Inhibition of Translation in Lysates of Mouse L Cells Infected with Vesicular Stomatitis Virus: Presence of a Defective Ribosome-Associated Factor†

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ABSTRACT: Lysates of L cells infected for 4 h with vesicular stomatitis virus were inhibited in their in vitro translational activity to about the same extent as protein synthesis was inhibited in vivo in infected L cells. Inhibition of translation occurred at the level of the ribosome as determined by reciprocal cross-reconstitution studies with polyribosomes and postribosomal supernatant fractions isolated from virus-infected and mock-infected cells. Inhibition of protein synthesis in reconstituted lysates of virus-infected cells was found to be at the level of initiation of translation as evidenced by reduction in incorporation into acid-precipitable proteins of formylatable [^{35}S]methionine. Ribosomes from virus-infected and mock-infected cells were exposed to 0.5 M KCl and fractionated by centrifugation into salt-washed polyribosomes and supernatant fractions containing ribosome-associated proteins; reciprocal reconstitution of translational activity by a mixing of salt-washed polyribosomes and ribosome-associated proteins revealed that the defect in initiation of translation was in the ribosome-associated proteins released by salt wash from the infected-cell ribosomes. Differential ammonium sulfate precipitation of the supernatant ribosome-associated proteins from virus-infected and mock-infected cells indicated by reciprocal reconstitution studies that the defective ribosomal initiation factor(s) was (were) present primarily in the 0–40% ammonium sulfate fraction that is considered to contain primarily eIF-3 and eIF-4B. These results are similar to those found in earlier studies of defective initiation factors responsible for impaired protein synthesis in cells infected with plus-strand viruses quite different from the rhabdovirus studied in these experiments.

Infection of animal cells with vesicular stomatitis virus (VSV),[1] a negative-strand RNA virus, results in inhibition of host-cell protein synthesis and the selective translation of viral mRNA (Mudd & Summers, 1970; Wertz & Youngner, 1972; McAllister & Wagner, 1976). This effect of VSV on host-cell translation appears to be independent of the inhibition by VSV of cellular RNA and DNA synthesis (McGowan & Wagner, 1981). The mechanism(s) by which VSV inhibits cellular protein synthesis has (have) not been elucidated although it is generally accepted that primary transcription of the VSV genome is required (McAllister & Wagner, 1976); however, the translational inhibitory transcript is less resistant to ultraviolet irradiation than the VS viral transcript that appears to inhibit cellular RNA synthesis (Marvaldi et al., 1978; McGowan & Wagner, 1981). Recent studies indicate that RNA newly transcribed by VSV inhibits in vitro translation in a reticulocyte lysate system (Thomas & Wagner, 1982).

The cellular target of the VSV inhibitor of protein synthesis has not been identified, but several studies have provided indirect evidence that inhibition occurs at the level of initiation of translation (Nuss & Koch, 1976; Stanners et al., 1977; Davis & Wertz, 1980). Possible mechanisms by which viruses inhibit cellular protein synthesis have been provided by studies with poliovirus and other plus-strand viruses, whose replicative strategy is very different from that of VSV. From various methods of fractionation and reconstitution of the translational machinery of uninfected and poliovirus-infected cell lysates, evidence has been provided implicating alteration postinfection of a 24K cap-binding protein that copurifies predominantly with a low-salt ribosomal-wash fraction containing eIF-3 and eIF-4B (Kaufmann et al., 1976; Rose et al., 1978; Helentjaris et al., 1979; Trachsel et al., 1980; Hansen & Ehrenfeld, 1981). Similar findings have also been reported for inhibition of host-cell protein synthesis by the plus-strand RNA Semliki Forest virus (Steeg et al., 1981).

We report here, by fractionation and reconstitution of uninfected and VSV-infected cell lysates, findings similar to those described for poliovirus-infected cells. We have obtained evidence by use of in vitro translation systems that VSV infection of mouse L cells results in reduced activity of a ribosomal salt-wash fraction. No attempt was made in these studies to determine the factors concerned with switching from host-cell protein synthesis to viral protein synthesis during the course of infection.

Materials and Methods

Chemicals. L-[^{35}S]Methionine (1053.2 Ci/mmol) and EnH^{3}ance were purchased from New England Nuclear, Boston, MA. Rabbit liver tRNA was obtained from GIBCO Laboratories, Grand Island, NY, and aminoacyl-tRNA synthetase from Sigma Chemical Co., St. Louis, MO.

Cells and Virus. Mouse L cell monolayers were grown in 150-cm^2 flasks at 37 °C in basal medium Eagle (BME) supplemented with 10% calf serum. The wild-type San Juan strain of the Indiana serotype of vesicular stomatitis (VS) virus was originally obtained from U.S. Agriculture Research Center, Beltsville, MD (Wagner et al., 1963). VS virus was grown in BHK-21 cells as previously described (Dubovi & Wagner, 1973).

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[1] Abbreviations: VSV, vesicular stomatitis virus; eIF, eukaryotic initiation factor; BME, basal medium Eagle; MOI, multiplicity of infection; $NaDodSO_4$, sodium dodecyl sulfate; pi, postinfection; pfu, plaque-forming units; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TNE, 0.01 M Tris (pH 8.2), 0.1 M NaCl, and 0.001 M EDTA.

