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Effect of Intracellular Vesicular Stomatitis Virus mRNA Concentration on the Inhibition of Host Cell Protein Synthesis

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Inhibition of host cellular protein synthesis by vesicular stomatitis virus (VSV) has been suggested to be primarily the result of competition for ribosomes between cellular and viral mRNAs (H. F. Lodish and M. Porter, J. Virol., 36:719–733, 1980; Lodish and Porter, J. Virol. 38:504–517, 1981). This hypothesis was investigated by regulating the extent of VSV mRNA synthesis through the use of defective interfering particles. Although intracellular VSV mRNA concentrations decreased by as much as a factor of 14 at high multiplicities of infection of defective interfering particles, the inhibition of host cell protein synthesis by VSV decreased by a maximum of only 10%. The data also indicated that under these conditions the protein-synthesizing capacity of the cells was not exhausted. We concluded that competition for cellular ribosomes could not have been the major factor in the inhibition of host cell protein synthesis by VSV. This conclusion was further supported by inhibition data obtained with VSV mutants. The *ts* G22 mutant, defective in replication but not in primary transcription, inhibited host protein synthesis at the nonpermissive temperature (39°C) to the same extent as did wild-type virus, even though it generated only 30 to 50% of the amount of viral mRNA as did wild-type virus. Conversely, in infections with the R1 mutant, which did not inhibit host cell protein synthesis, the amount of total and polysome-bound viral mRNA was indistinguishable from that obtained in infections by wild-type virus.

Infection of mammalian cells with vesicular stomatitis virus (VSV) results in the inhibition of cellular macromolecular synthesis (1, 12–14, 28, 29). Although it has become clear that transcription of at least some viral functions is required for this inhibition (12, 29), neither the nature of these functions nor their cellular target sites are known. Most of the inhibition of cellular protein synthesis by VSV takes place at the translational level (10). Stanners et al. (26) have isolated VSV mutants which are unable to inhibit host protein synthesis; however, the nature of the mutation was not established. Nuss et al. (16) have suggested that VSV mRNA might initiate protein synthesis more efficiently than does cellular mRNA and thus take over the protein-synthesizing components of the cell. Lodish and Porter (10) have determined the sizes of polysomes translating various cellular mRNAs in uninfected and VSV-infected cells and concluded that the inhibition of cellular protein synthesis is due primarily to competition for ribosomes by a large excess of viral mRNAs. Since the number of ribosomes in polysomes of viral and cellular mRNAs of comparable sizes was about the same in infected cells, these authors concluded that the efficiency of viral mRNA initiation could not significantly differ from that of cellular host mRNAs. The competitive nature of viral and host mRNA translation was also reported in a later publication by Lodish and Porter (11). A correlation between the intracellular viral mRNA concentration and the extent of cellular protein synthesis inhibition in infections with various VSV wild-type and mutant isolates was demonstrated.

Intracellular VSV mRNA concentrations are basically determined by two events, primary transcription by the incoming virion inoculum and amplified transcription during which newly synthesized viral nucleocapsids are utilized. Primary transcription usually contributes up to 10%, depending on the multiplicity of infection (MOI), of the fully amplified level of VSV mRNA (27). By inhibiting virion RNA replication to various degrees, intracellular VSV mRNA concentrations could be regulated in the range of 10 to 100% of the amplified value. This should make it possible to measure directly the inhibition of host cell protein synthesis as a function of VSV mRNA concentrations. Since chemical means of inhibiting virion RNA replication might affect cellular processes, regulation was accomplished by the use of defective inter-

fering (DI) particles. These particles inhibit virion replication without interfering with primary transcription (4, 17). Therefore, mixed infections of VSV and various MOIs of DI particles should be suitable for an examination of the dose effect of VSV mRNA on host protein synthesis inhibition. The results presented here show that inhibition of BHK host cell protein synthesis by VSV was essentially independent of DI particle concentration even when the intracellular VSV mRNA concentration dropped to 7.4% of its fully amplified value. We also found that the *ts* G22 mutant of VSV inhibited BHK cellular protein synthesis to a degree comparable to that by the wild-type virion even when the mutant genome was not replicated. Finally, we showed that the intracellular and polysomal concentrations of viral mRNAs during infection with the R1 mutant (26) were approximately the same as those during infection with the wild-type virion even though the mutant virus did not inhibit host cell protein synthesis during the first 4 h after infection.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cells (BHK-21, clone 13) were grown in monolayers as previously described (7, 21).

