Regulation of Protein Synthesis in Vesicular Stomatitis Virus-Infected Mouse L-929 Cells by Decreased Protein Synthesis Initiation Factor 2 Activity

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Infection of mouse L-cell spinner cultures by vesicular stomatitis virus (VSV) effected the selective translation of viral mRNA by 4 h after viral adsorption. Cell-free systems prepared from mock- and VSV-infected cells reflected this phenomenon; protein synthesis was reduced in the virus-infected cell lysate by approximately 75% compared with the mock-infected (control) lysate. This effect appeared to be specific to protein synthesis initiation since (i) methionine incorporation into protein from an exogenous preparation of initiator methionyl-tRNA gave completely analogous results and (ii) the addition of a ribosomal salt wash (containing protein synthesis initiation factors) stimulated protein synthesis by the infected cell lysate but had no effect on protein synthesis by the control. Micrococcal nuclease-treated (initiation-dependent) VSV-infected cell lysates were not able to translate L-cell mRNA unless they were supplemented with a ribosomal salt wash; a salt wash from ribosomes from uninfected cells effected a quicker recovery than a salt wash from ribosomes from infected cells. When salt wash preparations from ribosomes from uninfected and infected cells were tested for initiation factor 2 (eIF-2)-dependent ternary complex capacity with added GTP and initiator methionyl-tRNA, we found that the two preparations contained equivalent levels of eIF-2. However, initiation complex formation by the factor from virus-infected cells proceeded at a reduced initial rate compared with the control. When the lysates were supplemented with a partially purified eIF-2 preparation, recovery of activity by the infected cell lysate was observed. Mechanisms by which downward regulation of eIF-2 activity might direct the selective translation of viral mRNA in VSV-infected cells are proposed.

Appropriate host cell infection by vesicular stomatitis virus (VSV) results in the concurrent inhibition of cellular mRNA translation and accumulation of viral genome-encoded translation products (15, 16, 19, 20, 34; M. Jaye, W. Godchaux, and J. Lucas-Lenard, *Virology*, in press). Most frequently, infection results not only in this transition, but also in a decline of the capacity of the host cells for protein synthesis in general, regardless of the capped mRNA being translated (16, 19, 20, 38; Jaye et al., in press). Host cell mRNA's in infected cells remain fully competent for translation in exogenous systems, such as wheat germ (16) or reticulocyte lysates (Jaye et al., in press), and apparently compete for translation factors in these systems in proportion to their relative concentrations in the total mRNA pool.

This inhibition of cellular protein synthesis and subsequent cell death cannot be accounted for completely by the infection itself or by viral genome replication (18-20, 34). Therefore, the defect is presumably at the level of mRNA

translation. Experiments using defined UV irradiation of the VSV genome have shown that some transcription of the genomic RNA into mRNA is required for protein synthesis inhibition (18, 38); the expression of the N-protein gene (which is promoter proximal) and perhaps the adjacent NS-protein gene has been correlated directly with this inhibition (18).

A variety of mechanisms for the control of translation after VSV infection has been suggested. These include intrinsic differences between the virus-encoded mRNA and cellular mRNA in their efficiencies to bind factors necessary for translation (23), the synthesis of a viral gene product which appears to be required for both protein synthesis inhibition and cell killing (18, 34), and the simple inundation of cells with viral mRNA so that the stoichiometries of specific mRNA's determine their probabilities of translation (15, 16).

Information from a number of reports has indicated or suggested that the translational control evident is at the level of protein synthesis

initiation (3, 23, 34; Jaye et al., in press). Insofar as initiation factor activity appears to regulate protein synthesis in virus-infected cell lysates in other systems (5, 9, 26, 36) and since a great deal of information is available regarding the regulation of protein synthesis initiation by initiation factor 2 (eIF-2) activity in eucaryotes, we examined these parameters in VSV-infected L cells. We found that in VSV-infected L cells, some lesion for protein synthesis initiation does exist, and that this lesion may be localized to at least eIF-2 and is remedied by an exogenous supply of this factor. Furthermore, our data suggest that the various mechanisms proposed for host cell protein synthesis inhibition described above may not be mutually exclusive and, in fact, may all contribute in part to the protein synthesis profile generally observed after VSV infection.

