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Catalytic Utilization of eIF-2 and mRNA Binding Proteins Are Limiting in Lysates from Vesicular Stomatitis Virus Infected L Cells†

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ABSTRACT: Infection of mouse L cells by vesicular stomatitis virus results in the inhibition of cellular protein synthesis. Lysates prepared from these infected cells are impaired in their ability to translate endogenous or exogenous cellular and viral mRNAs. The ability of initiation factors from rabbit reticulocytes to stimulate protein synthesis in these lysates was examined. Preparations of eukaryotic initiation factor 2 (eIF-2) and the guanine nucleotide exchange factor (GEF) stimulated protein synthesis strongly in L cell lysates from infected cells but only slightly in lysates from mock-infected cells. Maximal stimulation was obtained when a fraction containing eukaryotic initiation factors 4B (eIF-4B) and 4F (eIF-4F) was also present. In lysates from infected cells, these initiation factors increased endogenous cellular mRNA translation on the average 2-fold. In contrast, endogenous viral mRNA translation was increased to a much greater extent: the M protein was stimulated 8-fold, NS 5-fold, N 2.5-fold, and G 12-fold. When fractions containing eIF-4B, eIF-4F, or eIF-4A were added to these lysates in the presence of eIF-2, all three stimulated translation. Fractions containing rabbit reticulocyte initiation factors eIF-3 and eIF-6 had no effect on translation in either lysate. The results suggest that lysates from infected L cells are defective in the catalytic utilization of eIF-2 and deficient in mRNA binding protein activity.

Infection of suitable host cells by vesicular stomatitis virus (VSV) results in a progressive inhibition of cellular protein synthesis (Jaye et al., 1982; Marvaldi et al., 1977, 1978; McAllister & Wagner, 1976; Stanners et al., 1977; Wertz & Youngner, 1972). The inhibition occurs at the level of polypeptide chain initiation (Jaye et al., 1982; Nuss et al., 1975; Stanners et al., 1977). Cellular messages remain intact and functional (Jaye et al., 1982; Lodish & Porter, 1980, 1981) but are translated at a reduced rate. The total mRNA content of infected cells rises dramatically due to the synthesis of viral mRNA. However, the protein synthetic machinery of the cell is incapable of translating the additional mRNAs because of the progressive inhibition of protein synthesis that affects the translation of both viral and cellular mRNAs (Lynch et al., 1981; Stanners et al., 1977; Gillies & Stollar, 1982). Any advantages in protein synthesis that the virus enjoys during this continuing deterioration of the protein synthesis machinery of the cell may result from the relative abundance of viral messages in the cell.

Lysates from VSV-infected L cells are also severely inhibited in their ability to translate either endogenous or exogenous cellular and viral mRNAs (Centrella & Lucas-Lenard, 1982; Jay et al., 1982). Purified initiation factors may be added to this cell-free system to determine which factor or factors are able to increase protein synthesis in the lysate.

In our previous work (Centrella & Lucas-Lenard, 1982), we showed that eIF-2 can stimulate protein synthesis in lysates from infected cells but not in lysates from uninfected cells. We interpreted these results as suggesting that VSV regulates protein synthesis through a decrease in eIF-2 activity. Regulation of eIF-2 activity in other systems is thought to occur

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through phosphorylation of the factor, which prevents its catalytic utilization (Voorma et al., 1982; Safer et al., 1982).

The results presented in this report suggest that the catalytic utilization of eIF-2 may be impaired in lysates from infected cells, as demonstrated by the ability of guanine nucleotide exchange factor [for a review, see Safer et al. (1982)] to relieve the translation inhibition seen in these lysates. In this paper we further suggest that in addition to the decreased catalytic utilization of eIF-2, the mRNA binding factors eIF-4B and eIF-4F may be limiting in lysates from infected cells. These factors interact directly with the cap structure at the 5′-terminus of eukaryotic messages (Edery et al., 1983) and are required for the efficient utilization of these messages (Grifo et al., 1983).

Materials and Methods

Cell Growth. Mouse L cells (L-929) were grown at 37 °C in spinner flasks in Eagle minimal essential medium supplemented with 10% fetal calf serum (complete medium). Cells were maintained at a density of approximately 6×10^5 cells/mL.

Growth of Virus and Infection of Cells. The heat-resistant strain of Indiana serotype HR-C VSV was used in this study. The stock preparations of VSV were grown at a multiplicity of infection (moi) of 10^{-3} plaque forming units (pfu) per cell in African green monkey kidney (Vero) cells and purified by centrifugation through 20–40% sucrose gradients as described by Wu & Lucas-Lenard (1980). Virus prepared in this way had a titer of approximately 1.5×10^{11} pfu/mL.

L cells grown in 1 L were collected by centrifugation, washed 3 times with phoshate-buffered saline (PBS), and then suspended at a density of 1×10^7 cells/mL in serum-free medium. Half of the cells were infected at an moi of approximately 50 pfu/cell. The remaining cells were mock-infected with PBS. Virus adsorption lasted for 45 min. at 37 °C, after which the cells were diluted with fresh complete medium to their original concentration (6×10^5 cells/mL). After 3.5 – 4 h the infected or mock-infected cells were collected by centrifugation for preparation of the cell-free extracts.