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Preparation of Mock-Infected and VSV-Infected Cell Lysates. Cell fractionation was carried out by modification of methods described by Kaufmann et al. (1976). L cell monolayers were grown to confluency in 150-cm^2 Corning flasks, and 10 of these were either mock infected with 1.0 mL of BME or infected with VSV (4×10^8 pfu) in 1.0 mL of BME to give a MOI of 10. After adsorption for 40 min at room temperature (~23 °C), 10 mL of BME with 10% calf serum was added to each flask. At 4 h postinfection, the cells from both mock-infected and VSV-infected monolayers were harvested in the presence of 0.25% trypsin and washed 3 times in phosphate-buffered saline (PBS). Cells were then resuspended in a volume of sterile water equivalent to that of packed cells and subjected to 20 strokes with a tight-fitting Dounce homogenizer. Each homogenate was then spun at 20000*g* for 15 min at 4 °C in a Sorvall SS-34 rotor to give mock-infected and VSV-infected postmitochondrial supernatant (S20) fractions, which were collected and frozen at −70 °C. The S20 fractions were further fractionated by subjecting equivalent volumes of either mock-infected or VSV-infected cells to centrifugation in a Beckman SW50.1 rotor at 200000*g* for 50 min at 4 °C. The resultant polyribosomal pellets were then resuspended in buffer A [10 mM Hepes/KOH (pH 7.1), 0.7 mM $Mg(OAc)_2$, 75 mM KCl, 6 mM 2-mercaptoethanol] at a fourth the original S20 fraction spun volume. The supernatants (S200) were recovered and frozen at −70 °C.

Preparation of Ribosomal Salt-Wash Fractions. Ribosomal pellets from either mock-infected or VSV-infected S20 fractions were prepared as above except that following centrifugation they were resuspended in buffer B [5 mM Hepes/KOH (pH 7.4), 4 mM $Mg(OAc)_2$, 500 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 250 mM sucrose]. After a frequent mixing of this resuspension at 4 °C for 30 min, the washed ribosomes were pelleted as above at 200000*g* and resuspended in buffer A at a fourth the original S20 fraction spun volume. The resulting supernatants were collected and dialyzed for 4 h against 500 volumes of buffer C [10 mM Hepes/KOH (pH 7.4), 44 mM KCl, 1.2 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 10% glycerol]. The dialysates were then used as uninfected and VSV-infected ribosomal salt-wash fractions.

Ammonium Sulfate Precipitates. These ribosomal salt-wash fractions were further fractionated by ammonium sulfate precipitation as described by Helentjaris et al. (1979). A saturated solution of $(NH_4)_2SO_4$ was added dropwise at 4 °C either to 2.8 mL of ribosomal salt wash from uninfected cells or to 2.6 mL of ribosomal salt wash from VSV-infected cells so as to achieve 40% $(NH_4)_2SO_4$ saturation. The wash fraction was stirred as the saturated $(NH_4)_2SO_4$ solution was added and then allowed to stand for 20 min at 4 °C with occasional stirring. The precipitate was collected by centrifugation at 9500 rpm for 20 min at 4 °C in a Sorval SS-34 rotor. To the supernatant fluid was then added in a similar fashion saturated ammonium sulfate solution to achieve 70% saturation; this was then treated and collected as described above. The 0–40% ammonium sulfate cut was designated fraction A and the 40–70% ammonium sulfate cut was designated fraction B. Protein determinations were made on each of the cell fractions as described by Lowry et al. (1951).

In Vitro Translation. In vitro translation reactions of either mock-infected or VSV-infected S20 lysates or translation reactions representing reconstitution of the various fractions of the S20 lysates were carried out at 25 °C in 25-μL volumes. Reactions contained 20 mM Hepes/KOH, pH 7.4, 85 mM K(OAc), 1.8 mM $Mg(OAc)_2$, 5 mM dithiothreitol, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 0.2 mg/mL creatine kinase, 0.4 mM spermidine phosphate, and unless otherwise noted, 19 amino acids at 0.005 mM each. Reactions contained either [^{35}S]methionine at 1.0 μCi/μL or, where indicated, 40 μg of rabbit liver tRNA containing f[^{35}S]-Met-tRNA$_f^{Met}$ added to give 0.1 μCi/μL.

In vitro translation of S20 lysates from either mock-infected or VSV-infected cells was carried out so that the lysates comprised 30% of the reactions by volume. These translation reactions were begun by the addition of lysate. Translation reactions with reconstituted components of the uninfected and infected cell lysates (i.e., polyribosome S200 fractions or polyribosomes with ribosomal wash fractions) were carried out at the protein concentrations of the various components as indicated in the figure legends. These reconstituted reactions were begun by the addition of polyribosomes to the reaction.

For reactions in which f[^{35}S]Met-tRNA$_f^{Met}$ was employed, the reactions were terminated by spotting and air-drying samples on Gelman Metricel GA-6 filters, which were then placed in cold (4 °C) 5% Cl_3CCOOH for 5 min. The filters were transferred to 5% Cl_3CCOOH, heated to 90 °C for 5 min, and washed twice with cold 5% Cl_3CCOOH and ethanol. After being air-dried, they were placed in Beckman Ready-Solv EP scintillation fluid and counted in a Beckman LS-230 scintillation counter. Reactions in which [^{35}S]methionine was used were terminated by transferring 2.5-μL samples into 25 μL of 0.1 N KOH and incubating at 32 °C for 30 min. They were then precipitated by the addition of 1 mL of 5% Cl_3CCOOH and collected on Whatman GF/A filters, washed with 5% Cl_3CCOOH and 95% ethanol, air-dried, and counted as described above.