MATERIALS AND METHODS

Chemicals. L-[³⁵S]methionine and a ³H-labeled L-amino acid mixture were purchased from New England Nuclear Corp. Phosphocellulose (type P-11) and DEAE-cellulose (type DE-52) were purchased from Whatman, Ltd.; benzoylated DEAE-cellulose was from Schwarz/Mann, Sephacryl S-200 was from Pharmacia Fine Chemicals, Inc., and oligodeoxythymidylic acid cellulose was from Collaborative Research, Inc. The membrane filters used had a pore size of 0.45 μ m (type HAWP; Millipore Corp.). Morpholinopropanesulfonic acid, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), Tris-hydrochloride, ATP, GTP, creatine phosphate, phosphoenolpyruvate, dithiothreitol (DTT), creatine phosphokinase, and pyruvate kinase were purchased from Sigma Chemical Co. Rabbit liver tRNA was purchased from GIBCO Laboratories. Micrococcal nuclease (*Staphylococcus aureus*) was purchased from Boehringer Mannheim Biochemicals. All other chemicals were reagent grade or better.

Cell growth. L-929 Spinner cell growth was at 37°C in Spinner flasks containing Eagle minimal essential medium (for suspension cultures) supplemented with serine, asparagine, and proline to 10 times the normal concentration and with calf serum and fetal calf serum (final concentration, 5% each). This medium was also supplemented with penicillin and streptomycin to preclude bacterial contamination.

VSV infection of Spinner cultures. Spinner culture L cells were collected by centrifugation and suspended to a concentration of 10^7 cells per ml in serum-free culture medium. Cultures were inoculated with Indiana serotype HR-C VSV at a multiplicity of infection of approximately 30 plaque-forming virus particles per cell. Adsorption was for 45 min at 37°C, after which each culture was diluted with fresh medium (containing the normal serum complement) to a concentration of 10^6 cells per ml. Infection proceeded at 37°C for an additional 3.5 h, after which the cells were collected and processed as described below. When mock-infected lysates were prepared, all procedures were identical to the procedures described above, except that buffered saline was substituted for the virus inoculum.

Cell fractionation. Cells were harvested by centrifuga-

tion and washed three times with washing buffer (146 mM NaCl, 11 mM dextrose, 35 mM Tris, pH 7.4), and the pellet was suspended in an equal volume of hypotonic buffer (15 mM KCl, 1.5 mM magnesium acetate, 2 mM DTT, 10 mM HEPES, pH 7.4). The cells were allowed to swell for 5 min and then lysed by Dounce homogenization with 20 strokes of a tight-fitting pestle. Post-mitochondrial supernatant fractions (lysates) were prepared by centrifugation of the homogenates at $22,000 \times g$ for 15 min at 4°C. These lysates were then stored (for later use in protein synthesis studies) by freezing at -70°C in small portions, or they were processed further. Ribosomes were isolated from the lysates by centrifugation at $160,000 \times g$ for 2.5 h at 4°C, and the resulting ribosome pellet was dissolved in salt washing buffer (500 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 0.1 mM EDTA, 250 mM sucrose, 5 mM HEPES, pH 7.4). The ribosome-free supernatant was stored at -70°C; this preparation was designated the S-100 fraction. A salt wash was prepared by ultracentrifuging the redissolved ribosome pellet at $160,000 \times g$ for 2.5 h at 4°C, and the supernatant was dialyzed against a solution containing 44 mM KCl, 1.2 mM magnesium acetate, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 10 mM HEPES (pH 7.4). This preparation was then stored in small portions at -70°C as a crude salt wash until it was used.

Initiation factor preparation. A salt wash prepared as described above from mouse Ehrlich Spinner cultures was fractionated further by ammonium sulfate precipitation. eIF-2 was purified from the fraction that precipitated between 40 and 70% saturation by sequential column chromatography on P-11 and DE-52 and by gel filtration on Sephacryl S-200, essentially as described by Safer et al. (32). eIF-2 was eluted from P-11 at approximately 0.6 M KCl and from DE-52 at approximately 0.2 M KCl. The columns were equilibrated with a solution containing 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol, and 20 mM morpholinopropanesulfonic acid, pH 7.2. Sephacryl fractionation was in the same buffer, except that 0.1 M KCl was used throughout.

Initiator [³⁵S]methionyl-tRNA preparation. Radioactive initiator methionyl-tRNA was prepared as described by Henshaw (10) and was stored at -70°C until column purification. The initiator species was separated from a tRNA pool-associated factor that was inhibitory to eIF-2-dependent ternary complex formation and protein synthesis initiation in reticulocyte lysates (11; Centrella, unpublished data) by chromatography on benzoylated DEAE-cellulose, as described previously (11).

mRNA preparations. Lysate fractions prepared as described above were extracted with an equal volume of phenol saturated with 0.1 M Tris (pH 9.0). The RNA in the aqueous phase was precipitated twice in 95% ethanol made 0.2 M in sodium acetate, and the RNA was collected each time by centrifugation at $16,500 \times g$ for 30 min at 0°C. The precipitate was then dissolved in a solution containing 500 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 10 mM Tris (pH 7.5), heated to 65°C for 5 min, and applied to oligodeoxythymidylic acid cellulose which had been equilibrated previously in the same buffer. The flow-through from the application was recycled through the resin bed two more times, and the bed was washed extensively with the same buffer. The bound fraction

was eluted with 10 mM Tris (pH 7.5), ethanol precipitated twice as described above, and dissolved in water to an RNA concentration of approximately 1 mg/ml.

