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Vesicular Stomatitis Virus mRNA and Inhibition of Translation of Cellular mRNA—Is There a P Function in Vesicular Stomatitis Virus?

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Infection of animal cells by vesicular stomatitis virus (VSV) results in inhibition of translation of cellular mRNA. We showed previously that, in BHK cells infected by the Glasgow isolate of VSV Indiana, this is due to competition during the initiation step of protein synthesis of viral and cellular mRNA for a constant, limiting number of ribosomes. We show here that infection of the same cells with the San Juan isolate of VSV resulted in a more rapid shutoff of host protein synthesis and that this was paralleled by a more rapid accumulation of viral mRNA. Extending our conclusion that shutoff is due to mRNA competition, we show further that the average size of polysomes translating viral and cellular mRNA was threefold smaller in cells infected by VSV San Juan than by VSV Glasgow, which, in turn, was about one-half that of uninfected cells. In all cases, cellular and viral mRNA's which encoded the same-sized polypeptides were found on the same-sized polysomes, a result indicating that the efficiency of translation of both types of mRNA's is about the same in the infected cell. Also, there was no preferential sequestration of viral or cellular mRNA's in ribonucleoprotein particles. Additional correlations between the levels of viral mRNA's and the inhibition of protein synthesis came from studies of three other wild-type VSV strains and also from studies with Vero and L cells. In particular, the rate of shutoff of L-cell protein synthesis after infection by any VSV isolate was slower than that in BHK cells, and this was correlated with a slower rate of accumulation of viral mRNA. VSV temperature-sensitive mutants which synthesized, at the nonpermissive temperature, no VSV mRNA failed to inhibit synthesis of cellular proteins. Stanners and co-workers (C. P. Stanners, A. M. Francoeur, and T. Lam, Cell 11:273-281, 1977) claimed that VSV mutant R1 inhibited synthesis of L cell protein synthesis less rapidly than did its parent wild-type strain HR. They concluded that this effect was due to a mutation in an unspecified VSV protein, "P." We found, in both L and BHK cells, that R1 infection resulted in a slightly slower inhibition of cellular mRNA translation than did HR infection and that this was correlated with a slightly reduced accumulation of VSV mRNA. The level of VSV mRNA, rather than any specific VSV protein, appeared to be the key factor in determining the rate of shutoff of host protein synthesis.

Infection of mammalian cells by vesicular stomatitis virus (VSV) results in inhibition of synthesis of cellular protein and RNA and eventual cell death. Neither replication of the genomic RNA nor production of infectious virus is necessary for the inhibition of cellular protein synthesis; however, some transcription of the negative-stranded viral genome into mRNA is essential (3, 4, 6-8). Much, if not all, of the inhibition of cellular protein synthesis is manifested at the translational level: after infection cellular mRNA remains intact and fully functional in in vitro translation systems, yet is translated in the cells at a decreasing rate (2).

At least three different mechanisms for this translational control have been proposed. Nuss et al. suggested that VSV mRNA initiates protein synthesis severalfold more efficiently than does cellular mRNA, and thus out-competes mRNA for ribosomes (9). Stanners et al. (12) have isolated a mutant of the HR (Winnipeg) wild-type isolate of the Indiana serotype of VSV. Infection of L cells with this mutant does not result in the rapid, precipitous inhibition of protein synthesis observed with the HR wild-type strain. They proposed that a specific viral gene product, termed P, is required for this inhibition and that the mutant, R1, is P^-.

More recently, we showed that in growing BHK cells over 80% of most of the predominant species of cellular mRNA are bound to polysomes, and over 60% remain on polysomes 4 h after VSV infection (2). The average size of polysomes translating individual cellular mRNA is reduced about two- to threefold after infection. For example, in uninfected cells, actin (molecular weight, 42,000) mRNA is found predominantly on polysomes with 12 ribosomes; 4 h after infection it is found on fivesomes, the same size as polysomes that are translating VSV N (molecular weight 52,000) and M (molecular weight 35,000) mRNA. We concluded 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 (about threefold more) than are present in growing cells. About 20 to 30% of each of the predominant cellular and viral mRNA's are present in ribonucleoprotein (RNP) particles in infected cells and are presumably inactive in protein synthesis. There is no preferential sequestration of cellular or viral mRNA's in RNPs after infection (2).

All of the above-mentioned studies were done with different isolates of VSV Indiana and in different cultured cells. As part of a systematic approach to the resolution of these differences, we have studied the effects of four commonly used wild-type VSV strains and also the R1 mutant on three of the cell lines used in these studies. Our results indicate that the rate and extent of inhibition is a function of both the cell line and virus used. In all cases tested, however, there was a good correlation between the accumulation of viral mRNA and the extent of inhibition of translation of cellular mRNA, thus extending our conclusions on the mechanism of translational control to other cell line-VSV strain combinations. Our studies also suggest that R1 may not be a simple mutant of the HR wild-type strain.

MATERIALS AND METHODS

Virus strains. The Glasgow isolate of VSV was obtained from Robin Weiss (Imperial Cancer Research Fund, London), who originally obtained it from Craig Pringle (Institute of Virology, Glasgow) (3). The San Juan isolate of VSV was obtained from Robert Lazzarini (National Institutes of Health), who obtained it from Martha Stampfer and Alice Huang in 1972. The Mudd-Summers strain was also obtained from Dr. Lazzarini. According to him (personal communication), it was first used at Harvard and then passed on in succession to Drs. Carver, Marcus, Summers, Mudd, and Holland before reaching him. Strains HR and R1 are described by Stanners et al. (12) and were obtained from C. Stanners (Ontario Cancer Institute). All viruses used in these studies were grown on Vero cells.

Animal cells and infections. Growth of suspension BHK cells and monolayer Vero cells and procedures for infection with VSV were detailed in previous papers (2, 3). Mouse L cells were obtained from C. Stanners and were cultured as monolayers in minimal essential α medium (GIBCO no. 410-1900) with 7% calf serum and 3% fetal calf serum. Infection of these with VSV used a protocol identical to that for Vero cells. In all cases, 10 PFU of VSV per cell was used.

Labeling of infected cells with [^{35}S]methionine and analysis of proteins by sodium dodecyl sulfate (SDS)-gel electrophoresis. Procedure for labeling and electrophoresis have been fully described in previous publications (2, 3).

Isolation of total cytoplasmic RNA. A previous publication detailed our procedures for isolation of polysomes and polysomal RNA from BHK cells and for cell-free protein synthesis (2). The procedure developed for extraction of RNA from BHK cells was used with only minor modifications for monolayer L cells. About 5×10^7 cells in three 150-mm-diameter plates were chilled on ice and washed several times with phosphate-buffered saline. The cells were scraped off the plates into phosphate-buffered saline and then treated as in the protocol for suspension cells (2). Note that total cytoplasmic RNA, not selected for polyadenylated components, was used. Thus, mRNA activities of RNA fragments are normalized to total cellular RNA, i.e., mostly rRNA, which is proportional to the number of cells fractionated.

RESULTS

Virus and cell strains. Much of this study is a comparison of the effects of five strains of VSV on three different host cells. Tracing the history of the common laboratory strains of VSV Indiana is only slightly less complicated than following the genealogy of Old Testament patriarchs, but our best attempt is presented in Materials and Methods. Strain R1 is a temperature-resistant revertant of temperature-sensitive (ts) mutant T1023, which is itself reported to be a ts mutant of the HR strain with a lesion in complementation group I (12). The BHK culture used was grown in suspension, whereas the Vero and L-cell cultures were maintained and infected as monolayers. Analysis by one-dimensional SDS-gels of the [^{35}S]methionine-labeled proteins synthesized by these (uninfected) cells reveals few similarities except for actin, which is in all cases the most predominant cell protein. Thus, to facilitate comparison of different cell lines, we have focused on the translation of actin mRNA. After infection of BHK cells by the Glasgow isolate of VSV, inhibition of actin synthesis parallels, we have shown, that of most cellular proteins (2).