Charging of tRNA$_f^{Met}$. Rabbit liver tRNA$_f^{Met}$ was charged with [^{35}S]methionine by the method of Takeishi et al. (1968), which has been modified slightly (Gupta et al., 1971) as described by Henshaw (1979). Each reaction contained 250 μCi/mL of [^{35}S]methionine (1.053.2 Ci/mmol), 100 mM sodium cacodylate (pH 7.4), 10 mM KCl, 2 mM ATP, 4 mg/mL stripped rabbit liver tRNA, and 0.18 mg/mL *Escherichia coli* mixed aminoacyl-tRNA synthetase. Incubation was carried out for 15 min at 37 °C. The reaction was then made 0.1 M in sodium acetate (pH 4.35), 2 volumes of TNE-saturated phenol were added, the suspension was mixed for 5 min at 4 °C, and the aqueous phase was recovered following low-speed centrifugation. The phenol phase was reextracted with 1 volume of 50 mM Na(OAc) (pH 5.0) and 5 mM $Mg(OAc)_2$. The aqueous extractions were then combined and were dialyzed for 6 h against 500 volumes of 0.5 M NaCl and 50 mM Na(OAc) (pH 5.0) and then dialyzed for 17 h against 500 volumes of 20 mM Na(OAc) (pH 5.0).

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis and Fluorography. Aliquots of 15 μL from in vitro translation reactions were combined with 50 μL of gel sample buffer containing 2% $NaDodSO_4$, 25 mM Tris (pH 6.8), 100 mM dithiothreitol, and 20% glycerol containing bromophenol blue. Samples were placed in boiling water for 3 min and then were loaded on a stacking gel consisting of 4% acrylamide, 0.2% bis(acrylamide), 0.1% $NaDodSO_4$, and 0.125 M Tris (pH 6.8) for electrophoresis by the method of Laemmli (1970) as modified by Carroll & Wagner (1978). Samples were run for 2.5 h at 200 V on a gel containing 12.5% acrylamide, 0.1% bis(acrylamide), 0.1% $NaDodSO_4$, and 0.375 M Tris (pH 8.7). The gels were fixed for 30 min and stained for 30 min in Coomassie blue. Following destaining for 20–24 h, they were placed in a commercially prepared fluorography reagent (EnH^{3}ance) for 1 h, washed, and then soaked in water for 2 h. Gels were then dried onto Whatman 3MM filter paper.

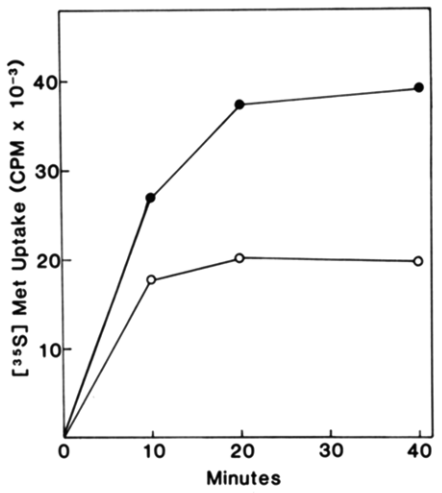

FIGURE 1: Comparative kinetics of cell-free protein synthesis in postmitochondrial (S20) lysates from mock-infected (●) and VSV-infected (○) L cells. As described under Materials and Methods, cells were infected with VSV at an MOI ~ 10 or mock-infected and lysates were prepared 4 h postinfection. Protein synthesis was measured by incorporation of [^{35}S]methionine (1.0 μCi/μL) in 25-μL complete translation mixtures in which VSV-infected or mock-infected lysates comprised 30% of the total volumes. Reactions were incubated at 31 °C and, at intervals thereafter, samples of 2.5 μL each were withdrawn and added to 25 μL of 0.1 N KOH and incubated at 31 °C for 30 min to deacylate tRNA. Samples were then precipitated with 1 mL of 5% Cl_3CCOOH, filtered, and washed, and radioactivity was counted by liquid scintillation spectrometry.

Fluorography was carried out with preflashed Kodak X-omatic AR film. Densitometry scans were obtained with an LKB Model 2202 Ultroscan laser densitometer and a Hewlett-Packard Model 3390A integrator.

Results

In Vitro Protein Synthesis by Postmitochondrial (S20) Lysates from Uninfected and VSV-Infected L Cells. Our initial experiments were designed to test the feasibility of using an in vitro translation system to simulate the in vivo conditions resulting from VSV infection of mouse L cells compared with uninfected cells. As described under Materials and Methods, we initially prepared postmitochondrial (S20) lysates from mock-infected and VSV-infected L cells 4 h pi and compared their endogenous translational ability by studying the relative capacity of these lysates to incorporate [^{35}S]methionine at 31 °C over a period of 40 min. Figure 1 demonstrates that the 4-h pi lysate from VSV-infected cells synthesized only ~50% as much acid-precipitable protein at 40 min as did the mock-infected cell lysate. These results were quite similar to the in vivo results reported for incorporation of [^{3}H]leucine in VSV-infected and mock-infected cells (Wertz & Youngner, 1972; McAllister & Wagner, 1976).