Protein synthesis in vitro. Lysate fractions prepared as described above were incubated at 28°C in small volumes (50 to 100 μ l) of a solution containing 80 mM potassium acetate, 3 mM magnesium acetate, 4 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, 1.5 mM DTT, 100 μ g of creatine phosphokinase per ml, and 30 mM HEPES (pH 7.4), unless noted otherwise. The lysates were supplemented with amino acids at concentrations of 10 μ M for the radioactive species and at concentrations of 50 to 100 μ M for the nonradioactive species. The lysates comprised 25 to 30% of the total reaction volume. When the lysates were supplemented with either the salt wash or initiation factor preparations, control lysates were supplemented with an identical buffering solution which did not contain the factors. The final salt, buffer, and component concentrations described above were attained by adding small amounts of concentrated stock solutions of each reagent. Incubation proceeded for different amounts of time (see figure legends), after which small samples were withdrawn, spotted onto Whatman no. 540 filter papers, air dried, and immersed in cold 5% trichloroacetic acid. The filters were heated at 90°C in 5% trichloroacetic acid for 5 min and then rinsed three times with acid, three times with 95% ethanol, and three times with acetone; they were then air dried and counted in a toluene-based scintillant in a liquid scintillation counter for determinations of radioactive amino acid incorporation into protein.

Nuclease treatment of lysates. Lysate fractions prepared as described above were supplemented with 1 mM calcium chloride and 10 μ g of micrococcal nuclease per ml (25). The preparations were incubated for 5 min at 22°C, after which the nuclease was inactivated by adding ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) (final concentration, 2 mM). The lysates were then supplemented with all of the other components necessary for protein synthesis and incubated as described above; however, all incubation mixtures were also supplemented with 40 μ g of tRNA per ml. The tRNA preparation used was commercial total rabbit liver tRNA further purified on benzoylated DEAE-cellulose by step elution from 0.4 to 0.6 M NaCl, which removed most of the endogenous initiation complex formation inhibitor present (11). A control was always run, in which the lysate was nuclease treated in the presence of ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid.

Initiation factor assay. Initiator [35 S]methionyl-tRNA bound to eIF-2 was determined by radioactive label retention on Millipore filters, essentially as described previously (11). Reactions were carried out in 200- to 250- μ l reaction mixtures containing 100 mM KCl, 1.5 mM magnesium acetate, 3.2 mM phosphoenolpyruvate, 0.2 mM GTP, 1 mM DTT, 4 activity units of pyruvate kinase, 50 μ g of bovine serum albumin, and 50 mM morpholinopropanesulfonic acid (pH 7.4). The preparations were incubated as noted in the figure legends, and reactions were terminated by rapid chilling on ice and filtration through 0.45- μ m Millipore filters without prior dilution of the reaction samples.

Polyacrylamide gel electrophoresis. Samples were diluted with an equal volume of twofold-concentrated buffer (2.3% sodium dodecyl sulfate, 10% glycerol, 5%

2-mercaptoethanol, 62.5 mM Tris, pH 6.8) and then heated at 90°C for 5 min. Approximately 100 μ g of protein was loaded into each well, and the preparations were electrophoresed on 10% polyacrylamide vertical slab gels (15 cm by 15 cm by 1.5 mm), essentially as described previously (19). The gels were dried onto Whatman no. 1 filter paper and autoradiographed at room temperature with X-Omat R film (Kodak).

RESULTS

Cell-free protein synthesis. Lysates prepared from both mock-infected and VSV-infected L-cell Spinner cultures were able to incorporate radioactive amino acids into proteins in a time-dependent manner; protein synthesis in the lysates was virtually complete after 90 min of incubation at 28°C (Fig. 1A). Both types of lysates showed optimal incorporation when the added magnesium concentration was 3 mM (Fig. 1B). Incorporation in the virus-infected cell lysate was approximately 25 to 30% of that in the control. An autoradiogram of the products synthesized (Fig. 2, lanes 1 and 4) demonstrated the following two reproducible findings: (i) most of the radioactivity incorporated by the virus-infected cell lysate (lane 4) was localized in a small number of bands which were not present in the control lysate (lane 1); and (ii) there was a great reduction in the synthesis of host-specific polypeptides in the virus-infected cell lysate. This was consistent with incorporation of radioactive amino acids into proteins in infected cells *in vivo* (15, 16, 19, 20, 24, 34). Furthermore, the use of an exogenous initiator [35 S]methionyl-tRNA preparation to follow protein synthesis initiation specifically in these lysates gave analogous re-

FIG. 1. Cell-free protein synthesis by lysates derived from mock-infected and VSV-infected L-929 cells. Incorporation of [35 S]methionine into protein was monitored as described in the text. The input lysates were 30% of the total reaction volume of 100 μ l (3.4 mg of protein per ml) and the [35 S]methionine concentration used was 0.05 mCi/ml. Incubation was at 28°C. Each point represents the average of duplicate 7- μ l samples. Symbols: ●, mock-infected cell lysate; ▲, VSV-infected cell lysate.

