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Translational Control of Protein Synthesis After Infection by Vesicular Stomatitis Virus

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Four hours after infection of BHK cells by vesicular stomatitis virus (VSV), the rate of total protein synthesis was about 65% that of uninfected cells and synthesis of the 12 to 15 predominant cellular polypeptides was reduced to a level about 25% that of control cells. As determined by in vitro translation of isolated RNA and both one- and two-dimensional gel analyses of the products, all predominant cellular mRNA's remained intact and translatable after infection. The total amount of translatable mRNA per cell increased about threefold after infection; this additional mRNA directed synthesis of the five VSV structural proteins. To determine the subcellular localization of cellular and viral mRNA before and after infection, RNA from various sizes of polysomes and nonpolysomal ribonucleoproteins (RNPs) was isolated from infected and noninfected cells and translated in vitro. Over 80% of most predominant species of cellular mRNA was bound to polysomes in control cells, and over 60% was bound in infected cells. Only 2 of the 12 predominant species of translatable cellular mRNA's were localized to the RNP fraction, both in infected and in uninfected cells. The average size of polysomes translating individual cellular mRNA's was reduced about two- to threefold after infection. For example, in uninfected cells, actin (molecular weight 42,000) mRNA was found predominantly on polysomes with 12 ribosomes; after infection it was found on polysomes with five ribosomes, the same size of polysomes that were translating VSV N (molecular weight 52,000) and M (molecular weight 35,000) mRNA. We conclude that the inhibition of cellular protein synthesis after VSV infection is due, in large measure, to competition for ribosomes by a large excess of viral mRNA. The efficiency of initiation of translation on cellular and viral mRNA's is about the same in infected cells; cellular ribosomes are simply distributed among more mRNA's than are present in growing cells. About 20 to 30% of each of the predominant cellular and viral mRNA's were present in RNP particles in infected cells and were presumably inactive in protein synthesis. There was no preferential sequestration of cellular or viral mRNA's in RNPs after infection.

Infection of animal cells with viruses frequently results in inhibition of translation of cellular mRNA and a switch to viral protein synthesis. The mechanism of this inhibition has been studied extensively with the picornaviruses poliovirus, EMC, and mengovirus, where after infection cellular mRNA remains fully intact (5, 20). Two very different types of explanations for the inhibition of translation of host mRNA have been proposed. After infection by poliovirus there is a rapid decline in the total rate of protein synthesis (3, 12). This is apparently accompanied by the inactivation of one or more factors required for initiation of protein synthesis, including eIF-3 (9) or a factor which binds to the m^7G residue which is at the 5′ end of most cellular mRNAs (39, 41). Inactivation of the latter factor would explain the preferential translation of poliovirus mRNA, which, unlike cellular mRNA, lacks a 5′ cap sequence (10, 32). By contrast, the total rate of protein synthesis after infection by EMC or mengovirus is not reduced significantly, and there is no evidence for change in the levels or specificity of any of the components required for protein synthesis (1, 8, 12, 18). Rather, EMC is believed to outcompete cellular mRNA's for the limiting numbers of ribosomes or initiation factors present in the cell (7, 11, 12). The above conclusions derive primarily from studies on the properties of cell-free protein-synthesizing extracts prepared from infected and uninfected cells and on competition between cellular and viral mRNA's for translation in various in vitro systems. It is not clear that these studies are directly applicable to mRNA in the intact cell.

In this paper, we describe a technique with which we can determine the size of polyribo-

somes or ribonucleoprotein (RNP) particles which contain the predominant species of translatable cellular and viral mRNA. In this manner one can deduce the rate of initiation of translation of all predominant mRNA's in the intact cell, as well as the fraction of each mRNA species which is sequestered in RNPs. These initial studies have focused on the inhibition of synthesis of BHK proteins after infection by vesicular stomatitis virus (VSV).

Infection of animal cells by VSV results in inhibition of synthesis of cellular proteins and RNA and in eventual cell death. Neither cell killing nor inhibition of cellular protein synthesis is a direct consequence of the infecting virion; at least some transcription of the negative-stranded viral genome into mRNA is essential. Neither replication of progeny virion RNAs nor production of infectious virions is necessary for manifestation of this inhibition (28–31, 40). One paper suggests that VSV mRNA initiates protein synthesis severalfold more efficiently than does cellular mRNA, and thus out-competes mRNA for ribosomes (33).

Here we demonstrate that cellular mRNA remains fully functional after VSV infection, and most remains attached to polyribosomes. Infection does result in a reduction in the rate of initiation of translation of all predominant species of cellular mRNA, but translation of cellular and viral mRNA's which encode for the same size of proteins occurs on the same size of polyribosomes. We conclude that the rates of initiation of protein synthesis on typical cellular and VSV mRNA's are the same, and that the large amount of viral mRNA synthesized after infection competes with cellular mRNA for ribosomes. This is responsible for much, but probably not all, of the inhibition of translation of cellular mRNA after infection. About 20 to 40% of all predominant species of cellular and viral mRNA's are found in RNPs in infected cells, but there is no preferential sequestration of any particular class of cellular or viral mRNA's after infection.

MATERIALS AND METHODS

Cell growth and infection. BHK-21 cells were grown in suspension culture to densities of between 5 $\times 10^5$ and 7 $\times 10^5$ per ml. For infection, the cells were recovered by centrifugation and suspended at 5 $\times 10^6$ cells/ml in culture medium also containing 1.5 mM $CaCl_2$ and 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5. A multiplicity of 10 PFU of the Glasgow isolate of the Indiana serotype of VSV, obtained originally from C. Pringle, Institute of Virology, Glasgow, was added, and the samples were incubated at 37°C for 30 min. Two volumes of culture medium, containing HEPES but no $CaCl_2$, was then added, and incubation at 37°C was continued.

Labeling of infected cells with [^{35}S]methionine. A sample of 6 ml of either mock-infected or VSV-infected cells was removed at the indicated times, and the cells were recovered by centrifugation. They were washed twice by centrifugation in growth medium lacking amino acids and $CaCl_2$ but containing 10% dialyzed fetal calf serum and 0.01 M HEPES, pH 7.5, and then resuspended in 1 ml of this medium. Duplicate samples of 0.2 ml of the cell suspension were added to 1.0 ml of prewarmed growth medium lacking $CaCl_2$ and containing 1% the normal concentration of unlabeled methionine and [^{35}S]methionine (10 μCi/ml, 100,000 mCi/mmol; The Radiochemical Centre). The samples were immediately incubated at 37°C, and 20-μl portions were removed, in duplicate, at intervals to monitor incorporation of radioactivity into trichloroacetic acid-precipitable protein. At the end of the incubation, generally 30 min, a 0.4-ml portion of the cell suspension was removed and centrifuged. The cell pellet was dissolved in 60 μl of lysis solution (0.01 M HEPES, pH 7.5, 0.01 M $MgAc_2$, 0.2% sodium dodecyl sulfate [SDS], and 0.25 μg of phenylmethylsulfonyl fluoride per ml); after lysis, 60 μl of a lysis solution containing 20 μg of DNase per ml (DPFF; Worthington Biochemicals Corp.) was added, and the samples were incubated at 37°C. After the viscosity had dropped, 60 μl of threefold-concentrated sample buffer for gel electrophoresis was added, and the samples were immediately plunged into a boiling water bath in preparation for gel electrophoresis.

