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The Viral Protein Sigma 3 Participates in Translation of Late Viral mRNA in Reovirus-Infected L Cells†

RÉAL LEMIEUX,‡* GUY LEMAY, AND STEWART MILLWARD
Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6

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Reovirus late (uncapped) mRNA was previously shown to be efficiently translated in vitro in extracts prepared from infected cells but not from uninfected cells. We demonstrated that different fractions from infected cells can stimulate translation of late viral mRNA when added to uninfected extracts. The activity of the different fractions correlated with their relative content of the σ 3 capsid protein; the fraction prepared by high-salt wash of the ribosomes had the highest specific activity. The activity present in this fraction was abolished by preincubation with an anti-σ 3 serum. Purified σ 3 protein also stimulated the translation of late viral mRNA, confirming that it was the factor involved. Altogether, these results suggest that this protein plays the role of a late-viral-mRNA-specific initiation factor. The absence of an inhibitory effect of σ 3 on the translation of other mRNAs indicates that this protein is not directly involved in the inhibition of host and early viral mRNA translation that occurs in infected cells but that a second mechanism is probably operative.

Infection of mouse L cells with reovirus type 3 results in a gradual inhibition of host protein synthesis, so that at late times postinfection, predominantly viral proteins are synthesized (23). Previous studies in our laboratory revealed that the protein synthesis machinery of the host cell is modified after reovirus infection, since the viral mRNA from late-infected cells is translated more efficiently in infected-L-cell extracts than in uninfected-L-cell extracts (8, 16). The finding that the late viral mRNA synthesized by progeny subviral particles (22) and present on polyribosomes in late-infected cells (16) has an uncapped pGp. . .-terminated 5′-end structure suggested that the modification of the translational machinery may consist of the inactivation of the cap-binding proteins (CBPs) by the reovirus, a precedent already established for poliovirus infection of HeLa cells (5, 20). However, for reovirus, several lines of evidence suggested that the inactivation of the CBPs could not fully account for the mRNA discrimination observed in the in vitro translation systems. First, unlike poliovirus RNA, late reovirus mRNA is not translated efficiently in extracts from uninfected cells, even in the presence of antibodies directed against the CBPs (19). Furthermore, the translation of late viral mRNA in extracts from infected cells is sensitive to inhibition by the cap analog m^7GTP (8). These findings indicated that an alteration in the activity of the CBPs might not be the only mechanism involved in the takeover of host protein synthesis during the late stages of infection of L cells with reovirus.

To shed light on the molecular basis of the mRNA discrimination observed in infected cells, we fractionated the in vitro cell-free systems in an attempt to characterize the factor responsible for the preferential translation of late viral mRNA in infected extracts. Results showed that the distribution of the factor activity among different fractions was similar to the distribution of the viral protein σ 3. The possibility that the viral capsid protein σ 3 participates in the translation of late viral mRNA was studied further by adding σ 3-specific antibodies or purified σ 3 protein to the translation systems. The results obtained clearly indicated that the presence of the σ 3 protein is essential for the efficient translation of the late viral mRNA in reovirus-infected cells. This effect of σ 3 probably plays an important role in the regulation of host protein synthesis in the late stages of reovirus infection, as previously suggested by mRNA competition experiments, showing that the late viral mRNA can easily outcompete the host cell mRNA for translation in extracts prepared from late-infected cells (8).

MATERIALS AND METHODS

Cells and virus. The mouse L cells were grown in suspension cultures at 37°C and infected with reovirus type 3 (Dearing strain) at 10 PFU/cell as previously described (8). The reovirus was purified by Freon extraction and isopycnic banding in CsCl gradients as described elsewhere (17).

For the in vivo labeling of viral proteins, 10^9 cells were infected with reovirus and incubated for 25 h at 31°C. The cells were centrifuged and suspended in 400 ml of methionine-free minimum essential medium containing 2% fetal calf serum (heat inactivated and dialyzed) and 20 ml of normal minimum essential medium (the final concentration of methionine was thus 1/20 of normal); [^{35}S]methionine was added (2 mCi at 1,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and incubation was continued for 4 h at 31°C. The incorporation of radioactive methionine was stopped by adding 1 liter of normal minimum essential medium, followed by an incubation of 1 h at 31°C. The cell fractions were prepared as described below for the unlabeled cells.