Preparation of Cell-Free Lysates from Mock-Infected and Infected Cells. Cells harvested as described above were washed 3 times with washing buffer and then suspended in hypotonic buffer for 8 min (Jaye et al., 1982). The swollen cells were lysed by using 20 strokes of a Dounce homogenizer and then centrifuged at 22000*g* for 15 min at 4 °C. The supernatant was adjusted to 1× HKMD with 10× HKMD (Jaye et al., 1982) and stored in small aliquots at −70 °C. The protein concentration of the lysates was determined by the method of Bradford (1976).

In Vitro Protein Synthesis in L Cell Lysates. The lysates, which usually constituted 20–40% of the total volume of the reaction mixture, were approximately 20–37 mg/mL. The reaction mixtures contained, in 25 μL, 18 mM potassium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (K-HEPES), pH 7.4, 72 mM potassium acetate, 3 mM magnesium acetate, 0.24 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 120 μg/mL creatine kinase, 10–20 μCi/mL [^{35}S]methionine (specific radioactivity, greater than 400 Ci/mmol), 100 μM each of all unlabeled amino acids except methionine, and 10 μM unlabeled methionine. After incubation for 30 min at 31 °C, a 2-μL sample from each reaction mixture was applied to a Whatman 540 filter paper disk to determine the amount of [^{35}S]methionine incorporated. The filters were placed in 5% trichloroacetic acid, boiled for 5 min, and then washed with 5% trichloroacetic acid followed by 95% ethanol and acetone. They were then dried and counted in a toluene-based scintillation cocktail in a liquid scintillation counter. Initiation factors were added in the amounts indicated in the figure and table legends.

Polyacrylamide Gel Electrophoresis of Proteins Labeled in Vitro. The products of the in vitro translation assays were analyzed according to the method of Laemmli (1970) in 10% polyacrylamide slab gels. Dried gels were subjected to autoradiography at room temperature with Kodak X-OMAT AR film. For analysis of the distribution of radioactivity in the specific polypeptides indicated in Figure 4, the bands were excised and incubated in vials contained 0.3 mL of NCS (Amersham Corp.) tissue solubilizer for 24 h at room temperature. Scintillation cocktail was then added to each sample for counting.

Purification of Initiation Factors. Rabbit reticulocyte initiation factors were isolated from the 0.5 M KCl ribosomal salt wash and further fractionated by phosphocellulose chromatography (Grifo et al., 1983). The phosphocellulose column was equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM dithiothreitol, 0.05 mM EDTA, and 10% glycerol. The unadsorbed fraction (K150) of the ribosomal salt wash contained initiation factors eIF-4A and eIF-4D (Grifo et al., 1983). The column was then washed with the above buffer containing 0.45 M KCl, followed by the same buffer containing 1 M KCl. Proteins eluting with 0.45 M KCl (K450 fraction) were subjected to sucrose gradient centrifugation in buffer containing 500 mM KCl as described by Grifo et al. (1983). This method allowed the separation of initiation factors eIF-4B and eIF-4F from eIF-3. The low molecular weight pool from the sucrose gradient fractionation, containing eIF-4B and eIF-4F, was further purified by adsorption to DEAE-cellulose in buffer containing 100 mM KCl. Both eIF-4B and eIF-4F were eluted with buffer containing 250 mM KCl. This fraction was made 0.5 M in KCl by the addition of 2 M KCl and applied to a Bio-Gel A-0.5 column (1.5 × 87 cm) equilibrated with buffer containing 0.5 M KCl (Grifo et al., 1983). Fractions containing eIF-4B and eIF-4F were pooled and dialyzed against buffer containing 0.1 M KCl and 15% polyethylene glycol.

Proteins eluting from phosphocellulose with 1.0 M KCl (K1000 fraction) were dialyzed against the above buffer containing 0.2 M KCl and applied to a CM-Sephadex C-50 column equilibrated with the same buffer (Mehta et al., 1983). The unadsorbed fraction contained a guanine nucleotide exchange activity (GEF), and eIF-2 was eluted by washing the column with buffer containing 0.4 M KCl. *Artemia* eIF-2 was isolated as described earlier (MacRae et al., 1979; Woodley et al., 1981). The reticulocyte (CM-Sephadex) and *Artemia* eIF-2 fractions were further purified by DEAE-Tris acryl and poly(C)-cellulose column chromatography as described by Mehta et al. (1983). The purity of the eIF-2 preparations obtained by these procedures was approximately 85%. Rabbit reticulocyte eIF-6 was partially purified from the postribosomal supernatant of lysates by an adaptation of the procedure described by Russell & Spremulli (1979) (C. L. Woodley and A. J. Wahba, unpublished results). The guanine nucleotide exchange factor (GEF) was prepared from the postribosomal supernatant of rabbit reticulocyte lysate as previously described (Mehta et al., 1983). Purity and subunit composition of the initiation factor preparations were determined by electrophoresis in polyacrylamide gels containing dodecyl sulfate (Laemmli, 1970).