Isolation of total cellular RNA. A sample of 5 $\times 10^7$ uninfected or VSV-infected cells was recovered by centrifugation, washed once in phosphate-buffered saline and once in low-salt buffer [0.01 M HEPES, pH 7.5, and 0.0015 M $Mg(OAc)_2$], and resuspended in 10 ml of the same buffer. The cell solution was incubated for 5 min at 37°C after addition of 0.2 ml of 10% SDS. Then 25 μg of DNase was added, and incubation was continued until the viscosity had dropped, generally 5 to 10 min. The following solutions were then added: 10 ml of phenol, 10 ml of chloroform (containing 4% isoamyl alcohol), 10 ml of water, 2 ml of buffer A (1 M NaCl–0.1 M Tris–0.01 M EDTA, pH 7.5), and 1.8 ml of 10% SDS. After vigorous shaking for 3 min, the phases were separated by centrifugation at 4°C. The organic phase was reextracted with a mixture of 10 ml of water, 1 ml of buffer A, and 1 ml of 10% SDS. The pooled aqueous layers were back-extracted with 20 ml of $CHCl_3$. RNA was precipitated with 2 volumes of ethanol and, after overnight storage at −20°C, was recovered by centrifugation. The RNA was dissolved in 4 ml of 0.4 M NaAc, pH 5.2, and precipitated with 2 volumes of ethanol. The recovered RNA pellet was again dissolved and precipitated with ethanol. Finally, it was dried under vacuum, dissolved in 0.5 ml of water, and stored in small portions at −70°C.

Isolation of polyribosomes. A postnuclear supernatant from 5 $\times 10^7$ [^{3}H]uridine-labeled cells was prepared, treated with detergents, and analyzed on a sucrose velocity gradient exactly as described previously (24, 25) except that centrifugation in the SW27 rotor was for 2 h at 27,000 rpm. Fractions of 1.5 ml

were collected into tubes containing 0.15 ml of 10% SDS, and a 50-μl sample was counted.

A 1.1-ml amount from two or three adjacent fractions was pooled and diluted to 4 ml; then 0.4 ml of buffer A was added. Extraction with phenol and $CHCl_3$ followed the protocol described above for total cellular RNA, except that proportionally smaller volumes of solutions were used. RNA was finally dissolved in 0.15 ml of water.

Cell-free protein synthesis. Wheat germ extracts (2) were preincubated with micrococcal nuclease before use, using the procedure of Pelham and Jackson (37), in order to reduce the background of endogenous protein synthesis. Reactions (25 μl) contained 20 mM HEPES, pH 7.5, 2.5 mM ATP, 0.3 mM GTP, 16.8 mM creatine phosphate, 60 μg of creatine phosphokinase per ml, 5 mM dithiothreitol, 30 mM KOAc, 1.5 mM $Mg(OAc)_2$, 0.125 mM each of 19 amino acids, 200 μCi of [^{35}S]methionine per ml (100,000 mCi/mmol), 0.76 mM spermidine, and 7.5 μl of wheat germ extract per reaction. The final concentrations of K^+ and Mg^{2+}, including that contributed by the wheat germ extract, were 66 and 3.0 mM, respectively.

Reactions (25 μl) contained up to 4 μg of RNA from cells or various polysomal fractions; protein synthesis was invariably proportional to the amount of RNA added in this range (see Fig. 3). Incubation was at 25°C for 90 min, and duplicate portions of 5 μl were taken to measure incorporation into protein.

One-dimensional and two-dimensional gel analysis of proteins. Conditions for analysis of viral or cellular proteins by electrophoresis through polyacrylamide gels containing SDS were described previously (14, 27) except that a 25-cm gel containing a linear 5 to 15% gradient of acrylamide was used. The dried gels were autoradiographed with Kodak SB-5 X-ray film. Two-dimensional gels were an adaption of the O'Farrell technique (15, 34). The two-dimensional gels were impregnated with the scintillant 2,5-diphenyloxazole (PPO) and exposed to prefogged film (16). Films were scanned with a Joyce-Loebl microdensitometer, using a wedge so that full-scale pen deflection represented 1.16 optical density units, a value within the linear range of the film and the densitometer.

RESULTS

Synthesis of cellular proteins after VSV infection. Four and one-half hours after infection of BHK cells by the Glasgow strain of the Indiana serotype of VSV, the total rate of incorporation of [^{35}S]methionine into protein was about 65 to 70% that of uninfected cells (Fig. 1). The rate of protein synthesis declined gradually throughout the early stages of infection and was about 85 and 75% that of control cells at 2.5 and 3.5 h, respectively (Fig. 1). Electrophoresis through long one-dimensional gradient polyacrylamide gels could resolve about 20 predominant cellular polypeptides and all five VSV structural proteins (Fig. 2, lanes A through F). Synthesis of all cellular polypeptides declined after VSV infection. Microdensitometry of the

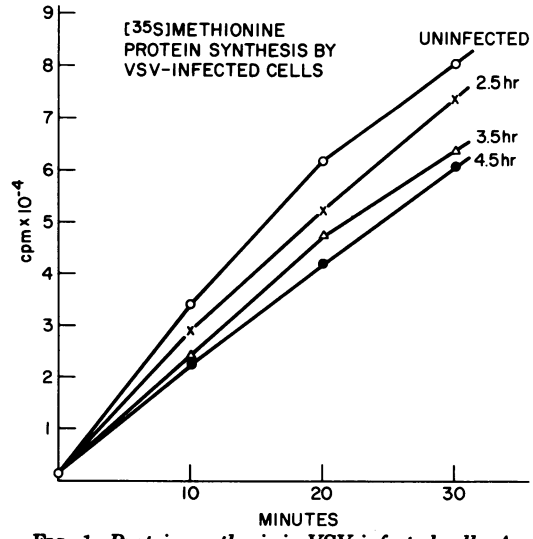

FIG. 1. *Protein synthesis in VSV-infected cells. As detailed in Materials and Methods, samples of VSV-infected cells at 2.5, 3.5, or 4.5 h after infection, or mock-infected cells, were labeled with [^{35}S]methionine. Indicated on the ordinate is the radioactivity incorporated by 5 μl of culture, equivalent to 8 × 10^3 cells.*

gels shown in Fig. 2 established that at 4.5 h after infection most of the predominant host polypeptides (lettered a through h) were synthesized at a rate about 25% that of mock-infected cells (Table 1); synthesis of some cellular polypeptides was inhibited to a greater or lesser extent than this value. Similar results were obtained after using two-dimensional gels to analyze synthesis of cellular and viral polypeptides (data not shown). The balance of the proteins synthesized in the infected cells were, of course, the viral structural proteins L, G, N, NS, and M (Fig. 2).

mRNA in infected cells. To quantitate the amount of translatable cellular and viral mRNA present in the infected cells, samples of infected and control cells were dissolved in buffer containing SDS. Total RNA was isolated and translated in an mRNA-dependent wheat germ cell-free system. The RNA was not fractionated into a polyadenylated component; thus, most of RNA used in these reactions was rRNA. The amount of [^{35}S]methionine-labeled protein synthesized was proportional to the amount of RNA added (Fig. 3).

