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Encephalomyocarditis Virus Infection of Mouse Plasmacytoma Cells

I. Inhibition of Cellular Protein Synthesis

CHARLES LAWRENCE AND ROBERT E. THACH

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University, Saint Louis, Missouri 63110

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Mouse plasmacytoma ascites tumor cells (MOPC 460) were efficiently infected with encephalomyocarditis virus. Inhibition of host protein synthesis was evident after 2 h and complete by 4 h postinfection. The mechanism by which virus infection results in inhibition of host cell protein synthesis was studied in vitro. Cell-free protein-synthesizing systems, prepared from uninfected and infected cells, were found to be equally active with respect to their abilities to translate cellular and viral mRNAs. The plasmacytoma cell-free system was also shown to be insensitive to the addition of double-stranded viral RNA. Host cellular mRNA was isolated from uninfected and infected cells. No difference in the amount or size distribution of the mRNA was detected. However, the mRNA from infected cells was translated only 46 to 49% as actively as that from uninfected cells. mRNA isolated from cells in which initiation of protein synthesis was inhibited with pactamycin was similarly inactivated. Simultaneous addition of viral RNA and cellular mRNA to the plasmacytoma cell-free system resulted in a complete suppression of the translation of the cellular message, whereas viral RNA was translated normally.

The infection of animal cells with picornaviruses results in an inhibition of host protein and RNA synthesis (2). We have investigated the virus-induced inhibition of cellular protein synthesis in encephalomyocarditis (EMC) virus-infected plasmacytoma ascites tumor cells. To study this problem in vitro, we have isolated a cellular mRNA from plasmacytoma tumors and analyzed the ability of a cell-free system made from infected cells to translate this message. We have also isolated this mRNA from infected cells and studied its translation in normal cell extracts. Finally, we have attempted to reconstruct in vitro the conditions found in the infected cytoplasm by simultaneously translating viral and cellular mRNAs.

MATERIALS AND METHODS

Plasmacytoma cell line. An MOPC 460 ascites tumor (obtained from R. Graff) was serially passaged by intraperitoneal injections of 0.2 ml of ascites fluid at 10-day intervals with BALB/c mice (also obtained from R. Graff). One mouse produced 3 to 5 ml of ascitic fluid containing approximately 2×10^8 to 3×10^8 tumor cells.

EMC virus. EMC virus stocks were grown in Krebs II ascites cells (initial cells and virus were generously provided to us by A. T. H. Burness). The procedures for growth (25) and purification (4) of EMC virus have been extensively described by other investigators and have been adopted in this laboratory.

The virus stock used in these experiments was obtained by low-multiplicity passage in Krebs ascites cells and had a titer of 5.1×10^5 hemagglutination units (HAU) per ml.

Infection of MOPC 460 ascites cells with EMC virus. Ascitic fluid, 9 to 14 days after the injection of the tumor cells, was diluted with 10 volumes of sterile ice-cold phosphate-buffered saline (PBS) without calcium or magnesium (Grand Island Biological Co., Grand Island, N.Y.), and the tumor cells were washed by repeated centrifugation at $200 \times g$ until free of contaminating erythrocytes. The cells were resuspended in Leibowitz L-15 medium (Schwartz/Mann) to a concentration of 4×10^6 or 5×10^6 cells per ml. Infections were initiated by adding an equal volume of 60% L-15 medium (0.6 volume L-15, 0.4 volume of water) containing the indicated amount of virus stock to give a final concentration of 80% L-15 medium and 2×10^6 or 2.5×10^6 cells per ml. The virus was allowed to attach at room temperature for 0.5 h in an Erlenmeyer flask with a capacity of 5 to 10 times that of the volume of the culture. After viral attachment, the flask was placed in a 37 C water bath and the cells were kept in suspension by gentle swirling. "Hours postinfection" used in the text refers to the time after the shift-up to 37 C. In the indicated experiments, the unattached virus was removed at 1.5 h postinfection by centrifugation of the cells and resuspension in fresh 100% L-15 medium to give the original cell concentra-

tion. All cultures contained 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 2% heat-inactivated horse serum (Grand Island Biological Co., Grand Island, N.Y.). In all experiments, an uninfected control was used and treated by the same procedures as the infected culture, except for the addition of virus.

In preliminary experiments, it was determined that cells were maximally infected with 0.5×10^{-2} HAU/cell. At this and higher virus inputs, 15 to 20% of the cells were resistant to infection as determined by the absence of cytopathic effect. In these experiments, a 4- to 20-fold excess of infecting virus was used to initiate infection.

Hemagglutination. EMC virus can agglutinate erythrocytes and the amount of hemagglutinating activity is proportional to the concentration of infectious virus (25). Portions of a culture to be assayed were withdrawn and frozen at −20 C, and the hemagglutinating activity of a 50-μliter sample was determined as described by Martin et al. (25). Human erythrocytes were used for the assay, as they gave higher and more reproducible titers than commercially prepared sheep erythrocytes.

Rate of protein synthesis in uninfected and infected cells. Cells were infected at a concentration of 2.5×10^6 cells per ml and 2.55×10^{-2} HAU/cell. Unattached virus was removed at 1.5 h postinfection. At various times, 0.2-ml portions were withdrawn and washed two times by centrifugation in ice-cold PBS. The cells were resuspended in 0.5 ml of Earle balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing 0.1 μCi of a mixture of 15 ^{14}C-labeled L-amino acids (New England Nuclear, Boston, Mass.) and incubated for 10 min at 37 C. Ice-cold PBS was added (3 ml), and the cells were immediately filtered through a glass-fiber filter (Whatman GF/C) and washed with 5% trichloroacetic acid, followed by washes of 95% ethanol and ethyl ether. The filters were dried, and radioactivity was determined in toluene scintillation fluid in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Synthesis of viral protein during infection. For polyacrylamide gel electrophoretic analysis of the proteins synthesized during infection, cells were infected at a concentration of 2.5×10^6 cells per ml and 2.04×10^{-2} HAU/cell. Unattached virus was removed at 1.5 h postinfection. At various times 0.2-ml portions were removed and washed two times by centrifugation in PBS. The cells were suspended in 0.5 ml of Earle balanced salt solution containing 5 μCi of [^{35}S]methionine (170 Ci/mmol, Amersham/Searle) and incubated for 5 min at 37 C. Cells were precipitated by the addition of 0.5 ml of 10% trichloroacetic acid. The precipitate was centrifuged at $860 \times g$ and washed two times with acetone. Residual acetone was removed by warming the precipitate at 37 C. The dried precipitate was dissolved in 50 μliters of sample buffer for electrophoresis and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, as described below.