The following isolates of the Indiana serotype of VSV were used in this study: MS (21); *ts* G22, obtained from C. R. Pringle (18); and R1, provided by C. P. Stanners (26). Viral inocula, freed of DI particles, were prepared by a fivefold clonal selection and enrichment in BHK cell monolayers at 39°C (MS and R1) or 31°C (*ts* G22) by the method of Stampfer et al. (25). After plaque purification, the *ts* G22 isolate exhibited a temperature-sensitive dependency of greater than two orders of magnitude. The R1 isolate has been previously reported to exhibit an attenuated cytopathic effect (26). However, after clonal selection, we observed the same degree of cytopathic effect with either R1 or its wild-type parent in BHK cells. In spite of this discrepancy, the clonally enriched R1 did not inhibit cellular protein synthesis as previously reported (26). The MS isolate was also used for a source of DI particles, VSI MS DI 0.23 (5′, 5%) (see reference 19 for nomenclature), which were obtained by undiluted passage of the viral inoculum.

Preparation and purification of virions, DI particles, and particle-bound RNAs. The isolation of virions and DI particles has been reported previously (2). Concentrations of DI particles were determined by absorbance measurements at 260 nm (A_{260}) with a Cary 14 spectrophotometer (Applied Physics Corp., Monrovia, Calif.). Effective DI particle MOIs were calculated as follows. Virion plaque assays indicated that one A_{260} unit corresponded to 3×10^9 PFU. Assuming that the same percentage of virion and DI particles is biologically active and correcting for the one-third difference in their sizes (20), we took one A_{260} unit to be equivalent to approximately 10^{10} active particles.

To radioactively label virion proteins, MS virus-infected cells were overlaid with serum- and methionine-free medium containing 4 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. After 18 to 24 h of incubation at 39°C, virions were isolated as described above and then pelleted at 35,000 rpm and 4°C for 90 min in a Beckman R40 rotor. Viral pellets were lysed and prepared for electrophoresis as described below for cellular pellets. MS virion RNA, radioactively labeled with tritium in all four bases, was prepared as previously described (22).

Interference assays. Interference by DI particles was quantitated as previously described (2) except that progeny virus was measured at 4 h instead of 18 h after infection.

Assay for intracellular protein synthesis. BHK confluent monolayers in 60-mm (5×10^6 cells) or 100-mm (1×10^7 cells) tissue culture plates were either virus infected at an MOI of 5 or mock infected at 39°C with occasional agitation. For experiments using DI particles, MS virions and appropriate dilutions of DI particles were allowed to adsorb simultaneously. After 30 min, the cells were overlaid with prewarmed medium and maintained at 39°C.

At various times after infection, the medium was replaced with methionine- and serum-free medium containing 0.5 to 1.0 μCi of [^{35}S]methionine. After 30 min (viral infections) or 45 min (DI particle infections), the cells were washed twice with cold phosphate-buffered saline and then overlaid with 5 ml of 10% trichloroacetic acid (TCA). After 5 min, the TCA was replaced with 5 ml of fresh 10% TCA for an additional 5 min. Intracellular pools of radioactive precursors were measured by dissolving 0.9 ml of the first TCA rinse in 6 ml of Aquasol (New England Nuclear) and counting in a Beckman LS-250 scintillation counter. The cells were then dehydrated with two 5-min washes of ethanol-ether (3:1), air dried, solubilized in 1 ml of freshly made 0.2% NaOH, added to 6 ml of Aquasol, and counted.

Preparation and gel electrophoresis of intracellular proteins. Infected and mock-infected cells in 60-mm plates were pulse-labeled for 30 min at 3 h postinfection with 4 μCi of [^{35}S]methionine per ml. The cells were rinsed twice with cold phosphate-buffered saline and then overlaid with 2 ml of cell resuspension buffer (25 mM Tris-hydrochloride [pH 7.9], 1 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.5 mM dithiothreitol) (24). After being scraped from the plates and pelleted at 2,500 rpm for 10 min in a Sorvall GLC-1 centrifuge, the cells were dissolved in 90 μl of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 10 mM magnesium acetate, 0.2% sodium dodecyl sulfate, 0.25 μg of phenylmethylsulfonyl fluoride per ml) (10) to which 90 μl of the same buffer containing 20 μg of DNase I (Worthington Diagnostics, Freehold, N.J.) per ml was added. After 1 h at 37°C, 90 μl of a protein-denaturing solution (30 mM Na_2PO_4, 7.5% sodium dodecyl sulfate, 15% [vol/vol] 2-mercaptoethanol, 30% [vol/vol] glycerol) (10) was added, and the samples were boiled for 3 min immediately before being loaded on gels. Separation of proteins was accomplished in 10% polyacrylamide slab gels by the method of Laemmli (6). Gels were either (i) fixed for 15 min in 10% acetic acid–5% glycerol, dried in a gel drier (Bio-Rad Laboratories, Richmond, Calif.), and autoradiographed with Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 48 to 96 h at −70°C or (ii) divided into individual lanes and cut

into 1-mm sections with a Mickle Gel Slicer (Brinkmann Instruments Inc., Westbury, N.Y.). The slices were dissolved overnight in 5 ml of 3% Protosol in Econofluor (New England Nuclear) at 37°C and counted.