The one-dimensional gel analysis of Fig. 2 (lanes H through L) makes the important point that all translatable cellular mRNA's remained intact and functional after infection. Per microgram of cellular RNA, preparations from infected and uninfected cells directed the synthesis

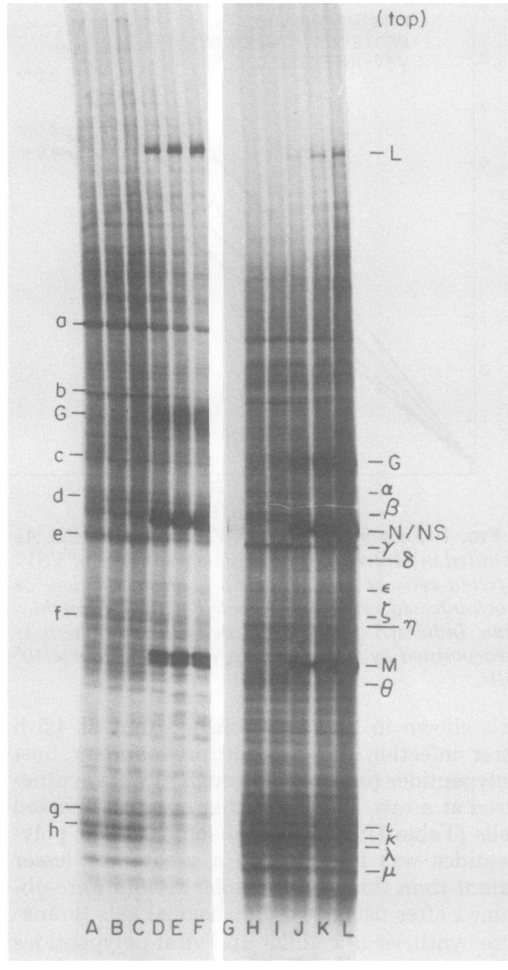

FIG. 2. *Gel analysis of proteins synthesized in infected cells and of cell-free translation products of RNA from infected cells. A linear 10 to 15% gradient polyacrylamide gel was used. (Lanes A–F) Proteins synthesized during a 30-min labeling period with [^{35}S]methionine, beginning at the indicated times. A, Mock-infected cells, 2.5 h; B, mock-infected cells, 3.5 h; C, mock-infected cells, 4.5 h; D, VSV-infected cells, 2.5 h; E, VSV-infected cells, 3.5 h; F, VSV-infected cells, 4.5 h. The letters a through h indicate predominant species of cellular proteins whose synthesis is quantitated in Table 1. (Lanes G–L) Proteins synthesized in a wheat germ cell-free system using 2 μg of the indicated RNAs per 25-μl reaction. A 5-μl sample of the reaction, containing in brackets the indicated amount of acid-precipitable radioactivity, was analyzed. G, No added RNA (21,000 cpm); H, RNA from mock-infected cells, 2.5 h (190,000 cpm); I, RNA from mock-infected cells, 4.5 h (194,000 cpm); J, RNA from VSV-infected cells, 2.5 h (236,000 cpm); K, RNA from VSV-infected cells, 3.5 h (310,000 cpm); L, RNA from VSV-infected cells, 4.5 h (580,000 cpm). The Greek letters denote predominant translation products of cellular mRNA, and the capital letters are the VS^{V} structural proteins.*

TABLE 1. *Synthesis of some predominant cellular proteins after VSV infection*[a]

Cells	Synthesis relative to uninfected cells							
	a	b	c	d	e	f	g	h
Uninfected	65	34	27	24	57	20	23	32
Infected								
2.5 h[b]	0.61	0.58	0.80	0.71	0.58	0.81	0.79	0.42
3.5 h	0.39	0.31	0.53	0.38	0.36	0.51	0.50	0.27
4.5 h	0.23	0.18	0.33	0.29	0.20	0.31	0.38	0.19

[a] The gel radioautograms of the gels depicted in Fig. 2 (lanes A through F) were scanned with a Joyce Loebl microdensitometer, using a full-scale absorbance of 1.16 optical density units, a value within the linear range of the film. Lower-case letters denote polypeptides. The values for uninfected cells are the areas, in arbitrary units, for the various polypeptides; these values are proportional to the amount of radioactivity in these proteins, and are thus a measure of their relative rates of synthesis. The values for infected cells are the areas, relative to the values for growing cells.

[b] Time after infection.

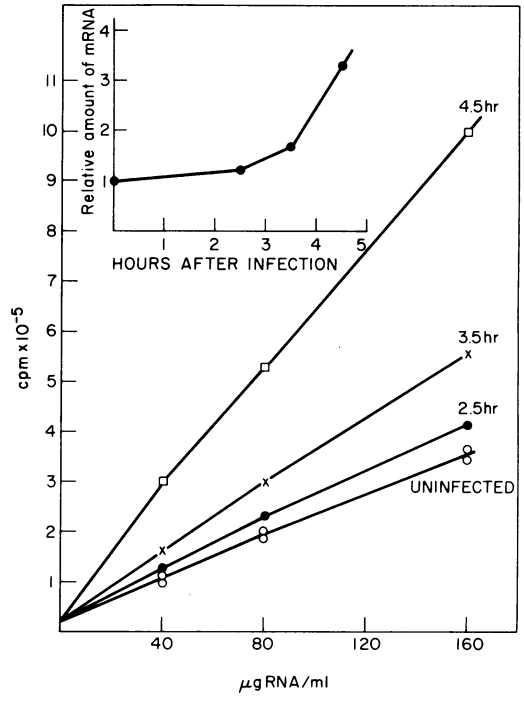

FIG. 3. *Relative amount of translatable mRNA in VSV-infected cells. As detailed in Materials and Methods, total RNA was isolated from mock-infected cells, in duplicate, and from cells at 2.5, 3.5, and 4.5 h after VSV infection. Translation was in a wheat germ cell-free system; indicated is the amount of [^{35}S]methionine incorporated into protein per 5 μl of reaction. (Inset) The relative amount of translatable RNA, determined from the initial slopes of the curves in the main figure, relative to that in uninfected cells, is plotted versus the time after infection.*

of equal amounts of all predominant cellular polypeptides. The additional translation activity present in RNA from infected cells directed synthesis of the five VSV structural proteins, plus a few other polypeptides which may have been incomplete or degraded products of these. Many of the translation products of cellular mRNA comigrated with predominant protein species synthesized by the growing cells. A notable example is actin (polypeptide γ), but conclusive identification of these cellular species must await peptide mapping.

Microdensitometry of the radioautograms established that the synthesis of all predominant species of cellular and viral proteins was proportional to the amount of RNA added. Thus, the amount of protein synthesized in vitro, within the range of mRNA concentrations used, is a valid measure of the amount of corresponding mRNA activity in the sample.

Translation products of many more cellular mRNA species could be resolved by two-dimensional gel electrophoresis (arrows, Fig. 4b). Again, mRNA isolated at 4 h after VSV infection