Initiation Factor Assays. eIF-2 activity was determined by ternary complex formation in 75-μL reaction mixtures containing 0.2 mM GTP, 1–2 μg of poly(C)-cellulose fraction of

eIF-2, 3 pmol of yeast [^{35}S]Met-tRNA$_f$, and other components as previously described (MacRae et al., 1979). For assay of GEF activity, the replacement of eIF-2 bound [^{3}H]GDP with free GTP was determined in 75-μL reaction mixtures containing 1 mM Mg^{2+}, 0.8 pmol of eIF-2·[^{3}H]GDP, 0.2 mM GTP, and 0.08–0.1 pmol of GEF. After incubation for 10 min the reaction was terminated with cold buffer, filtered through nitrocellulose membranes, and the amount of eIF-2·[^{3}H]GDP retained was determined by liquid scintillation counting (Mehta et al., 1983). Under these conditions greater than 90% nucleotide exchange is observed. eIF-6, isolated from the postribosomal supernatant of rabbit reticulocyte lysates, was assayed in 75-μL reaction mixtures for its ability to prevent the Mg^{2+}-induced reassociation of ribosomal subunits. 80S ribosomes (3 pmol) isolated from dormant *Artemia* embryos were dissociated in the presence of buffer containing 100 mM KCl, 1 mM Mg^{2+}, and eIF-6 and incubated 5 min at 30 °C, and the Mg^{2+} concentration was adjusted to 5 mM. The level of dissociation was determined by fractionation of the reaction mixtures on sucrose gradients containing 5 mM Mg^{2+} (Russell & Spremulli, 1979). With the partially purified eIF-6 preparation used in these assays, approximately 10 μg of eIF-6 produced 1.5 pmol of ribosomal subunits. eIF-3 was assayed in a reconstituted translation system containing in 35 μL 1.2 μg of *Artemia* poly(A)-rich mRNA, 0.4 A_{260} unit of ribosomes from dormant *Artemia* embryos, 2 μM [^{3}H]leucine (3,060 cpm/pmol), 3 mM magnesium acetate, 100 mM KCl, 1 mM ATP, 0.2 mM GTP, 1 mM dithiothreitol, 0.5 μg of *Artemia* eIF-2, 25 μg of the 50–70% ammonium sulfate fraction of reticulocyte ribosomal salt wash, 1 μg of eIF-3, 0.3 μg of eIF-4B, 0.2 μg of eIF-4A, and other components as previously described (Woodley et al., 1981). eIF-4A, eIF-4B, and eIF-4F were assayed by their ability to bind ^{3}H-labeled poly(A)-rich mRNA, isolated from Novikoff ascites tumor cells, to nitrocellulose membranes (Grifo et al., 1983). Incubation mixtures contained in 75 μL 0.5 μg of [^{3}H]uridine-labeled ascites poly(A)-rich mRNA (46 000 cpm/μg), 5 mM Mg^{2+}, 1 mM ATP/Mg^{2+}, and, as appropriate, 3.4 μg of eIF-4A, 1.5 μg of eIF-4B, and 1.6 μg of eIF-4F. Samples of eIF-4A, eIF-4B, and eIF-4F, kindly provided by W. C. Merrick, were compared with our factor preparations in each assay, as well as by analysis in dodecyl sulfate/polyacrylamide gels. Our initiation factor preparations behaved identically with those obtained from W. C. Merrick.

Results

Effect of Various Fractions of the Ribosomal Salt Wash on Translation in Lysates from Mock-Infected and Infected L Cells. As shown by Jaye et al. (1982), cell-free lysates from infected cells, which are inhibited in their protein synthetic capacity in vivo, also showed decreased ability to translate endogenous and exogenous mRNAs in vitro. Since eIF-2 stimulated translation in extracts from infected but not mock-infected cells, it was concluded that a regulation of eIF-2 activity occurs in VSV-infected cells. However, eIF-2 was the only initiation factor examined, and it was thought important to test the specificity of the effect.

To determine if other initiation factors are involved with protein synthesis regulation in VSV-infected lysates, the ribosomal salt wash from rabbit reticulocyte ribosomes was fractionated by phosphocellulose chromatography as described under Materials and Methods section. Fractions eluting at 0.15 M KCl (containing eIF-4A and eIF-4D), 0.45 M KCl (containing eIF-4B, eIF-4F, and eIF-3), and 1 M KCl (containing eIF-2, eIF-5, and eIF-4C) were added to lysates from mock-infected or infected cells. None of the phosphocellulose

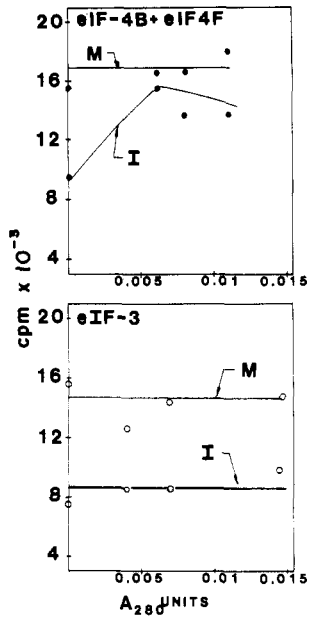

FIGURE 1: Ability of eIF-4B/eIF-4F and eIF-3 from reticulocyte ribosomal salt wash to stimulate incorporation of [^{35}S]methionine in lysates from mock-infected and infected L cells. L cells were mock-infected or infected with VSV at an moi of 50 pfu/cell. Lysates from these cells were prepared as described under Materials and Methods. The same amount of L cell protein (180 μg/25 μL of incubation mixture) was used in each reaction mixture. Symbols: (●) fraction after sucrose gradient analysis containing eIF-4B and eIF-4F; (○) fraction after sucrose gradient analysis containing eIF-3. The incubation was for 30 min.

