may be explained if the non-preincubated extract contains a double-stranded RNase that degrades contaminating double-stranded RNA in the viral RNA preparation, but which is inactivated during the preincubation step. We plan to test this possibility.

Using a non-preincubated extract, mengovirus RNA was added at a concentration of 60 μg/ml, and globin mRNA was added at 20 μg/ml. At this molar ratio of 1:4, respectively, mengovirus RNA completely inhibited the translation of globin mRNA (Fig. 10). This is in contrast to the simultaneous translation of mengovirus and crystallin mRNA's at a molar ratio of 1:3, respectively. Under these conditions crystallin mRNA was still translated, although to a lesser extent than in the absence of mengovirus RNA. These results could be interpreted as suggesting that crystallin has a higher affinity for initiation factors than globin mRNA or that a certain number of mengovirus RNA molecules must be present for total suppression of nonviral mRNA translation.

These results suggest that, late in infection when there is a large concentration of viral

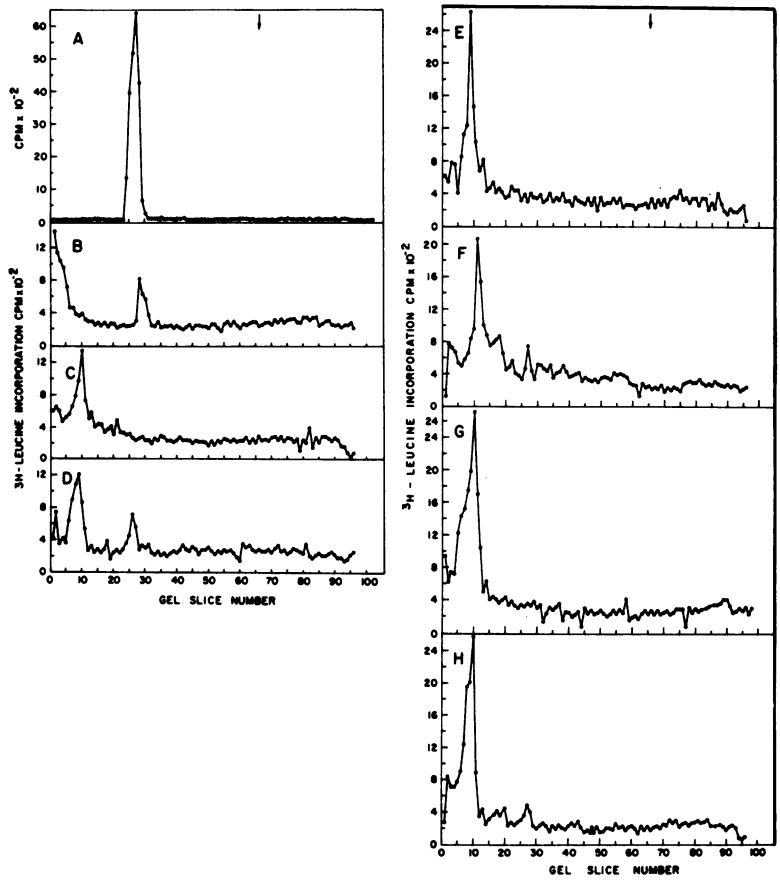

FIG. 9. *Competition experiments. Crystallin mRNA was kept constant at a concentration of 20 μg/ml, whereas mengovirus mRNA concentration was varied. (A) Lens crystallin marker, prepared as described in the text. (B) Crystallin mRNA at above concentration. (C) Mengovirus mRNA (6 μg/ml) added. (D) Both messengers, at above concentrations, added simultaneously. (E) Mengovirus mRNA (12 μg/ml) added alone. (F) Mengovirus mRNA (12 μg/ml) and crystallin (20 μg/ml) added simultaneously. (G) Mengovirus mRNA (24 μg/ml) by itself. (H) Both messengers added simultaneously. Mengovirus mRNA concentration, 24 μg/ml; crystallin mRNA concentration, 20 μg/ml.*