Figure 2 compares by polyacrylamide gel electrophoresis and fluorography the proteins synthesized during 30 min of the in vitro translation reactions for mock-infected and VSV-infected L cell lysates, as well as in vivo protein synthesis in mock-infected and VSV-infected L cells. Reactions containing 30% lysate from VSV-infected cells exhibited substantially less protein-synthesizing capacity than did comparative lysates from mock-infected cells. Actin was the only readily identifiable endogenous cellular protein, and its synthesis was markedly inhibited (see Figure 2). Laser density scanning and integration revealed 60–70% inhibition of protein synthesis by the VSV-infected cell lysate compared with the comparable mock-infected cell lysate (data not shown). Both mock-infected and VSV-infected cell lysates were found to translate

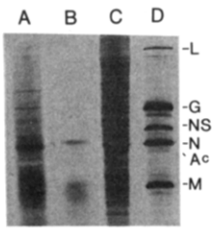

FIGURE 2: Comparative electrophoretic analysis of endogenous proteins synthesized in vitro by (A) mock-infected L cell lysates (S20) and (B) VSV-infected L cell lysates and proteins synthesized in vivo in (C) mock-infected L cells and (D) VSV-infected L cells. As described under Materials and Methods, the lysates obtained at 4 h pi were translated in vitro in the presence of [^{35}S]methionine (1 μCi/μL), comprised 30% of the total reaction volume, and were incubated for 30 min at 31 °C. In vivo protein synthesis was determined in L cell monolayers mock-infected or infected with VSV at a multiplicity of infection of 10 pfu/cell; at 4 h pi cells were labeled for 30 min with ^{14}C-labeled amino acids (5 μCi/mL), following which they were solubilized and sheared samples were suspended in Laemmli buffer. All samples were electrophoresed on 12.5% polyacrylamide gels in the presence of $NaDodSO_4$. Markers for actin (Ac) and VSV proteins are shown.

viral mRNA that had been transcribed in vitro (data not shown). VS viral proteins could be detected among the proteins synthesized by VSV-infected cell lysates (Figure 2). As expected, the VS viral N protein was the most readily detectable because the N-protein mRNA is most abundant after VSV transcription (Iverson & Rose, 1981). On comparing the proteins synthesized in vivo by mock-infected and VSV-infected L cells labeled for 30 min with [^{35}S]methionine at 4 h pi, we found by electrophoresis and fluorography strikingly similar polyacrylamide gel profiles to those shown in the in vitro electropherograms (see Figure 2). We feel confident, therefore, that inhibition of translation in lysates of VSV-infected cells mirrors the events in vivo that lead to inhibition of cellular protein synthesis in L cells infected with VSV.

Comparative Capacity To Initiate Translation by Ribosomal Fractions from Mock-Infected and VSV-Infected Cells. We next attempted to confirm that the defect in protein-synthesizing activity of VSV-infected cell lysates was at the level of initiation of translation. We also hoped to locate the defect in one of two crude cell fractions. For these purposes, lysates from mock-infected or VSV-infected cells were separated into polyribosome and S200 supernatant fractions, which were tested in various reconstituted combinations for their capacity to incorporate formylatable [^{35}S]methionine from f[^{35}S]Met-tRNA$_f^{Met}$ into Cl_3CCOOH-precipitable material in the in vitro translation system.

Figure 3A summarizes the data on the translational initiation activity of polyribosomes from mock-infected and VSV-infected cell lysates reconstituted with their S200 supernatants. As shown in Figure 3A, unwashed polyribosomes from infected cell lysates incorporated formylatable [^{35}S]-methionine with an efficiency of only 51% and 66% when reconstituted with the S200 postribosomal supernatants from mock-infected or VSV-infected cell lysates, respectively, when compared to the efficiency of mock-infected polyribosomes. In fact, the S200 fraction from infected-cell lysates, when recombined with mock-infected cell polyribosomes, incorporated formylatable [^{35}S]methionine at a level slightly higher than that of the control (Figure 3A). These data serve to confirm the hypothesis that the translational defect in VSV-infected cells is at the level of initiation and the site of the translational defect is at the level of the ribosome rather than a postribosomal cytoplasmic factor. These data are also in agreement with those of Nuss & Koch (1976), who found that