Preparation of RNA. Reovirus capped mRNA was synthesized in vitro with reovirus cores and was purified by Sephadex G-100 chromatography as described elsewhere (15). The uninfected L-cell mRNA and the reovirus late mRNA were extracted from the polyribosomal fraction of uninfected and late-infected (30 h at 31°C) cells, as described previously (16). The tobacco mosaic virus (TMV) RNA was

* Corresponding author.

† This report is dedicated to the memory of Stewart Millward, who died on 19 September 1985.

‡ Present address: Canadian Red Cross, Quebec, Quebec, Canada G1K 2L4.

prepared as described previously (8). The concentration of RNA was determined by A_{260}, assuming an $E_{1\ mg/ml}^{1\ cm}$ of 25.

Preparation and fractionation of L-cell extracts. The S-10 extracts were prepared from 2×10^9 uninfected and reovirus-infected (30 h at 31°C) L cells as previously described (8, 14). Immediately after their preparation, the S-10 extracts were centrifuged at 4°C for 1 h at 50,000 rpm in a model 50 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant which constituted the 200,000 × *g* supernatant fraction was designated S-200. The ribosomal pellets and aliquots of S-200 were frozen in liquid nitrogen and stored at −70°C. Subsequently, the ribosomal pellets were thawed in the palm of the hand and suspended at 4°C in about 0.6 ml of HKM buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–KOH [pH 7.5], 10 mM potassium acetate, 1.5 mM magnesium acetate) containing 1 mM dithiothreitol (DTT). After 1 h of incubation at 4°C with frequent homogenization with a Pasteur pipette, the suspension was clarified by a centrifugation of 1 min at 12,000 × *g* in an Eppendorf microcentrifuge. The supernatant was adjusted to 0.5 M KCl by adding one-fifth the volume of 3 M KCl, incubated for 15 min at 4°C, and centrifuged at 4°C for 1 h at 200,000 × *g*. The supernatant (about 0.8 ml) constituted the ribosomal salt-washed protein (RSW) fraction and was immediately dialyzed at 4°C for 4 h against 1 liter of RSW buffer (10 mM HEPES–KOH [pH 7.5], 100 mM KCl, 0.05 mM EDTA, 6 mM 2-mercaptoethanol). The dialysis buffer was changed three times. After dialysis, the RSW fraction was clarified by a centrifugation of 1 min at 12,000 × *g*, aliquoted, and stored in liquid nitrogen. The pellet of ribosomes (salt-washed ribosomes) was suspended as above in 0.8 ml of HKM-DTT buffer, clarified, aliquoted, frozen in liquid nitrogen, and stored at −70°C.

The concentration of ribosomes was estimated by measuring the optical density at 260 nm, assuming an $E_{1\ mg/ml}^{1\ cm}$ of 12. The concentration of protein in the various fractions was measured with the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard protein.

In vitro translation systems. The conditions for in vitro translation of the various types of mRNA in nuclease-treated S-10 extracts have been described previously (8, 14). For the supplementation assays, the standard incubation mixture consisted of the following: 6 μl of nuclease-treated extract prepared from uninfected cells, 4 μl of the master mix solution, 6 μl of water or other additions, and 4 μl of the fractions (S-200, RSW, and ribosomes) containing the indicated amounts of proteins; the final volume was 20 μl. The polyribosomal RNA from infected cells was added to a final concentration of 200 μg/ml. The master mix solution (5×) contained 50 mM HEPES–KOH (pH 7.5), 500 mM potassium acetate, 2.5 mM magnesium acetate, 250 μM spermidine, 5 mM DTT, 50 mM creatine phosphate, 2 mg of creatine phosphokinase per ml, 2.5 mM GTP (pH 7.0), 100 μM each of 19 amino acids (minus methionine), and 2 mCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear) per ml. Before its addition in the supplementation assay, the S-200 supernatant was dialyzed overnight at 4°C against 500 volumes of a solution consisting of 10 mM HEPES–KOH (pH 7.5), 100 mM potassium acetate, 0.5 mM magnesium acetate, and 1 mM DTT to adjust the concentration of salts and lower the cold methionine pool. The concentration of the potassium and magnesium salts present in the master mix solution was decreased according to the amount of these salts present in the dialyzed fractions added. Also, the ribosomal fractions were treated with micrococcal nuclease, as described previously (14), to inactivate the mRNA present in the salt-washed ribosomes. Incubation of the translation reactions was at 30°C for 2 h. The amount of [^{35}S]methionine incorporated into proteins was determined by trichloroacetic acid precipitation as described previously (14).