Purification of a cellular mRNA. Solid MOPC 460 tumors grown in BALB/c mice (generously provided for us by E. Simms) were collected in 2.5 volumes of ice-cold buffer containing 0.05 M Tris-hydrochloride (pH 7.4), 0.025 M KCl, 0.005 M $MgCl_2$, 0.007 M β-mercaptoethanol, 0.8 M sucrose, and 0.5 mg of heparin per ml (Sigma Chemical Co., St. Louis, Mo.). The tumors were dispersed in a Waring blender at low speed for 1 min and at high speed for 0.5 min at 4 C. The dispersed tumor was homogenized by five strokes in a motor-driven Teflon-glass homogenizer, and the homogenate was centrifuged at $15,000 \times g$ for 20 min. The supernatant was mixed with an equal volume of 0.2 M Tris-hydrochloride (pH 9.0), 0.2 M NaCl, 0.01 M EDTA, and 2% SDS, and this mixture was extracted twice at room temperature with an equal volume of water-saturated phenol-chloroform-isoamyl alcohol (50:50:1). RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 20% potassium acetate (pH 5.5) and 2.5 volumes of 95% ethanol, and stored at −20 C. A polyadenylic acid [poly(A)]-containing fraction was obtained by chromatography on oligodeoxythymidylic acid (oligo-dT) cellulose as described by Swan et al. (39). The proportion of the total RNA which bound to cellulose was about 1.1%. The poly(A)-containing fraction eluted at low salt was precipitated by the addition of 0.1 volume of 4 M NaCl and 2.5 volumes of 95% ethanol, collected by centrifugation, and dissolved in 1% SDS. Approximately 540 absorbancy units at 260 nm (A_{260}) of RNA were layered on five 17-ml 5 to 30% (wt/wt) sucrose gradients containing 0.01 M Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 0.001 M EDTA, and 1% SDS. Centrifugation, at 20 C, was for 18.5 h at 26,000 rpm in a Spinco SW27 rotor. The 10*S* region was pooled, precipitated with ethanol, and recentrifuged on two SDS-sucrose gradients as described above, except that centrifugation was for 21 h. The indicated fractions were pooled and the RNA was precipitated as above, dissolved in water, and reprecipitated with ethanol to remove any residual SDS. The RNA was finally dissolved in sterile water for use in cell-free protein synthesis and stored at −20 C.

EMC viral RNA, rabbit globin mRNA, and EMC replicative form (RF). EMC viral RNA was prepared from virus grown in Krebs II ascites tumor cells by the method of Boime and Aviv (4). The RNA extracted from purified virus was purified further by SDS-sucrose gradient sedimentation, as described above for the preparation of cellular mRNA except that centrifugation was performed for 12 h. The 35*S* RNA species was pooled and prepared for use in cell-free protein synthesis, as described above.

The rabbit globin mRNA used was the poly(A)-containing fraction of RNA from rabbit reticulocytes (prepared by P. Yau).

EMC RF (prepared by D. Dobbertin) was purified from Kreb's cells infected for 5 h with EMC virus by a method described previously (S. S. Thach, D. Dobbertin, C. Lawrence, F. Golini, and R. E. Thach, Proc. Nat. Acad. Sci. U.S.A., in press).

Preparation of cytoplasmic extracts for cell-free protein synthesis. Uninfected or infected plasmacytoma cells were washed two times by centrifugation in an ice-cold solution of 0.035 M Tris-hydrochloride (pH 7.5) and 0.14 M NaCl. The cells were resuspended in 2 volumes of Dounce buffer (0.01 M Tris-hydrochloride, [pH 7.5], 0.01 M KCl, and 0.0015

M magnesium acetate) and homogenized with 25 strokes in a tight-fitting Dounce homogenizer. The homogenate was adjusted to 0.03 M Tris-hydrochloride (pH 7.5), 0.12 M KCl, 0.005 M magnesium acetate, and 0.007 M β-mercaptoethanol by the addition of 0.1 volume of a concentrated solution, and then centrifuged at 30,000 × *g* for 10 min. The supernatant was apportioned and stored in liquid N_2.

Preincubated Krebs ascites cell cytoplasmic extract was prepared by the procedure of Boime and Aviv (4).

Cell-free protein synthesis. Reaction mixtures for cell-free protein synthesis contained 0.03 M Tris-hydrochloride (pH 7.5), KCl as indicated below, 0.0035 M magnesium acetate, 0.007 M β-mercaptoethanol, 10^{-3} M ATP, 10^{-4} M GTP, 6×10^{-4} M CTP, 0.01 M creatine phosphate, 0.16 mg of creatine kinase per ml (Boehringer-Mannheim), 19 amino acids (methionine omitted; 4×10^{-5} M each), 3 μCi of [^{35}S]methionine (195 Ci/mmol, Amersham/Searle), mRNA as indicated, and 0.5 A_{260} of cytoplasmic extract in a final volume of 50 μliters. Translation of cellular and globin mRNA was performed at 85 mM KCl and viral RNA was translated at 115 mM KCl. When both cellular and viral messages were present simultaneously, the reaction mixture contained 104 mM KCl. Efficient translation of each message alone occurs at this intermediate concentration. Incubations were performed at 30 C for 2.5 h during which incorporation into messenger-dependent polypeptides is linear in the plasmacytoma cell-free system. Addition of tRNA was not required for activity of either the plasmacytoma or Krebs cell-free systems.

Analysis of the cell-free reaction products by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (22). Protein synthesis reactions were stopped by the addition of 100 μliters of 1% SDS. Protein was precipitated by the addition of 2 ml of acetone and collected by centrifugation. The acetone was removed by aspiration and the dried precipitate was dissolved in 40 μliters of sample buffer containing 5% 2-mercaptoethanol and heated for 2 min at 90 to 95 C. Twenty-five-μliter samples were layered on a slab gel consisting of a 4% acrylamide stacking gel and a 7.5 to 20% acrylamide linear gradient separating gel (5). Electrophoresis was performed at 100 V for 6 h. The gel was fixed in a solution of 25% ethanol plus 10% acetic acid followed by 7% acetic acid, and the fixed gel was dried under vacuum onto Whatman no. 1 chromatography paper. Autoradiography was performed with Kodak RP/R54 X-ray film for 1 to 3 days. Radioactivity in various bands was determined by cutting out the desired region of the gel and incubating it for 2 h at 70 C with 1.0 ml of 30% NH_4OH plus 15% H_2O_2. The eluted radioactivity was quantitated in a Packard Tri-Carb liquid scintillation counter using Brays scintillation solution. Radioactivity was determined by scanning the exposed X-ray film in a Joyce-Loebl double-beam scanning microdensitometer (see Fig. 10). The net density in a messenger-dependent band was determined by subtracting the density of the appropriate minus-messenger control. The data presented in Fig. 10 are the relative densities calculated by dividing the net density by the maximal density in the experiment.