fractions stimulated significantly incorporation of [^{35}S]-methionine in the mock-infected lysate. The K1000 fraction, on the other hand, significantly increased protein synthesis in the lysates from infected cells (data not shown). This result was not unexpected, since the K1000 fraction contains eIF-2. The K450 fraction also stimulated [^{35}S]methionine incorporation but to a much lesser extent (data not shown). In order to determine whether the slight stimulation by the latter fraction was significant, this fraction was purified further by sucrose gradient centrifugation. This procedure separated the K450 preparation into two fractions, a low-M_r pool containing eIF-4B and eIF-4F and a high-M_r fraction containing eIF-3. As shown in Figure 1, the eIF-3 fraction had no effect on translation in these lysates. However, the fraction containing the eIF-4B and eIF-4F increased incorporation of [^{35}S]-methionine in lysates from infected but not mock-infected cells (see also Figure 3 for a larger effect by the eIF-4B/eIF-4F fraction).

A partially purified fraction from the postribosomal supernatant containing eIF-6 (Russell & Spremulli, 1979; Valenzuela et al., 1982) was also tested for its ability to increase protein synthesis in infected lysates. No effect was observed (data not shown).

Because the preparation of eIF-2 used to stimulate protein synthesis in lysates from infected cells was only partially pure (Centrella & Lucas-Lenard, 1982), the effect of more highly purified fractions from two sources, rabbit reticulocytes and *Artemia*, was examined. The preparations contained the three usual eIF-2 subunits as shown by polyacrylamide gel electrophoresis (Woodley et al., 1981) and were estimated to be approximately 85% pure (Mehta et al., 1983). Both preparations were equally effective in stimulating incorporation of [^{35}S]methionine in lysates from infected cells (data not shown). The same factors, in contrast, had little effect on amino acid incorporation in lysates from mock-infected cells. The ability

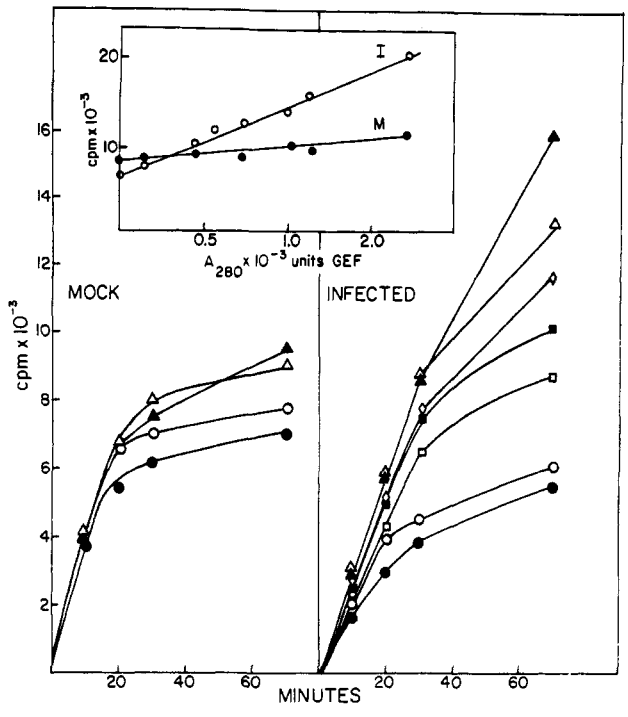

FIGURE 2: Effect of GEF on [^{35}S]methionine incorporation in lysates from mock-infected and VSV-infected L cells. The reaction mixture (25 μL) contained 0.14 mg of lysate protein and GEF as indicated. The K^+ concentration was 120 mM, and in addition, 4.5% of glycerol and 0.096 mM EDTA were present in the reaction mixture. Symbols: (●) no factor added; (○) 0.145 × 10^{-3} A_{280} unit of GEF; (□) 0.435 × 10^{-3} A_{280} unit of GEF; (■) 0.725 × 10^{-3} A_{280} unit of GEF; (◇) 1.015 × 10^{-3} A_{280} unit of GEF; (△) 1.16 × 10^{-3} A_{280} unit of GEF; (▲) 1.74 × 10^{-3} A_{280} unit of GEF. Two-microliter aliquots were removed at each time point for radioactivity measurement. Insert: The abscissa represents the amount of GEF A_{280} units added per 25-μL incubation. Incubation was for 120 min.