BHK cells infected by different strains of VSV. Infection of BHK cells with four of the

five virus strains tested (Glasgow, Mudd-Summers, HR, and R1) resulted in similar kinetics of inhibition of synthesis of actin and of most cellular proteins (Fig. 1 and 2). At 2.5 h after infection, the rate of actin synthesis was 60 to 75% that of mock-infected cells, and at 4 h it was 25 to 30%. Infection by VSV San Juan, by contrast, resulted in a much more rapid inhibition of synthesis of predominant cellular proteins (Fig. 2–4); actin synthesis was reduced to 45% of the control value at 2.5 h after infection, 1 h sooner than after infection by the other VSV isolates. Inhibition of synthesis of other predominant cellular proteins also occurred about 1 h sooner after infection by VSV San Juan than VSV Glasgow (Fig. 4). In all cases, by 5 h postinfection actin synthesis was less than 10% of control levels (data not shown). This result, together with microscope studies on these cells, indicated that over 90%, and probably over 98%, were infected with VSV.

The inhibition of cellular protein synthesis after infection of BHK cells by VSV Glasgow is due, we have shown (2), to the competition of cellular and viral mRNA for the constant, limiting number of functional ribosomes. Were this to apply to infection of BHK cells by VSV San Juan, then one would expect a more rapid rate of accumulation of viral mRNA after infection by VSV San Juan. Figure 3 and Table 1 show that this was the case. In this study, a constant, limiting amount of cytoplasmic RNA was translated in a wheat germ cell-free system. The one-dimensional gel analysis of Fig. 3 (lanes 11 to 18) makes the important point that all translatable cellular mRNA's remained intact and functional after infection by both VSV Glasgow and VSV San Juan. Per microgram of cellular RNA, preparations from infected and uninfected cells directed the synthesis of equal amounts of all predominant cellular polypeptides. 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. 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. The amounts of both total translatable mRNA and viral mRNA increased steadily from 2.5 h after infection, and did so about 1 h earlier after infection by VSV San Juan than by VSV Glasgow (Table 1). Therefore, the differential accumulation of viral mRNA paralleled the differential inhibition of host protein synthesis by the two strains. The increased level of VSV mRNA's in San Juan-infected cells was paralleled by an

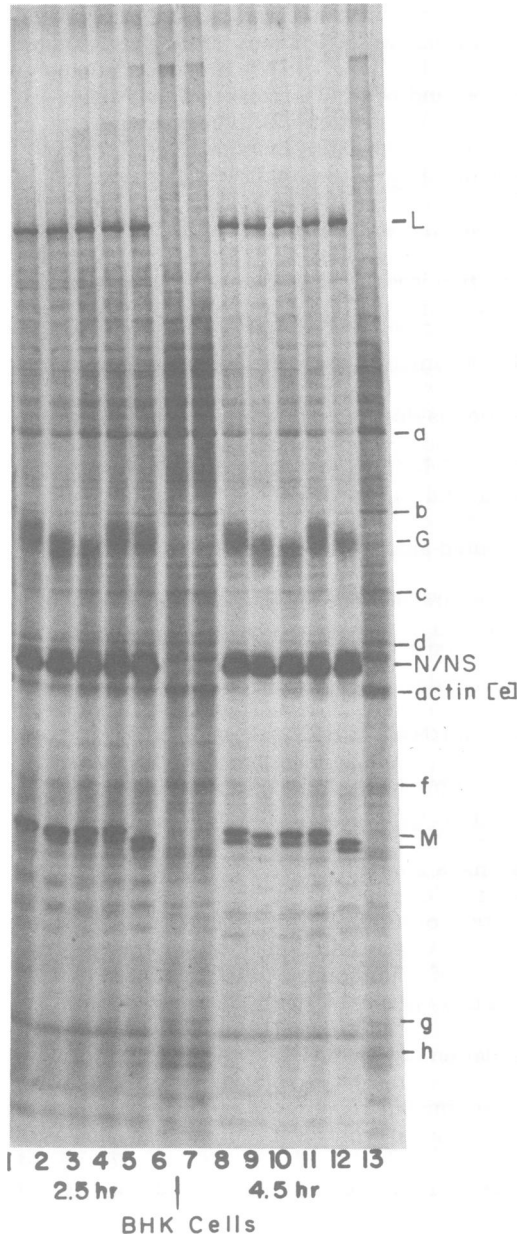


FIG. 1. *Gel analysis of protein synthesis in VSV-infected BHK cells. As detailed in Materials and Methods, samples of VSV-infected BHK cells were labeled with [^{35}S]methionine for 30 min beginning at 2.5 h (lanes 1–6) or 4.5 h (lanes 7–13) after infection. (Lanes 6, 7, and 13) Mock infected; (1 and 8) VSV Glasgow; (2 and 9) VSV San Juan; (3 and 10) VSV Mudd-Summers; (4 and 11) VSV HR; (5 and 12) VSV R1. Samples of the cell lysates were analyzed by electrophoresis through a linear 10 to 15% polyacrylamide gel containing SDS. Shown is a radioautogram of the dried gel. The upper case letters on the right denote VSV proteins, and the lower case letters indicate predominant species of cellular polypeptides.*

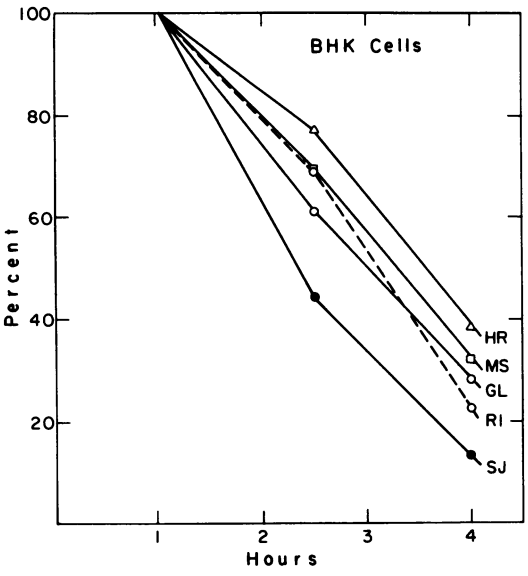


FIG. 2. *Synthesis of actin in BHK cells infected by different VSV strains. The radioautograms of Fig. 1 were scanned with a microdensitometer under conditions where the darkening of the film was strictly proportional to the amount of radioactive protein. The amount of radioactivity in the actin polypeptide, relative to that of mock-infected cells, is plotted as a function of time after infection. Although not shown in Fig. 1, there was no reduction of actin synthesis at 1 h after infection of any of the strains used. Symbols:* (●) *VSV San Juan (SJ):* (-O-) *VSV Glasgow (GL):* (△) *VSV HR (HR);* (□) *VSV Mudd-Summers (NS);* (--O--) *VSV R1 (R1).*

accelerated rate of synthesis of VSV proteins compared with infection by VSV Glasgow (compare lanes 4 and 7 and lanes 5 and 8 of Fig. 3).

The amount of viral mRNA synthesized after infection of BHK cells by VSV strains HR4 and R1 was similar to that in VSV Glasgow-infected cells (Table 2). Together with our finding (Fig. 1 and 2) that the kinetics of inhibition of cellular protein synthesis are the same, this is consistent with our conclusion that host shutoff is primarily a function of the amount of translatable viral mRNA.