To study the effect of the immune sera on the in vitro translation of reovirus mRNA, the RSWs (2 μg) were first incubated for 15 min at 4°C alone or with 1 μl of control or immune sera. The goat immune serum against reovirus type 3 was obtained from MA Bioproducts (Walkersville, Md.). The rabbit monospecific serum against the σ 3 protein was kindly provided by P. W. K. Lee (Calgary, Alberta, Canada). Before their use, the sera were dialyzed overnight at 4°C against 500 volumes of a solution of 10 mM HEPES–KOH (pH 7.5) and 50 mM potassium acetate to adjust the concentration of salts.

Poly(I)-poly(C) chromatography. The σ 3 protein was purified from the RSW fraction of late-infected cells by affinity chromatography on poly(I)-poly(C) cellulose, essentially as described previously (3). The RSW fraction was adjusted to 200 mM KCl and 5 mM $MgCl_2$ and applied to a 2-ml column of poly(I)-poly(C) cellulose. The column was washed sequentially with the RSW buffer containing 200 and 400 mM NaCl. The bound material was then eluted with a solution consisting of 10 mM HEPES–KOH (pH 7.5), 1.0 M NaCl, 5 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. Under these conditions, about 1% of the RSWs bound firmly to the poly(I)-poly(C) column and were eluted with the 1.0 M NaCl buffer. The various fractions were dialyzed overnight at 4°C against 2 liters of RSW buffer and stored in aliquots in liquid nitrogen.

SDS-polyacrylamide gel electrophoresis. To analyze the proteins synthesized in the in vitro translation system, 5-μl portions of the various translation mixtures were diluted with 25 μl of sample buffer, heated for 2 min at 100°C, and electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel in the Laemmli buffer system (4). After the electrophoresis (20 h at 50 V), the gels were fixed, dried on Whatman 3MM filter paper, and exposed for 3 days against Kodak X-Omat AR-5 film. The analysis of the proteins present in the cell fractions was done by electrophoresis on SDS–10% polyacrylamide gels in the Laemmli buffer system or in the phosphate-SDS-urea buffer system described previously (17). The proteins were stained with Coomassie brilliant blue or with silver reagent (Bio-Rad silver stain kit). Standard proteins of known molecular weights were obtained from Bio-Rad.

RESULTS

Stimulation of late viral mRNA translation in extracts from uninfected cells. We previously showed that the late reovirus mRNA is translated much more efficiently in extracts prepared from late-infected cells than in extracts from uninfected cells (8, 16). To determine the molecular basis for this mRNA discrimination, we fractionated the extracts into the 200,000 × *g* supernatant (S-200), the RSW, and the ribosomes. The results obtained with the reconstituted cell-free systems suggested that the factor responsible for the efficient translation of reovirus late mRNA was present in the three fractions prepared from infected cells (data not shown). Since the fractions prepared from uninfected cells did not inhibit the translation of this type of mRNA, we studied the possibility of supplementing the crude uninfected S-10 extracts with the fractions prepared from infected S-10 extracts