Isolation of intracellular and polysome-bound RNAs. Total intracellular RNA was isolated at various times after viral infection. Cell monolayers in 100-mm plates were washed three times with phosphate-buffered saline and then lysed with 5 ml of E buffer (40 mM Tris-acetate [pH 7.2], 20 mM sodium acetate, 1 mM EDTA) containing 1% sodium dodecyl sulfate. The lysed cells were extracted twice with an equal volume of phenol-$CHCl_3$ (1:1) and once with an equal volume of $CHCl_3$. The RNA species were then precipitated with 3 volumes of ice-cold ethanol in the presence of 300 mM sodium acetate at −20°C. After at least 8 h, the RNA was collected by centrifugation at 9,000 rpm at 4°C for 20 min in a Sorvall RC2-B centrifuge, dried under nitrogen, and dissolved in water. The optical density of the RNA samples was determined at 260 nm. The RNAs were then reprecipitated with ethanol dissolved in the appropriate volume of water and stored at −70°C until used.

Polysomes were isolated according to the procedures of Lodish and Froshauer (9), with the following modifications: emetine treatment (to inhibit elongation) was omitted, cell monolayers were used instead of cells in suspension, and polysomes were not fractionated on sucrose gradients.

At 3.5 h postinfection, the cell monolayers were washed, scraped off the plates, and poured into chilled Dounce homogenizers. The cells were allowed to swell for 10 min and then were homogenized 40 times as described previously (9). The detergent-treated postnuclear fraction was layered on a 0.5- to 1.0-ml pad of 40 mM HEPES (pH 7.5)–80 mM NaCl–2.5 mM magnesium acetate containing 15% sucrose in SW50.1 nitrocellulose tubes. Polysomes were pelleted by centrifugation at 45,000 rpm in a Beckman SW50.1 rotor for 1.5 h, the time required to sediment 80S ribosomes at 5°C. After centrifugation, the pellets were suspended in 1 ml of water (for TCA precipitations) or 3 ml of extraction buffer (10 mM Tris-hydrochloride [pH 7.5], 100 mM NaCl, 10 mM EDTA) containing 1% sodium dodecyl sulfate (for extraction of RNA).

Quantitation of viral intracellular and polysome-bound mRNAs. Viral intracellular mRNAs were quantitated by annealing to a ^3H-labeled virion RNA as described previously (22). When less than 40% of the virion RNA was rendered RNase resistant, there was a direct relationship between the amount of annealed virion RNA and the amount of complementary mRNA species. The results (expressed as counts per minute) were then extrapolated to the total amount of viral mRNA per infected cell sample and normalized by correcting for variations in the total cellular RNA content as determined by A_{260} readings. Polysome-bound viral mRNA species were also compared in a similar manner, except that the A_{260} of RNAs was used as the correction factor.

Polysome-bound viral mRNAs were also quantitated by labeling RNA in the presence of actinomycin D as described below. Cell monolayers were treated for 30 min before infection with medium containing 7.5 μg of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were then mock infected or infected with MS or R1 virions at an MOI of 5 for 30 min at 39°C. Medium containing 7.5 μg of actinomycin D per ml was then added, and incubation was continued at 39°C for 1 h. After this time, medium containing 7.5 μg of actinomycin D and 5 μCi each of [5,6-^3H]uridine (38 Ci/mmol) (New England Nuclear) and [2,8-^3H]adenosine (37 Ci/mmol) (ICN Chemical and Radioisotope Division, Irvine, Calif.) per ml was added. Incubation was continued for an additional 2.5 h, at which time polysomes were isolated as described above. The polysomes were then suspended in 1 ml of water, precipitated with 1 ml of 10% TCA and 100 μg of yeast RNA, collected on 2.8-cm glass fiber filters, washed, dried, and counted.

RESULTS

Effect of DI particles on the inhibition of host protein synthesis by VSV. When BHK cells were infected with a high MOI of DI particles, cellular protein synthesis was partially inhibited even in the absence of VSV (Table 1, DI particle MOIs of 28 and 56). This inhibition was not due to virion contamination since cells infected with the number of PFU corresponding to the level of contamination in these inocula did not differ from mock-infected controls in their protein-synthesizing activity. It is more likely that the high MOI of DI particles caused extensive damage to the cell membrane, primarily through interactions with the G protein of the particle spikes (15, 20). The interfering ability of these DI particle concentrations with VSV yields from

TABLE 1. Effect of DI particles of various interfering ability on BHK host cell protein synthesis

DI particle MOI[a]	Methionine incorporation (cpm)	Protein synthesis (% of control)	Log reduction of infectivity (PFU/ml)[b]
0	164,000	100	
1.8	160,000	98	1.76
3.5	157,000	96	2.16
7	165,000	101	2.76
14	151,000	92	ND[c]
28	135,000	82	3.39
56	110,000	67	3.48

[a] MOIs were determined by A_{260} measurements. Virion plaque assays indicated that one A_{260} unit corresponded to 3×10^9 PFU. Assuming that the same fraction of virions and DI particles was biologically active and correcting for the difference in size, the MOI is based on the value of 10^{10} particles per A_{260} unit.