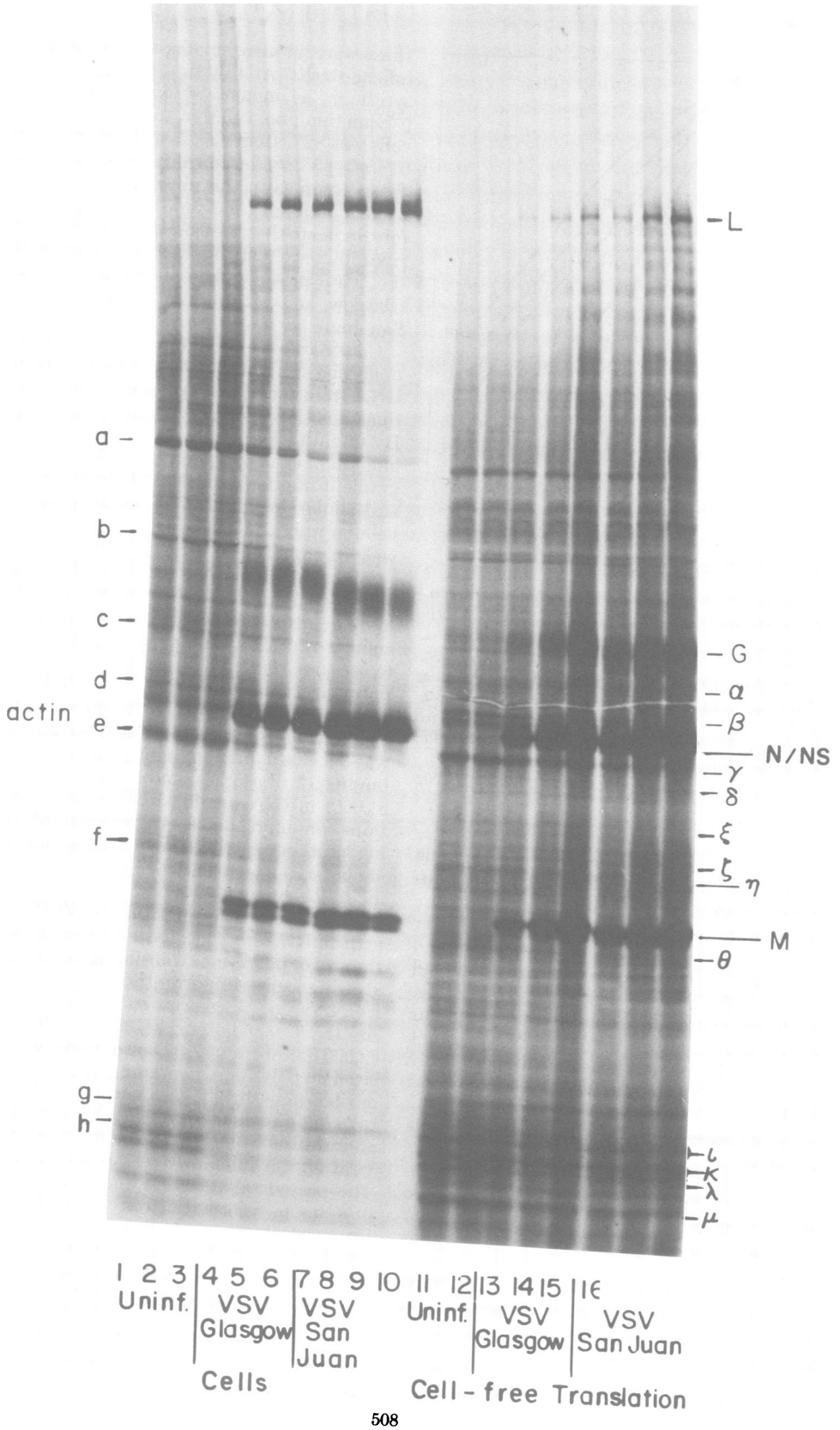
Is the increased level of viral mRNA in cells infected by VSV San Juan, relative to VSV Glasgow, indeed the cause of the enhanced inhibition of translation of cellular mRNA? To answer this question, we studied the subcellular distribution of the predominant cellular mRNA's and of the viral mRNA 3 h after infection. Figure 5 shows that 83% of ^{3}H-labeled BHK ribosomes were on polysomes in uninfected cells, and 75% were on polysomes after infection by either virus. However, the average size of polysomes was smaller in the VSV San Juan-infected cells than in those infected by VSV Glasgow: these were, in turn, smaller than those in mock-infected cells. At this time the rate of protein synthesis, as measured by incorporation of [^{35}S]methionine, relative to that of uninfected cells was 82% after VSV San Juan infection and 87% after VSV Glasgow infection. Thus, most of the polysomes in the infected cells must have been functional. The profiles in Fig. 5a, therefore, indicate that the same number of functional ribosomes are distributed among more mRNA's (i.e., viral plus cellular) after infection by VSV Glasgow, and yet more after infection by VSV San Juan.

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 (2). By quantitating with a microdensitometer the amount of each polypeptide synthesized by each gradient fraction, it is possible to determine the subcellular localization of many predominant cellular mRNA's encoding proteins of molecular weights from 20,000 to 70,000 (Fig. 6).

With few exceptions (proteins β, δ, and ζ in Fig. 3), in uninfected cells over 85% of the translatable mRNA encoding the 12 predominant cellular proteins was bound to polysomes (Fig. 6; see also reference 2). In general, the size of polysomes translating any given cellular mRNA was 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 6 to 8, whereas mRNA's encoding proteins ι, κ, λ, and μ (molecular weight, about 20,000) were enriched in fractions containing 5 ribosomes.

Three hours after infection by VSV Glasgow, actin mRNA was localized on hexasomes, 50% the size of polysomes translating actin mRNA in growing cells. Importantly, viral and cellular mRNA's encoding proteins of about the same size (N, 52,000 daltons; M, 35,000 daltons; actin, 42,000 daltons: and θ, 31,000 daltons) were translated on polysomes containing the same number of ribosomes, in this case four to six (Fig. 6). 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. The polysomal mRNA's encoding the smaller proteins (ι, κ, λ, and μ) were enriched in structures containing two to three ribosomes, a value significantly smaller than the fivesomes which translate these mRNA's in growing cells.

The size of polysomes translating all predominant cellular and viral mRNA's was much smaller after infection by VSV San Juan than by VSV Glasgow. Actin mRNA, to cite one example, was localized predominantly to di- and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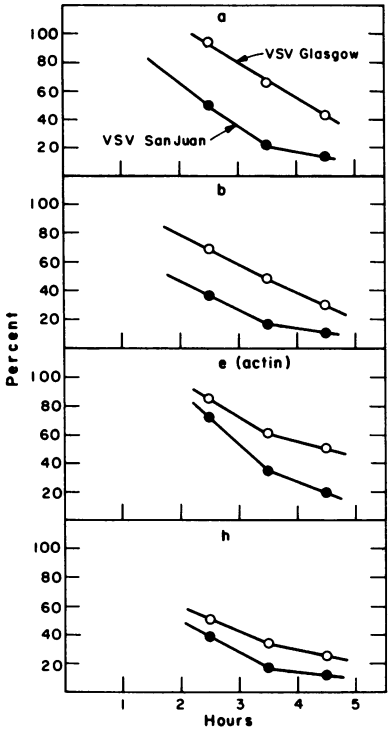


FIG. 4. *Synthesis of BHK proteins after infection with VSV Glasgow or VSV San Juan. The autoradiogram of Fig. 3 (lanes 1-9) was scanned with a microdensitometer. The areas under the peaks corresponding to four predominant cellular proteins, a, b, c (actin), and h, were determined and normalized to the value of mock-infected cells.*

trisomes, only 17 to 25% the size of polysomes translating actin mRNA in growing cells. Again, the viral and cellular mRNA's encoding proteins of the same size (N, M, actin, and θ) were translated on polysomes containing the same number of ribosomes, here two to three.