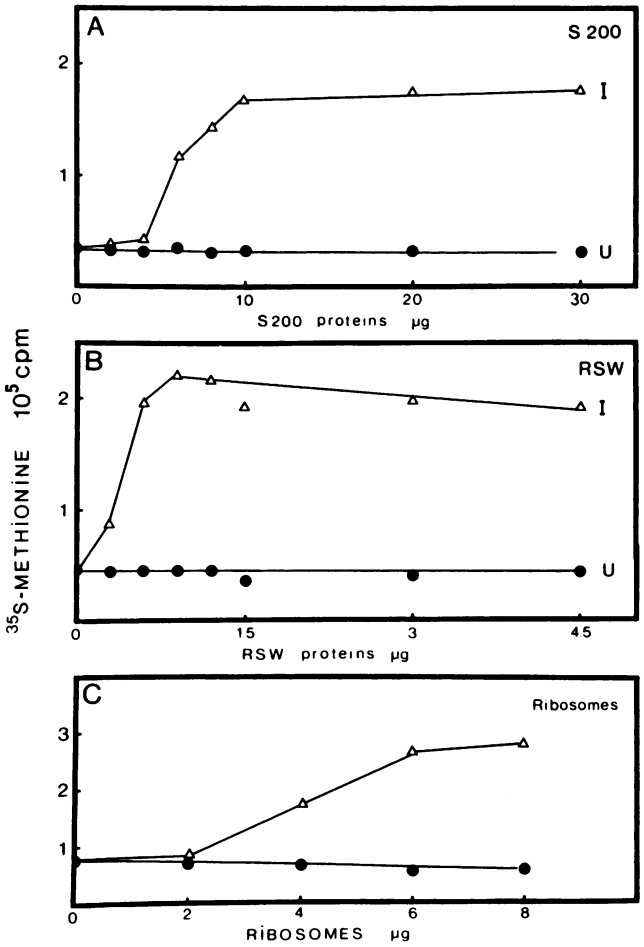

FIG. 1. Translation of late-infected cell polyribosomal RNA in nuclease-treated extracts prepared from uninfected cells. Shown is the effect of increasing amounts of proteins present in the S-200 fraction (A), RSW fraction (B), and ribosome fraction (C) prepared from uninfected (U; ●) and late-infected (I; △) cells. The values of incorporation of [^{35}S]methionine into proteins were determined from 5-µl aliquots.

to determine if the addition of these fractions could stimulate the translation of late viral mRNA in the uninfected extracts.

The effect of each of the three fractions on the translation efficiency of late reovirus mRNA in the uninfected extract is shown in Fig. 1. The addition of increasing amounts of the S-200, RSW, and ribosome fractions prepared from uninfected cells did not significantly affect the translation of late viral mRNA in the uninfected extract. This was expected, since the amount of total polysomal RNA added to the extract (200 µg/ml) was undersaturating (data not shown; 8). However, the addition of similar amounts of any of the three fractions prepared from infected cells produced dramatic increases in the translation efficiency of late viral mRNA. The curves obtained were sigmoidal and reached a plateau corresponding to a ca. fivefold stimulation of translation. This result shows that the factor responsible for the efficient translation of the late viral mRNA was present in the three fractions prepared from infected extracts. The finding that the stimulation of translation of late viral mRNA was dependent on the amount of the three fractions added to the uninfected extract (Fig. 1) allowed us to calculate the distribution of the factor activity. One unit of the factor was defined as the amount of protein that stimulated the translation of late viral mRNA threefold when added to the uninfected extract. The results are summarized in Table 1. The amount of protein per unit of factor varied in the three fractions, ranging from 5.5 µg for the S-200 to 4.6 µg for the ribosomes to 0.46 µg for the RSW. The assay for protein concentration allowed the quantitation of the total units present in the fractions. In this experiment, 51% of the factor was present in the RSW fraction, 44% was in the S-200 fraction, and only 5% was in the ribosome fraction (Table 1). The presence of 50% of the factor activity in the RSW fraction was probably a minimum, since it was found to be around 60% in two other fractionation experiments (data not shown). Also, the specific activity of the RSW fraction for the factor activity was 13-fold higher than that of the S-200 fraction.

The proteins synthesized in the uninfected extract in the presence of the RSW fraction were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). In the absence of exogenous RNA, no radioactive protein bands were detected, indicating that the RSW preparations did not contain significant amounts of mRNA (Fig. 2, lanes 1 to 3). In the presence of reovirus late mRNA, the addition of the RSW fraction from infected cells resulted in a dramatic increase in the synthesis of viral proteins (Fig. 2, lanes 10 to 12). Similar results were obtained with in vitro synthesized reovirus uncapped mRNA (data not shown). This result shows that the increased efficiency of translation of this type of mRNA in the presence of the infected-cell fractions (Fig. 1) corresponds to the synthesis of authentic viral proteins. Furthermore, the addition of the RSW fraction from uninfected or infected cells did not affect the translation efficiencies of several types of mRNA, including TMV RNA (Fig. 2, lanes 4, to 6), L-cell mRNA (Fig. 2, lanes 7 to 9), reovirus capped mRNA (see Fig. 4, lanes 13 and 14), globin mRNA, and poliovirus RNA (data not shown), indicating that the stimulation observed in the presence of the RSW fraction from infected cells is specific for reovirus late mRNA.

Additional experiments suggested that the molecule(s) present in the RSW fraction of infected cells and responsible for the stimulation of translation of late viral mRNA is probably proteinaceous, since it was retained after dialysis and was resistant to nuclease treatment but was rapidly inactivated at 50°C. Results previously obtained in our laboratory (D. Skup and S. Millward, unpublished observations) showed that antibodies against reovirus proteins inhibit the translation of reovirus uncapped mRNA in extracts from late-infected cells. This observation, combined with the high specificity of the factor activity, led us to study the possibility that a viral protein might directly participate in the translation of late viral mRNA in reovirus-infected cells.