Isolation of mRNA from untreated, infected, or pactamycin-treated cells. Untreated, infected, or pactamycin-treated cells were collected by centrifugation and washed with ice-cold PBS. The cells were suspended in 2 volumes of Dounce buffer and homogenized with 25 strokes of a tight-fitting Dounce homogenizer. The homogenate was adjusted to 0.03 M Tris-hydrochloride (pH 7.5), 0.12 M KCl, and 0.005 M magnesium acetate and was centrifuged at 15,000 × *g* for 10 min. A poly(A)-containing RNA fraction was obtained by SDS-phenol extraction and oligo-dT cellulose chromatography, as described above for the purification of cellular mRNA. This RNA was layered on a 4.2-ml 5 to 30% (wt/wt) sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.5), 0.1 M KCl, and 0.001 M EDTA. Centrifugation was performed at 55,000 rpm for 4 h at 2 C in a Spinco SW56 rotor.

RESULTS

Effect of infection on cellular protein synthesis and demonstration of viral protein synthesis. The rate of protein synthesis during infection of MOPC 460 plasmacytoma cells by EMC virus was estimated by pulse-labeling portions of uninfected and infected cultures with a mixture of 15 ^{14}C-labeled L-amino acids, and by determining the incorporation of label into acid-precipitable radioactivity. A decline in the rate of protein synthesis began between 1 and 2 h postinfection, and this inhibition continued until about 5 h postinfection (Fig. 1). After 5 h of infection, no further inhibition occurred and a residual level of protein synthe-

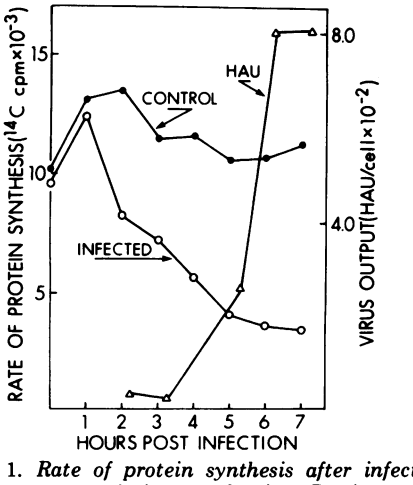

FIG. 1. *Rate of protein synthesis after infection and time course of virus production. Portions of an uninfected and an infected culture were pulse-labeled with ^{14}C-labeled L-amino acids at various times after infection and assayed for incorporated radioactivity. Unattached virus was removed at 1.5 h postinfection, and portions of the infected culture were taken at various times for determination of hemagglutination activity.*

sis remained. Some of this residual protein synthesis probably occurred in the population of cells which remained resistant to infection.

To further analyze the proteins synthesized during infection, cells were pulse-labeled with [^{35}S]methionine at various times postinfection, and the total cellular protein was analyzed by polyacrylamide gel electrophoresis. As is evident in Fig. 2, the viral inhibition of protein synthesis was due to a coordinate inhibition in the synthesis of all the cellular proteins. At 3 and particularly 4 h postinfection, a large amount of the label was incorporated into a population of polypeptides not previously present. Three of these new polypeptides (indicated by arrows) have molecular weights of approximately 10^5, 8.2×10^4, and 3.7×10^4, which correspond closely to the molecular weights of the three primary translation products of the viral message observed by Butterworth et al. (6) in HeLa cells infected with EMC. Much of the protein synthesis observed at 3 and 4 h postinfection (Fig. 2), therefore, appeared to be virus specified, and the inhibition of cellular protein synthesis was actually complete by 4 h postinfection.

Viral RNA synthesis during infection. Viral RNA synthesis in infected cells was monitered by measuring the actinomycin D-resistant accumulation of [^{3}H]uridine into acid-precipitable radioactivity. Viral RNA was maximally synthesized between 3 and 4.5 h postinfection, but newly synthesized viral RNA could be detected as early as 2 h postinfection. Synthesis of viral double-stranded RNA (dsRNA) was barely detectable at that time (unpublished data).

Isolation of a cellular mRNA. To quantitatively study the ability of a cell-free protein-synthesizing system to translate cellular mRNA, a relatively pure messenger was necessary. We initially attempted to purify the mRNA for immunoglobulin light chain from MOPC 460 cells; however, it became apparent that another cellular mRNA could be more easily obtained in higher purity by a very simple purification scheme.

Cytoplasmic RNA was prepared by SDS-

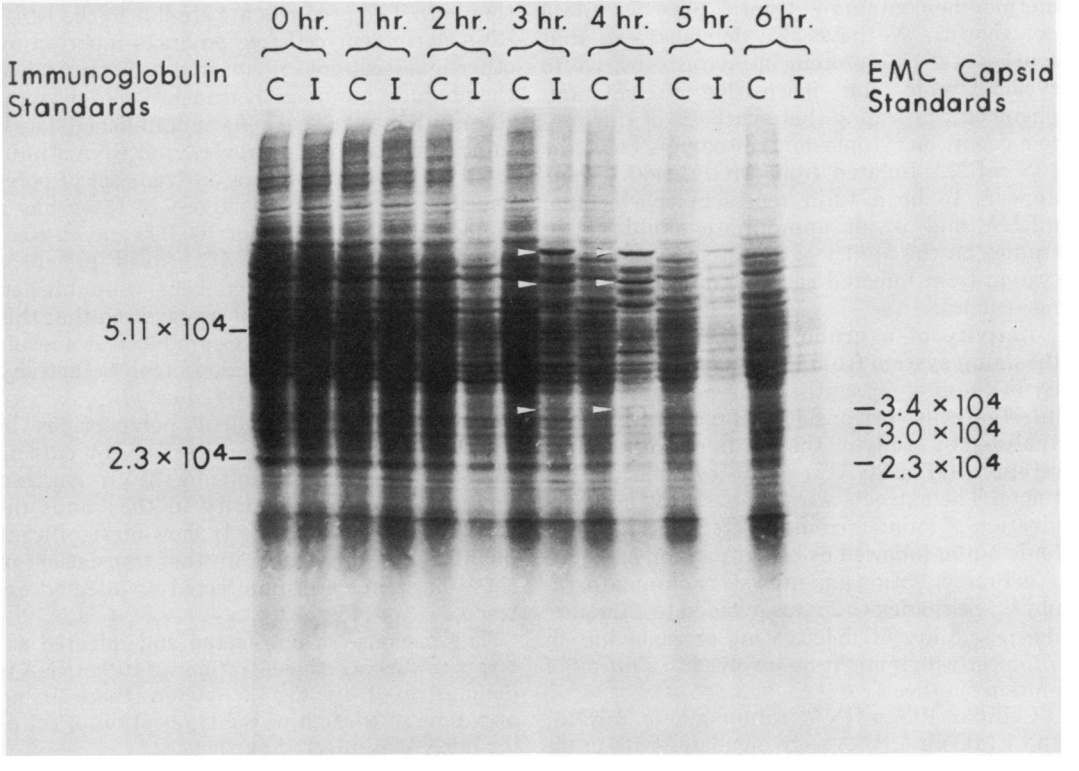

FIG. 2. *Polyacrylamide gel electrophoresis of proteins synthesized during infection. Portions of control (C) and infected (I) cultures were pulse-labeled with [^{35}S]methionine at the indicated times after infection and prepared for electrophoresis. The figure presented is an autoradiograph of the gel described. Unlabeled marker proteins were also run in the gel, and the position of their migration and molecular weight are indicated (right side of gel: EMC capsid polypeptides; left side of gel: purified myeloma protein from MOPC 460 tumors). The arrows indicate presumptive viral polypeptides synthesized in infected cells.*