[b] Results are expressed as log of the difference between the number of infectious particles produced by BHK cells coinfected with virions (5 PFU per cell) and DI particles and the number produced by cells infected with virions only. In the absence of DI particles, the yield of virions was 1.3×10^7 PFU/ml at 4 h postinfection.

[c] ND, Not determined.

doubly infected cells (5 PFU of VSV per cell) is also shown in Table 1. The decrease in viral yields ranged from 1.8 to 3.5 logs, which was approximately equivalent to a 98.3 to 99.97% inhibition.

In Table 2, the relationship among VSV inhibition of host cell protein synthesis, DI particle concentration, and intracellular virus mRNA concentration is shown. The latter was determined by annealing samples of RNA from infected cells with a radioactively labeled virion RNA probe and then measuring RNase A- and T1-resistant counts. The annealings were performed with concentration ranges of cellular RNA which gave a linear annealing response, i.e., up to 40% of the saturation level of the probe. These data were extrapolated to give the relative mRNA concentrations, which are expressed as counts per minute of VSV RNA probe annealed by the RNA content of two 60-mm plates of cells after normalization by A_{260} values (Table 2). These data are also expressed as a percentage of the VSV mRNA synthesized in the absence of DI particles. At very high MOIs of DI particles, the mRNA levels dropped to 7% of the amount synthesized by virus only. This rate of mRNA synthesis is consistent with that of virion primary transcription (27), which is not inhibited by DI particles (4, 17). The higher value of 11% obtained in the presence of cycloheximide (Table 2) might be due to incomplete inhibition of protein synthesis (8). Indeed, a slight incorporation of methionine was observed under these conditions (Table 2). In spite of the 14-fold change in VSV mRNA concentration, the incorporation of [^{35}S]methionine into proteins by the cells remained the same over the entire range of DI particle concentrations and did not differ appreciably from the incorporation by cells infected with virion alone (Table 2).

The ^{35}S incorporation experiments did not necessarily prove that host protein synthesis was unaffected by the VSV mRNA levels. Since the DI particles inhibited VSV replication, viral protein synthesis was probably diminished in the doubly infected cells and could have been compensated for by increased cellular protein synthesis. This is exactly what would be expected if the ribosomes were in limiting supply. It was, therefore, necessary to analyze the synthesis of cellular and viral proteins separately. Figure 1 shows profiles of pulse-labeled cellular and viral proteins separated by polyacrylamide gel electrophoresis. Each lane was loaded with extracts corresponding to 5×10^5 cells and labeled with [^{35}S]methionine as described above. Lane a contained extracts from uninfected cells, and lane f was loaded with purified VSV. All other lanes contained extracts from cells infected with VSV (5 PFU per cell) and pulse-labeled 3 h after infection; the extracts shown in lanes c, d, and e were isolated from cells also infected with 59, 15, and 3.7 DI particles per cell, respectively.

An examination of lanes a and b of Fig. 1 demonstrated the strong inhibition of cellular protein synthesis by VSV in this experiment. Bands corresponding to cellular proteins are barely discernible in lane b. The four strong bands in lane b which corresponded to viral proteins L, G, N, and M (N and NS were not

TABLE 2. Incorporation of methionine in BHK cells infected with VSV[a] in the presence and absence of DI particles[b]

DI particle MOI[c]	Methionine incorporation (cpm)	Protein synthesis (% of control)	VSV mRNA concn (cpm)[d]	Viral mRNA synthesis (% of control)
0	114,000	69	1.19×10^5	100.0
1.8	102,000	62	3.21×10^4	27.0
3.5	93,000	56	1.68×10^4	14.0
7	102,000	62	1.30×10^4	11.0
14	ND[e]	ND	1.06×10^4	8.9
28	104,000	64	8.86×10^3	7.4
56	101,000	61	8.33×10^3	7.0
0 (No VSV)	164,000	100	0.00	0.0
0 (+ cycloheximide[f])	2,900	2	1.30×10^4	11.0

[a] VSV concentration, 5 PFU per cell.
[b] All measurements were made 4 h after infection.
[c] DI particle concentrations were determined as described for Table 1.
[d] The relative concentration of viral mRNA is expressed as the total ^{3}H counts of virion RNA which could be rendered RNase resistant after annealing. This value has been normalized for 10^7 cells by measurements of A_{260} of cellular RNA. The results were corrected for nonspecific annealing by subtracting the ^{3}H counts of virion RNA made RNase resistant after annealing with uninfected intracellular RNA.
[e] ND, Not determined.
[f] Cycloheximide concentration, 50 μg/ml.