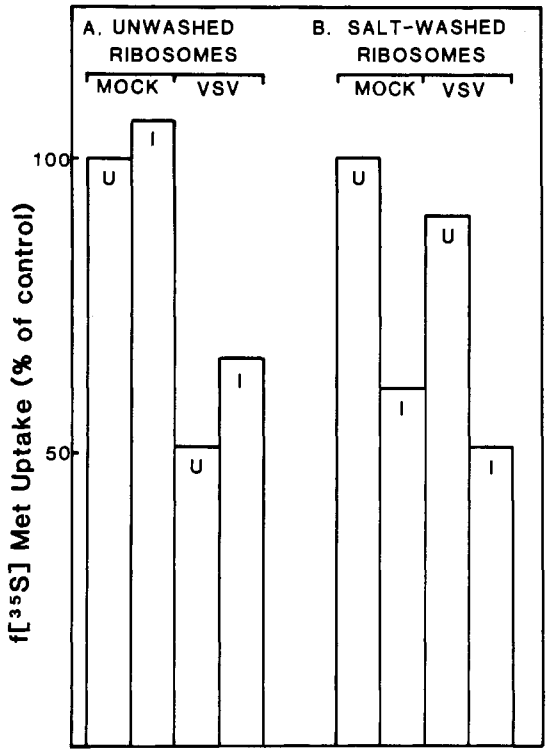

FIGURE 3: Comparative ability to initiate translation in mock-infected or VSV-infected L cell lysates after reciprocal reconstitution of (A) unwashed ribosomes mixed with S200 postribosomal supernatants from mock-infected (U) or VSV-infected (I) cells and (B) salt-washed ribosomes (exposed to 0.5 M KCl) from mock-infected or VSV-infected cells mixed with salt-wash fractions from ribosomes of mock-infected (U) or VSV-infected (I) cells. Initiation of translation was measured by incorporation of formylatable [^{35}S]methionine during incubation with charged f[^{35}S]Met-tRNA$_f^{Met}$ in an in vitro translation reaction as described under Materials and Methods, except that all amino acids were omitted other than unlabeled methionine (0.05 mM). In the case of unwashed ribosomes (A), 4 μL of mock-infected or VSV-infected polyribosomes was mixed with 98 μg/mL corresponding or reciprocal S200 fractions. In the case of salt-washed ribosomes (B), each translation reaction contained 3.3 μL of salt-washed polyribosomes, 98 μg/mL mock-infected postribosomal fraction, and 528 μg/mL protein released from salt-washed ribosomes. All translation reactions were performed in 25-μL volumes for 30 min at 31 °C, and duplicate 7.0-μL aliquots were spotted on Gelman Metricel GA-6 nitrocellulose filters, precipitated with cold 5% Cl_3-CCOOH, and treated with 5% Cl_3CCOOH at 90 °C for 5 min to deacylate charged tRNA$_f^{Met}$ before counting ^{35}S radioactivity by liquid scintillation spectroscopy. In the case of unwashed ribosomes (A), the mock-infected polyribosomes mixed with the mock-infected S200 fraction incorporated 3704 cpms/7 μL, which was taken as the 100% base-line control level for translation in this system. In the case of salt-washed ribosomes (B), the mock-infected polyribosomes mixed with the mock-infected ribosomal salt-wash incorporated 6288 cpm/7 μL, which was taken as the 100% base-line control level for translation in this system. Mock-infected and VSV-infected polyribosomes were stored at 5.1 mg/mL and 5.5 mg/mL, respectively.

protein synthesis in VSV-infected MPC-11 myeloma cells is inhibited at the stage of initiation of translation.

Having located the lesion in the ribosome of VSV-infected cells, we next set out to determine whether ribosome-associated protein factors were responsible for the defect. Such defects in initiation factors had previously been demonstrated for impaired regulation of protein synthesis in host cells infected with other viruses (Rose et al., 1978; Trachsel et al., 1980; Hansen & Ehrenfeld, 1981). Therefore, polyribosomes from mock-infected and VSV-infected cells were washed with 0.5 M KCl (buffer B) and separated by centrifugation at 200000g into salt-washed polyribosomes and salt-wash supernatant fractions that were then reconstituted in various combinations to study initiation of protein synthesis by incorporation of

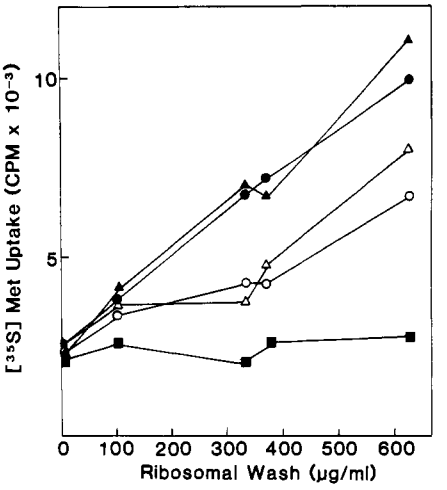

FIGURE 4: Effect of increasing concentrations of protein released by salt wash from mock-infected or VSV-infected cells to restore translational activity to salt-washed polyribosomes from mock-infected and VSV-infected cells. As described under Materials and Methods, ribosomes were treated with buffer B containing 0.5 M KCl to prepare salt-washed polyribosomes and salt-wash fractions. Increasing concentrations of protein released from ribosomes of mock-infected cells were added to salt-washed polyribosomes from mock-infected cells (●) or VSV-infected cells (▲), and increasing concentrations of salt-wash protein released from ribosomes of VSV-infected cells were added to salt-washed polyribosomes from mock-infected cells (○) and VSV-infected cells (△). The proteins released from ribosomes of mock-infected cells in the absence of polyribosomes (■) served as a control. Reaction mixtures of 25 μL each with increasing amounts of protein from ribosomal salt washes (shown on the abscissa) were incubated in the presence of [^{35}S]methionine at 31 °C for 30 min, and duplicate 2.5-μL aliquots were placed in 25 μL of 0.1 N KOH, incubated for 30 min at 31 °C, and precipitated with 1 mL of 5% Cl_3CCOOH. The precipitates were filtered and washed, and the radioactivity was counted by scintillation spectrometry. Mock-infected and VSV-infected cell salt-wash ribosomes were stored at 3.9 and 4.1 mg/mL, respectively.

formylatable [^{35}S]methionine. Figure 3B reveals that the defect in initiation of translation resides in the salt-wash fraction rather than the salt-washed polyribosomes. The salt-wash fraction from infected-cell ribosomes exhibited only 61% of the activity of the mock-infected salt-wash fraction when reconstituted with salt-washed polyribosomes from mock-infected cells. Likewise, when reconstituted with salt-washed ribosomes from VSV-infected cells, the salt-wash fraction from mock-infected cells stimulated incorporation of formylatable [^{35}S]methionine by ~180% when compared with the salt-wash fraction released from infected-cell ribosomes. These results were confirmed by laser densitometry scans and integration of autoradiographs prepared from polyacrylamide gel electrophoresis of proteins synthesized in vitro by salt-washed polyribosomes reconstituted with salt-wash fractions (data not shown).

These experiments indicate that the defect in initiation of protein synthesis in VSV-infected cells is a ribosome-associated factor(s) that is (are) released by a washing with 0.5 M KCl and that factors released from ribosomes of uninfected cells can restore much of the translational activity to infected-cell polyribosomes.