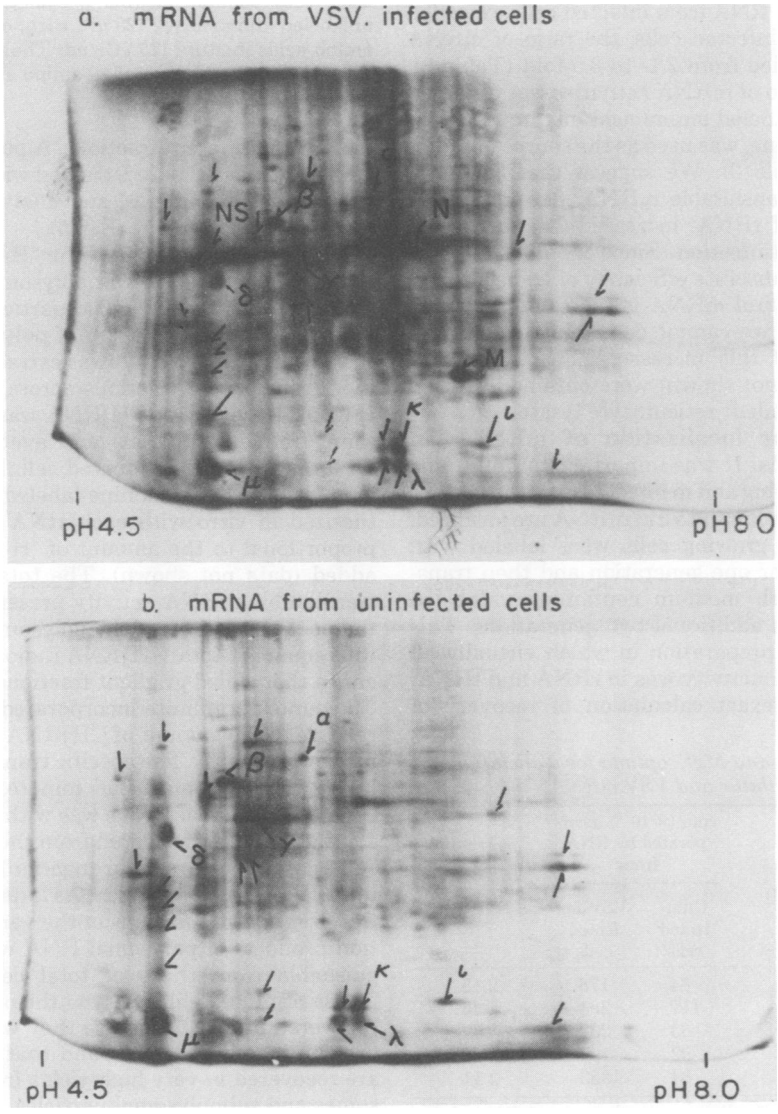

FIG. 4. *Two-dimensional gel electrophoresis of translation products of RNA from uninfected cells (b) or from cells at 4 h after infection (a). The acidic end (pH about 4.5) is on the left; the basic end (pH 8.0) is on the right (see reference 15). The viral proteins N (molecular weight 52,000) and M (molecular weight 35,000) provide internal molecular weight standards. Arrowed are some of the predominant cellular translation products which do not overlap the major viral proteins.*

was as efficient in directing in vitro synthesis of all of predominant cellular proteins as was RNA from growing cells (Fig. 4a). We conclude that cellular mRNA is stable after VSV infection and remains fully translatable.

RNA from cells at 4.5 h postinfection directed synthesis of 3.5 times as much protein, per microgram of total RNA, as did RNA from uninfected cells. The amount of translatable activity increased steadily from 2.5 h after infection (Fig. 3, inset). Translation of cellular and viral mRNA did have slightly different optimal K^+ and Mg^{2+} concentrations (Table 2). Under all conditions, translation of RNA from infected cells exceeded that from uninfected cells; the ratio of mRNA activities varied from 2.1- to 3.5-fold (Table 2). A similar ratio of mRNA activities was obtained when a ^{14}C-labeled amino acid mix, rather than [^{35}S]methionine, was used as the source of radioactivity (Table 3). We suggest that the total amount of translatable mRNA, per cell or per microgram of rRNA, increased 2.5- to 3.5-fold by 4.5 h postinfection. Since we do not know whether the absolute efficiency of translation of cellular and viral mRNA is the same in this in vitro system, we cannot determine exactly the magnitude of this increase. However, similar results (data not shown) were obtained with an mRNA-dependent reticulocyte lysate.

Subcellular localization of mRNA's in growing cells. It was important to determine where in growing and in infected cells the various species of cellular and viral mRNA are localized. To this end, growing cells were labeled with [^3H]uridine for one generation and then transferred to fresh medium containing unlabeled uridine for an additional two generations. This resulted in a preparation in which virtually all of the ^3H radioactivity was in rRNA and tRNA, and allowed exact calculation of recovery of RNA from different fractions. A postnuclear supernatant was prepared, treated with detergents to dissolve membranes, and analyzed on a sucrose velocity gradient (Fig. 5).

TABLE 2. *K^+ and Mg^{2+} optima for translation of cellular and VSV mRNA*[a]

K^+ (mM)	Mg^{2+} (mM)	cpm ($\times 10^{-3}$) incorporated by RNA from:		VSV/uninfected
		Uninfected cells	VSV-infected cells	
36	3.0	84	178	2.12
66	3.0	117	284	2.43
96	3.0	63	233	3.67
66	2.5	92	192	2.08
66	3.5	84	263	3.11

[a] Reactions (25 μl) contained 2 μg of RNA from either uninfected or VSV-infected cells and the indicated concentrations of K^+ and $Mg(OAc)_2$. Included is the 36 mM KCl and 1.5 mM Mg^{2+} contained in the wheat germ extract.

TABLE 3. *Comparison of mRNA activities using [^{35}S]methionine and ^{14}C-labeled amino acid mixture*[a]

Radioactive amino acid	cpm incorporated with RNA from:		VSV/uninfected
	Uninfected cells	VSV-infected cells	
[^{35}S]methionine	222,900	593,800	2.66
^{14}C-mixture	3,850	8,681	2.25

[a] Reactions (25 μl) containing 4 μg of total RNA from growing cells, or cells at 4 h after infection, were used; they contained either [^{35}S]methionine and 19 unlabeled amino acids, 125 μM each; or a ^{14}C-labeled amino acids mixture (25 μCi/ml; The Radiochemical Centre) and 12 μM each of 20 amino acids. Duplicate portions of 5 μl were counted.

In growing cells, 83% of the ^3H-labeled ribosome radioactivity was in polysomes (fractions 1 to 22), and 17% was in 80S particles (fractions 23 to 26). The average size of polysomes is five to eight ribosomes. RNA was extracted and purified from pooled fractions across the gradient (Fig. 5). Recovery of [^3H]RNA was roughly the same from all fractions and averaged 51.6 ± 7.1%. As with unfractionated cellular RNA, the amount of [^{35}S]methionine-labeled protein synthesized in vitro with each RNA fraction was proportional to the amount of ^3H radioactivity added (data not shown). The total amount of translatable mRNA activity present in each of the pooled fractions of the polysome gradient is thus equal to: (total [^3H]RNA radioactivity present in the pooled gradient fractions) × (ratio of ^{35}S counts per minute incorporated into protein relative to the amount of [^3H]RNA added to the in vitro reaction). The specific translation activity ([^{35}S]/[^3H]counts per minute) of the recovered polysomal RNAs was within a factor of 1.5 that of RNA recovered from the postnuclear supernatant (before centrifugation) or from the total cells (data not shown). As is detailed below, the sizes and proportions of the various translation products of polysomal RNA were indistinguishable from those of total cellular RNA. These results established that the polysomes are recovered intact (otherwise they would contain broken, inactive mRNA) and that all mRNA's are recovered in very high yields from the polysomes and subpolysomal particles.

Of the mRNA activity in growing cells, 82% was bound to ribosomes (Fig. 5, fractions 1 to 26; pooled fractions A to H); 18% sedimented slower than 80S ribosomes (fractions I to K) and