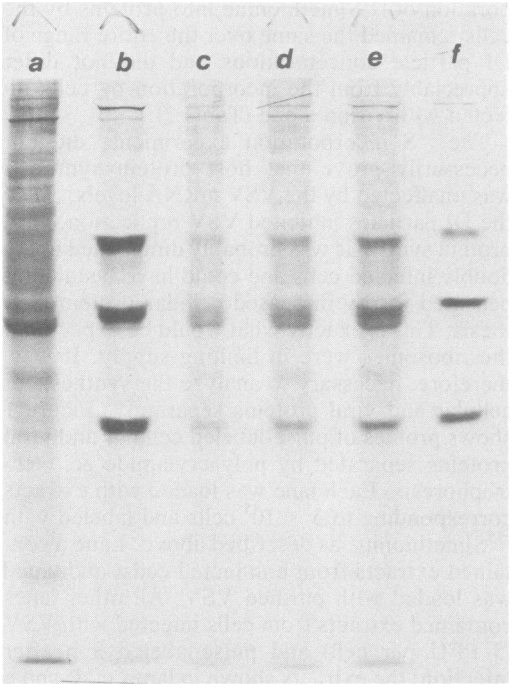

FIG. 1. Gel analysis of proteins synthesized in BHK cells, either mock infected, infected with VSV, or doubly infected with VSV and DI particles. As described in the text, the cells were labeled with [^{35}S]methionine for 30 min beginning 3 h after infection. Cell extracts from equivalent numbers of cells were electrophoresed through a 10% polyacrylamide gel (6). Lanes: a, mock-infected cells; b, c, d, and e, cells infected with 5 PFU of VSV per cell and DI particle MOIs of 0, 59, 15, and 3.7, respectively; f, marker VSV proteins from purified virions.

separated) indicated that extensive viral protein synthesis had taken place. The addition of DI particles to the VSV inoculum had a very small effect on the intensity of the bands which corresponded to cellular proteins. Even at an MOI of 15 (lane d), when the synthesis of viral proteins was severely inhibited and the intracellular VSV mRNA concentration was suppressed to approximately 9% of its normal value (Table 2), the intensity of the bands corresponding to cellular proteins increased only marginally.

To quantitate the effect of DI particles on the inhibition of cellular protein synthesis by VSV, the gels of each lane were sliced, and the radioactive content was determined. Due to the presence of viral protein bands in lanes b through e of Fig. 1, the radioactivity corresponding to cellular and viral proteins was corrected as follows. Since 30% of the cellular proteins in lane a of Fig. 1 (uninfected cells) were in the corresponding positions of viral proteins, the radioactivity in lanes b through e of Fig. 1, representing cellular protein bands only, was divided by 0.70. These data, which represent a relative measure of true cellular protein synthesis, are shown in Table 3, as are the amounts of VSV proteins synthesized relative to total cellular proteins under the various conditions of this experiment. It should be noted that the values for the VSV proteins have been corrected for the presence of cellular proteins by subtracting 30% of the respective cellular protein radioactivities (Table 3). At DI particle MOIs of 15 and 59, the data illustrate that even though intracellular VSV mRNA was suppressed to the level of primary transcription (Table 2), cellular protein synthesis was enhanced by only 10% over that taking place in the absence of DI particles and in the presence of a 10-fold-greater VSV mRNA content. This seems to be the maximum level of competition that may occur between cellular and viral mRNA in this concentration range.

Inhibition of protein synthesis by VSV mutants. Group II temperature-sensitive mutants of the Indiana serotype of VSV are defective at the

TABLE 3. Inhibition of BHK host cell protein synthesis by VSV[a] in the presence and absence of DI particles

DI particle MOI[b]	Methionine incorporation into cellular proteins (cpm)[c]	Cellular protein synthesis (% of control)	Methionine incorporation into VSV proteins (cpm)[d]	VSV protein synthesis (% of total protein synthesis)
0	42,500	14	101,000	70
3.5	59,000	19	38,000	39
15	70,000	23	24,000	25
59	74,000	24	26,000	26
0 (No VSV)	310,000	100		

[a] VSV concentration, 5 PFU/cell.
[b] See Table 1 for MOI determinations.
[c] Cells were labeled at between 3 and 3.5 h postinfection. The counts were obtained from sliced gels such as that shown in Fig. 1. Thirty percent of the control counts (no VSV) were in areas corresponding to viral protein bands. The numbers were obtained after appropriate corrections.
[d] These numbers were obtained after subtracting 30% of the respective cellular protein counts from the determined values.