To extend these results, we attempted to quantitate the relative capacity of salt-wash factors released from ribosomes of VSV-infected and mock-infected cells to restore translational activity to salt-washed polyribosomes from infected or uninfected L cells. To this end, we added to salt-washed polyribosomes increasing concentrations of proteins released from uninfected or VSV-infected cell polyribosomes and measured incorporation of [^{35}S]methionine into acid-precipitable proteins

in the in vitro translation system. Figure 4 reveals quite similar linear incorporation of [^{35}S]methionine into proteins when increasing concentrations of ribosomal wash fractions from mock-infected cells were added to salt-washed polyribosomes derived from either mock-infected or VSV-infected cells. In contrast, protein fractions released by 0.5 M KCl from ribosomes of VSV-infected cells exhibited greatly reduced capacity to restore translational activity to salt-washed polyribosomes obtained from either mock-infected or VSV-infected cells, particularly at protein concentrations of 300–400 μg/mL. It is quite evident from the data in Figure 4 that the degree of protein-synthesis stimulation by salt-released protein factors from either mock-infected or VSV-infected cells is independent of whether salt-washed polyribosomes are derived from mock-infected or VSV-infected cells. So that we could rule out incorporation of [^{35}S]methionine by contaminating components, such as uncentrifuged ribosomes, that might be present in the salt-wash fractions, mock-translation reactions were also carried out at increasing concentrations of salt washes in the absence of added polyribosomes; as shown in Figure 4, background counts remained relatively low despite increasing salt-wash concentrations. Polyacrylamide gel analysis of these mock reactions revealed no detectable protein synthesis as measured by fluorography (data not shown).

These data further support evidence presented in Figure 3B that the translational defect in VSV-infected cells resides in the proteins associated with polyribosomes that are released by 0.5 M KCl rather than in the salt-washed polyribosomes themselves.

Comparative Protein Synthesis by Salt-Washed and Unwashed Polyribosomes from VSV-Infected L Cells following Stimulation by the Proteins Released by 0.5 M KCl from Uninfected-Cell Ribosomes. Centrella & Lucas-Lenard (1982) recently reported that protein synthesis by S20 lysates of VSV-infected cells was considerably enhanced by addition of salt-wash initiation factors released from uninfected L cell ribosomes. This result was somewhat surprising since one would expect that endogenous and presumably inactive initiation factors on infected-cell polyribosomes might compete with initiation factors added from uninfected-cell ribosomes. It was incumbent upon us to repeat this experiment by testing unwashed S20 infected-cell lysates as well as comparing the effect of salt-wash proteins from uninfected-cell ribosomes on reconstituting the translational activity of infected-cell polyribosomes salt depleted of iniation factors, a control experiment not reported by Centrella & Lucas-Lenard (1982). Therefore, we compared unwashed ribosomes and 0.5 M KCl washed ribosomes from VSV-infected cells for their capacity to incorporate [^{35}S]methionine into Cl_3CCOOH-precipitable proteins after addition of the salt-wash fraction liberated by 0.5 M KCl from uninfected-cell ribosomes, under conditions similar to those described in the legend for Figure 4.

Figure 5 shows that increasing amounts of proteins from uninfected-cell ribosomes did enhance the translational activity of unwashed polyribosomes from VSV-infected cells, but even the addition of 628 μg/mL of the salt-wash fraction enhanced protein synthesis only 1.3-fold above the level for no added salt-wash proteins. In sharp contrast, addition of salt-wash proteins from uninfected-cell ribosomes progressively enhanced translation of salt-washed polyribosomes from VSV-infected cells to a level of 4.6-fold above that of the salt-washed polyribosomes to which no protein was added.

These results are consistent with the hypothesis that a defective ribosome-associated factor(s) is (are) present on unwashed polyribosomes from VSV-infected cells and this defective factor competes effectively with active initiation factors from uninfected-cell ribosomes. Removal of the defective factors by treating infected-cell polyribosomes with 0.5 M KCl presumably allows the added nondefective ribosomal protein factor to gain access to ribosomal sites and markedly stimulate protein synthesis. Apparently, a limited number of sites are available for binding of active initiation factors to unwashed polyribosomes from VSV-infected cells. These results are also consistent with our finding that the salt-wash fractions released from VSV-infected cell ribosomes are defective in their translation-promoting activity.

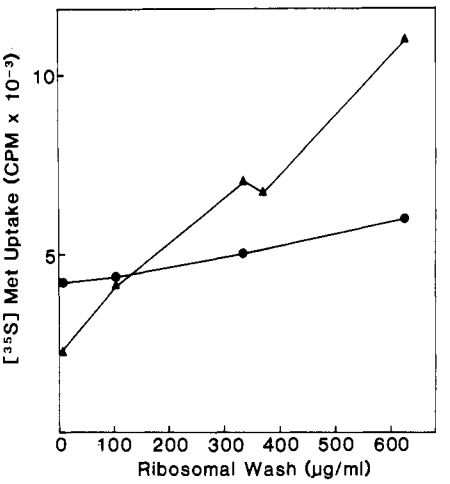

FIGURE 5: Comparative translational activity of salt-washed (▲) or unwashed (●) polyribosomes from VSV-infected cells reconstituted with increasing amounts of proteins released by 0.5 M KCl from ribosomes of mock-infected cells. Each reaction mixture of 25 μL contained 3.3 μL of either salt- (0.5 M KCl) washed polyribosomes or unwashed polyribosomes, 98 μg/mL S200 fraction from unwashed mock-infected cells, and increasing concentrations of protein released from ribosomes of mock-infected cells as shown on the abscissa. Translation reactions were incubated in the presence of [^{35}S]methionine (1.0 μCi/μL) and assayed as described in the legend for Figure 4.