Together with the data of Fig. 3 and 4, the study in Fig. 6 indicated that the reduction after infection in the number of ribosomes translating each molecule of actin mRNA and other species of cellular mRNA was 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 twofold increase in the total amount of translatable mRNA 3 h after infection by VSV Glasgow observed in this experiment was reflected in a twofold reduction in the number of ribosomes per actin mRNA. The fivefold increase found in this experiment after VSV San Juan infection resulted in a proportionately larger reduction in the number of ribosomes translating actin mRNA, and thus in the rate of actin synthesis. In neither case did there 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 fractions of viral mRNA's and typical cellular mRNA's were in RNPs (Table 3).

Vero cells. Inhibition of cellular protein synthesis by all strains of VSV occurred at a somewhat faster rate in Vero cells than in BHK cells (Fig. 7 and 8). The rate of synthesis of total protein 3 h after infection was greater than 80% of that obtained in uninfected cells (see figure legends). Depending on the virus strain, actin synthesis was reduced to 15 to 30% of the control value by 3 h. In all cases, by 5 h actin synthesis was less than 10% of normal, and all of the cells had rounded and partly detached from the plate. Thus, well over 90% of the cells were infected. Note, however, that the relative efficiency of the different VSV strains to inhibit cellular protein synthesis differed in the two types of cells. In BHK cells, VSV San Juan inhibited more rapidly than did VSV Glasgow, whereas in Vero cells, both inhibited at about the same rate (Fig. 8). However, in both Vero and BHK cells, shutoff by VSV HR and R1 strains was slightly slower than by VSV Glasgow (compare Fig. 8 and 2). Because the differences among VSV strains was less pronounced in Vero cells than in BHK cells, we did not attempt to correlate the different profiles of shutoff of Vero proteins by

FIG. 3. *Gel analysis of proteins synthesized in infected BHK cells and of cell-free translation products of RNA from infected cells. A linear 10 to 15% gradient polyacrylamide gel was used. (Lanes 1-9) Proteins synthesized during a 30-min labeling period with [^{35}S]methionine, beginning at the indicated time. (1) Mock-infected cells, 2.5 h; (2) 3.5 h; (3) 4.5 h; (4) VSV Glasgow-infected cells, 2.5 h; (5) 3.5 h; (6) 4.5 h; (7) VSV San Juan-infected cells, 2.5 h; (8) 3.5 h; (9) 4.5 h. As in Fig. 1, the lower-case letters represent predominant species of host proteins. (Lanes 10-18) 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 was analyzed. Table 1 presents the total amount of acid-precipitable radioactivity incorporated per reaction, as well as the relative amount of VSV proteins made. (10) RNA from mock-infected cells, 2.5 h; (11) 3.5 h; (12) 4.5 h; (13) RNA from VSV-Glasgow-infected cells, 2.5 h; (14) 3.5 h; (15) 4.5 h; (16) RNA from VSV San Juan-infected cells, 2.5 h; (17) 3.5 h; (18) 4.5 h. The Greek letters on the right denote predominant translation products of cellular mRNA, and the capital letters are the VSV structural proteins.*

the various strains (which are reproducible) with the levels of VSV mRNA's.

Mouse L cells. The rate of inhibition of cellular protein synthesis after VSV infection was much slower in L cells (Fig. 9 and 10) and in either Vero or BHK cells. At 3 h, synthesis of actin in L cells was 70 to 100% of that of uninfected cells, depending on the VSV strain used. In all of our experiments, shutoff of actin synthesis after infection of L cells by VSV R1 was slightly less rapid than after infection by VSV HR, although this difference was not observed in the two other cell lines tested (Fig. 2 and 8). In all cases, the total amount of protein synthesis at any time up to 5 h of infection was greater than 90% that of uninfected cells (data not shown).

TABLE 1. *Messenger activity in RNA from VSV-infected BHK cells*[a]

Cell	mRNA activity		Relative amt of VSV mRNA	
	[^{35}S]methionine incorporated (10^5 cpm/μg of RNA)	Normalized	N/NS	M
Uninfected	4.18	1.00		
VSV Glasgow				
2.5 h	4.98	1.19	1.00	1.00
3.5 h	6.99	1.67	3.50	3.62
4.5 h	13.86	3.31	11.50	12.10
VSV San Juan				
2.5 h	8.41	2.01	4.85	5.31
3.5 h	12.98	3.10	10.85	11.10
4.5 h	20.59	4.92	19.63	21.60

[a] Total cellular RNA was extracted from BHK cells at the indicated times after infection. Wheat germ cell-free protein synthesis reactions (25 μl) containing 1, 2, or 4 μg of total cytoplasmic RNA were used, and duplicate 5-μl samples were assayed for acid-precipitable radioactivity. For all RNAs, the amount of [^{35}S]methionine incorporated into acid-precipitable radioactivity was proportional to the amount of RNA added; shown in column 2 is the total amount of ^{35}S radioactivity incorporated in the entire 25-μl reaction. In column 3, the results are normalized to the mRNA activity of RNA from uninfected cells. Each of these reactions was analyzed by SDS-gel electrophoresis, and the amount of N/NS and M proteins was determined from the area under the peak of the microdensitometer scan of the radioautogram. These values are normalized to the value characteristic of RNA from cells 2.5 h after infection by VSV Glasgow.

Two types of comparisons indicated that the shutoff of synthesis of L-cell protein was correlated with the level of VSV mRNA. First, VSV mRNA accumulated to a slightly lower level after infection of L cells by VSV R1 than by VSV HR (Table 2); this corresponded to a slightly slower rate of inhibition of L-cell actin synthesis by VSV R1. Second, with all three VSV strains tested (Glasgow, HR, and R1), much less translatable VSV mRNA accumulated after infection in L cells than in BHK cells (Table 2); this correlated well with the reduced shutoff observed in L cells. Note that the amount of translatable mRNA (per milligram of cytoplasmic RNA) recovered from uninfected L cells and BHK cells was the same and that the predominant species of translatable L-cell mRNA also remained fully functional after VSV infection (Fig. 11).

VSV ts mutants. As another approach to investigating the role of VSV mRNA in inhibition of cellular protein synthesis, we used ts VSV mutants in each of the five complementation groups (10, 13). All of the mutants used were

TABLE 2. *Messenger activity in RNA from VSV-infected L cells and BHK cells*[a]

Cells	Virus	Time after infection (h)	Total mRNA activity		Relative amount of VSV mRNA	
			[^{35}S]methionine incorporated (10^5 cpm/μg of RNA)	Normalized	N/NS	M
BHK	None		2.60	1.00		
	VSV Glasgow	3	6.11	2.35	1.00	1.00
		4	8.40	3.23	1.58	1.68
	VSV HR	4	9.31	3.58	1.88	1.94
	VSV R1	4	7.46	2.87	1.39	1.36
L cells	None		2.20	1.00		
	VSV Glasgow	3	3.26	1.48	0.46	0.41
		4	4.36	1.98	0.86	0.79
	VSV HR	3	4.03	1.83	0.68	0.64
		4	5.02	2.28	1.03	0.97
	VSV R1	3	3.32	1.51	0.53	0.44
		4	4.49	2.04	0.99	0.96

[a] Analysis of the total cellular RNAs was detailed in the legend to Table 1. Values for total mRNA activity are normalized to that obtained with RNA from uninfected BHK or L cells. In all cases, the amount of viral N/NS and M mRNA activity is normalized to that obtained from RNA isolated 3 h after infection of BHK cells with VSV Glasgow.