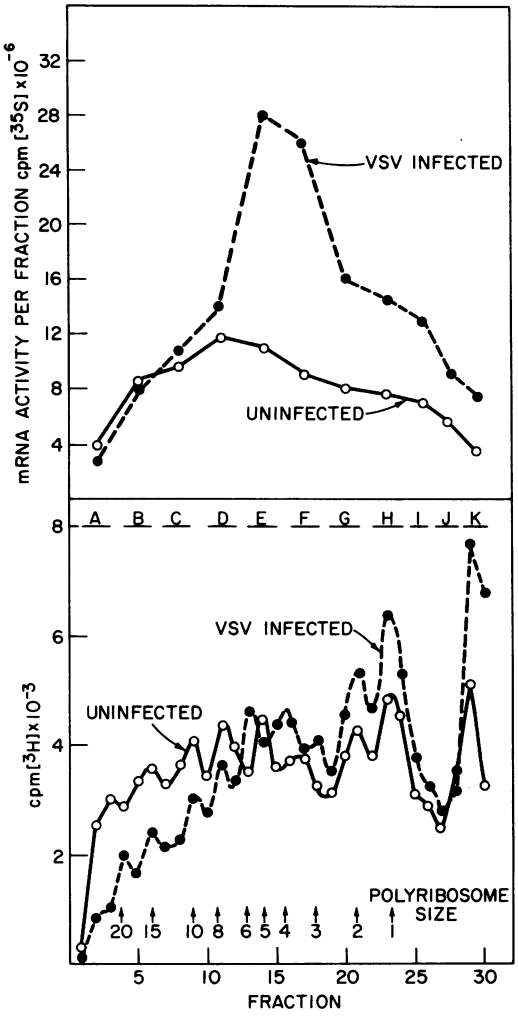

FIG. 5. *Subcellular localization of ribosomes and translatable mRNA in growing and VSV-infected cells. A sample of 6 × 10⁷ BHK cells was grown for 20 h in the presence of 1.5 mCi of [³H]uridine (The Radiochemical Centre), harvested by centrifugation, and resuspended in 1 liter of medium. After growth for an additional two generations, half of the cells were mock infected and half were infected with VSV. Four hours after infection, a postnuclear supernatant from the cultures was prepared and centrifuged through a 15 to 30% (wt/vol) sucrose gradient, as detailed in Materials and Methods. Shown in the bottom panel is the ³H radioactivity per 50-μl portion of fractions from the gradient of the extract from uninfected or infected cells. The size of polyribosomes was determined by extrapolation of the profile of an extract from rabbit reticulocytes (22) analyzed in parallel. As indicated by the bars, 1.1 ml from two or three adjacent fractions was pooled. RNA was then isolated and translated in a wheat germ cell-free system. Two different amounts of RNA were used, generally about 1.5 and 3.0% of the total RNA recovered from each fraction. Within this range, protein synthesis was invariably proportional to the amount of RNA added (see Fig. 3). Plotted on the ordinate in the top panel is the total amount of translatable mRNA per pooled fraction; this is the product of (cpm [³⁵S]methionine incorporated per cpm [³H]RNA added) and (total amount of [³H]-RNA on the pooled fractions). Since recoveries of [³H]RNA were very similar in all fractions, the correction for differential recovery of [³H]RNA in the different fractions is small and does not affect the position of any of the data points shown by more than 10%.*

was presumably in RNP complexes. The peak of mRNA activity was associated with fractions containing about eight ribosomes per mRNA. The specific translation activity ([³⁵S]incorporation per [³H]RNA) of RNA isolated from the different polysome fractions A to G differed by no more than 50%, as can be calculated from the data in Fig. 5.

Considerably more information is obtained by analyzing the translation products directed by RNA from individual polysome and RNP fractions on a long polyacrylamide gradient gel (Fig. 6). By quantitating with a microdensitometer the amount of each polypeptide synthesized by each gradient fraction, it is possible to determine the subcellular localization of each of 12 predominant cellular mRNA's encoding proteins of molecular weights from 20,000 to 70,000 (Fig. 6 and 7).

With three exceptions (proteins β, δ, and ζ), over 85% of the translatable mRNA encoding the 12 predominant cellular proteins was bound to polysomes (Fig. 6 and 7; Table 4). In general, the size of polysomes translating any given cellular mRNA is proportional to the size of the protein product. Actin mRNA (γ, molecular weight 43,000) was localized predominantly in fractions containing 12 ribosomes, and θ (molecular weight 31,000) mRNA was localized in polysome fractions containing 8, whereas mRNA's encoding proteins ι, κ, λ, and μ (molecular weight about 20,000) were enriched in fractions containing 5 ribosomes.

The majority of the mRNA activity encoding proteins δ and ζ was found in messenger RNP (mRNA) particles which sedimented slower than 80S monosomes (Figs. 6 and 7). The balance appeared to be in small polysomes; whether these mRNA's were actually translated in cells is not known. A similar sequestration of a small subset of translatable mRNA's into RNP particles has been observed in Vero cells (19). About 25% of the mRNA activity encoding a protein comigrating with β (molecular weight 55,000) was localized in RNPs; the balance was localized on rather large polyribosomes (Fig. 6 and 7). Analysis of the translation products on two-di-

mensional gels indicates that both polysomal and RNP RNAs directed synthesis of the same β, δ, and ζ polypeptides (data not shown). In the Discussion, we propose explanations for this unusual sequestration of a significant portion of a species of mRNA in growing cells.

The translation of both polysomal and RNP RNA, either from uninfected or infected cells, was unaffected by the presence of 1 mM *S*-adenosylhomocysteine, an inhibitor of mRNA capping (data not shown). Thus, the translation of these RNAs is not dependent on capping by the wheat germ extract, and presumably these RNAs are capped in situ.

Localization of cellular and viral mRNA in infected cells. Four hours after VSV infection, 78% of the ribosomes were polysomal. The weight-average size of polysomes (about four ribosomes per mRNA) is much smaller than the average of seven found in growing cells. After infection, 78% of the total amount of translatable cytoplasmic mRNA sedimented with, or faster than, 80S ribosome monomers. The peak of total mRNA activity was associated with polysomes containing three to four ribosomes, much smaller than is found in growing cells.

The three predominant species of viral mRNA, encoding G (molecular weight 62,000), N (molecular weight 52,000), and M (molecular weight 35,000) proteins, were localized predom-

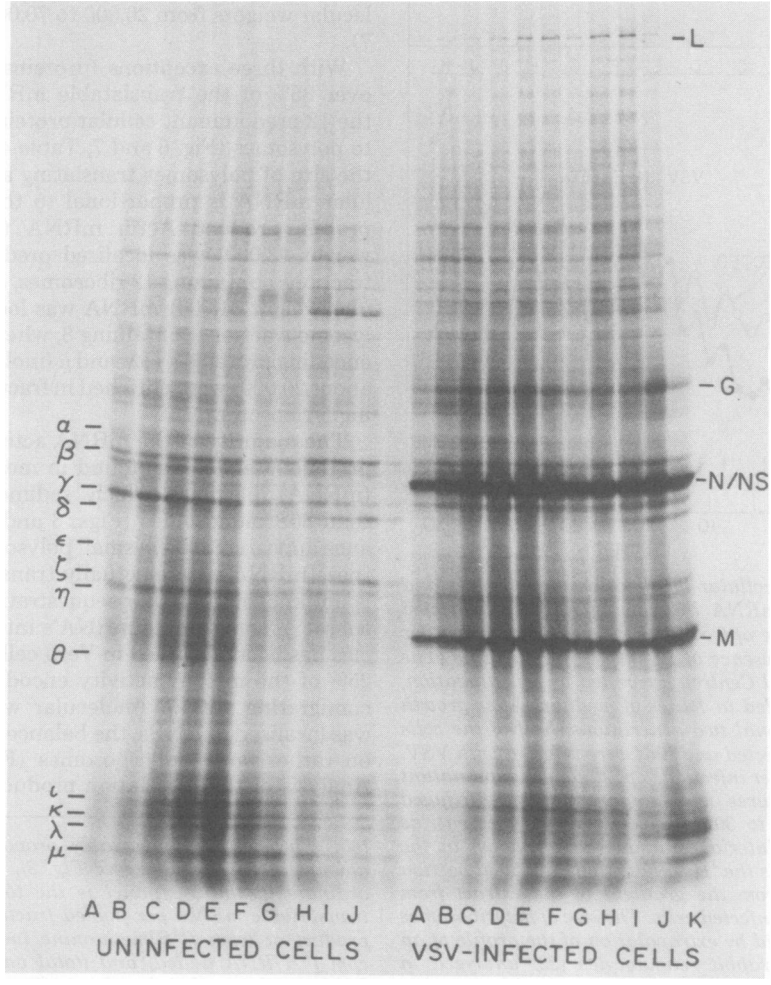

FIG. 6. *Cell-free translation products of RNA from subcellular fractions. (Left) Uninfected cells; (right) VSV-infected cells. RNA from different fractions of the polysome gradient of Fig. 5 was translated in a wheat germ cell-free system, and the products were resolved by electrophoresis through a 10 to 15% gradient polyacrylamide gel. In all cases, an amount of recovered RNA, generally 1.3%, equivalent to precisely 0.76% of the total RNA present in the initial pooled fraction, was added to a 25-μl cell-free reaction.*