phenol extraction of a post-nuclear supernatant from solid MOPC 460 tumors, and a poly(A)-containing fraction was obtained by oligo-dT cellulose chromatography. This fraction was sedimented in an SDS-sucrose gradient (Fig. 3A). Various fractions from this gradient were tested in a cell-free protein-synthesizing system, and the products were analyzed by SDS-polyacrylamide gel electrophoresis. Whereas most fractions stimulated incorporation of radioactive amino acids into a number of polypeptides, RNA from the 10*S* region of the gradient stimulated incorporation into only one major polypeptide. This RNA was purified further by sedimentation in an additional SDS-sucrose gradient (Fig. 3B). The translation of this mRNA fraction in a cell-free system from MOPC 460 ascites cells is demonstrated in Fig. 4b. The polypeptide synthesized has a molecular weight of approximately 20,000, and co-electrophoreses with a protein found in the plasmacytoma cells. The apparent identity of this cellular protein with the polypeptide synthesized in response to the 10*S* mRNA in vitro was indicated by two-dimensional mapping of the methionine-containing tryptic peptides (data not shown). We have also demonstrated that synthesis of this protein in vivo is sensitive to cycloheximide and insensitive to chloramphenicol, confirming that synthesis of this protein occurs on cytoplasmic ribosomes. Thus, the 10*S* mRNA isolated from MOPC 460 tumors appears to be a valid representative cellular mRNA and is an appropriate standard for studies on the ability of a protein-synthesizing system from infected cells to translate cellular messenger.

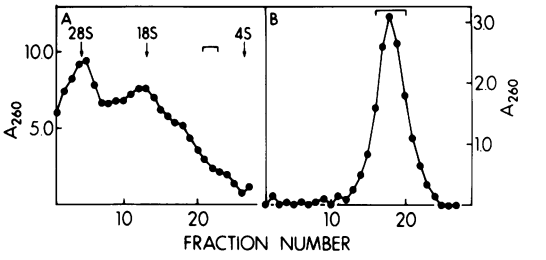

FIG. 3. *Purification of cellular 10S mRNA. (A) Poly(A)-containing RNA fraction isolated from MOPC 460 tumors was sedimented in a SDS-sucrose gradient. The position of rRNA species in a parallel gradient is indicated. The 10S region (indicated by bracket in A) was pooled and recentrifuged in another SDS-sucrose gradient which is shown in B. The 10S region (indicated by bracket in B) from this second gradient was pooled and processed for use in cell-free protein synthesis.*

Activity of a crude cell-free protein-synthesizing system from infected cells. The ability of the protein-synthesizing machinery from infected cells to translate cellular mRNA was analyzed by studying the cell-free translation of cellular 10*S* mRNA in crude cytoplasmic extracts. The extracts were prepared by homogenization of uninfected or infected cells in hypotonic buffer followed by centrifugation at 30,000 $\times$ *g*. Preincubation and dialysis or chromatography on Sephadex G-25 was avoided to eliminate the possibility of inactivating or removing an inhibitor which might be involved in shut-off of protein synthesis.

Cellular 10*S* mRNA, rabbit globin mRNA, and EMC viral RNA were incubated with crude extracts of uninfected and infected cells in the presence of [^{35}S]methionine, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiograph of this experiment is shown in Fig. 4. No qualitative difference could be observed in the translation of 10*S* mRNA and globin mRNA in uninfected or infected extracts. In uninfected extracts, viral RNA stimulated incorporation into a large polypeptide with a molecular weight of about 110,000 and a number of smaller polypeptides (Fig. 4d). The products are similar to the EMC RNA-dependent cell-free products reported by other investigators (5, 20), and are identical to the products obtained by translation of the viral RNA in Krebs cell extracts (unpublished data). In infected extracts, however, viral RNA stimulated incorporation into a different set of polypeptides (Fig. 4h). The largest of these has a molecular weight of about 100,000 and co-electrophoreses with the EMC capsid precursor found in vivo in infected cells (unpublished data). Preliminary evidence suggested that this new set of polypeptides was produced as a result of a post-translational endoproteolytic activity present in extracts of infected cells.

The messenger-dependent polypeptides in Fig. 4 were analyzed quantitatively by cutting them out of the gel, solubilizing the protein, and measuring the radioactivity in the bands directly. The results (Table 1) show no significant quantitative difference in the translation of these messengers in uninfected or infected extracts.

The response of uninfected and infected extracts to varying concentrations of 10*S* mRNA is demonstrated in Fig. 5. Again there is no apparent restriction of the translation of cellular mRNA in infected extracts.

Lack of sensitivity of plasmacytoma cell-free protein synthesis to EMC RF. Since dsRNA has been implicated in the viral-induced shut-off of protein synthesis, we attempted to demonstrate an effect of EMC RF on in vitro protein synthesis in the MOPC 460