Analysis of viral proteins in infected-cell fractions. Since the activity stimulating the translation of late viral mRNA was present in different amounts in the three fractions (S-200, RSW, and ribosomes) prepared from infected cells (Fig. 1), we compared the protein composition of these fractions by SDS-polyacrylamide gel electrophoresis (Fig. 3). The left part (Fig. 3A) is a photograph of a Coomassie brilliant blue-stained gel showing the total protein composition of the corresponding fractions prepared from uninfected and late-infected cells. For each fraction, the amount of protein analyzed (50 to 70 µg of protein) was not proportional to the distribution obtained after the fractionation procedure. Under the centrifugation conditions used, about 85% of the proteins present in the starting extract were isolated in the

TABLE 1. Subcellular distribution of the reovirus factor activity in infected cells

Cell fraction	Protein amt (μg/U)	Protein concn (mg/ml)	Vol (ml)	Total protein (mg)	Total U[a] (%)[b]	Sp act (U/mg of protein)
S-200	5.5	9.5	6	57	10,360 (44)	182
RSW	0.42	6.6	0.75	5	11,900 (51)	2,380
Salt-washed ribosomes	4.6	9.8	0.6	5.9	1,280 (5)	226

[a] One unit of the factor corresponds to the amount of proteins that stimulated the translation of late reovirus mRNA threefold in an uninfected-L-cell extract.
[b] Percentage of total units present in each fraction.

S-200 supernatant, and only about 7.5% of the proteins were isolated in the RSW and ribosome fractions. Because of this distribution, most of the proteins present in the crude extracts (Fig. 3A, lanes 1 and 2) were also present in the S-200 fraction (Fig. 3A, lanes 3 and 4), while the ribosome fraction (Fig. 3A, lanes 5 and 6) contained mostly low-molecular-weight proteins and the RSW fraction (Fig. 3A, lanes 7 and 8) contained mostly high-molecular-weight proteins. A close comparison of the protein patterns showed that the distribution of cellular proteins was similar in the corresponding fractions prepared from uninfected and late-infected cells. However, the fractions from infected cells contained three additional classes of proteins with molecular weights around 140,000, 75,000 and 42,000, corresponding to the λ, μ, and σ classes of viral proteins, respectively. Comparison of the distribution of these proteins in the cell fractions revealed that a viral protein with a molecular weight around 42,000, later shown to comigrate with the σ 3 protein present in reovirus, behaved unexpectedly during the fractionation procedure. Although the S-200 fraction contained 85% of the extract proteins, the amount of σ 3 protein present in this fraction was greatly reduced compared with the amount present in the crude extract (Fig. 3A, cf. lane 2 to 4). Conversely, this protein was greatly enriched in the RSW fraction from infected cells (Fig. 3A, lane 8). The relative distribution of this protein in the three fractions of infected cells is thus similar to that of the factor which stimulated translation of the late viral mRNA in extracts from uninfected cells. This σ 3 protein represents one of the smallest proteins present in the RSW fraction, suggesting that its presence in this fraction is not due solely to the centrifugation steps but to its association with fast-sedimenting material (perhaps the polyribosomes).

To better characterize the viral proteins present in the three fractions, infected cells were labeled at late times postinfection with [^{35}S]methionine and fractionated into the S-200, ribosome, and RSW fractions. Proportional amounts