FIG. 2. Autoradiogram of proteins synthesized by cell-free lysates from mock-infected and VSV-infected L-929 cells. Details were as described in the legend to Fig. 1, except that the [35 S]methionine concentration used was 0.15 mCi/ml; incubation was for 90 min. Lanes 1 through 3, Mock-infected cell lysate; lanes 4 through 6, VSV-infected cell lysate; lanes 1 and 4, unsupplemented lysates; lanes 2 and 5, lysates supplemented with L-cell ribosomal salt wash; lanes 3 and 6, lysates supplemented with eIF-2 preparation. Viral proteins were identified by an *in vivo*-labeled marker run in a separate lane. Equal amounts of lysate protein were loaded onto each lane. Exposure was for 4 days.

sults (Fig. 3); in this case incorporation in the virus-infected cell lysate was approximately 25% of that in the mock-infected control. A comparison of several experiments in which [35 S]methionine, a 3 H-amino acid mixture, and initiator [35 S]methionyl-tRNA were used to follow protein synthesis *in vitro* showed that the results were completely reproducible between different lysates and with different protein precursors (data not shown).

It is important to note that the level of protein synthesis in lysates from virus-infected cells varied from about 50 to 20% of the control value, depending to a large degree on the growth conditions of the cell culture before infection. In addition, viral proteins L and G were translated

to a lesser degree relative to the other viral proteins in the cell-free system, compared with *in vivo*-labeled cultures. A longer exposure of the gel shown in Fig. 2 did produce all five distinctive viral bands, but this exposure is not shown here since it also produced complete overexposure of the products of the mock-infected cell lysate. Therefore, although the absolute ratio of protein synthesis by cell-free lysates from VSV- and mock-infected cultures may have been slightly reduced relative to the *in vivo* situation, the relative decreases in the synthesis of host-specific polypeptides were virtually identical (24). We chose a very reproducible well-shut-off system for study since this was the phenomenon in which we were interested.

Ribosomal salt wash studies. Ribosomes prepared by differential centrifugation from L-cell lysates were extracted with 0.5 M KCl; the ribosome-free supernatant of this salt wash was then dialyzed and tested for its effect on protein synthesis by both mock-infected and VSV-infected cell lysates. Figure 4 shows that the salt wash had no significant effect on the mock-infected cell lysate, but that it supported a twofold increase in amino acid incorporation by

FIG. 3. Cell-free incorporation of initiator [35 S]methionyl-tRNA into protein by lysates derived from mock-infected and VSV-infected L-929 cells. Incorporation of [35 S]methionine into protein was as described in the legend to Fig. 1, except that the system was supplemented with excess (50 μ M) methionine to minimize incorporation of free [35 S]methionine into internal polypeptide sites in the event of chemical deacylation of charged tRNA *in vitro*. The concentration of initiator [35 S]methionyl-tRNA used was 0.76 nM (973 μ Ci/nmol). Symbols: $\bullet$, mock-infected cell lysate; $\blacktriangle$, VSV-infected cell lysate.

FIG. 4. Cell-free protein synthesis by lysates derived from mock-infected and VSV-infected L-929 cells with the addition of ribosomal salt wash. Details were as described as in the legend to Fig. 1, except that the [³⁵S]methionine concentration used was 0.09 mCi/ml. The salt wash from ribosomes from growing L-929 cells was 40% of the total reaction volume (1.2 mg of protein per ml); lysates were 30% of the total reaction volume (4.8 mg of protein per ml). The open symbols represent unsupplemented lysates, and the solid symbols represent lysates incubated in the presence of salt wash. Symbols: ○ and ●, mock-infected L-cell lysates; △ and ▲, VSV-infected L-cell lysates.

the VSV-infected cell lysate. An autoradiogram of this effect is shown in Fig. 2, lanes 2 and 5. Other analogous experiments showed that the amount of recovery was dependent on the amount of salt wash added (Table 1); this increase could be enhanced further with greater salt wash concentrations (data not shown).