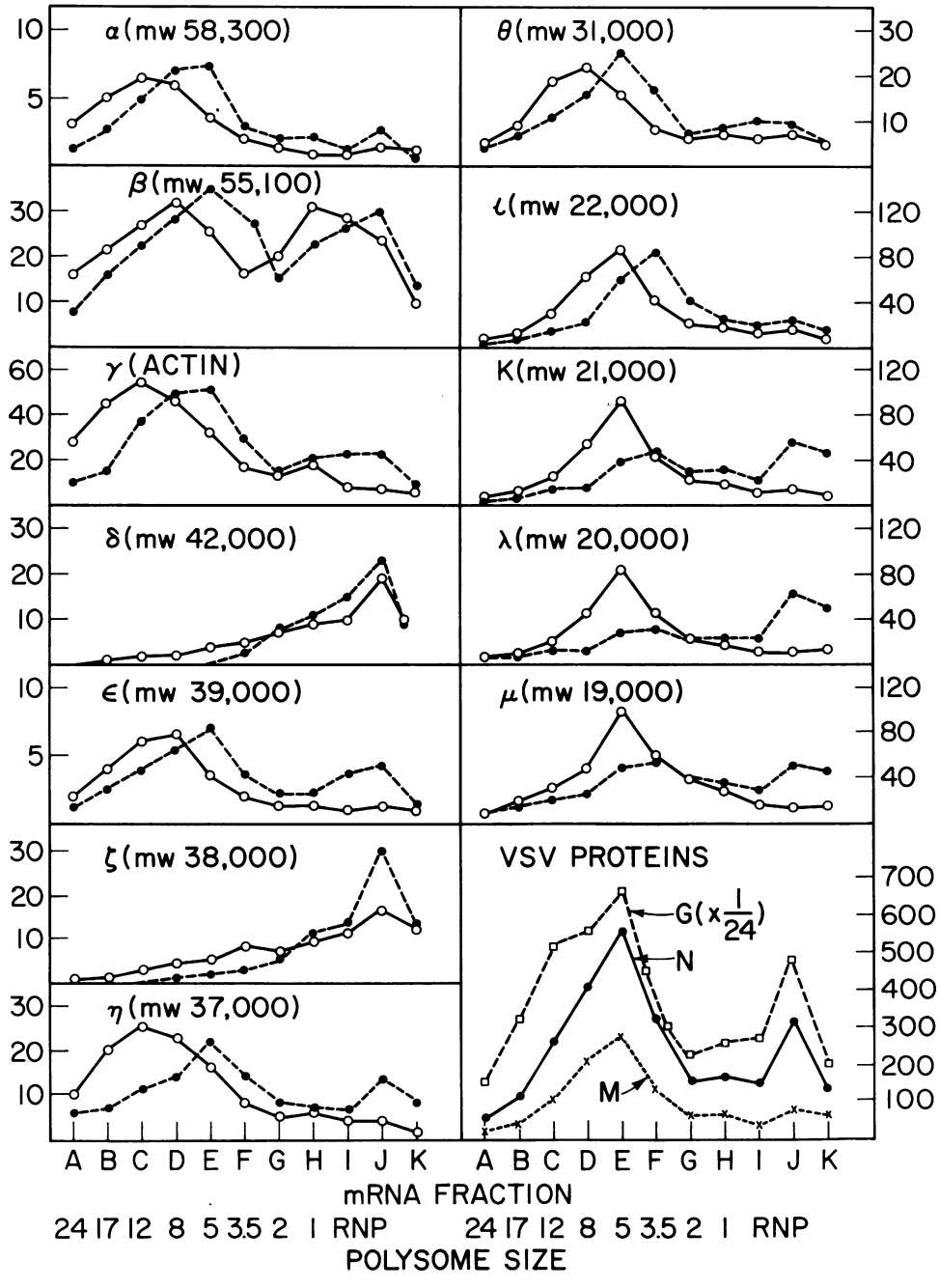

FIG. 7. *Subcellular localization of specific cellular and viral mRNA. Three different exposures of the gel shown in Fig. 6 were scanned with a Joyce-Loebl microdensitometer, and the areas of the polypeptide bands indicated were determined. A 1-h exposure was used for the VSV G and M proteins; a 24- or 48-h exposure was used for calculation of cellular bands and VSV G. The areas (in arbitrary units) shown on the ordinate are normalized to the equivalent of a 24-h exposure of the film; thus the values for the different polypeptides are proportional to their relative extents of synthesis in the wheat germ extract. The average size of the gradient fractions from which the RNA was isolated was taken from Fig. 7.*

TABLE 4. *Fraction of viral and cellular mRNA's in RNP particles*[a]

mRNA	Expt 1		Expt 2	
	Uninfected cells	VSV-infected cells	Uninfected cells	VSV-infected cells
α	0.10	0.13	0.11	0.20
β	0.25	0.29	0.30	0.28
γ	0.08	0.20	0.09	0.17
δ	0.57	0.69	0.60	0.64
ε	0.10	0.25	0.12	0.16
ζ	0.50	0.71	0.45	0.65
η	0.08	0.24	0.16	0.18
θ	0.17	0.20	0.24	0.24
ι	0.11	0.19	0.18	0.21
κ	0.12	0.40	0.13	0.48
λ	0.12	0.50	0.14	0.44
μ	0.11	0.33	0.13	0.31
G		0.26		0.24
N/NS		0.23		0.22
M		0.22		0.19
Total	0.18	0.22	0.13	0.22

[a] For experiment 1, the data in Fig. 7 were used to calculate the fraction of mRNA for each of the 12 cellular and 3 viral mRNA's which is localized in the RNP fraction (gradient fractions 25 through 30; pools I, J, and K). The value for total mRNA was calculated from the data in Fig. 6. Experiment 2 is a duplicate experiment.

inantly on pentasomes (Fig. 6 and 7). As befits an mRNA encoding a protein of higher molecular weight, G mRNA was found, on the average, on slightly heavier polysomes than was M or N mRNA (Fig. 6 and 7; 25). Nonetheless, these three mRNA's were translated by polysomes that were much smaller than mRNA's encoding proteins of this size in uninfected cells. Additionally, between 20 and 26% of each of these species of VSV mRNA's was apparently unbound to polysomes and sedimented with RNP particles (Table 4). The average size of a polysome translating a given mRNA species is proportional to the size of the translated mRNA sequence and to the rate of initiation of protein synthesis, and is inversely proportional to the rate of chain elongation. We conclude that the rate of initiation of translation on these VSV mRNA molecules, relative to the rate of elongation, is about two- to threefold lower than would be expected for mRNA's of this size in uninfected cells.

Figures 6 and 7 and Table 4 make several important points concerning the localization of the 12 predominant species of cellular mRNA after VSV infection. First, the majority of each of these species of cellular mRNA's (with the exception of δ and ζ, discussed above) remained localized to polysomes. The fraction of mRNA's found in the RNP fraction did, in most cases, increase. The fraction of mRNA encoding proteins γ (actin) and θ bound to RNPs increased about twofold, for instance, yet still 80% of the mRNA remained polysomal. In the case of mRNA's encoding some of the smaller proteins, ι, κ, λ, and μ, as much as 30 to 50% was found in RNP particles, a two- to threefold increase over the value characteristic of uninfected cells. Again, the translation of RNP RNA is unaffected by *S*-adenosylhomocysteine (data not shown).

Second, and most important, the average size of the polysomes translating each species of cellular mRNA was only 30 to 50% of that characteristic of uninfected cells. As one example, actin mRNA was translated predominantly on pentasomes, 40% the size of polysomes translating actin mRNA in growing cells. The polysomal mRNA's encoding proteins ι, κ, λ, and μ (molecular weight about 20,000) were enriched in structures containing two to four ribosomes, a value significantly smaller than the pentasomes which translate these mRNA's in growing cells. Note that about 25% of the mRNA encoding protein β sedimented with RNP particles in both uninfected and infected cells. However, the average size of the β polysomes in infected cells was only 50% that of uninfected cells.