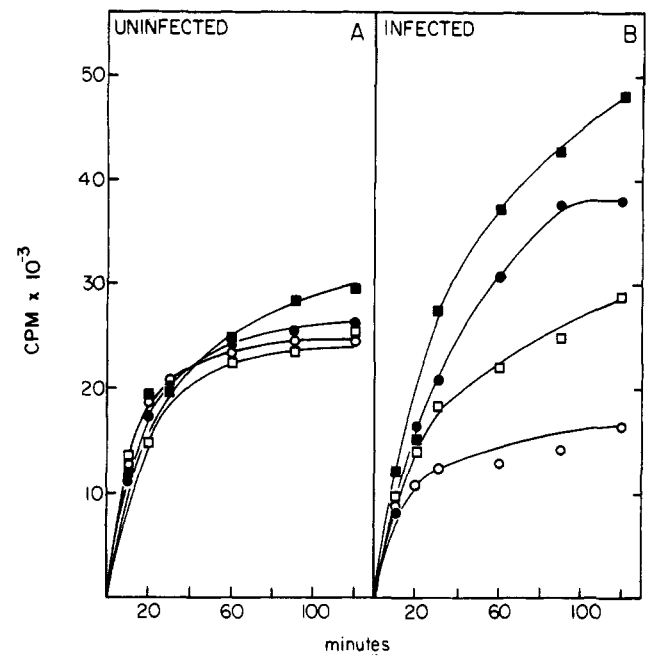

FIGURE 3: Time course of incorporation of [^{35}S]methionine into protein by lysates from mock-infected and infected L cells in the presence of eIF-2 and a phosphocellulose column fraction containing eIF-4B and eIF-4F. The reaction conditions were as described under Materials and Methods, except that 120 mM potassium acetate, 0.96 mM 2-mercaptoethanol, 4.5% glycerol, and 0.096 mM EDTA were present in the reaction mixture. Symbols: (○) no factors added; (●) 3.5 μg of purified rabbit reticulocyte eIF-2 fraction added; (□) 9.6 μg of eIF-4B/eIF-4F fraction added; (■) 3.5 μg of eIF-2 and 9.6 μg of eIF-4B/eIF-4F added.

of highly purified preparations of eIF-2 to reconstitute amino acid incorporation in extracts of infected cells suggests that the stimulatory activity in the K1000 fraction was not due to eIF-4C or eIF-5 which may be present in this fraction.

Because the nucleotide exchange factor (GEF) is capable of rescuing initiation in hemin-deprived reticulocyte lysates (Amesz et al., 1979; Grace et al., 1982; Panniers & Henshaw, 1983; Safer et al., 1982; Matts et al., 1983), the effect of different concentrations of this factor on the kinetics of [^{35}S]methionine incorporation by L cell lysates from both mock-infected and infected cells was examined. The results, shown in Figure 2, indicated a substantial stimulation of translation in both lysates, but particularly in the lysate from infected cells. The greater stimulation of translation in the latter lysate appeared to result from an extended period of initiation: in the lysate from mock-infected cells, incorporation was linear for, at most, only 20 min, whereas, in the lysate from infected cells, incorporation remained linear for at least 40 min when the highest amount of the nucleotide exchange factor was added. The effect on amino acid incorporation of GEF as a function of factor concentration is shown in the insert to Figure 2.

Time Course of [^{35}S]Methionine Incorporation in Extracts from Infected and Mock-Infected Cells. As shown in Figure 1, a fraction containing both eIF-4F and eIF-4B also stimulated [^{35}S]methionine incorporation in lysates from infected cells. Therefore, the effect of adding both eIF-2 and the sucrose gradient fraction containing eIF-4F and eIF-4B on incorporation of [^{35}S]methionine by these lysates as a function of time was examined. As shown in Figure 3, both fractions were required for maximal stimulation of translation in the lysate from infected cells. Furthermore, neither fraction alone had any significant stimulatory effect on the lysate from mock-infected cells. The two fractions together, however, stimulated translation in the lysate from mock-infected cells but not to the same extent as in the lysates from infected cells. Increasing the level of either the eIF-2 or eIF-4B/4F fraction alone in the incubation mixture did not stimulate translation to the same extent as with the two fractions together (data not shown).

It is important to emphasize that the time course of incorporation of [^{35}S]methionine was quite different in the lysates from mock-infected and infected cells (see Figure 3). The incorporation leveled off early in the lysates from both mock-infected and infected cells in the absence of added initiation factors. However, in the presence of both eIF-2 and the fraction containing eIF-4B and eIF-4F, the reaction continued for an extended period, particularly in the case of the lysate from infected cells. The fact that more radioactive methionine was incorporated into the lysate from infected cells than in the lysate from mock-infected cells was attributed at least partially, to this additional period of protein synthesis.