These lysates were also treated with micrococcal nuclease to digest the bulk of their endogenous message pools; they were then supplemented with L-cell polyadenylated mRNA or L-cell ribosomal salt wash or both. The data from two such experiments (Table 2) showed that this mRNA could be translated by the nuclease-treated mock-infected cell lysate, but not (or only very poorly) by the VSV-infected cell lysate. To a small degree, the salt wash was able to stimulate translation of the low, residual levels of mRNA in these nuclease-treated lysates, but much more stimulation was observed when mRNA was added to them. In all situations the VSV-infected cell lysate was stimulated to a greater extent by the salt wash than the control was. Most importantly, added L-cell mRNA

could be translated adequately by the VSV-infected cell lysate only when it was supplemented with added ribosomal salt wash.

It appeared that the salt wash was able to restore some missing or inactive factor or activity to the VSV-infected cell lysate and that this factor or activity enhanced the translation of either endogenous or exogenous supplies of mRNA; therefore, we compared salt washes from ribosomes from uninfected and infected cells. Figure 5 shows that when the nuclease-treated virus-infected cell lysate was provided with L-cell mRNA, enhanced translation occurred only when the system was also supplemented simultaneously with the salt wash. However, it was apparent that recovery was effected immediately by the salt wash from ribosomes from uninfected cells but was delayed somewhat when the salt wash from VSV-infected cells was used. This delay may have resulted from the compound trauma of displacing the initiation factor of relatively decreased activity already present on the cellular ribosomes by a similarly less active factor found in the ribosomal salt wash prepared from the same source. Recovery by factor derived from infected cells may have been a consequence of its reactivation *in vitro* after removal from the ribosomes, and some critical concentration of active factor may have been required before recovery was apparent.

eIF-2 studies. Previous data from polysome profiles and transit time studies (34; Jaye, et al., *in press*) and the data presented above for both initiator methionyl-tRNA incorporation and salt wash stimulation indicated that the lesion in protein synthesis in VSV-infected L cells was at the level of initiation. Modulation of protein synthesis initiation by control of eIF-2 activity (which is responsible for the stoichiometric

TABLE 1. Cell-free protein synthesis by lysates derived from mock-infected and VSV-infected L-929 cells with the addition of increasing levels of ribosomal salt wash^a

Salt wash added (μg of protein per ml)	Mock-infected cells		VSV-infected cells	
	Radioactivity (cpm)	Ratio ^b	Radioactivity (cpm)	Ratio ^b
0	15,814	1.00	3,545	1.00
225	15,151	0.96	4,442	1.25
450	15,426	0.98	5,103	1.44
975	15,852	1.00	6,376	1.80

^a Incorporation was monitored as described in the legend to Fig. 1. Incubation was for 60 min at 28°C. Lysates contained 5.2 mg of protein per ml, and the concentration of [³⁵S]methionine used was 0.09 mCi/ml.

^b Ratio of the radioactive amino acid incorporation by lysates in the absence of the salt wash to the incorporation in the presence of the salt wash.

TABLE 2. mRNA-dependent cell-free protein synthesis by lysates from mock-infected and VSV-infected mouse L-929 cells^a

Expt	Treatment	Mock-infected cells		VSV-infected cells	
		Radioactivity (cpm)	Change (-fold)	Radioactivity (cpm)	Change (-fold)
1 ^b	None	392,460		113,196	
	Nuclease alone	15,000	1.0	3,384	1.0
	Nuclease + mRNA	28,536	1.9	3,804	1.1
	Nuclease + salt wash	16,766	1.1	4,332	1.3
	Nuclease + mRNA + salt wash	38,976	2.6	19,836	5.9
2 ^c	None	206,771		70,205	
	Nuclease alone	6,924	1.0	458	1.0
	Nuclease + mRNA	17,550	2.5	427	0.9
	Nuclease + salt wash	15,006	2.2	2,105	4.6
	Nuclease + mRNA + salt wash	43,133	6.2	18,080	39.5

^a Details as described in the text.^b Lysates contained 4.8 mg of protein per ml, and the salt wash contained 1.2 mg of protein per ml. Incubation was with 20 µg of L-929 cell polyadenylic acid-containing mRNA per ml; the [³⁵S]methionine concentration used was 0.12 mCi/ml.^c Lysates contained 4.0 mg of protein per ml, and the salt wash contained 1.2 mg of protein per ml. Incubation was with 30 µg of L-929 cell polyadenylic acid-containing mRNA per ml; the [³⁵S]methionine concentration used was 0.21 mCi/ml.