Translation-Inducing Activity of Fractionated Salt Washes from Ribosomes of Uninfected and VSV-Infected Cells. Fractionation of ribosomal salt washes by differential precipitation with ammonium sulfate has been found by Benne & Hershey (1976) and Benne et al. (1976, 1977, 1978) to separate initiation factors into two groups: fraction A [0–40% $(NH_4)_2SO_4$ cut contains primarily eIF-3 and eIF-4B] and fraction B [40–70% $(NH_4)_2SO_4$ cut contains primarily eIF-1, eIF-2, eIF-4A, eIF-4C, eIF-4D, and eIF-5]. As described under Materials and Methods, ribosomal salt washes (0.5 M KCl) from mock-infected and VSV-infected L cells were fractionated by the $(NH_4)_2SO_4$ technique into these two groups of initiation factors. These two fractions (A and B) were then mixed in various combinations in order to reconstitute the translational activity of 0.5 M KCl washed polyribosomes from mock-infected cells. Proteins labeled with [^{35}S]methionine in these in vitro translation reactions were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis and fluorography. Two major protein bands, the molecular weights of which were ~36 000 and ~40 000, were apparent and represented ~80% of the product synthesized by each translation reaction. These two bands in each fluorogram were scanned by soft-laser densitometry, and the area under both peaks was integrated. The 40K protein is presumed to be actin, but neither of the two major proteins can be identified directly.

Figure 6 compares the densitometry scans of the 36K and 40K proteins synthesized by salt-washed polyribosomes from uninfected L cells reconstituted with various combinations of fractions A and B derived from salt-washed ribosomes of

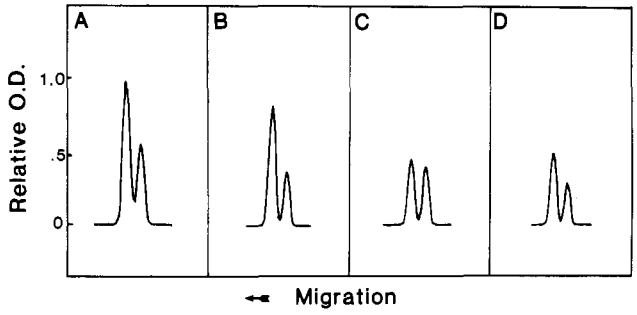

FIGURE 6: Comparative densitometry tracings of electrophoretic fluorograms of 40K and 36K proteins synthesized by salt-washed polyribosomes from mock-infected cells reconstituted with various combinations of two ammonium sulfate fractions of salt-wash ribosomal proteins from uninfected and VSV-infected cells. Proteins from ribosomal salt washes (0.5 M KCl) from either mock-infected or VSV-infected cells were fractionated by precipitation with 0–40% $(NH_4)_2SO_4$ (fraction A) or 40–70% $(NH_4)_2SO_4$ (fraction B), as described under Materials and Methods. In vitro translation reactions of 25 μL each were reconstituted by adding 4 μL of salt-washed polyribosomes from mock-infected cells, 98 μg/mL S200 fraction of mock-infected cells, 566 μg/mL fraction A, and 716 μg/mL fraction B in the following combinations: (Panel A) mock-infected cell fractions A and B; (panel B) mock-infected cell fraction A and VSV-infected cell fraction B; (panel C) VSV-infected cell fraction A and mock-infected cell fraction B; (panel D) VSV-infected cell fractions A and B. Reactions were incubated at 31 °C for 30 min and then subjected to electrophoresis on a 12.5% polyacrylamide gel prior to fluorography and densitometry scanning as described under Materials and Methods.

uninfected and 4-h VSV-infected cells. If integration of the two peaks resulting from reconstitution with fractions A and B from the mock-infected salt wash is considered 100% translation (control, Figure 6A), incorporation of [^{35}S]-methionine was reduced to 66% in the presence of mock-infected cell fraction A and VSV-infected cell fraction B (Figure 6B), to 46% translation in the presence of VSV-infected cell fraction A and mock-infected cell fraction B (Figure 6C), and to 41% translation in the presence of VSV-infected cell fractions A and B (Figure 6D). These results were confirmed by excising these bands from the gels and subjecting them to liquid scintillation spectrometry (data not shown).

These data provide additional evidence for reduction in translation-iniation activity in ribosomal proteins derived from cells infected with VSV. The iniation factor(s) responsible for this altered translational activity may be present in both fractions A and B but coprecipitates predominantly with infected-cell fraction A [0–40% $(NH_4)_2SO_4$ cut], which contains primarily eIF-3 and eIF-4B.

Discussion

Inhibition of host-cell protein synthesis following infection with vesicular stomatitis virus has been well documented (Mudd & Summers, 1970; Wertz & Youngner, 1972; Baxt & Bablanian, 1976; McAllister & Wagner, 1976; Otto & Lucas-Lenard, 1980). This postinfection inhibition of protein synthesis appears to involve two phenomena: (i) a switch from host-cell protein synthesis to predominantly VSV protein synthesis and (ii) a linear decrease with time in overall protein synthesis reaching levels of 50–70% inhibition by 4 h postinfection (Wertz & Youngner, 1972; McAllister & Wagner, 1976). In the present studies we focused our efforts on in vitro studies to identify the factors concerned with the second phenomenon, the ribosome target(s) for the action of VSV that leads to attenuation of overall translation. These experiments led us to implicate ribosome-associated proteins that coprecipitate predominantly with initiation factors eIF-3 and eIF-4B. No attempt was made here to investigate the first phenomenon concerned with a switch in translation from cellular to viral messengers; these latter experiments require a different set of protocols that will be the subject of later investigations.