In order to determine whether the stimulation of incorporation by the two initiation factor fractions resulted from a uniform or specific increase in protein synthesis, samples from the 120-min time point in the experiment shown in Figure 3 were analyzed by polyacrylamide gel electrophoresis, and the autoradiograph of the results is presented in Figure 4. The bands marked by numbers were excised from the gel and their content of ^{35}S-labeled polypeptides analyzed by liquid scintillation spectroscopy. The radioactivity in host protein bands 2, 3, 5–7, 10, 12, and 13 were summed, and the total counts in these proteins under various conditions are shown in Table I. As can be seen, host cell protein synthesis in infected cell lysates was only 45% of that in mock-infected lysates. In the

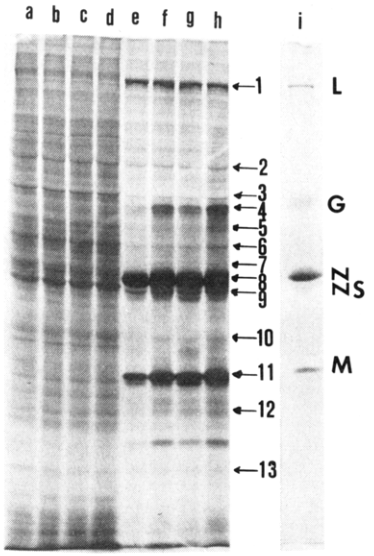

FIGURE 4: Polyacrylamide gel electrophoretic analysis of products synthesized in lysates from mock-infected and infected cells supplemented with eIF-2 and the eIF-4B/eIF-4F fraction. The 120-min time point from Figure 3 was used for this analysis, which was carried out as specified under Materials and Methods. Twenty microliters of the reaction mixtures was taken for electrophoretic analysis. Lanes a–d, reaction mixtures containing lysate from mock-infected cells; lanes e–h, reaction mixtures containing lysate from infected cells. Lanes a and e, no addition; lanes b and f, plus 3.2 μg of eIF-2; lanes c and g, plus 9.6 μg of eIF-4B/eIf-4F fraction; lanes d and h, plus 3.2 μg of eIF-2 and 9.6 μg of eIF-4B/eIF-4F fraction. i, VSV marker proteins containing N, NS, M, G, and L proteins. The numbers refer to the host and viral proteins that were excised for the analyses shown in Tables I and II.

Table I: Effects of eIF-2 and the eIF-4B/eIF-4F Fraction on Cellular Protein Synthesis in Extracts from Mock-Infected and VSV-Infected Cells

conditions[a]	mock infected (cpm)	infected (cpm)
no additions	12120	5463 (45)
+eIF-2	11832	6967 (59)
+eIF-4B/eIF-4F fraction	12748	7671 (60)
eIF-2 and eIF-4B/eIF-4F fraction	13922	11845 (85)

[a] The host bands designated in Figure 4 were excised and their radioactivity was determined as described under Materials and Methods. The numbers in parentheses represent the percent activity relative to the corresponding value for mock-infected cells. The amount of factor used is the same as that specified in Figure 3.

presence of saturating quantities of eIF-2 or the fraction containing eIF-4B and eIF-4F, protein synthesis activity was increased to 59 and 60%, respectively, of the mock-infected value. However, in the presence of both fractions, translation of host mRNAs was nearly completely recovered (85% of the mock infected). Although not every cellular protein band was excised from the gel and counted, we assume that those chosen were representative of the total.

The effect of these fractions of viral protein synthesis, except for the L protein, was very large as shown in Table II. The eIF-2 stimulated the synthesis of viral proteins G, N, NS, and M at least 2-fold. The increase by the eIF-4B/eIF-4F fraction was somewhat less. However, when both fractions were added to the lysate from infected cells, maximal stimulation was obtained (3.1-fold overall). When the blank values, indicated in the footnotes to Table II, were subtracted, one sees that addition of the combined initiation factor preparations stimulated synthesis of N protein 2.5-fold, NS 5-fold, M 8-fold, G 12-fold, and L not at all. The linear kinetics of incorporation of [^{35}S]methionine in the presence of the two initiation factor

Table II: Effect of eIF-2 and the eIF-4B/eIF-4F Fraction on Protein Synthesis in Extracts from Infected Cells

viral[a] protein	after infection (cpm)	+eIF-2 (cpm)	+eIF-4B/eIF-4F (cpm)	+eIF-2 and eIF-4B/eIF-4F (cpm)
L	2408[b]	2222	2078	2263
G	803	2453	1377	3137
N	14662	36561	22034	36645
NS	1776	3666	3673	6494
M	3498	12635	9117	23658
total	23147	57537 (2.5)	38279 (1.7)	72197 (3.1)

[a] The bands corresponding to VSV proteins (designated in Figure 4) were excised and their radioactivity was determined. The numbers in parentheses represent the *x*-fold stimulation. [b] The amount of radioactivity in host bands comigrating with viral bands could not be determined directly. To estimate the cpm contributed by host proteins, the radioactivity in the corresponding bands in the gel from the mock-infected lysate was measured and the values were reduced to accout for 55% protein synthesis inhibition. The estimates are (L) 644, (G) 662, (N) 1002, (NS) 908, and (M) 632 cpm.