binding of initiator methionyl-tRNA to the 40S ribosomal subunit) has been found in a variety of systems in which overall protein synthesis appears to be regulated (1, 2, 6, 14, 17, 28, 35, 37; M. H. Vaughan, D. W. Friele, and T. Jacobowicz, *Fed. Proc.* 40:1550, 1981). Since the ribosomal salt wash contained all of the known protein synthesis initiation factors, it was important to examine eIF-2 activity in VSV-infected cells. When salt washes from ribosomes from both uninfected and infected cells were tested for the ability to form an initiation (ternary) complex by the eIF-2 present in them with added GTP and initiator methionyl-tRNA, we observed two phenomena. First, when lower levels of salt wash were incubated at slightly reduced temperatures to moderate the reaction rate, the salt wash from ribosomes from infected cells supported ternary complex formation at a reduced initial rate compared with the control (Fig. 6A). And second, when the reaction was carried out at slightly higher protein concentrations and temperatures and for an extended period of time, both salt washes were able to carry the reaction to the same extent. Again, the preparation derived from virus-infected cells was somewhat slower initially (Fig. 6B). This could not be attributed simply to a depletion of the initiator methionyl-tRNA substrate used to monitor complex formation since this substrate was present in great excess and greater amounts of [³⁵S]methionyl-tRNA could be bound to the ternary complex when higher levels of the salt wash factor were tested (data not shown). Also, the eIF-2 activity found in the soluble fraction of the cells (the ribosome-free cell supernatant or S-100) was lower after VSV infection. In fact,

there appeared to be a decrease in both the rate and the extent of ternary complex formation by eIF-2 in the VSV-infected L-cell S-100 compared with the rate and extent in mock-infected cultures (Fig. 6C).

A partially purified (65% homogeneous) preparation of eIF-2 obtained from large batch Spinner cultures of mouse Ehrlich cells was used to supplement the protein-synthesizing lysates prepared from both mock- and VSV-infected mouse L cells. As in the salt wash studies, enhancement of protein synthesis in the infected L-cell lysate was achieved with this purified preparation (Fig. 7). The same amount of eIF-2 had no significant effect on the protein-synthesizing capacity of the mock-infected lysate. Heat denaturation of the eIF-2 preparation completely destroyed its ability to restore translation in virus-infected cell lysates. Inactivation of the initiation factor preparation was verified by a ternary complex formation assay (Fig. 7, insert). The amount of eIF-2 used increased incorporation in the virus-infected cell lysate from about 30% of the control value at 130 min of incubation in the absence of the factor to 90% in its presence. We calculated that this amount of eIF-2 was about two to four times the amount of initiation factor bound to the ribosomes and already present in the lysate. A similar recovery was achieved by adding L-cell salt-wash-derived eIF-2 of approximately 70% homogeneity (data not shown).

DISCUSSION

The results described above suggested that protein synthesis inhibition in VSV-infected

FIG. 5. Kinetics of mRNA-dependent cell-free protein synthesis by lysates from VSV-infected L-929 cells in the presence of salt wash from mock-infected or VSV-infected cells. Details were as described in the text. Lysates were 25% of the total reaction volume (4.0 mg of protein per ml), and the salt wash was 40% of the total reaction volume (1.2 mg of protein per ml). Incubation was with 30 μ g of L-929 cell polyadenylic acid-containing mRNA per ml; the [35 S]methionine concentration used was 0.13 mCi/ml. Symbols: ●, VSV-infected cell lysate with no addition; ▲, VSV-infected cell lysate plus L-cell polyadenylic acid-containing mRNA; ▼, VSV-infected cell lysate plus L-cell polyadenylic acid-containing mRNA and L-cell ribosomal salt wash; ■, VSV-infected cell lysate plus L-cell polyadenylic acid-containing mRNA and VSV-infected cell ribosomal salt wash.

mouse L cells results from a specific lesion in the protein-synthesizing machinery at the level of initiation; this is in agreement with the results of Stanners et al. (34) and Jaye et al. (in press). Regulation of protein synthesis after virus infection at the initiation step has been detected or suspected in a number of other systems (5, 7-9, 26, 33, 36) and apparently involves either inactivation of some protein synthesis initiation factor (8, 9, 26, 36) or some intrinsic differences between cellular and viral mRNA's, allowing the latter to compete successfully for a limited pool of initiation factor (5, 23, 33). The reduction in protein synthesis which we observed may be accounted for by an mRNA-independent mechanism, since regulation was observed at the level of eIF-2-GTP-initiator methionyl-tRNA (ternary) complex formation, an event preceding binding of mRNA to the 40S ribosomal subunit. Our data are consistent with an overall reduction in

protein synthesis resulting from this lesion and subsequent competition (perhaps on a stoichiometric basis) for the limited amount of active initiation factor-ribosome complex formed.