Almost all studies to date on the mechanisms by which VSV inhibits protein synthesis have been done with in vivo systems. These studies demonstrate that infection by VSV does not result in degradation of host-cell mRNA (Nishioka & Silverstein, 1978; Lodish & Porter, 1980) and that the minimal requirement for inhibition of cell protein synthesis is primary transcription of the VSV genome as determined by the inability of temperature-sensitive mutants phenotypically restricted in transcription to inhibit protein synthesis at the nonpermissive temperature (McAllister & Wagner, 1976). Marvaldi et al. (1978) have demonstrated by ultraviolet-inactivation studies for mapping the VSV genome that inhibition of protein synthesis requires unimpaired transcription of the N gene and possibly NS gene as well. Of course, inhibition of VSV transcription also results in inhibition of VSV protein synthesis, which, in theory, could be involved in inhibition of cellular protein synthesis (Wertz & Youngner, 1972).

Although quite limited in scope, a few in vivo studies have attempted to identify possible host-cell targets for inhibition of protein synthesis following VSV infection. Nuss & Koch (1976) reported that the pattern of protein-synthesis inhibition following VSV infection of MPC-11 myeloma cells is similar to that observed following exposure of these cells to hypertonic media; these data were interpreted as indirect evidence that inhibition of host-cell protein synthesis by VSV occurs at the level of initiation of translation. Wertz & Youngner (1972) described a small-plaque mutant (S2) of VSV, which causes rapid inhibition of protein synthesis in L cells to levels of 90% inhibition in 2 h. Further studies on this S2 mutant by Davis & Wertz (1980) indicated that S2-mutant transcripts are associated with polyribosomes smaller than those associated with VSV wild-type transcripts; this reduction in polysome size was interpreted as resulting from a decreased efficiency in initiation of protein synthesis and not as an effect on chain elongation, functional alteration of transcripts, or competition by excess viral transcripts for a limited number of ribosomes. Stanners et al. (1977) described another VSV mutant, in this case derived from the HR strain of the Indiana serotype, which was unable to inhibit cellular protein synthesis. By comparison, cells infected with a revertant of this HR mutant were able to inhibit protein synthesis and contained a greater number of free ribosomes than did cells infected with the mutant virus, suggesting a decrease in initiation of translation in those cells infected with the revertant as compared to those infected with the mutant. All these studies collectively provide indirect evidence that inhibition of protein synthesis by VSV occurs at the level of initiation of translation.

Other investigators studying in vitro inhibition of protein synthesis by quite different positive-strand RNA viruses have used cell fractionation and reconstitution techniques with some success to pinpoint the target for inhibition of protein synthesis; in general, these studies reveal alteration in initiation factor(s) associated with ribosomal proteins (Kaufmann et al., 1976; Helentjaris et al., 1979; Steeg et al., 1981). The indirect evidence for a defect in initiation of translation in cells infected with VSV led us to apply similar techniques of fractionation and reconstitution of the translation machinery in uninfected and VSV-infected L cells. Our results presented here are similar to those found for other virus–host-cell systems, as follows: (1) lysates of VSV-infected L cells show reduced capacity to synthesize proteins compared to mock-infected control cells; (2) this inhibition occurs at the level of initiation of translation; (3) reconstitution studies reveal that the factors

associated with impaired protein synthesis appear in the fraction released from infected-cell ribosomes washed with 0.5 M KCl; and (4) the responsible initiation factor(s) was (were) detected most readily in the 0–40% ammonium sulfate precipitate that contains predominantly eIF-3 and eIF-4B. Subsequent to the completion of our experiments, a paper by Centrella & Lucas-Lenard (1982) was published and reported similar results on defective incorporation into protein of methionine from initiation methionyl-tRNA; they conclude that the defective initiation factor is eIF-2, but no studies were reported on fractionation of salt-wash proteins from VSV-infected or uninfected cells, nor were data reported on reconstitution of translational activity in salt-washed polyribosomes depleted of initiation factors.

Studies on cytopathogenicity of polioviruses indicate that infected cells lose considerable activity in a ribosomal salt-wash fraction that contained eIF-3 and eIF-4B (Rose et al., 1978; Helentjaris et al., 1979). Preliminary studies indicate similar findings with neuroblastoma cells late in infection with Semliki Forest togavirus (Steeg et al., 1981). In both of these systems, infection results in eventual loss of translation that is dependent on the 5′-terminal cap structure of mRNA.

The studies reported here suggest that the translational lesion in VSV-infected L cells resides in the ribosomal salt-wash fraction. There is a slight inhibition (20–25%) that can be ascribed to the ammonium sulfate precipitated fraction B, but it appears that the factor responsible for most of the inhibitory effect of VSV on protein synthesis coprecipitates with fraction A containing eIF-3 and eIF-4B. Rose & Lodish (1976) had previously shown that the 5′-terminal 7-methylguanosine is not obligatory for binding to ribosomes of VSV mRNA. Further studies will be required to determine more definitively whether eIF-3, eIF-4B, a cap-binding protein, or some other ribosomal factor altered by infection with VSV is responsible for the resulting inhibition of host-cell protein synthesis. We have clearly demonstrated, however, that the negative-strand VSV rhabdovirus compromises translation at the level of initiation by ribosomal factors in a manner that may be similar to that caused by positive-strand viruses, which have a quite dissimilar transcriptional and replicative strategy.

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