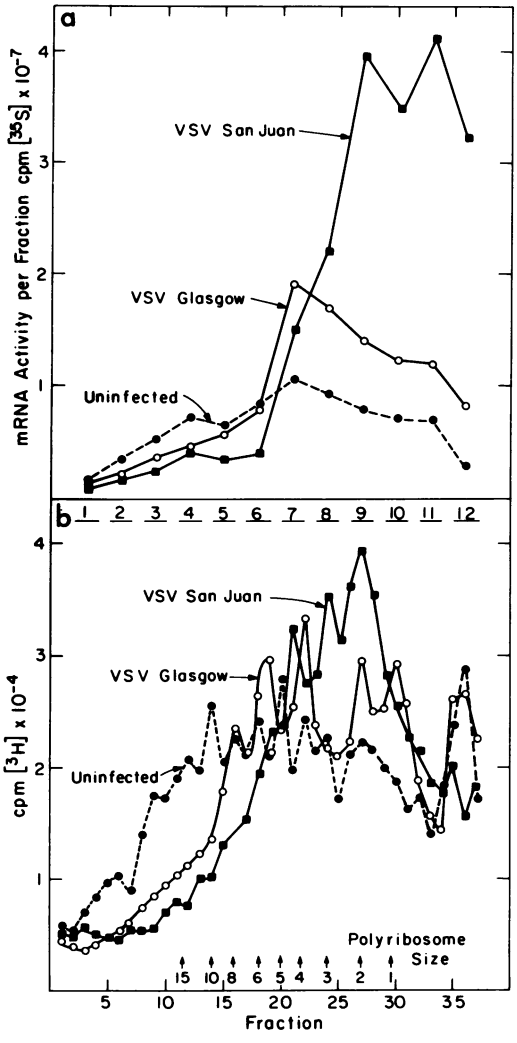


FIG. 5. *Subcellular localization of ribosomes and translatable mRNA in growing BHK cells and in cells 3 h after infection by VSV Glasgow and VSV San Juan. A sample of 6 × 10⁷ BHK cells was grown for 20 h in the presence of 1.5 mCi of [³H]uridine (The Radiochemical Centre) and harvested by centrifugation. One-third of the cells were mock infected, and one-third each were infected with VSV Glasgow and VSV San Juan. Three hours after infection, a postnuclear supernatant from the cultures was prepared and centrifuged through a 15 to 30% (wt/vol) sucrose gradient, as detailed previously (2). Shown in panel b 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 analyzed in parallel. As indicated by the brackets, 1.0 ml from 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 (cf. reference 2). Plotted on the ordinate in panel a is the total amount of translatable mRNA per pooled fraction: this is the product of cpm of [³⁵S]methionine incorporated/cpm of [³H]RNA added and total amount of [³H]RNA in the pooled fractions. Since recoveries of [³H]RNA were very similar in all fractions and averaged 85%, 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%.*

derived from the Glasgow isolate of VSV. Three of the mutants made little or no VSV mRNA at 39.5°C, the nonpermissive temperature: *ts*G114(I), *ts*G22(II); and *ts*G41(IV). Infection of BHK cells (Fig. 12) or Vero cells (data not shown) at 39.5°C by these mutants had no effect at all on the rate of synthesis of predominant cellular proteins. Shutoff of cellular proteins did occur, by contrast, after infection by the two RNA⁺ mutants used: *ts*G33(III) and *ts*L513(V) (Fig. 12). The lower rate of VSV protein synthesis in *ts*L514(V)-infected cells, relative to infection by the wild type, was a consequence of increased cell death; at 3 h these cells synthesized wild-type levels of VSV proteins (14). Again, there was an excellent correlation between synthesis of VSV mRNA and inhibition of translation of cellular mRNA. A corollary of this finding is that the infecting particles themselves, at least up to a level of 10 PFU/cell, had no effect on mRNA translation.

DISCUSSION

Depending on the isolate of VSV Indiana and on the host cell used, there are marked differences in the kinetics of inhibition of cellular protein synthesis. In all cases tested, this inhibition was manifest at the translational level: the amount of translatable mRNA's for the predominant cellular proteins which can be extracted from the cells remains unchanged after infection, whereas the rate of synthesis of cellular proteins decreases steadily.

We emphasize again (2) that we have used in vitro synthesis of discrete viral and cellular proteins as a measure of the amount of corresponding mRNA in the infected cells. Especially in the case of VSV RNAs, this technique is preferable to the more commonly used hybridization, gel separation, or isotope incorporation techniques to quantitate VSV mRNA, since it measures the biologically relevant parameter—its ability to direct protein synthesis. Not all VSV-specific polyadenylated RNAs are functional mRNA's, and thus physical measurements can seriously overestimate the amount of VSV mRNA. A

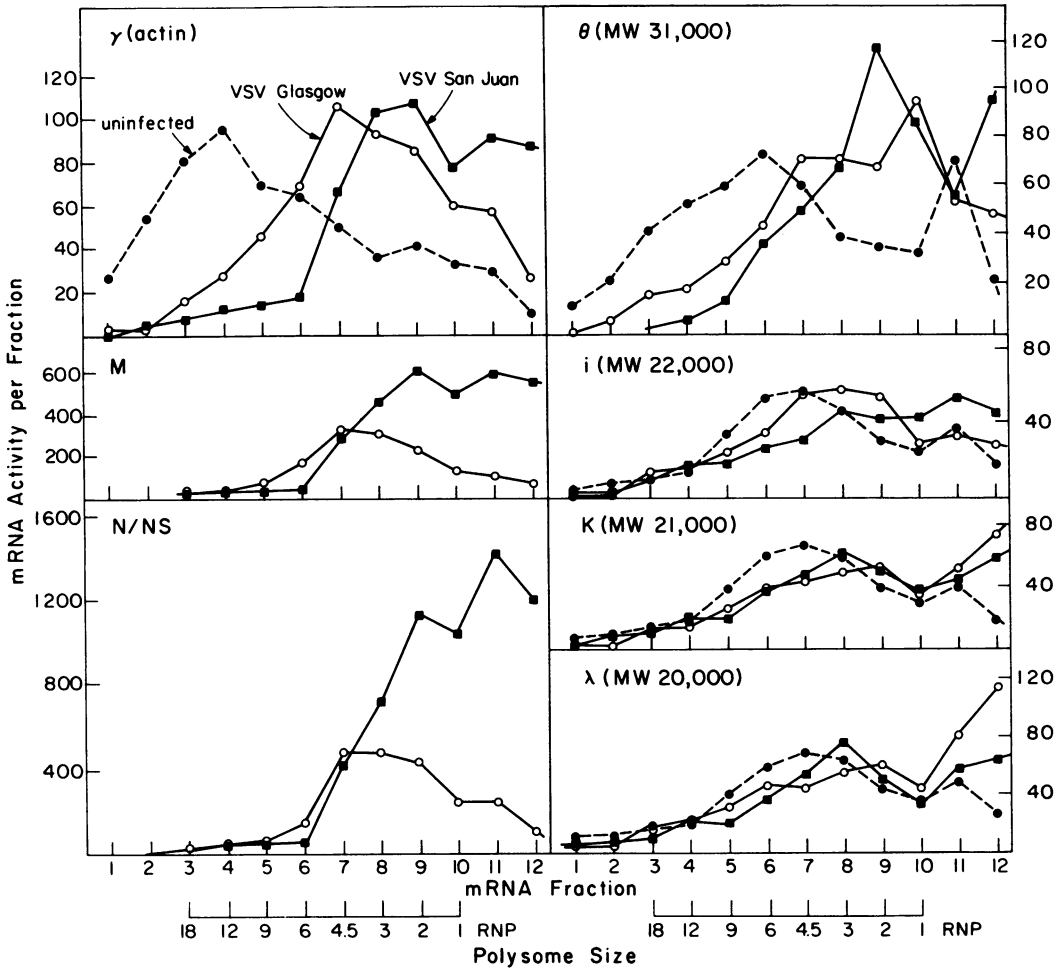


FIG. 6. *Subcellular localization of specific cellular and viral mRNA. RNA from different fractions of the polysome gradient of Fig. 5 was translated in a wheat germ cell-free system, and the products were resolved by electrophoresis through a 10 to 15% gradient polyacrylamide gel (cf. Fig. 3). In all cases, an amount of recovered RNA, equivalent to 0.76% of the total RNA present in the initial pooled fraction, was added to a 25-μl cell-free reaction. Three different exposures of the gel of the translation products were scanned with a Joyce-Loebl microdensitometer, and the areas of the polypeptide bands indicated (cf. Fig. 3) were determined. A 1-h exposure was used for the VSV G and M protein; a 24- or 48-h exposure was used for calculation of cellular bands. 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. 4.*

significant fraction of normal VSV 13 to 18S polyadenylated RNAs possess the 5′ termini pppA and pppG in infected cells. These are not found on polysomes and are presumably inactive as messengers (11). In cells infected at the nonpermissive temperature by ts mutants defective in the M protein, there is a vast overproduction of VSV polyadenylated RNAs (1, 5). Gel electrophoresis and hybridization studies indicate that they are indistinguishable from normal VSV mRNA (1, 5). However, they are undermethylated and are not found on polyribosomes (5).