Regulation of ternary complex formation and, therefore, of the ensuing steps in protein synthesis initiation is under study in a variety of systems in which protein synthesis rates appear to be regulated (1, 6, 11, 14, 17, 26, 28, 35, 37; Vaughan et al., *Fed. Proc.* 40:1550, 1981). Whether this regulation proceeds by chemical modification (phosphorylation [2, 6, 28, 37]), accessory factor activity (1, 2, 4, 27), some combination of these two mechanisms, or some other mechanism (11) in VSV-infected cells is under investigation. It does appear that the downward regulation of this factor is not irreversible since the same total amount of ternary complex-forming activity may be obtained by factor freed from ribosomes from uninfected and infected cells. The reversibility may be due to some unstable chemical modification (phosphorylation?) which is susceptible to reversal (by endogenous phosphatase activity? [35; S. T. Wong, W. Mastopalo, and E. C. Henshaw, *Fed. Proc.* 40:1550, 1981]) after salt wash removal from the ribosomes.

It has been shown that small amounts of double-stranded RNA are able to activate a kinase which specifically phosphorylates eIF-2 and that this activity copurifies with some factor which is responsible for inhibition of protein synthesis by the reticulocyte lysate system (29). However, this type of mechanism is not likely to be operating during VSV infection, since no detectable levels of double-stranded RNA have been observed in VSV-infected cells (22); on the other hand, this possibility cannot be eliminated entirely, since the lower limits of double-stranded RNA needed to activate the kinase are not known and may be below the sensitivity of the technique used to make the measurements.

If accessory factors are important in determining eIF-2 activity in cells, they most likely function by a reversible mechanism in which they are in equilibrium with free eIF-2. This equilibrium may be disturbed after VSV infection; some virus-determined function may inhibit binding of accessory factor to eIF-2 directly by competition with one for the other or by some other modification which reduces their affinity for each other. Again, this alteration may be stable when eIF-2 is bound to the 40S subunit but reversible upon its removal. Nevertheless, even a reversible inhibition of eIF-2 activity which alters only the rate of ternary complex formation in infected cell lysates would be consistent with the results observed *in vivo*. If ternary complex formation can be the limiting step in protein synthesis (and this is a likely

FIG. 6. Kinetics of eIF-2-directed ternary complex formation by S-100 or salt wash derived from ribosomes from mock-infected and VSV-infected cells. Ternary complex formation was determined as described in the text. (A) Initial rate determination. Incubation was at 25°C and 26 μ g of salt wash-derived protein per ml (final concentration). The initiator [³⁵S]methionyl-tRNA concentration was 732 Ci/mmol (input level, 121 nCi). (B) Extent determination. Incubation was at 30°C with 50 μ g of salt wash-derived protein per ml (final concentration). The initiator [³⁵S]methionyl-tRNA concentration was 731 Ci/mmol (input level, 125 nCi). (C) Ribosome free-cell supernatant (S-100) eIF-2 activity. Incubation was at 28°C with 1.3 mg of S-100 protein per ml (final concentration). The initiator [³⁵S]methionyl-tRNA concentration was 1,038 Ci/mmol (input level, 73 nCi). (A and B) Symbols: ●, mock-infected cell ribosomal salt wash; ▲, VSV-infected cell ribosomal salt wash. (C) Symbols: ●, mock-infected cell S-100 eIF-2; ▲, VSV-infected cell S-100 eIF-2.

possibility since the binding of initiator tRNA to the 40S subunit occurs extremely inefficiently in the absence of ternary complex formation), then the likelihood that a decreased rate of complex formation alone may in turn regulate the overall level of polypeptide production in cells becomes very significant.

Recent evidence obtained by Henshaw and co-workers has indicated that there are large differences between ribosome-bound eIF-2 and soluble eIF-2 with regard to the phosphorylation state of the factor; whereas soluble eIF-2 is phosphorylated almost entirely *in vivo*, eIF-2 derived from the ribosome fraction is mostly unphosphorylated (Wong et al., Fed. Proc. 40:1550, 1981). Other studies (M. Centrella and E. C. Henshaw, unpublished data) have shown that soluble eIF-2 activity increases after dialysis, incubation, or subsequent column purification; these findings suggest the loss or inactivation of some modifying function or factor which reduces soluble eIF-2 activity. The data presented in this paper indicate that the eIF-2 found in the S-100 fraction from VSV-infected L cells is deficient with regard to both rate and extent of ternary complex formation; whatever function allows the downward regulation of eIF-2 activity in VSV-infected cells, it is likely to be found in

this cell compartment since the lesion apparently persists in the eIF-2 found here.

The addition of partially purified eIF-2 to an infected cell lysate causes almost complete recovery of the ability to synthesize protein. A polyacrylamide gel analysis of a virus-infected cell lysate supplemented with either total salt wash or this eIF-2 preparation shows no apparent selective stimulation of host cell mRNA; the proteins which are translated in the unsupplemented lysate all appear to be stimulated equally (Fig. 2). This also is consistent with an mRNA-independent lesion in protein synthesis, but it does not exclude the possibility that added initiation factor has simply acquired some excess viral function from the infected cell lysate, which programs it for the viral mRNA present there in such abundance (15, 16; Jaye et al., *in press*).