nonpermissive temperatures in virion RNA replication but not in primary transcription (27). If high concentrations of viral mRNAs are required for inhibition of host protein synthesis, then mutants belonging to this group should not influence host protein synthesis at 39°C but should behave like the wild type at 31°C. Protein synthesis in cells infected with mutant *ts* G22 at 39°C was compared with that in cells infected with wild-type virus by measuring the incorporation of [^{35}S]methionine during 30-min pulses. The data, expressed as percent methioine incorporation as compared with that in uninfected cells, are shown in Fig. 2. It is clear that the *ts* G22 mutant inhibited protein synthesis as efficiently as did the wild-type virus. However, the viral mRNA content of wild-type- and mutant-infected cells was not the same. At 3 and 4 h after infection, the VSV mRNA content of the mutant-infected cells was two- to threefold lower than that of the wild-type-infected cells (Table 4). As in the case of cells doubly infected with DI particles and with wild-type VSV, the lower level of intracellular viral mRNA had no effect on the ability of the virus to inhibit host protein synthesis.

The effect of the R1 mutant on host protein synthesis was also examined. In agreement with previous reports (26), we found that in cells infected with this virus, protein synthesis was not inhibited during the first 3 h after infection.

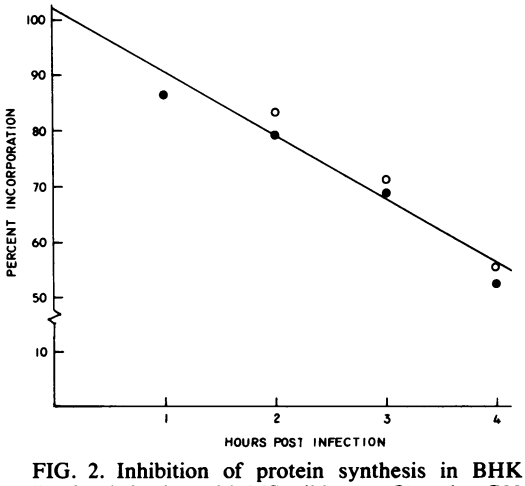

FIG. 2. Inhibition of protein synthesis in BHK cells after infection with MS wild-type (●) and *ts* G22 mutant (○) VSV at 39°C and 5 PFU per cell. After infection the cells were maintained at 39°C and pulse-labeled with [^{35}S]methionine (1 μCi/ml) for 30 min at hourly intervals. TCA-precipitable counts were determined as described in the text. The counts were compared with those obtained from similarly labeled mock-infected cells and are expressed as percentages of incorporation by the uninfected cells. All determinations represent an average of two cell plates.

TABLE 4. Relative amounts of VSV mRNAs in cells infected with wild-type (MS) and mutant viruses

Time after infection (h)	Relative concn of viral mRNA (cpm)[a]			
	MS	*ts* G22	MS	R1
1	2.87×10^3	7.82×10^3	1.59×10^4	1.35×10^4
2	2.23×10^4	2.36×10^4	7.90×10^4	7.99×10^4
3	4.64×10^4	1.91×10^4	1.32×10^5	1.42×10^5
4	7.74×10^4	2.42×10^4	1.67×10^5	2.07×10^5

[a] The relative concentrations of viral mRNA are expressed as RNase-resistant counts of ^{3}H-labeled virion RNA after annealing as described for Table 2.

One hour later, protein synthesis increased considerably above the value of the noninfected cells (Fig. 3). This increase could be due to a significant contribution of viral proteins to the total cellular protein synthesis. On the other hand, the wild-type virus inhibited total protein synthesis at 4 h postinfection by approximately 45%. Because of a probable contribution by viral proteins to total protein synthesis, this value of

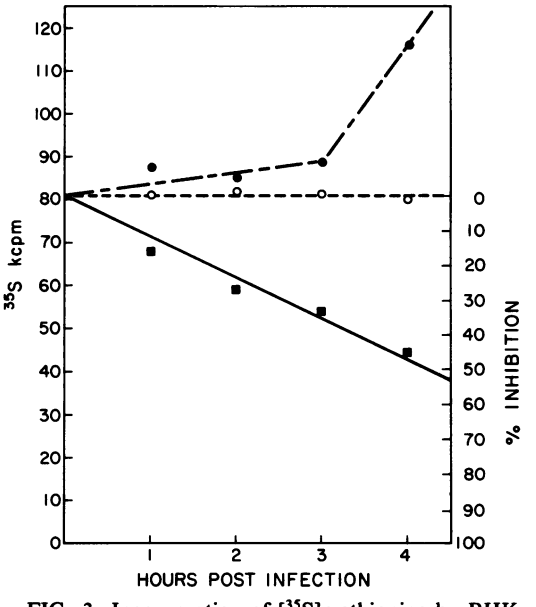

FIG. 3. Incorporation of [^{35}S]methionine by BHK cells mock infected (○) or infected with MS wild-type (■) or R1 mutant (●) VSV. Cells were infected at 5 PFU per cell and incubated at 39°C. Duplicate plates were pulse-labeled for 30 min at hourly intervals with [^{35}S]methionine (1 μCi/ml), and TCA-precipitable counts were determined as described in the text. The average values of [^{35}S]methionine incorporation are plotted on the left ordinate as a function of time postinfection. The right ordinate represents percent inhibition of protein synthesis in cells infected with MS wild-type VSV as compared with that in mock-infected cells.