Previously, we studied the mechanism of this inhibition in BHK cells infected by the Glasgow isolate of VSV Indiana (2). At 4 h the total amount of translatable mRNA (viral plus cellular) was about threefold that of growing cells. Most species of cellular and viral mRNA were localized on polysomes. Infection did result in a reduction (about threefold) in the rate of initiation of translation of all predominant species of cellular mRNA, but translation of cellular and

TABLE 3. *Fraction of viral and cellular mRNA's in RNP particles*[a]

mRNA	Fraction of mRNA		
	Uninfected cells	VSV Glasgow-infected cells	VSV San Juan-infected cells
α	0.19	0.14	0.20
γ	0.07	0.14	0.30
θ	0.18	0.20	0.29
ι	0.16	0.18	0.29
κ	0.15	0.31	0.26
λ	0.17	0.34	0.28
N/NS		0.16	0.39
M		0.13	0.37

[a] The data in Fig. 6 were used to calculate the fraction of mRNA activity for each of six cellular and two viral mRNA's which is localized in the RNP faction (gradient fractions 32 to 37; pools 11 and 12).

viral mRNA's which encoded for the same-size proteins occurred on the same size of polyribosomes. We concluded 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 a constant number of ribosomes. Our results are in disagreement with the prediction of Nuss et al. (9), 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 present results extend our conclusions to additional VSV strain-host cell combinations. First, inhibition of cellular protein synthesis is dependent on synthesis of VSV mRNA. After infection at the nonpermissive temperature by VSV ts mutants defective in synthesis of VSV mRNA, there was no inhibition at all of synthesis of cellular proteins (Fig. 12). Identical results were obtained with mutants defective in the N (complementation group IV), NS (group II), or L (group I) proteins. Mutants defective in the M or G protein, by contrast, made close to normal levels of VSV mRNA and inhibited synthesis of cellular proteins with normal kinetics (Fig. 12 and data not shown).

Second, in BHK cells, three of the four VSV isolates tested [Mudd-Summers, HR (Winnipeg), and R] inhibited synthesis of cellular proteins at about the same rate as did the Glasgow strain of VSV Indiana. Similarly, the amount of VSV mRNA accumulated after infection by these strains was approximately the same, as was the rate of VSV protein synthesis in the infected cells (Fig. 1). By contrast, infection of BHK cells by the San Juan isolate of VSV resulted in inhibition of synthesis of all cellular proteins, and actin in particular, about 45 to 60 min earlier than did infection by the other vi-

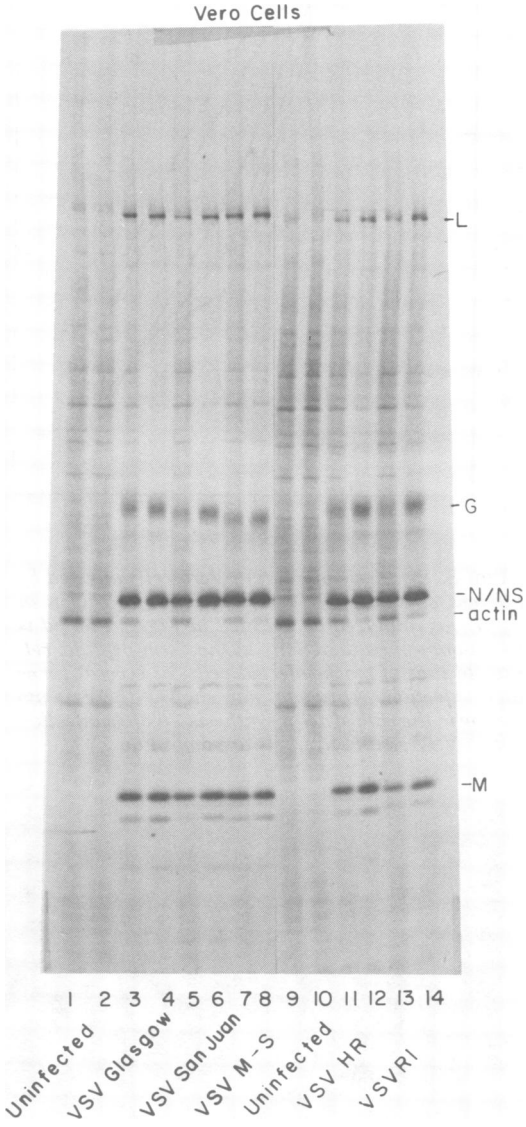


FIG. 7. *Gel analysis of proteins synthesized in Vero cells infected by different strains of VSV. Samples of infected or mock-infected Vero cells were labeled for 30 min with [^{35}S]methionine beginning at 2 h (odd-numbered lanes) or 3 h (even-numbered lanes) after infection. Analysis of the samples was as described in the legend to Fig. 1. In parentheses is given the total amount of [^{35}S]methionine incorporated into protein 3 h after infection relative to the value of uninfected cells. (1 and 2) Uninfected cells (1.00); (3 and 4) infected by VSV Glasgow (0.90); (5 and 6) infected by VSV San Juan (0.85); (7 and 8) infected by VSV Mudd-Summers (0.81); (9 and 10) infected by VSV HR (0.81); (11 and 12) infected by VSV R1 (0.91).*

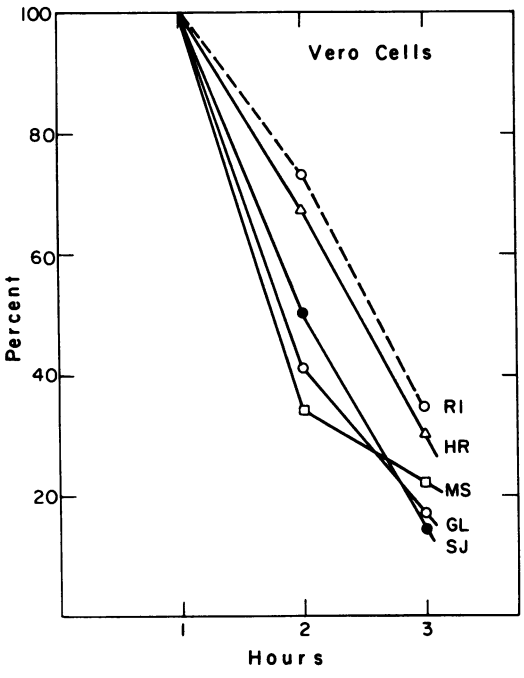


FIG. 8. *Synthesis of actin in Vero cells infected by different strains of VSV. The gel profile shown in Fig. 7 was analyzed as detailed in Fig. 2. Plotted is the amount of radioactivity in the actin peptide, relative to that of mock-infected cells, at several times after infection. Although not shown, there is no change in the rate of actin synthesis 1 h after infection by any VSV strain. Symbols are as in Fig. 2.*