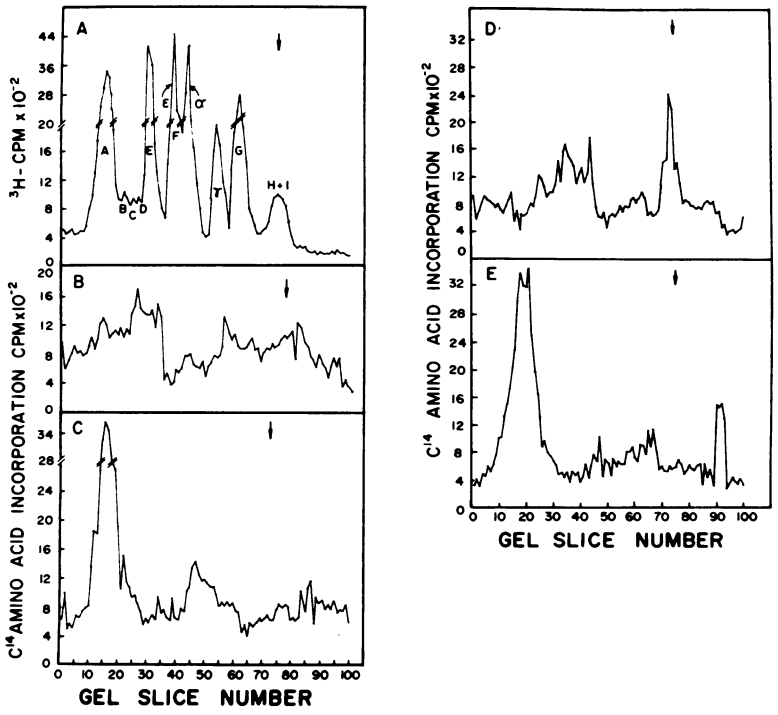

FIG. 10. *Competition experiments in non-preincubated, uninfected EAT cell-free extracts. Globin mRNA was added at a concentration of 20 μg/ml; mengovirus mRNA was added at 60 μg/ml. (A) Viral polypeptide marker. (B) Endogeneous incorporation; no messenger RNA added. (C) Mengovirus mRNA added. (D) Globin mRNA added. (E) Both mengovirus and globin mRNA added simultaneously.*

mRNA's in the cell, the displacement of host mRNA's from polysomes could result from competition by viral RNAs with host RNAs. The relevance of competition to early shutoff of host protein synthesis, however, is questionable, since there would not be sufficient mengovirus RNA molecules present to out-compete host mRNA's. Also, shutoff of host protein synthesis takes place in the presence of azetidine, which inhibits viral RNA synthesis over 99.7% (10), in cells infected with mutants of poliovirus which are defective in viral RNA synthesis (21) and in cells treated with guanidine, an inhibitor of poliovirus RNA replication (2).

Recently, evidence has been presented for the existence of a considerable amount of short-lived mRNA species in HeLa cells (18). According to Puckett et al. (18), most of these short-lived messages do not contain a poly(A) tail. Thus, in our studies (9) on the fate of host poly(A)-containing mRNA's in mengovirus-infected L cells, we would not have detected their presence. It is not yet known whether mengovirus infection results in the shutoff of these species of RNA but, if so, it might contribute to the early disappearance of host polysomes. However, early shutoff of host protein synthesis cannot be explained solely on this basis, and we are still searching for a putative inhibitor of nonviral mRNA translation.

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LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. 69:1408-1412.
2. **Bablanian, R.** 1972. Depression of macromolecular synthesis in cells infected with guanidine dependent poliovirus under restrictive conditions. Virology 47:255-259.
3. **Baltimore, D.** 1969. The replication of picornaviruses, p. 101-176. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, Inc., New York.