We emphasize that viral and cellular mRNA's encoding proteins of about the same size (N, 52,000 daltons; M, 35,000 daltons; actin, 42,000; and θ, 31,000) were translated on polysomes containing the same number of ribosomes, in this case five (Fig. 6 and 7). We take this as evidence that the rate of polypeptide chain initiation, relative to elongation, on typical viral and cellular mRNA's is about the same. Recall that there was only a 10% decrease in the number of ribosomes on polysomes after infection. Thus, the two- to threefold reduction after infection in the number of ribosomes translating each molecule of actin mRNA and other species of cellular mRNA is a reflection, primarily, of the increased amount of total translatable mRNA species (predominantly viral mRNA) in the cell. Virtually the same number of ribosomes were distributed among a larger number of mRNA molecules. Thus, the average size of polysomes in infected cells was only 40 to 50% that of growing cells, and the overall rate of chain initiation, per mRNA molecule, was reduced. There did not appear to be preferential translation of viral mRNA's or of cellular mRNA's in the infected cells, nor was there preferential sequestration of particular cellular RNAs into inactive RNPs; about the same fraction of viral mRNA's and typical cellular mRNA's were in RNPs (Table 4).

Results very similar to those in Fig. 6 and 7 have been obtained by in vitro translation in

mRNA-dependent reticulocyte lysates (data not shown); thus, the choice of the wheat germ cell-free lysate system does not appear to have affected the above results and conclusions in any significant way.

DISCUSSION

Our principal conclusion is that inhibition of cellular protein synthesis after infection by VSV is due to competition for ribosomes by a large (about two- to threefold) excess of viral mRNA. As judged by the sizes of polysomes which translate typical mRNA's for cellular (γ, actin, molecular weight 42,000; θ, molecular weight 30,000) and viral (N, molecular weight 52,000; M, molecular weight 35,000) proteins, the rate of translation initiation and elongation on the two classes of mRNA's is the same. Furthermore, whereas about 20% of each species of cellular and viral mRNA is localized in RNPs, there does not appear to be preferential sequestration of particular species of mRNA after infection. Before discussing these results and their implications in more detail, it is necessary to consider first some of the more technical aspects of the present work and some details concerning mRNA translation in growing cells.

Assays for cellular and viral mRNA. We have used in vitro synthesis of discrete cellular and viral proteins as an assay for the amount of corresponding mRNA. For the present study, this technique is preferable to the more commonly used hybridization techniques to quantitate RNA, since it measures the biologically relevant property of mRNA—its ability to direct protein synthesis. Hybridization measures mRNA sequences, not necessarily mRNA function. For instance, a significant fraction of all VSV mRNA's possess the 5′ termini pppA and pppG. These are not found on polysomes in infected cells and are presumably inactive or poorly active as messengers (38). Hybridization and RNA gel analyses would wrongly score these as viral mRNA's. Moreover, these assays could not distinguish nontranslatable (e.g., uncapped, nicked) derivatives of cellular mRNA's which might be produced after infection. The presence of cellular mRNA sequences in RNPs, for instance, would not necessarily indicate that these are translatable. The in vitro translation assay does have several potential problems. It is not known whether all mRNA's are extracted from the cell in an undegraded form. It is also possible that some mRNA's may not be capped in the cell, and therefore presumably be untranslated, yet be capped and then translated by the wheat germ extract. However, the profile of the translation products of RNAs from both polysomes and RNPs is unaltered if pm^{7}G, an inhibitor of capping, is included in the in vitro reaction (data not shown).

To the extent that the amount of specific protein produced is proportional to the amount of mRNA added, the in vitro assay is a valid measure of a particular mRNA species. This assay is unaffected by the presence of extraneous RNAs, such as rRNA, which is present here in vast excess over the amount of mRNA. Providing the total amount of mRNA added is low, there appears to be little competition in vitro of different mRNA's for ribosomes. Thus, this in vitro translation assay can be used to quantitate the relative amount of any given translatable mRNA species in several populations of RNAs. This assay does not measure the absolute amount of any given mRNA in a sample, since we cannot be sure that two different species of mRNA will direct, in a cell-free synthesis reaction, production of the same amount of polypeptide product. Thus, our estimate that the ratio of viral to cellular mRNA at 4.5 h after infection is 2.1:1 to 3.5:1 may be subject to some error. In this connection, though, it is important to note that the relative translation of viral and cellular mRNA is not affected significantly by alterations in the K^+ and Mg^{2+} concentrations of the in vitro reaction (Table 2). Detailed analyses of the translation products on one-dimensional SDS-gels have established, furthermore, that the relative translatability of individual species of cellular and viral mRNA's is similarly unaffected by variations in the K^+ and Mg^{2+} concentration (data not shown).

Our analysis has focused on 12 predominant species of cellular mRNA (α through μ). Their translation products are resolved on one-dimensional gradient SDS-gels from other proteins, including viral polypeptides. When analyzed by two-dimensional gel electrophoresis, each of these 12 bands focused into one or two discrete polypeptide species (Fig. 4); note that not all of the 12 proteins focused on these gels and that each is presumably a single protein of a group of closely related polypeptides (e.g., actin). Our experience is that quantitation of these species can be achieved most easily and reliably by scanning the autoradiograms of the one-dimensional gels.

Subcellular localization and translation of mRNA in growing BHK cells. As judged by the size of polysomes on which they are found, 10 of the 12 predominant species of mRNA (all except δ and ζ) appear to be translated at a high efficiency in growing cells. As standards of comparison, ovalbumin (molecular weight 40,000) mRNA in hen oviduct cells is translated on polysomes containing an average of 12 ribosomes (35). β Globin (molecular weight

16,600) mRNA in rabbit reticulocytes is found predominantly on pentasomes, whereas α globin mRNA is translated on tri- and tetrasomes (4, 22). This is a result of the fact that each molecule of β globin mRNA initiates protein synthesis 1.5 times more often than does each α globin mRNA (23, 26), although the structural features in the mRNA's which are responsible for these differences are not known with certainty (36). Thus actin (molecular weight 42,000), ϵ (molecular weight 39,000), and ζ (molecular weight 37,000) mRNA's are translated on polysomes which are the approximate size of those translating ovalbumin, whereas mRNA's encoding proteins ι, κ, λ, and μ (molecular weight about 20,000) are found on polyribosomes of the same size, or slightly larger, than those translating α and β globin mRNA in the reticulocyte. Since the rate of polypeptide chain elongation in reticulocytes and oviduct magnum cells (about 5 amino acids/s) is similar to that in most growing tissue culture cells and presumably also in BHK cells (35), it is reasonable to conclude that the rate of chain initiation on these 10 BHK mRNA's is comparable to that on α and β globin mRNA and ovalbumin mRNA in reticulocytes or oviduct cells (about 9 ribosomes/mRNA per min at 37°C). To be certain of this conclusion, however, it would be necessary to measure directly the rate of elongation of each of these BHK peptides, but this has not been attempted.

RNP particles in growing cells. At least three cellular mRNA's are exceptions to the above conclusions. Over half of the mRNA's encoding two proteins (δ and ζ) sediment slower than polysomes and are presumably in RNPs. The balance of these species are in very small polysomes, containing only one or two ribosomes. There are at least two very different explanations for this: (i) these mRNA's are preferentially sequestered or inactivated by some protein or other entity, much in the way mRNA's are believed to be stored in sea urchin eggs (13); or (ii) these mRNA's are intrinsically very inefficient in their ability to initiate protein synthesis. Thus, for stochastic reasons, at any time a significant fraction of these mRNA's will find themselves unbound to any active ribosomes.