ruses (Fig. 2 and 4). This inhibition was correlated with an increase in the rate of accumulation of VSV mRNA and in an acceleration of the rate of VSV protein synthesis: the same amount of VSV mRNA was accumulated in the cell 45 min sooner after infection by VSV San Juan than by VSV Glasgow. (The history of the laboratory strain of VSV San Juan is highly relevant [A. Huang, personal communication]. The strain, as originally obtained in 1963 from the American Type Culture collection, formed relatively small plaques and yielded a stock of virus of about 10^8 PFU/ml. To select for a variant which grew to a higher titer, Dr. Huang passaged the virus in CHO cells 17 times in succession at low multiplicities of infection. After this, the virus was cloned repeatedly. The yield of infectious virus in a single-step growth curve was increased about 10-fold by this enrichment procedure, and it is apparent that a more rapidly replicating variant [at least in some cells] was selected. The differences between this isolate and the other VSV wild-type strains is most pronounced in the BHK cell line.) The effects of this increased amount of mRNA on the subcellular distribution of cellular (and viral) mRNA are in complete accord with our conclusion that viral and cellular mRNA's compete on an equal basis during the initiation step of protein synthesis for a limiting number of ribosomal subunits. At 3 h after infection by either virus, at least 75% of cellular ribosomes remain in polysomes, and the total rate of total protein synthe-

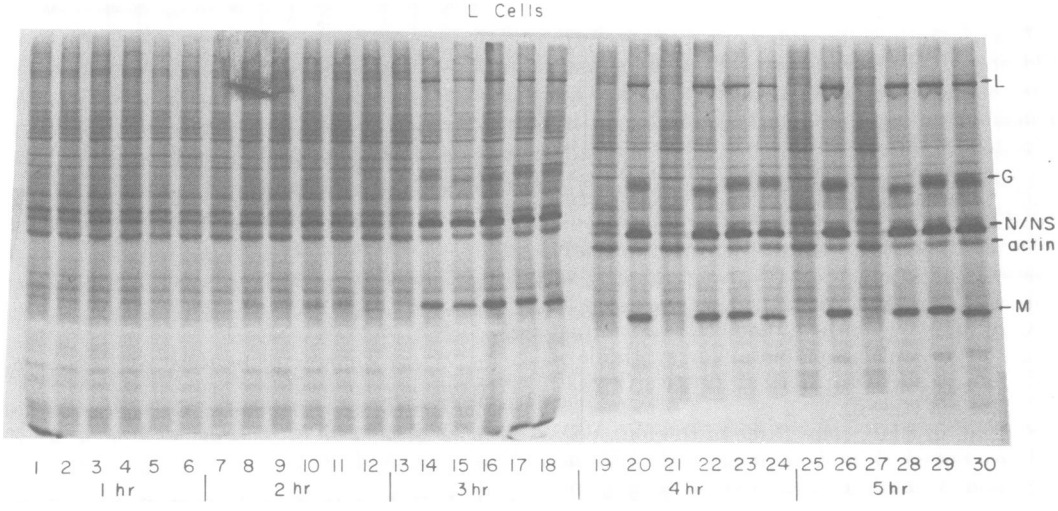


FIG. 9. *Gel analysis of proteins synthesized in L cells by different strains of VSV. Samples of mock-infected or VSV-infected cells were labeled for 30 min with [^{35}S]methionine beginning at 1 h (lanes 1–6), 2 h (7–12), 3 h (13–18), 4 h (19–24), or 5 h (25–30) after infection. Total cellular protein was analyzed by electrophoresis through a 10% polyacrylamide gel containing SDS; shown is a radioautogram of the dried gel. (Lanes 1, 7, 13, 19, 21, 25, 27) Uninfected cells; (2, 8, 14, 20, 26) infected by VSV Glasgow; (3, 9, 15) infected by VSV San Juan; (4, 10, 16, 22, 28) infected by VSV Mudd-Summers; (5, 11, 17, 23, 29) infected by VSV HR; (6, 12, 18, 24, 30) infected by VSV R1.*

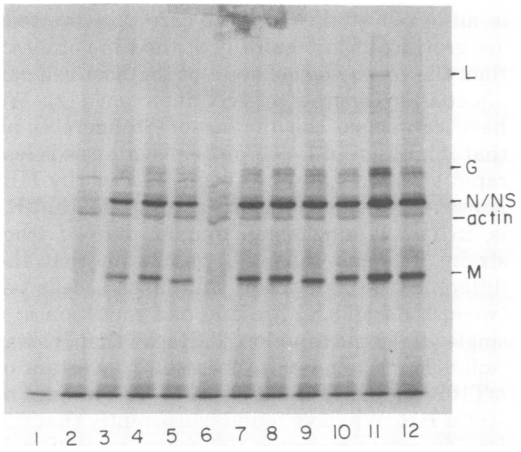


FIG. 10. *Cell-free translation products of RNA from VSV-infected L cells. Proteins were synthesized in a wheat germ cell-free synthesis reaction, using 1 μg of the indicated RNAs per 25-μl reaction. A 5-μl sample of the reaction was analyzed on a 10% polyacrylamide gel containing SDS. Table 2 presents the total amount of acid-precipitable radioactivity incorporated per reaction, as well as the relative amounts of VSV proteins produced. (Lane 1) No RNA; (2) RNA from mock-infected L cells, 3 h; (3) RNA from VSV Glasgow-infected L cells, 3 h; (4) RNA from VSV HR-infected L cells, 3 h; (5) RNA from VSV R1-infected L cells, 3 h; (6) RNA from mock-infected L cells, 4 h; (7) RNA from VSV Glasgow-infected L cells, 4 h; (8) RNA from VSV HR-infected L cells, 4 h; (9) RNA from VSV R1-infected L cells, 4 h; (10) RNA from a different preparation of VSV Glasgow-infected L cells, 4 h; (11) RNA from VSV Glasgow-infected BHK cells, 4 h; (12) RNA from VSV Glasgow-infected BHK cells, 3 h.*

sis is at least 80% that of growing cells. Thus, the vast majority of ribosomes are functioning in protein synthesis. However, the average size of polysomes translating cellular and viral mRNA's is much smaller in the VSV San Juan-infected BHK cells. After infection by VSV Glasgow, mRNA's for two typical cellular proteins of molecular weight of 30,000 to 40,000, θ and actin, are found to be pentasomes, the same size as polysomes which are translating VSV N (52,000) and M (35,000) mRNA. This is 40 to 50% the size of polysome on which is found actin mRNA in growing cells, a result consistent with the twofold increase in total amount of functional mRNA per cell. In the VSV San Juan-infected cells, by contrast, actin, θ, N, and M mRNA's are all localized to di- or trisomes—one-sixth the number of ribosomes per actin or θ mRNA found in growing cells. This corresponds to the five- to sixfold increase in total translatable mRNA per cell observed in this experiment.

Although our results on infection of mouse L cells by VSV are different in several respects from those using BHK cells, they are consistent with the notion that host shutoff is a consequence of mRNA competition for limiting ribosomes. First, for all VSV strains studied the kinetics of host shutoff are slower in L cells than in BHK cells: about 1 h in the case of inhibition of translation of actin mRNA. This is paralleled by a correspondingly slower rate of accumulation of VSV mRNA. In particular, the amount of VSV mRNA found 3 h after infection of BHK cells with VSV Glasgow is about the same as at 4 h after infection of L cells by the same virus preparation (Table 2).