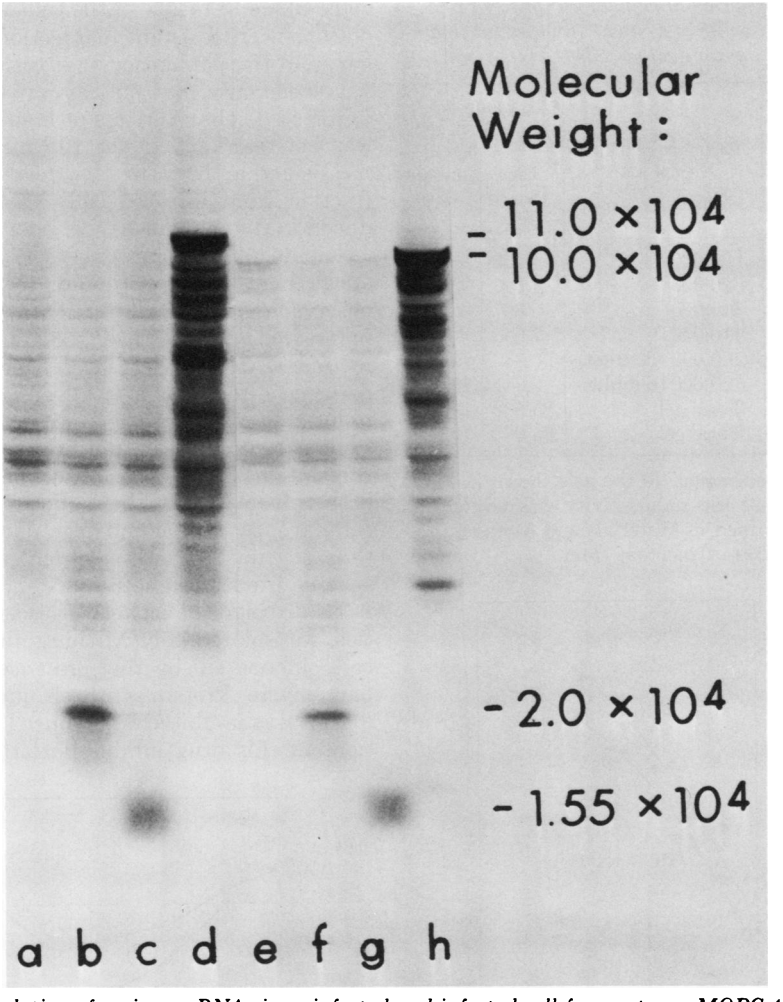

FIG. 4. *Translation of various mRNAs in uninfected and infected cell-free systems. MOPC 460 ascites cells were infected with EMC virus (5 × 10⁻² HAU/cell) or incubated without virus for 4 h. The cells were harvested and cytoplasmic extracts were prepared. mRNAs (as indicated) were incubated in reactions containing either uninfected or infected cytoplasmic extract and [³⁵S]methionine. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. (a–d) Uninfected extracts; (e–h) infected extracts. Additions: (a and e) no RNA added; (b and f) 0.037 A_{260} 10S mRNA added; (c and g) 0.052 A_{260} rabbit globin mRNA added; (d and h) 0.05 A_{260} EMC RNA added.*

cell-free system. Figure 6 compares the response of cell-free systems from MOPC 460 cells and from Krebs II ascites cells to EMC RF. The translation of cellular mRNA in the plasmacytoma system is not significantly affected by EMC RF, whereas translation in the Krebs system can be inhibited up to 75%. The insensitivity of the plasmacytoma system to EMC RF is apparently not due to degradation of the dsRNA since incubation of radioactive EMC RF with plasmacytoma extracts does not render the radioactivity acid soluble (unpublished data), as has been reported for Krebs cell extracts (34). The plasmacytoma cell-free system is also insensitive to polyinosinic acid:polycytodylic acid in concentrations ranging from 10^{-4} to 100 μg/ml (unpublished data).

Activity of cellular mRNA from infected cells. Poly(A)-containing RNA was isolated from uninfected and infected cells after 4 h of incubation. The yield of poly(A)-containing RNA was 1.8% and 2.0% of the total RNA from uninfected and infected cells, respectively. The RNA was analyzed by sucrose gradient sedimentation, and Fig. 7 demonstrates that there was no apparent difference in the size distribution of the RNA isolated from uninfected or infected cells. This is consistent with a recent

TABLE 1. *Translation of cellular 10S mRNA and EMC RNA in cell-free systems from uninfected and infected cells*

mRNA	Region of gel eluted (mol wt)	^{35}S counts/min eluted from gel[a]	
		Uninfected cells[b]	Infected cells
-mRNA	20,000	762	446
+ 10S mRNA	20,000	4,626	3,826
-mRNA	110,000	867	
+ EMC RNA	110,000	7,695	
-mRNA	100,000 (doublet)		1,303
+ EMC RNA	100,000 (doublet)		16,007
-mRNA	Total gel	25,983	19,888
+ EMC RNA	Total gel	85,065	77,159

[a] The indicated regions of the gels shown in Fig. 4 were cut out and the radioactivity was eluted and counted as described in Materials and Methods.

[b] Where cell-free extract was from.

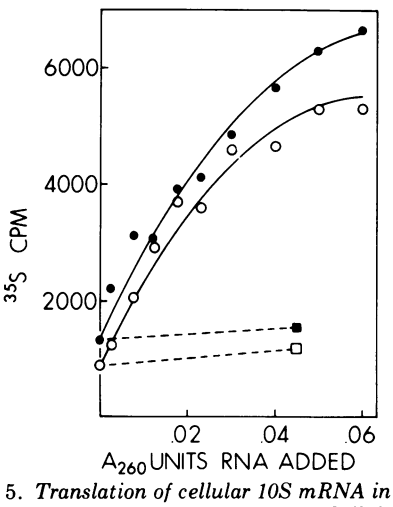

FIG. 5. *Translation of cellular 10S mRNA in uninfected and infected cell-free systems. Cellular 10S mRNA was incubated with cytoplasmic extracts from uninfected or infected cells and [^{35}S]methionine. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The 20,000-molecular weight region of each gel was cut out, and the radioactivity was eluted and counted (see Fig. 4). Symbols: radioactivity stimulated by 10S mRNA (circles), or rRNA (squares), in uninfected extract (solid symbols) or infected extract (open symbols).*

study of the poly(A)-containing RNA isolated from mengovirus-infected L cells (8).

The 10S region of the gradient was assayed in a cell-free protein-synthesizing system to determine the activity of the cellular 10S mRNA. As shown in Fig. 8A, the 10S message from infected cells was 57% as active as that from uninfected cells. Since 15 to 20% of the cells in our infected cultures were resistant to infection, the messenger from the population that was infected may have been only 46 to 49% as active as that from uninfected cells. The loss of messenger activity was not restricted to the 10S mRNA since all messenger activity in the poly(A)-containing fraction from infected cells was similarly reduced (data not shown).

The decrease of messenger activity described above may have accounted, in part, for the observed decline in the rate of protein synthesis in infected cells. However, a distinction must be made as to whether messenger inactivation occurred directly as a result of a specific viral-induced function, or whether it occurred secondarily as a result of an inhibition of host mRNA translation. In the latter case, the untranslated host mRNA would be subjected to normal cellular inactivation and degradation processes, thus accounting for the observed decrease in specific translation activity.