Neither of these interpretations can explain the peculiar subcellular localization of the mRNA for β, assuming that it is a single protein and a single species of mRNA: 25% sediments with the RNP fraction, and 75% sediments with large polyribosomes (8 to 11 per mRNA). If the average translating mRNA contains eight ribosomes, then on a stochastic basis at any one time exp(-8), or 0.0003, of the mRNA would be expected to be unbound to any ribosomes; this value is clearly lower than that (0.25) observed with β mRNA. One explanation postulates the existence of an mRNA "recruitment" or "stabilization" factor (17, 21) involved in the initiation step of protein synthesis. The binding of such a factor to mRNA would be a prerequisite for chain initiation, and the affinity of the factor for mRNA would be such that the half-life of the factor-mRNA complex would be much larger than the transit time for chain elongation. That is, if an mRNA molecule is bound to such a factor, it would initiate several rounds of protein synthesis and be incorporated into a large polysome. If an mRNA lacks such a factor, it would be inactive, although it could acquire the factor at a later time and then be incorporated into a polysome. The amount of such a hypothetical factor would set a limit on the number of mRNA molecules that could be translated at any instant. Different mRNA's may have different rate constants for binding to such a factor; one would imagine that some, such as the mRNA for protein β, would bind relatively poorly in competition with other cellular mRNA's, so that, at any time, 25% would not have a factor attached, and thus would be localized in the nonpolysomal mRNP fraction. It is worth noting that several cellular mRNA's, such as those encoding proteins α, γ, and θ, are present in mRNP's in amounts greater than those expected on a stochastic basis; possibly the above explanation applies to these as well. [In growing cells, each actin mRNA is bound to 12 ribosomes. Applying Poisson's equation, one would expect, on a random basis, that exp(-12), or 0.00001, of these mRNA's would be unattached to ribosomes at any instant. This is clearly lower than the observed value of 0.11 (Table 4).]

Translation of mRNA's in VSV-infected cells. There does appear to be a reduction in the overall rate of protein synthesis after VSV infection, although the reasons for this are obscure. Assuming that the rate of incorporation of [^{35}S]methionine (Fig. 1) or ^{14}C-labeled amino acids (not shown) into protein is a valid measure of the rate of protein synthesis, it would appear that this value is reduced 35% by 4.5 h after infection. Additionally, a significantly higher fraction of ribosomes sediment as monosomes after infecton (22 versus 17% in growing cells) and are presumably inactive in mRNA translation. One problem is that we are not sure whether all cells in the population respond homogeneously to VSV infection. It is possible that some fraction of the cells are totally inactive in synthesis of either viral or cellular proteins; all of the mRNA in them could be in inactive RNPs.

In the following discussion we will assume, for simplicity, that the rate of elongation of polypeptide chains is not significantly reduced after infection. It is possible that some reduction in this rate does occur, and that this accounts for some, or all, of the reduction in the total rate of polypeptide synthesis; nonetheless, this assumption does not affect the following conclusions. Since all cellular mRNA's are found, after infection, on smaller polysomes than in growing cells, it is clear that there is a reduction in the rate of initiation of protein synthesis per mRNA molecule relative to the rate of chain elongation.

The total amount of translatable mRNA per cell increases about threefold after VSV infection. This extra viral mRNA is largely localized to polysomes (Fig. 7); previous work indicated that all species of viral mRNA are translated at the same efficiency (6, 25, 42). Our results show that viral mRNA competes with the preexisting cellular mRNA for ribosomes in the initiation step of protein synthesis. There does not appear to be preferential translation of viral or cellular mRNA's; rather, viral and cellular mRNA's encoding the same-sized proteins are found on the same-sized polysomes. The two- to threefold reduction in the size of polysomes translating predominant species of cellular mRNA is accounted for by the presence of a two- to threefold increase in the total amount of mRNA per cell. Clearly, competition by the "extra" viral mRNA's for a constant number of ribosomes accounts for a two- to threefold reduction in the synthesis of major cellular proteins.

We have not yet attempted VSV-cellular mRNA competition experiments in in vitro translation systems, and it is not at all clear that these studies would be relevant to the situation in the intact cell. Our conclusions differ from those of Nuss et al. (33), who concluded, on the basis of the effects of hypertonic treatment of cells, that VSV mRNA is translated in preference to host mRNA. However, neither the mechanism of inhibition of protein synthesis by hypertonic treatment nor the relevance of these results to mRNA translation in normal media is at all clear.

The actual reduction in the rate of synthesis of most cellular proteins appears to be greater than this, about fourfold. It thus appears that mRNA competition can explain some, but probably not all, of the inhibition of translation of cellular mRNA which occurs after infection.

Other possible factors which could reduce the rate of translation of cellular mRNA's after infection include: (i) reduction in the rate of polypeptide chain elongation; (ii) death of a certain fraction of cells; and (iii) sequestration into RNPs of a significant fraction (about 25%) of cell species of cellular and viral mRNA's. The last point deserves some additional comment. These mRNP's could be localized only to the dead, inactive cells. Alternatively, they could have an explanation similar to that advocated above for β mRNA in growing cells. The amount of this postulated mRNA recruitment factor would be in excess over the number of mRNA molecules present in growing cells, but might become limiting after infection, due to the large increase in the pool of translatable mRNA. This would account for the presence of about 25% of all cellular and viral mRNA's in RNPs after infection if the amount of active factor was sufficient to bind to only 75% of the total (viral plus cellular) mRNA's. Whatever the mechanism for increase in the level of RNPs after infection, it is significant that there is no difference in the extent of sequestration of typical viral and cellular mRNA species (Table 4).

Possible differences between strains of VSV Indiana. All of the published data on the Glasgow isolate of the Indiana serotype of VSV used in these studies are consistent with the notion that mRNA competition is a major determining factor in inhibiting translation of cellular mRNA. In particular, temperature-sensitive mutants in three complementation groups which do not induce the synthesis of viral mRNA at the nonpermissive temperature [tsG11(I); tsG114(I); tsG22(II); tsG41(IV)] do not kill infected cells, neither do they inhibit synthesis of cellular proteins, for periods of up to 3 days (27, 28, 30; H. F. Lodish, unpublished data). At the permissive temperature synthesis of viral mRNA, inhibition of host protein synthesis, and cell killing proceed normally. Mutants which direct the synthesis of normal levels of viral mRNA [tsG31(III); tsG33(III); tsL511(V); tsL513(V)] inhibit synthesis of cellular proteins with kinetics indistinguishable from that of wild-type VSV, at both permissive and nonpermissive temperatures (30; Lodish, unpublished data). There are ts mutants in complementation group IV which induce, at the nonpermissive temperature, reduced amounts of viral mRNA (27); these do inhibit synthesis of cellular proteins, but only after 8 to 10 h of infection (G. Strous and H. F. Lodish, manuscript in preparation). Thus, synthesis of viral mRNA appears both necessary and sufficient for inhibition of cellular protein synthesis.

Published work from other laboratories would suggest that other wild-type isolates of VSV, in cell lines other than that used here, might inhibit synthesis of cellular proteins by a mechanism different from, or in addition to, the one pro-

posed here. Infection of some cells by the HR (Winnipeg) wild-type isolate of VSV Indiana results in an inhibition of total protein synthesis both quicker and more extreme than found in the present studies. Additionally, a temperature-sensitive mutant (W10) of the HR strain inhibits synthesis of cellular protein at the nonpermissive temperature, even though no, or very little, viral mRNA is made (27). Stanners et al. (40) have isolated and characterized a mutant of the HR strain, infection with which does not result in the rapid, precipitous inhibition of protein synthesis observed with the HR wild type. In fact, infection by this mutant appears to resemble, in these respects, infection by our Glasgow wild-type strain (H. F. Lodish and M. Porter, our unpublished data). It will be of interest to study a variety of VSV isolates in the same cell line, and also the same wild-type isolates in different cell lines. In particular, it is important to study the fate of cellular mRNA under these varied conditions, as it is possible that there are multiple mechanisms by which VSV can inhibit the synthesis of cellular proteins.

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