Table III: Effect of eIF-4B, eIF-4F, and eIF-4A on [^{35}S]Methionine Incorporation in Lysates from Mock-Infected and Infected Cells

conditions[a]	mock infected (cpm)	infected (cpm)
no additions	37164	28872
+eIF-2 (0.62 μg)	39740	58854
+eIF-2 (0.62 μg) and eIF-4F (0.5 μg)	42587	66133
+eIF-2 (0.62 μg) and eIF-4B (0.9 μg)	42583	64156
+eIF-2 (0.62 μg) and eIF-4A (2.0 μg)	41385	67242

[a] The lysates used in this experiment were prepared according to Brown et al. (1983). The composition of the incubation mixture was the same as that described under Materials and Methods, except that the salt concentration was raised to 140 mM. The volume of the incubation mixture was 25 μL, and the temperature of the reaction was 30 °C. The samples represent the 60-min time point. The multiplicity of infection was 5 pfu/cell, and monolayer cultures of L cells were used. The samples represent the 5-h time point after infection, at which time in vivo there was a 30% inhibition of protein synthesis, as determined by [^{35}S]methionine incorporation.

fractions shown in Figure 3 suggests that message is undergoing several rounds of initiation.

In order to determine whether eIF-4B or eIF-4F was responsible for the stimulation of viral protein synthesis seen in Figure 4 and Table II, we separated these factors using the method of Grifo et al. (1983) and added them separately to lysates from infected and mock-infected cells. As shown in Table III, in the presence of eIF-2 both eIF-4B and eIF-4F stimulated translation in the mock-infected and virus-infected lysates. However, the stimulation in the latter lysate was greater than the stimulation in the former lysate. Since eIF-4F contains a subunit that may be equivalent to eIF-4A (Grifo et al., 1983), the effect on these lysates of eIF-4A (kindly donated by W. C. Merrick) was examined. As shown in Table III, eIF-4A was as effective as eIF-4F in stimulating lysates from both infected and mock-infected cells.

Cell Specificity of Enhanced Viral mRNA Translation in Lysates from Infected Cells. In order to determine if the large stimulation of endogenous viral mRNA translation seen in lysates of infected cells in the presence of initiation factors eIF-2 (or GEF and eIF-4B/eIF-4F) (see Figures 2 and 3) was specific to L cell lysates, similar lysates were prepared from uninfected HeLa cells and HeLa cells infected with VSV. Figure 5 shows that the combination eIF-2/eIF-4B/eIF-4F fraction stimulated incorporation of [^{35}S]methionine nearly 5-fold in the lysate from infected cells and only 2-fold in the lysate from uninfected cells, consistent with the results obtained with the L cell lysates. As in the case of L cells, the stimulation resulted primarily from an increase in viral protein synthesis

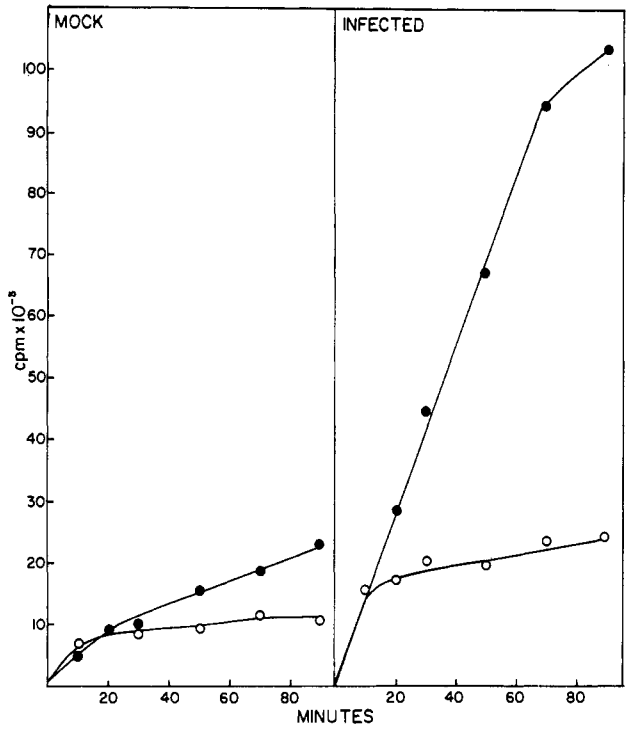

FIGURE 5: Effect of initiation factors on translation of endogenous mRNA in lysates from mock-infected and VSV-infected HeLa cells. Lysates from HeLa cells grown in suspension culture were prepared as described for L cells under Materials and Methods. The reaction conditions were those described under Materials and Methods and Figure 2. Symbols: (○) no additions; (●) plus 9.6 μg of reticulocyte eIF-4B/eIF-4F fraction and 3 μg of *Artemia* eIF-2.

(data not shown), suggesting that the effects seen in L cell lysates are not cell specific.

Discussion

As shown here and in previous reports from this laboratory (Centrella & Lucas-Lenard, 1982; Dunigan & Lucas-Lenard, 1983; Jaye et al., 1982), lysates from VSV-infected cells are severely inhibited in their ability to translate both cellular and viral messages. Rabbit reticulocyte lysates contain components that are capable of increasing the translation of these endogenous messages. In this paper we have attempted to identify those components that stimulate maximally both viral and cellular protein synthesis in the lysates from infected cells. Our previous results (Centrella & Lucas-Lenard, 1982) indicated that addition of eIF-2 increases translation in lysates from infected L cells. The data in this report demonstrate that in addition to eIF-2, other rabbit reticulocyte fractions containing GEF and mRNA binding proteins eIF-4B, eIF-4F, and eIF-4A also stimulate translation.