To distinguish between these two possibilities, we isolated poly(A)-containing RNA from cells incubated in the presence of 10^{-7} M pactamycin. Protein synthesis under these conditions was inhibited 80%. Since low concentrations of this drug inhibit initiation of protein

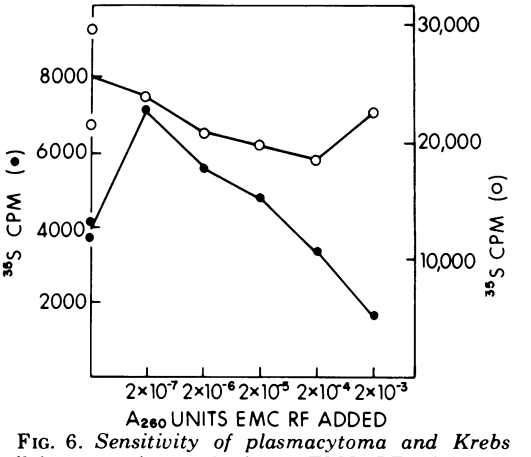

FIG. 6. *Sensitivity of plasmacytoma and Krebs cell-free protein synthesis to EMC RF (dsRNA). Varying amounts of EMC RF and 0.034 A_{260} of 10S mRNA were added to reactions containing either Krebs or uninfected plasmacytoma cell cytoplasmic extracts and [^{35}S]methionine. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The 20,000-molecular weight region of each gel was cut out, and the radioacitivy was eluted and counted. A minus-messenger background of 1,484 and 12,764 counts/min was subtracted from the Krebs and plasmacytoma 20,000-molecular weight region, respectively. Symbols: ●, radioactivity stimulated by 10S mRNA in Krebs extracts; ○, plasmacytoma extracts.*

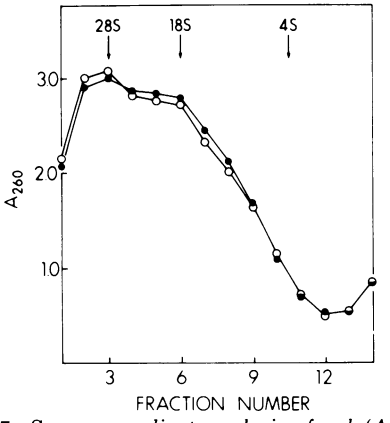

FIG. 7. *Sucrose gradient analysis of poly(A)-containing RNA from uninfected and infected cells. MOPC 460 ascites cells were infected with EMC virus (10×10^{-2} HAU/cell) or incubated without virus for 4 h. The cells were harvested and a poly(A)-containing RNA fraction was prepared and analyzed by sucrose gradient centrifugation. The position of rRNA standards in a parallel gradient is indicated. Symbols: ●, poly(A)-containing RNA from uninfected cells; ○, infected cells.*

synthesis (7), we can study the effects of blocking initiation on mRNA activity independent of virus infection. The yield of poly(A)-containing RNA was 1.9 and 1.5% from untreated and pactamycin-treated cells, respectively, and sucrose gradient analysis revealed no detectable differences in the size distribution of the RNA (data not shown). However, Fig. 8B demonstrates that an inactivation of the mRNA took place in pactamycin-treated cells similar to that which took place during viral infection.

It is interesting that under two different conditions where initiation of protein synthesis was inhibited, virus infection and pactamycin treatment, a functional inactivation of mRNA occurred in the absence of detectable physical alteration of the mRNA. Although the significance of this observation in terms of mRNA metabolism is not understood, it suggests that such a functional inactivation may be a preliminary event in mRNA degradation.

Simultaneous cell-free translation of viral RNA and cellular RNA. Translation of exogenously added cellular mRNA in an infected cytoplasmic extract is not an accurate reconstruction of the infected cell cytoplasm, since much of the endogenous microsomal cellular and viral messenger is removed by centrifugation at 30,000 × *g*. Furthermore, the extensive dilution of the cytoplasm, which takes place in preparing the cell-free system, lowers the concentration of both protein synthesis factors and endogenous mRNA so that any effect of, for example, the presence of viral RNA on the translation of cellular mRNA may be eliminated.

Therefore, the effect of the presence of viral RNA on the translation of cellular 10*S* mRNA was studied by adding both mRNAs in saturating amounts to a cytoplasmic extract and analyzing the products by SDS-polyacrylamide gel electrophoresis. The translation of cellular 10*S* mRNA was suppressed by the presence of viral RNA whereas translation of the latter was unaffected (Fig. 9). This phenomenon was investigated more quantitatively by varying viral RNA concentration while holding cellular mRNA concentration constant (Fig. 10A), or by varying cellular mRNA concentration and holding viral RNA constant (Fig. 10B). The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity in the cellular 10*S* mRNA-dependent polypeptide and in a viral RNA-dependent polypeptide was

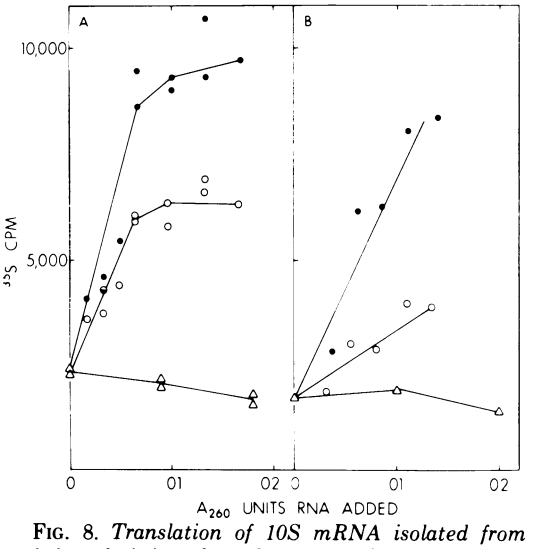

FIG. 8. *Translation of 10S mRNA isolated from uninfected, infected, and pactamycin-treated cells. (A) Varying amounts of fraction 9 from Fig. 5 were added to a reaction containing preincubated Krebs cell cytoplasmic extract and [^{35}S]methionine. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The 20,000-molecular weight region was cut out and the radioactivity eluted and counted. Symbols: ●, radioactivity stimulated by uninfected 10S mRNA; ○, infected 10S mRNA; △, rRNA. (B) Plasmacytoma ascites cells were incubated at a concentration of 3 × 10^6 cells per ml for 2.5 h at 37 C in medium containing no additions or 10^{-7} M pactamycin. Poly(A)-containing RNA was isolated from these cells and analyzed as in Fig. 7. The mRNA sedimenting at 10S was assayed for activity as described in A. Symbols: ●, radioactivity stimulated by 10S mRNA from untreated cells; ○, pactamycin-treated cells; △, rRNA.*