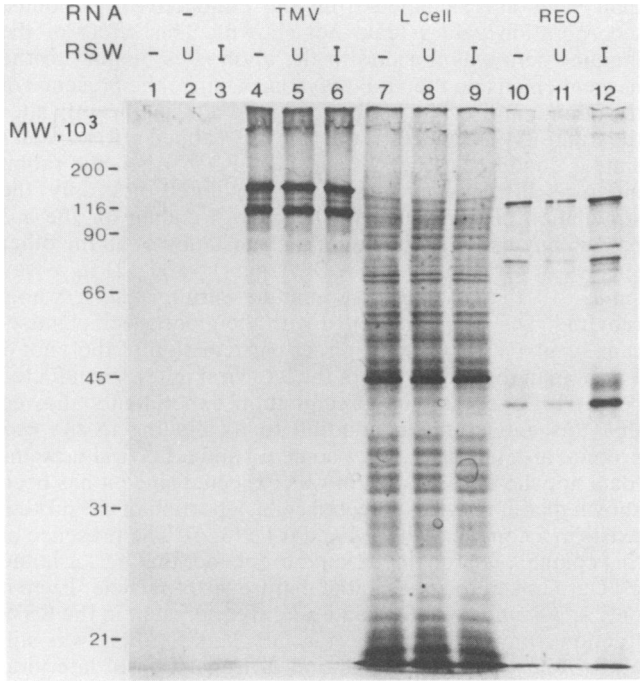

FIG. 2. SDS-polyacrylamide gel electrophoresis of the proteins synthesized in the uninfected-cell extract. The extracts were incubated in the absence of exogenous RNA (−) (lanes 1 to 3) and in the presence of TMV RNA (lanes 4 to 6), uninfected-L-cell polyribosomal RNA (L cell) (lanes 7 to 9), and late-infected-cell polyribosomal RNA (REO) (lanes 10 to 12) in the absence of RSW (−) and in the presence of 2 μg of the RSW fraction prepared from uninfected cells (U) and late-infected cells (I). Similar volumes of each reaction were analyzed on gels, followed by autoradiography. The migration of proteins of known molecular weight is shown on the left.

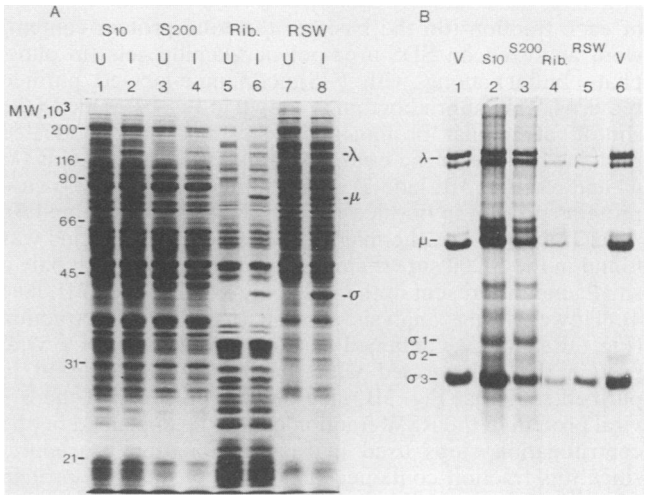

FIG. 3. SDS-polyacrylamide gel electrophoresis of the proteins present in the fractions prepared from the infected-cell extracts. (A) Similar amounts (50 to 70 μg) of the proteins of each fraction were analyzed on an SDS–10% polyacrylamide gel in the Laemmli buffer system; the proteins were then stained with Coomassie brilliant blue. Electrophoresis was done on whole extract (lanes 1 and 2), S-200 supernatant (lanes 3 and 4), salt-washed ribosomes (lanes 5 and 6), and RSW (lanes 7 and 8) prepared from uninfected cells (U) and late-infected cells (I). The migration of proteins of known molecular weight is indicated on the left side, and the reovirus proteins are indicated on the right side. (B) Proportional amounts of fractions prepared from [^{35}S]methionine-labeled late-infected cells were analyzed on a 7.5% polyacrylamide gel in the phosphate-SDS-urea gel system; shown here is an autoradiogram of the dried gel. V, Purified [^{35}S]methionine-labeled reovirus; S10, whole extract; S200, S-200 supernatant; Rib., salt-washed ribosomes. The reovirus proteins are identified on the left side.