inhibition is, if anything, too low. If a correction based on the R1 data was to be made, the inhibition by the wild type would amount to about 65%.

To determine whether the inability of the R1 mutant to inhibit host protein synthesis was due to lower intracellular concentrations of viral mRNA than those in the wild-type infection, annealing experiments with a radioactive VSV virion RNA probe were performed. The relative concentrations of wild-type and R1 mRNAs isolated from cells run in parallel with the inhibition measurements (Fig. 3) are shown in Table 4. No significant differences between viral mRNA concentrations in wild-type and R1 mutant infections were observed up to 4 h after infection.

The determinations of intracellular VSV mRNA by the hybridization techniques (Table 4) do not necessarily represent biologically active mRNAs capable of binding to ribosomes. To determine whether the R1 mutant produced mostly inactive mRNAs which did not compete with cellular mRNAs for ribosomes, we investigated the polysome-bound fraction of wild-type and R1 mRNA. These experiments were performed in two ways. In the first experiment, actinomycin D-treated cells infected with either wild-type or R1 virus were labeled at 1.5 to 3.5 h postinfection with [^{3}H]uridine and [^{3}H]adenosine, and polysomes were isolated as described above. The incorporation of radioactive counts from an equivalent number of cells (based on A_{260} measurements) from wild-type- and R1 virus-infected cells were 23,900 and 25,300 cpm, respectively. In the second experiment, the actinomycin D and the radioactive nucleoside pulse were omitted to eliminate any possible effect on polysome composition due to immediate inactivation of cellular transcription. The isolated polysomes were phenol-$CHCl_3$ extracted, and the VSV mRNA content was determined by annealing with a radioactive virion RNA probe. The number of RNase-resistant counts (normalized to 10^7 cells) obtained from wild-type infections was 7.49×10^4 cpm, and that from R1 infections was 8.94×10^4 cpm. Under these conditions, the inhibition of cellular protein synthesis as measured by [^{35}S]methionine incorporation was 53% for wild-type and 6% for R1 virus infections. These data indicated that a comparable quantity of viral mRNAs was present in the polysome fraction of wild-type- and of R1 virus-infected cells.

DISCUSSION

The intracellular regulation of VSV mRNA levels by means of DI particles as described in this work eliminated the necessity to use chemical means of regulation which might have introduced unwanted side effects on cellular metabolism. This procedure also made it possible to follow the correlation between VSV mRNA content and inhibition of cellular protein synthesis in infections by the same wild-type VSV isolate, rather than to correlate these parameters in different wild-type isolates (11). It was not clear whether the increase in intracellular VSV mRNA with decreasing DI particle-to-cell ratios in the inocula (Table 2) was due to a larger number of cells not infected with DI particles at all or to a general increase in VSV mRNA synthesis in all cells. The kinetic interpretation of DI particle interference by Bellett and Cooper (3) and more recently by Sekellick and Marcus (23) would suggest that total inhibition of viral amplification was accomplished by a single DI particle per cell. Therefore, increasing the DI particle MOI should have had no further effect on the VSV RNA concentration in the cells already doubly infected, and the observed decrease in VSV mRNA would be entirely due to infection of cells which were free of DI particles at the lower MOIs. Unfortunately, the experimental data were not accurate enough to distinguish between the two possibilities. Thus, at a DI particle MOI of 1.8, the VSV mRNA concentration was 27% of its amplified value (Table 2). The zero term of the Poisson distribution predicts that 16.4% of the cells would be free of DI particles while still infected with VSV and that 83.5% would be infected with both particles. The predicted VSV mRNA content based on this statistic is $(0.164 + 0.835 \times 0.07) \times 100 = 22.3\%$. Considering the error in the determinations of MOI and intracellular mRNA content, this value is not very different from the experimentally determined 27%.

In any case, when all cells were infected either with DI particles and virion or with virion alone, the 7 or 100% level of VSV mRNA, respectively, was a true reflection of the intracellular contents. Under these conditions, even though the VSV mRNA content increased 14-fold, the incorporation of [^{35}S]methionine into TCA-precipitable counts remained the same. To ensure that the DI particles or virus did not influence [^{35}S]methionine uptake by the cells, TCA-soluble counts were also measured in washed cells and were found to be identical in noninfected, in VSV-infected, and in VSV- and DI particle-infected cells (data not shown).