The amount of initiation factor added has been calculated to be at most only two- to fourfold more than the amount present on the ribosomes contained in the amount of lysate tested. This suggests that no artifact resulting from nonspecific protein interactions was introduced. In fact, very much less total protein was added to effect recovery which was greater than that which could be obtained by an unfractionated

FIG. 7. Cell-free protein synthesis by lysates derived from mock-infected and VSV-infected L-929 cells with the addition of eIF-2. Details were as described in the legend to Fig. 1, except that the [³⁵S]methionine concentration used was 0.18 mCi/ml. eIF-2 was prepared as described in the text and was tested at an input level of 5.1 nM with or without heating at 48°C for 5 min to inactivate the factor. The open symbols indicate mock-infected L-cell lysate (○, lysate with no addition; Δ, lysate plus eIF-2; ▽, lysate plus heat-denatured eIF-2). The closed symbols indicate VSV-infected L-cell lysate (●, lysate with no addition; ▲, lysate plus eIF-2; ▼, lysate plus heat-denatured eIF-2). (Insert) Ternary complex formation activity by eIF-2 preparations without (○) or with (▲) heat denaturation. Incubation was at 28°C for 30 min; the initiator [³⁵S]methionyl-tRNA concentration was 850 Ci/mmol (input level, 357 nCi).

salt wash (which achieved a comparatively limited recovery for the technical reason that there was no room for more salt wash volume in the incubation system). Also, it might be expected that slightly more factor should be necessary since added factor must displace the less active initiation factor which is already present in the system.

We determined that the eIF-2 used in our experiments was about 65% homogeneous; this was done by adding enough protein to a polyacrylamide gel to pick up background bands. Since this preparation was admittedly only 65% homogeneous (the remainder was spread among a small number of minor bands), some other factor may have been present which accounted for the protein synthesis recovery observed. This other factor would have been present in somewhat smaller quantities, and much less

than stoichiometric amounts would be expected to be required for recovery.

Total protein synthesis is reduced in VSV-infected cells in the presence of increasing amounts of total (cellular plus viral) mRNA (15, 20, 34; Jaye et al., in press). Using difference analysis of electropherograms which depicted virus-infected and uninfected cellular protein synthesis, McAllister and Wagner (20) have shown that there is both a progressive decline in host protein synthesis and a concomitant rise in virus-specific proteins; more importantly, the data that these authors derived from an analysis of this sort clearly indicated that this decline in cellular protein synthesis could not be explained by a simple substitution by virus-specific translation products.

There is no direct evidence regarding some special property inherent in native VSV mRNA to ensure its translation above that of host mRNA other than relative abundance. In fact, preferential translation of virus-encoded mRNA in a factor-limited system could be explained as due to simple stoichiometric competition, but in such a situation competition could only be considered secondary to the translational machinery lesion. Since ribosomes themselves do not appear to be limiting (34; Jaye et al., in press) but fewer initiation complexes can form in the event of decreased eIF-2 activity, competition for a limited number of "activated" 40S subunits would produce a result consistent with the phenomena observed. This mRNA-independent function of eIF-2 is sufficient to explain the data, but the problem may be compounded by the mRNA-dependent function of this factor observed by Kaempfer et al. (12, 13, 31). These authors present strong evidence that eIF-2 recognizes with a high degree of affinity both the cap structure of mRNA (13) and some internal mRNA site (12, 13). In addition, although certain mRNA's are inhibited almost entirely for translation after cap removal (21, 30), Rose and Lodish (30) have shown that VSV mRNA is much less so. These data suggest that, as Nuss et al. (23) reported, some difference in initiation efficiency that is inherent to a particular mRNA type may explain why certain viral mRNA's are translated preferentially in infected cells. In fact, internal mRNA recognition sites for eIF-2 may determine the translational efficiency of many subclasses of mRNA. This may become very important when various mRNA types coexist in a more-or-less equivalent ratio or when a small number of mRNA's from newly expressed genes must compete with an overwhelming number of preexisting messages to produce a regulatory function (such as early in VSV infection, when only a small number of primary transcripts are produced which code for proteins necessary for

subsequent viral RNA amplification and, therefore, secondary viral mRNA transcription). When one class is somewhat more abundant (as is the case late in VSV infection), differences in efficiency may simply be an additional contributing factor toward selecting that mRNA. When eIF-2 activity is limited, as suggested here, competition for this downward-regulated factor by both stoichiometry and efficiency differences may overwhelmingly favor the synthesis of VSV genome-encoded translation products.

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