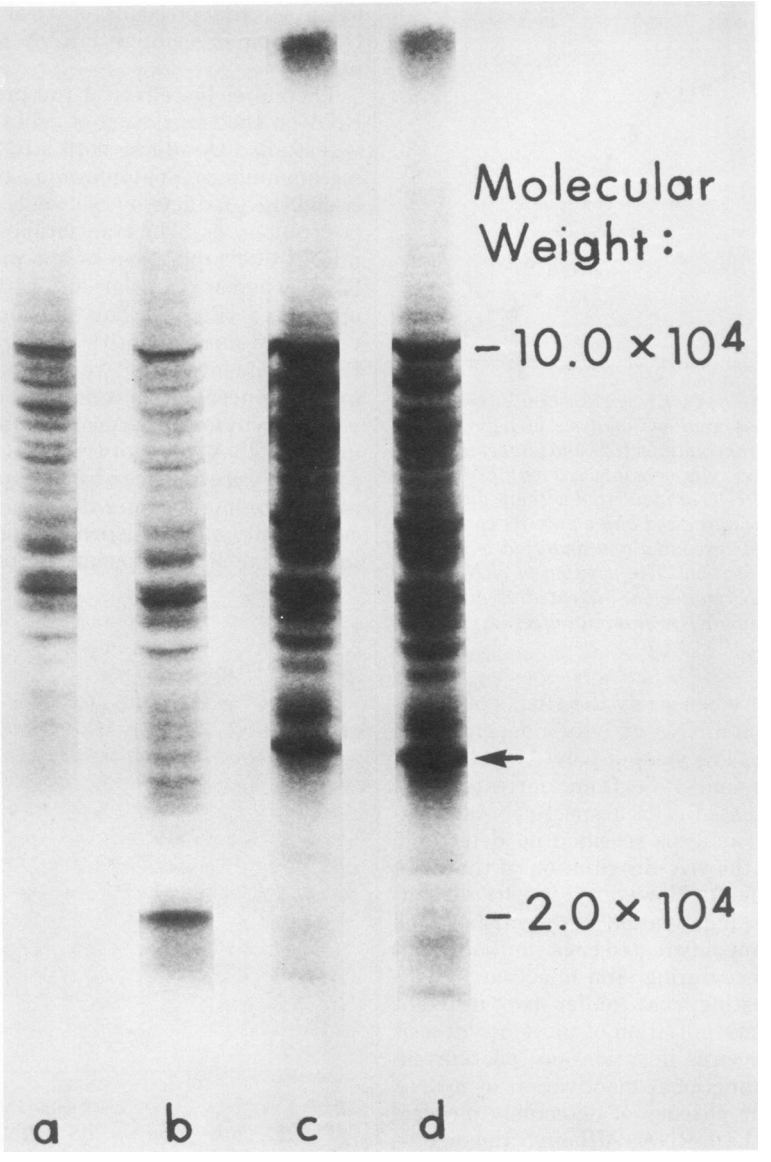

FIG. 9. *Simultaneous translation of EMC viral RNA and cellular 10S mRNA. Varying amounts of mRNAs were added to a reaction containing infected MOPC 460 cytoplasmic extract (see Fig. 4) and [^{35}S]methionine. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Additions: (a) no RNA added; (b) 0.03 A_{260} 10S mRNA; (c) 0.05 A_{260} EMC RNA; (d) 0.03 A_{260} 10S mRNA plus 0.05 A_{260} EMC RNA.*

determined. The presence of viral RNA could completely suppress the translation of cellular mRNA, whereas the presence of cellular mRNA had minimal effect on viral RNA translation (Fig. 10). Fig. 10A indicates that viral RNA was somewhat more effective in inhibiting the translation of cellular mRNA in infected extracts than in uninfected extracts. This was probably due to the presence of some endogenous viral message in these extracts. The most straightforward interpretation of these experiments is that viral RNA has a high affinity for a cellular factor necessary for the translation of cellular mRNA, possibly an initiation factor.

In other experiments, we demonstrated that viral RNA will suppress the translation of cellular mRNA species other than 10S and of rabbit globin mRNA, suggesting that the dominance of viral RNA is general with respect to cellular mRNAs. Interestingly, the cellular 10S

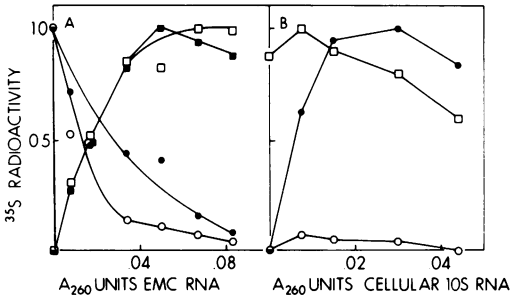

FIG. 10. *Simultaneous translation of EMC viral RNA and cellular 10S mRNA. Reactions contained varying amounts of RNA, uninfected or infected plasmacytoma cytoplasmic extract, and [^{35}S]methionine. Reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The radioactivity in a viral RNA-dependent polypeptide (indicated by arrow in Fig. 9) and in the 10S mRNA-dependent polypeptide was determined as described in Materials and Methods. Symbols: (A) □, reactions contained infected cytoplasmic extract plus viral RNA; ■, ○, viral RNA and 0.03 A_{260} 10S RNA: ●, contained uninfected cytoplasmic extract plus viral RNA and 0.03 A_{260} 10S mRNA; radioactivity incorporated into viral protein, (squares); and cellular 20,000-molecular weight protein, (circles). (B) ●, reactions contained infected cytoplasmic extract plus cellular mRNA; ○, □, cellular mRNA and 0.05 A_{260} EMC RNA; radioactivity incorporated into viral protein, (squares); and cellular 20,000-molecular weight protein, (circles). The relative radioactivity of 1.0 for the cellular 20,000-molecular weight polypeptide and for the viral polypeptide corresponds to about 2,000 and 4,000 counts/min, respectively. The background radioactivity which is subtracted from this data is less than 500 counts/min.*

mRNA will suppress the translation of globin mRNA when both messages are present in a cell-free reaction (unpublished data), indicating that 10S mRNA is neither an abnormally weak message nor seriously inactivated by our purification scheme.

To compare our in vitro results to events that take place in vivo, we have made calculations of the concentrations of viral and cellular mRNA molecules present in our reaction mixtures as well as in the cell cytoplasm in vivo. The concentration of viral RNA necessary to achieve maximal in vitro protein synthesis and maximal inhibition of cellular mRNA translation is about 3×10^{-8} M. The concentration of cellular 10S mRNA necessary to achieve maximal translation in vitro is about 1.5×10^{-7} M. We have estimated that the volume of free cytoplasm in an MOPC 460 cell is approximately 6×10^{-13} liters. The above in vitro concentrations are therefore equivalent to 10^4 molecules of viral RNA and 5×10^4 molecules of cellular mRNA per cell cytoplasm. We have estimated that the total number of viral RNA molecules synthesized in an infected plasmacytoma cell is about 10^5 and the total number of poly(A)-containing cellular mRNA molecules per cell is about 1.5×10^5. These values indicate that the mRNA concentrations used in vitro are within the physiological range and, therefore, that our experimental results are likely to reflect the translation of viral and cellular mRNA in vivo.

DISCUSSION

One feature common to many types of virulent viruses is their ability to inhibit the synthesis of host cell proteins during infection. Viruses for which this property has been demonstrated include picorna (2), Sindbis (36), Newcastle disease (16), vesicular stomatitis (41), adeno (14), herpes (3), and vaccinia viruses (27). Since viral proteins must be synthesized in cells infected with these viruses under conditions where synthesis of cellular proteins is suppressed, this phenomenon offers the opportunity to study a unique type of translational control. EMC virus was chosen for this study because the replication of picornaviruses has been described in some detail (2), because EMC virus can be grown and purified in large quantities (4), and because the RNA extracted from purified virions is an active messenger in cell-free protein-synthesizing systems (5, 20). The MOPC 460 mouse plasmacytoma cell line was chosen as a suitable host cell because the cells can be grown efficiently in mice in either ascites or solid tumor form. The former are useful for studying viral infections in vivo and for the preparation of active cell-free systems for in vitro studies of protein or RNA synthesis. The latter is convenient starting material for large-scale purification of subcellular components such as mRNA and RNA polymerases.