The incorporation data did not exclude the possibility that in doubly infected cells host protein synthesis actually increased over that in cells infected with virus alone but this increase was exactly compensated for by the decrease in synthesis of viral proteins due to interference. The polyacrylamide gel electrophoresis profiles (Fig. 1) visually demonstrated that this was not the case. Moreover, from the sliced gels, a

quantitative determination of the contribution of cellular and viral protein synthesis to total protein synthesis was also made (Table 3). These data indicated that inhibition of cellular protein synthesis was only slightly dependent on intracellular levels of viral mRNA, probably not exceeding about 10% of the total inhibiting activity in the range of mRNA concentrations measured. This 10% may therefore reflect the extent of competition for ribosomes by very high levels of VSV mRNA. Although these data do not prove conclusively that the competition for all available ribosomes had not already taken place when VSV mRNA levels reached 7% of their amplified value, an examination of the polyacrylamide gel profiles in Fig. 1 is informative in relation to this question. In lanes c, d, and e, it can be seen that as the DI particle concentration was decreasing (and VSV mRNA increasing), viral protein synthesis strongly increased. This clearly indicated that the protein-synthesizing ability of the cells represented in lane d (VSV mRNA, 9%) was not exhausted and that all available ribosomes could not have been fully utilized in translation. This conclusion can also be reached by examining the data in Table 3. In no case do the radioactive counts obtained from infected cells add up to the levels of radioactivity in the proteins of noninfected cells.

That concentrations of intracellular VSV mRNA of between 7 and 100% of the amplified level had only marginal effects on the inhibition of protein synthesis was further confirmed by the data obtained with VSV mutants *ts* G22 and R1. The R1 mutant, which in our hands did not inhibit incorporation of [^{35}S]methionine up to 4 h after infection (see also reference 26), generated the same amount of mRNA as did the wild type. However, as pointed out by Lodish and Porter (10, 11), comparisons of these kinds should not be based on hybridization assays, since a large and variable fraction of the VSV mRNAs might be translationally inactive and would not participate in any competition for ribosome binding. To examine whether the wild type and the R1 mutant generated comparable amounts of active mRNAs, polysomes were isolated from both types of infection under equivalent conditions. No difference in the VSV mRNA content of the polysomal fraction in the two infections was observed. This result was also consistent with the observation that the yields of R1 and wild-type VSV virions were comparable (data not shown), whereas a lower viral mRNA concentration in R1 infections might lead to relatively lower yields of this virus.

The polysomal fraction of viral mRNA was not measured in the experiments with DI particle- and VSV-infected cells. The criticism of the hybridization assay in those experiments would only be valid if the DI particles preferentially inhibited the generation of inactive mRNAs while the total content of active RNA remained the same at the 7 and 100% levels of hybridizable mRNA. Such an interpretation is not plausible in view of the severe interference with viral protein synthesis (Fig. 1 and Table 3). A possible effect of DI particles on mRNA sequestration is also eliminated by the data in Table 1. At least up to an MOI of 14, DI particle infections in the absence of VSV had very little effect on host cell protein synthesis.

Recent investigations of the inhibition of cellular protein synthesis by VSV have led to the conclusion that VSV mRNAs have no advantage in the initiation or elongation process of polypeptide synthesis. These conclusions were based on the average polysome size of active cellular and viral mRNAs of comparable lengths (10). However, in the infected cell, chain initiation rates are reduced equally for both types of mRNAs, as reflected in generally reduced sizes of polysomes. This has been interpreted as being consistent with the hypothesis of a limiting supply of ribosomes in the infected cells when a large excess of viral mRNAs is generated. The lack of correlation between high levels of viral mRNAs and the extent of inhibition of cellular protein synthesis demonstrated in this paper conflicts with that interpretation. Other differences between infected and noninfected cells must serve as a basis for the inhibition. It should be noted that recent investigations of the inhibition of cellular protein synthesis by encephalomyocarditis virus in L cells have also eliminated competition, inactivation of cellular mRNAs, and inactivation of cap recognition functions as possible mechanisms of the inhibition. Instead, it was proposed that the reduced activity in infected cells is due to reversible changes (5). The in vitro assays would not register such reversible changes, especially if they were caused by an imbalance of the low-molecular-weight components for which the in vitro translation system has been optimized. Similarly, sequestration of the cellular mRNA could also be reversible so that when total RNA is isolated from infected and noninfected cells, an in vitro translational assay using mRNAs from the two types of cells would not reveal any differences. The answer to these problems may therefore not be obtainable through the use of in vitro systems and may require new approaches.

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