An additional correlation emerges from a comparison of the infection by L cells by VSV strains HR4 and R1. As Stanners et al. (12) reported, inhibition of cell protein synthesis is less after R1 infection than after HR infection. However, the consistent difference we find (Fig. 11) is not as marked as that previously described (10). Nonetheless, the reduced level of VSV mRNA which accumulates by 3 h in R1-infected cells relative to HR4-infected cells correlates with the reduced inhibition of actin mRNA translation.

Stanners et al. (12) found a much more rapid inhibition of L-cell protein synthesis after infection by the HR strain than we have observed. Since we have used cell lines and virus grown from stocks provided by C. P. Stanners, we have

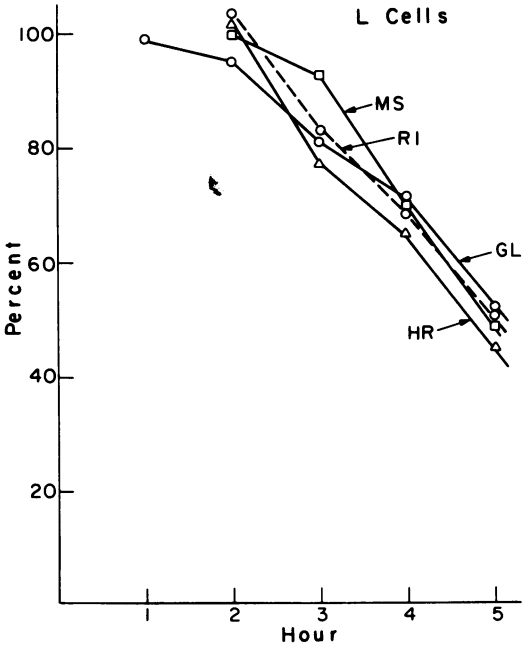
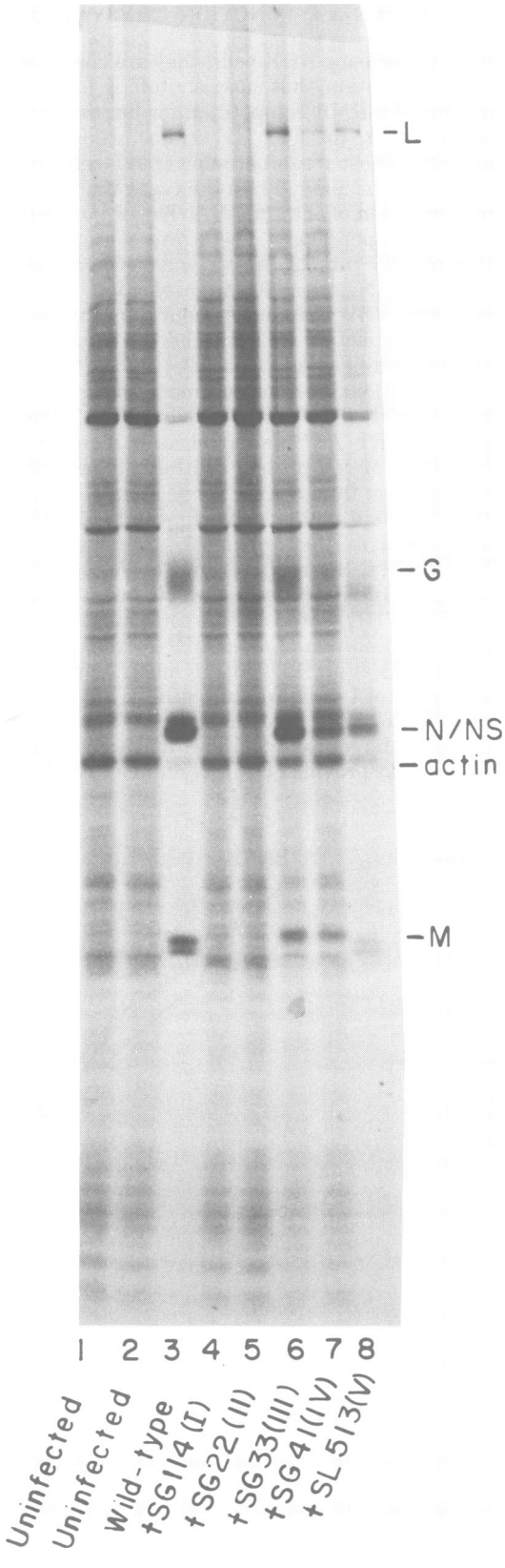


FIG. 11. *Synthesis of actin in L cells infected by four strains of VSV. The gel radioautogram of the gel in Fig. 9 was analyzed as detailed in the legend to Fig. 2. Symbols are also described there.*



no explanation for this difference, except that we are culturing the cells as monolayers. Also, in all of our studies we take care that the cells are growing logarithmically at the time of infection; the physiological state of the host cell can affect the response to VSV infection (12). We have confirmed the finding of Stanners et al. that inhibition of L-cell protein synthesis is less rapid after infection by strain R1 than by HR. However, we question their assumption that R1 is, in fact, a single-step mutant derived from strain HR, and thus the conclusion that the differences in kinetics of shutoff of L cells between HR4 and R1 are due to a mutation in a single viral gene function, P. Recall that R1 was isolated as a temperature-resistant revertant of *ts*T1023(I), which is supposed to be a mutant of strain HR. We have found consistently that the R1 M protein migrates 10% faster on SDS-gels than does the HR M protein. This is true for M proteins synthesized in BHK, L, and Vero cells (Fig. 1, 7, and 9). It is also the case for HR and R1 M proteins synthesized in a wheat germ cell-free system (Fig. 10). It is difficult to imagine how a single-step mutation could result in such a large shift in gel mobility. Clarification of this point will require isolation of P^- mutations directly from a known wild-type strain.

We do not know, in detail, why the kinetics of inhibition of cellular proteins synthesis varies with the host cell and with the strain of VSV used. There is, as noted above, a good correlation between the kinetics of host shutoff and the accumulation of viral mRNA, but it is not clear why the rate of accumulation of viral mRNA should be so dependent on the host cell line and on the strain of VSV. Our preliminary studies indicate that the inhibition of actin synthesis 3 h after infection of BHK cells by 10 or 50 PFU/cell is not significantly (10%) different, so differential adsorption or penetration of the virions is unlikely to be involved. As noted in the text, in all cases all of the cells are infected by VSV. Whereas a VSV virion contains its own RNA polymerase activity which will, in vitro, direct synthesis of all five VSV mRNA's, it is not known whether, in the infected cells, specific host proteins (which might be present also in the virion in minute amounts) are also essential, nor is it known how the intracellular levels of

FIG. 12. *Gel analysis synthesis of proteins synthesized in BHK cells infected at 40.0°C by ts mutants of VSV. As in Fig. 1, cells were infected by the indicated mutants and labeled with [^{35}S]methionine 4 h after infection at 40°C. Electrophoresis of the labeled protein utilized at 10 to 15% gradient polyacrylamide containing SDS. (Lanes 1 and 2) Uninfected cells; (3) infection by wild-type VSV Glasgow; (4) infection by tsG114 (I): (5) infection by tsG22 (II); (6) infection by tsG33 (III); (7) infection by tsG41 (IV); (8) infection by tsL513 (V).*

ribonucleoside triphosphates or SAdoMet in the cells might influence the rate of viral RNA transcription.

Taken together, our results indicate that inhibition of cellular protein synthesis is primarily a consequence of competition by viral and cellular mRNA for a constant limiting number of ribosomes. Shutoff clearly is dependent on synthesis of viral mRNA. However, our data on the subcellular distribution of mRNA's after infection and on the correlation between the levels of viral mRNA and inhibition of cellular mRNA translation are only semiquantitative. It is difficult to eliminate rigorously other factors which could reduce the rate of translation of cellular mRNA's after infection. Previously we listed some of these: (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. These mRNA's could be localized only to the dead, inactive cells. 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.

ACKNOWLEDGMENT

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