Inhibition of host protein synthesis during picornavirus infection is characterized by a gradual disaggregation of the polysomes synthesizing cellular proteins (11, 30, 37). However, the rate of polypeptide elongation on the remaining polysomes is unchanged during disaggregation (37), and cellular mRNA appears to be unaltered physically during infection (8, 21, 42), suggesting that inhibition of protein synthesis takes place at the level of initiation. (The activity of this cellular mRNA was not analyzed directly, however.) At high multiplicities of infection, host shut-off is induced in the presence of drugs which prevent viral replication such as guanidine and penicillamine (1, 17, 26, 31), and in the presence of amino acid analogues

such as fluorophenylalanine and azetidine (9, 17). Use of the latter drugs results in both synthesis of nonfunctional viral proteins and an inhibition of viral replication. This would suggest that a component of the infecting virion, if present in high enough concentration, can cause the inhibition of host protein synthesis.

An alternative model for the shut-off of host protein synthesis has recently been proposed, namely that viral dsRNA (RF, formed as a by-product of replication) specifically inhibits translation of host mRNAs (12). dsRNA is a potent inhibitor of initiation of protein synthesis in reticulocyte cell-free systems (12) and a somewhat less potent inhibitor in other cell-free systems (34). Furthermore, dsRNA is cytotoxic and can inhibit protein synthesis in vivo when added exogenously to tissue culture cells (10). It has been proposed that dsRNA inhibits protein synthesis by binding to an initiation factor (19) or by stimulating the production of an inhibitor (23). However, certain evidence suggests that the notion of dsRNA involvement in viral induced host shut-off must be viewed with caution: (i) inhibition by dsRNA in vitro is nonselective with respect to the translation of viral and cellular mRNAs (34); (ii) as mentioned above, host shut-off can occur at high multiplicities of infection in the absence of replication, and presumably replication is necessary for dsRNA synthesis; (iii) the lack of sensitivity of the plasmacytoma cell-free system to EMC RF (Fig. 6) suggests that some cell types can be resistant to the effects of dsRNA and yet demonstrate a pronounced inhibition of protein synthesis when infected by picornavirus (this has also been shown to be true for chicken embryo fibroblasts which are shut off by infection with Sindbis virus but whose cell-free protein synthesis is insensitive to dsRNA (35); (iv) it has recently been demonstrated that EMC viral RNA is replicated through single-stranded structures (28; Thach et al., in press) implying that the amount of truly dsRNA early in infection may be very small. For these reasons, we are inclined to seek an alternative explanation for the shut-off phenomenon.

It is apparent that protein synthesis during EMC infection of MOPC 460 cells is characterized by two distinct phenomena: (i) a general inhibition of protein synthesis; and (ii) a switch-over to the synthesis of viral protein (Fig. 1 and 2). However, our study of the activity of the crude cell-free protein-synthesizing system from infected cells indicates neither a general debility in protein synthetic capacity nor a specific restriction against translation of cellular mRNA (Fig. 4 and 5, Table 1). This result has a number of implications. First, it is evident that the total cellular protein-synthesizing machinery, including ribosomes, tRNA, initiation and elongation factors, and aminoacyl-tRNA synthetases, is unaffected by infection. Moreover, it seems likely that no specific inhibitor of translation or viral-induced mechanism for inactivating cellular mRNA is present in the cell-free system. Apparently, the mechanisms which cause the overall decline in protein synthetic activity and which cause switch-over from host to viral protein synthesis have been lost or masked in the cell-free reaction mixtures.

One important factor which may contribute to the overall decline in protein synthesis, especially very late in infection, could be a depletion of the cellular energy pools (ATP, GTP, etc.). In fact, the rate of polypeptide elongation on virus-specific polysomes has been shown to decrease late in infection (38), thus supporting this concept.

An alternative explanation for the general inhibition of protein synthesis is that the ribosomal transit time (per codon) on viral RNA might be intrinsically longer than that of the average cellular mRNA. Since viral RNA replaces most of the cellular mRNA in polysomes during infection, the result would be a lowering of the total rate of protein synthesis. The rate of elongation on poliovirus and EMC-specific polysomes in vivo has been estimated to be 205 to 250 amino acids per min (33). In contrast, the in vivo rates of elongation of alpha- and beta-hemoglobin chains (molecular weight 15,500) are 600 and 400 amino acids per min, respectively (18); ovalbumin (molecular weight 42,000) and conalbumin (molecular weight 76,000) are 320 and 224 amino acids per min, respectively (in chicken oviducts at hen body temperature) (29); and collagen precursor (molecular weight 115,000) is 209 amino acids per min (40). In addition, the average rate of elongation in Chinese hamster ovary cells (weight average molecular weight 85,000) is 420 amino acids per min (13). Thus, different mRNAs can be translated with different elongation rates in the same cell type or tissue and, in general, long mRNAs are translated at lower specific rates than shorter ones. This suggests that the replacement of cellular mRNA by viral RNA on polysomes could itself account for much of the general decline in protein synthetic activity during infection.

The switch-over to synthesis of viral protein during infection is probably caused by the

ability of viral RNA to out-compete cellular mRNA for a component necessary for the initiation of protein synthesis (Fig. 10). This property of viral RNA is also likely to be indirectly responsible for the low specific activity of cellular mRNA isolated during infection. Thus, as the amount of viral RNA increases during infection, the rate of initiation on cellular mRNA will decline, possibly resulting in a greater availability of cellular mRNA to the normal cellular processes of mRNA inactivation and degradation.

Our results are consistent with much of the published literature dealing with host shut-off. At high multiplicities of infection, sufficient viral RNA may be present in the cell to induce the decrease in cellular protein synthesis even in the absence of replication (17). The acceleration of viral induced shut-off of protein synthesis caused by actinomycin D (24) may be due to its effect directly or indirectly on initiation of protein synthesis (15, 32). When the rate of initiation is nonspecifically decreased by an agent such as actinomycin D, the ability of viral RNA to exclude cellular RNA from the protein synthesis machinery may be greatly enhanced.

The ability to utilize cellular protein synthesis machinery more efficiently than cellular mRNA may be a general property of the mRNA of the virulent viruses which suppress cellular protein synthesis. This would be a logical way to insure that a maximal amount of viral specified protein will be synthesized from a minimal amount of mRNA. The general inhibition of protein synthesis in cells infected with such virulent viruses may be only a secondary and relatively unimportant consequence of the efficient translation of viral RNA.

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