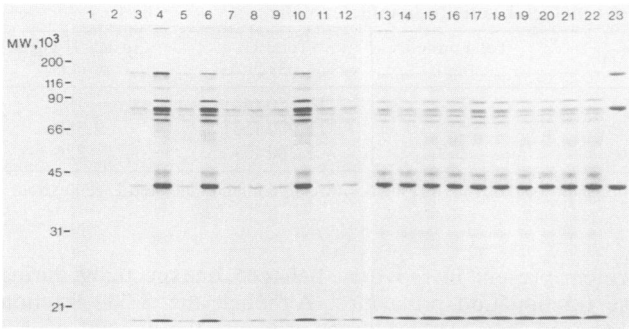

FIG. 4. Translation products of the polyribosomal late viral mRNA and the reovirus capped mRNA in uninfected-cell extracts in the presence of RSW and control or immune sera. Similar volumes (5 μl) of the translation mixtures were analyzed on an SDS–10% polyacrylamide gel, followed by autoradiography. Electrophoresis was done on translation products in the absence of exogenous RNA (lanes 1 and 2), in the presence of 200 μg of late-infected-cell polyribosomal RNA per ml (lanes 3 to 12), and in the presence of 20 μg of reovirus capped mRNA (synthesized in vitro by reovirus cores) per ml (lanes 13 to 22). The translation reaction was done in the presence of RSW (2 μg) prepared from uninfected cells in the odd-numbered lanes (lanes 1 to 21) and from late-infected cells in the even-numbered lanes. The RSWs were preincubated alone (lanes 3, 4, 13, and 14) and in the presence of goat control serum (lanes 5, 6, 15, and 16), goat antireovirus serum (lanes 7, 8, 17, and 18), rabbit control serum (lanes 9, 10, 19, and 20), and anti-σ 3 serum (lanes 11, 12, 21, and 22). Proteins from [^{35}S]methionine-labeled reovirus are shown in lane 23. The migration of proteins of known molecular weight is shown on the left side.

of each fraction (on the basis of the total protein content) were analyzed on SDS-urea-polyacrylamide gels (in phosphate buffer) along with [^{35}S]methionine-labeled purified reovirus. The autoradiogram is shown in Fig. 3B. Due to the shutoff of cellular protein synthesis, most of the labeled proteins present in the crude extract were viral proteins (λ, μ, and σ) (Fig. 3B, lane 2) comigrating with authentic viral proteins present in purified reovirus (Fig. 3B, lane 1 and 6). After fractionation, the majority of these viral proteins was found in the S-200 supernatant (Fig. 3B, lane 3), with only a small amount present in the ribosome fraction (Fig. 3B, lane 4). However, the analysis showed that the RSW fraction (Fig. 3B, lane 5) contained a significant amount of a viral protein that comigrated with the σ 3 protein present in purified reovirus (Fig. 3B, lane 6). The presence of the σ 3 viral protein in the RSW fraction cannot be explained by the centrifugation steps used in the fractionation procedure, since this fraction contained only 7.5% of the total protein content of the crude extract and low amounts of the viral proteins of higher molecular weight (λ and μ). These observations thus suggested that, in the crude extract, a proportion of the σ 3 protein was associated with fast-sedimenting structures (perhaps the polyribosomes) and that this association is sensitive to the high-salt medium used to prepare the RSW fraction. To determine if the presence of the σ 3 protein in the RSW fraction is necessary for the activity of the factor that stimulated the translation of the late viral mRNA, we studied the effect of antibodies against reovirus proteins on the in vitro translation of late reovirus mRNA.