4. **Baltimore, D., R. M. Franklin, and J. Callender.** 1963. Mengovirus-induced inhibition of host ribonucleic acid and protein synthesis. Biochim. Biophys. Acta 76:425-430.
5. **Boime, I., and H. Aviv.** 1973. Cell-free synthesis of viral proteins in Krebs II ascites tumor system, p. 188-216. *In* A. I. Laskin and J. A. Last (ed.), Nucleic acid biosynthesis. Marcel Dekker, Inc., New York.
6. **Burr, H., and J. B. Lingrel.** 1971. PolyA sequences at the 3'-termini of rabbit globin mRNAs. Nature (London) New Biol. 233:41-43.
7. **Butterworth, B. E., L. Hall, M. Stoltzfus, and R. Rueckert.** 1971. Virus specific proteins synthesized in encephalomyocarditis virus-infected HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 68:3083-3087.
8. **Cassel, W. L., and K. M. McCaskill.** 1974. Ehrlich ascites tumor preservation for fifteen years—a simple method. Appl. Microbiol. 28:726.
9. **Colby, D. S., V. Finnerty, and J. Lucas-Lenard.** 1974. Fate of mRNA of L-cells infected with mengovirus. J. Virol. 13:858-869.
10. **Collins, F. D., and W. K. Roberts.** 1972. Mechanism of mengo virus-induced cell injury in L cells: use of inhibitors of protein synthesis to dissociate virus-specific events. J. Virol. 10:969-978.
11. **Franklin, R. M., and D. Baltimore.** 1962. Patterns of macromolecular synthesis in normal and virus infected mammalian cells. Cold Spring Harbor Symp. Quant. Biol. 27:175-195.
12. **Gould, H. J., and P. H. Hamlyn.** 1973. The molecular weight of rabbit globin messenger RNA's. FEBS Lett. 30:301-304.
13. **Lawrence, C., and R. E. Thach.** 1974. Encephalomyocarditis virus infection of mouse plasmacytoma cells. I. Inhibition of cellular protein synthesis. J. Virol. 14:598-610.
14. **Lodish, H. F.** 1974. Model for the regulation of mRNA translation applied to haemoglobin synthesis. Nature (London) 251:385-388.
15. **Loening, U. E.** 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 113:131-139.
16. **Muthukrishnan, S., G. W. Both, Y. Furuichi, and A. J. Shatkin.** 1975. 5'-terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature (London) 155:33-37.
17. **Piatigorsky, J., P. Zelenka, and R. T. Simpson.** 1974. Molecular weight and subunit structure of delta-crystallin from embryonic chick lens fibers. Exp. Eye Res. 18:435-446.
18. **Puckett, L., S. Chambers, and J. E. Darnell.** 1975. Short-lived messenger RNA in HeLa cells and its impact on the kinetics of accumulation of cytoplasmic polyadenylate. Proc. Natl. Acad. Sci. U.S.A. 72:389-393.
19. **Robertson, H. D., and M. B. Mathews.** 1973. Double-stranded RNA as an inhibitor of protein synthesis and as a substrate for a nuclease in extracts of Krebs II ascites cells. Proc. Natl. Acad. Sci. U.S.A. 70:225-229.
20. **Scraba, D. G., P. Hostvedt, and J. S. Colter.** 1970. Physical and chemical studies of mengovirus variants. III. Absorbance-temperature profiles, sedimentation in dextran sulfate gradients, and total/infectious particle ratios. Can. J. Biochem. 48:412-417.
21. **Steiner-Pryor, A., and P. D. Cooper.** 1973. Temperature-sensitive polio-virus mutants defective in repression of host protein synthesis are also defective in structural protein. J. Gen. Virol. 21:215-225.
22. **Zelenka, P., and J. Piatigorsky.** 1974. Isolation and in vitro translation of crystallin mRNA from embryonic chick lens fibers. Proc. Natl. Acad. Sci. U.S.A. 71:1896-1900.
23. **Ziola, B. R., and D. G. Scraba.** 1974. Structure of the mengo virion. I. Polypeptide and ribonucleate components of the virus particle. Virology 57:531-542.