Because eIF-2 stimulated translation in the lysate from infected cells but not that in mock-infected cells [see Centrella & Lucas-Lenard (1982), Figure 3 and Tables I and II], we concluded that infection of L cells by VSV results in the regulation of eIF-2 function. The observation that the guanine nucleotide exchange factor (GEF) can substitute for eIF-2 in restoring protein synthesis in lysates from VSV-infected cells supports our earlier interpretation and strongly suggests that upon VSV infection eIF-2 may become phosphorylated and consequently unable to recycle. Under various conditions, including the presence of double-stranded RNA (Hunter et al., 1975; Farrell et al., 1977), interferon (Roberts et al., 1976), and heme deficiency (Farrell et al., 1977), a protein kinase is activated that phosphorylates the α-subunit of eIF-2. When the α-subunit of eIF-2 is phosphorylated, GEF binds to the eIF-2–GDP binary complex, but GTP is unable to displace the GDP as occurs during nucleotide exchange with the nonphosphorylated factor. eIF-2 cannot bind GTP and Met-tRNA$_f$ until the GDP from the previous cycle is removed. Work is currently in progress to determine whether the phosphorylation state of eIF-2 is altered in L cells after VSV infection.

As shown in Figures 1 and 3 and Tables I and II, the mRNA binding proteins alone stimulated translation in lysates from infected cells but not lysates from uninfected cells. These results suggested that the infected lysates are deficient in mRNA binding factors. This would not be an unusual case, for in Semliki Forest virus-infected cell lysates, there is a decreased level of both eIF-4B and eIF-4F activity (van Steeg et al., 1981). The apparent limitation in the mRNA binding proteins in VSV-infected lysates may result from the large increase in the mRNA content of these cells as the result of viral mRNA synthesis. Lysates from infected cells reflect this abundance of viral messages (see Figures 2, 3, and 5). In some cases the template activity of the lysates exceeds 3-fold that of lysates from uninfected cells (Jaye et al., 1982; Lodish & Porter, 1980). There may simply not be enough binding protein present in these lysates to saturate the system.

A curious aspect of these experiments is that the eIF-2 and mRNA binding factors stimulated viral protein synthesis to a much greater extent than cellular protein synthesis. These results suggest that viral mRNA translation is more inhibited than cellular mRNA translation. The observation that viral, as well as cellular, protein synthesis is inhibited in infected lysates is consistent with the results of Lynch et al. (1981), who noticed that VSV mRNAs on polysomes at 3 h after infection are not translated in vivo as efficiently as mRNAs at 2 h after infection in Chinese hamster ovary (CHO) cells. Although it might be argued that the inhibition of viral mRNA translation results from a reduced efficiency of the host cell translational system to translate VSV mRNAs later in infection, more recent evidence points to a different mechanism (Rosen et al., 1982, 1984). Rosen et al. (1982, 1984) have found that with increasing time of infection, an mRNA–ribonucleoprotein particle (mRNP) accumulates in VSV-infected CHO cells that contains all five VSV mRNAs and, almost exclusively, viral N protein. These authors have further noted that by 4.5 h after infection, 97% of the viral mRNA in vivo is associated with the mRNP and only 3% is on polysomes. The authors have suggested that the mRNP serves as an organelle that sequesters the excess VSV messages that are synthesized during secondary transcription.

Interestingly, Rosen et al. (1982, 1984) have also found that the viral mRNP inhibits in vitro protein synthesis, more specifically, decreases ternary (eIF-2–GTP–Met-tRNA) complex formation. The inhibition is partially reversed by the addition of purified eIF-2.

Besides the possibility mentioned earlier that eIF-2 in L cells may be regulated through phosphorylation of the eIF-2 α-subunit, the above discussed model must also be considered. It is possible that in L cells infected by VSV, similar viral mRNPs form that somehow decrease eIF-2 and mRNA binding protein activity. Indeed, the large increase in the translation of VSV messages in lysates from infected cells upon addition of eIF-2 and the mRNA binding proteins could result from the activation of such translationally sequestered viral mRNPs.

It should be pointed out that the inhibition of protein synthesis by such viral mRNPs could only account for part of the decrease in translation seen in VSV-infected cells. As shown by Dunigan & Lucas-Lenard (1983), expression of only the

first 40 or so nucleotides from the 3'-end of the viral genome is sufficient to decrease protein synthesis of L cells significantly.

It should be noted that two different methods were used to disrupt cells and to prepare the lysates in these experiments: one involved hypotonic lysis of the cells and the other a detergent treatment (Brown et al., 1983). In both cases the results were similar, indicating that they were not a function of the technique used in the preparation of the lysates. These data also suggest that the stimulatory effect of the eIF-2 and mRNA binding proteins is not cell specific, for the same enhancement was observed in lysates from HeLa cells as in lysates from L cells (see Figure 5).

In summary, the results presented in this report lead us to conclude that VSV-infected L cell lysates are defective in the catalytic utilization of eIF-2, as suggested by the strong dependence on added GEF. Although contamination of the mRNA binding protein preparations with other initiation factors cannot be ruled out at this point, these data suggest that mRNA binding protein activity including eIF-4A, eIF-4B, and eIF-4F may also be limiting in viral-infected cell lysates.

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