In vitro translation of reovirus mRNA in the presence of antibodies against reovirus proteins. The effect of antisera against reovirus proteins was studied in the supplementation system described in Fig. 1, with RSW preparations from infected cells to supplement an extract from uninfected cells. The proteins synthesized in vitro were then analyzed by gel electrophoresis, followed by autoradiography. A photograph of the autoradiogram is shown in Fig. 4. The reaction mixtures were incubated in the presence of reovirus late mRNA (Fig. 4, lanes 3 to 12) or reovirus capped mRNA synthesized in vitro by reovirus cores (Fig. 4, lanes 13 to 22). To study the effect of the antisera, the RSW preparations were first incubated alone or with the various sera before their addition to the translation systems. In the absence of antiserum, the translation efficiency of reovirus late mRNA was much higher in the presence of the RSW fraction from infected cells than in the presence of the RSW fraction from uninfected cells (Fig. 4, lanes 3 and 4), as already seen in Fig. 1 and 2. However, the two RSW preparations did not affect the translation products of the reovirus capped mRNA (Fig. 4, lanes 13 and 14), which are known to be preferentially translated in extracts from uninfected cells (8, 15). The translation products of both types of mRNA comigrated with authentic viral proteins present in purified reovirus (Fig. 4, lane 23). For late viral mRNA, pretreatment of the RSW fractions with the nonimmune sera from goat or rabbit did not effect the stimulation of translation observed in the presence of the RSW fraction from infected cells (cf. lanes 5 and 9 with lanes 6 and 10 [Fig. 4]). In the presence of the immune sera, the results obtained were clearly different. The goat antiserum against whole reovirus (Fig. 2, lanes 7 and 8) and the rabbit monospecific antiserum against σ 3 (Fig. 2, lanes 11 and 12) inhibited most of the stimulation of translation of late viral mRNA observed in the presence of the RSW from late-infected cells. The inhibition was greater than 95%, as calculated from the radioactive methionine incorporation values (data not shown). This effect of the immune sera was not due to the antibodies binding to the nascent chains on the viral polysomes, since the presence of the nonimmune or the immune sera did not significantly alter the translation products of the reovirus capped mRNA under similar conditions (Fig. 4, lanes 12 to 22). Also, the rabbit monospecific serum against σ 3 inhibited not only the stimulation of translation of the mRNA coding for the σ 3 protein but also the stimulation of translation of all the other species of viral mRNA (Fig. 4, lanes 11 and 12) in a way similar to that of the goat immune serum against whole reovirus. The results obtained with the monospecific antiserum against σ 3 suggested direct participation of the viral σ 3 protein in the translation of the late viral mRNA in infected cells. However, immunoprecipitation experiments showed that this antiserum, in addition to its binding to the σ 3 protein, also interacted with some μ 1 and μ 1 c viral proteins (data not shown). This result was expected since it has been shown that in reovirus-infected cells, a portion of σ 3 protein exists in a complex with μ 1 and μ 1 c (3, 6). The presence of this complex is probably related to the fact that σ 3, μ 1, and μ 1 c are linked together in the mature virus particle. Even if only small amounts of μ 1 and μ 1 c were present in the RSW fraction from infected cells (Fig. 3B, lane 5), there was still a possibility that the stimulation of translation of late viral mRNA was due to the activity of the σ 3-μ 1-μ 1 c complex, instead of the native σ 3 protein. However, separation of the free σ 3 protein from the σ 3-μ 1-μ 1 c complex by sedimentation in sucrose gradients showed that the factor responsible for stimulatory activity cosedimented with native σ 3, with no activity present in the fractions containing a similar amount of σ 3 present in the higher-molecular-weight σ 3-μ 1-μ 1 c complex (data not shown). Since the free σ 3 protein can be purified from cell extracts by using its known affinity for double-stranded RNA (3), we have further

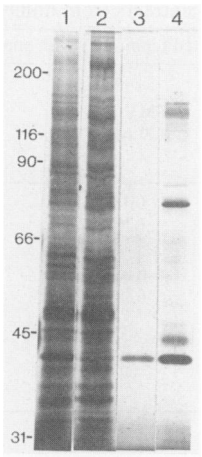

FIG. 5. SDS-polyacrylamide gel of the proteins present in the fractions of the poly(I)-poly(C) cellulose chromatography. The resolved proteins were stained with silver nitrate. Lane 1, RSW from infected cells (starting material); lane 2, unbound material (flowthrough); lane 3, 1.0 M NaCl eluate; lane 4, purified reovirus. The migration of proteins of known molecular weight is shown on the left side.

studied the effect of purified σ 3 protein on the translation of several types of mRNA in uninfected cell extracts.

Effect of purified σ 3 protein on the translation of mRNA in uninfected extracts. The σ 3 protein was purified from the RSW fraction of late-infected cells by chromatography on poly(I)-poly(C) cellulose (3) (Fig. 5). The proteins resolved by SDS-polyacrylamide gel electrophoresis were stained with silver nitrate, instead of Coomassie brilliant blue, because of the low amount of protein present in the 1.0 M NaCl eluate. The analysis showed that the 1.0 M NaCl eluate contained a single protein band which comigrated with the σ 3 protein present in purified reovirus (Fig. 5, lanes 3 and 4). The unbound material (flowthrough) contained a greatly reduced amount of the σ 3 protein compared with the starting material (Fig. 5, cf. lanes 1 and 2). The effect of the various fractions on the translation efficiencies of several types of mRNA in uninfected extracts is shown in Table 2. The results obtained in experiment 1 showed that the poly(I)-poly(C) chromatography almost completely abolished the capacity of the RSW from infected cells to stimulate the translation of late viral mRNA in uninfected cells. The finding (see above) that the factor responsible for the stimulatory activity cosedimented with free σ 3 in sucrose gradients indicates that most of the residual σ 3 in the flowthrough might be in the form of a σ 3-μ 1-μ 1 c complex. The addition of the purified σ 3 protein present in the 1.0 M NaCl eluate resulted in a clear stimulation of translation of this type of mRNA (fourfold in experiment 1 and twofold in experiment 2). In experiment 1 (Table 2), the stimulation of translation of the late viral mRNA by the purified σ 3 protein was similar to that obtained in the presence of the crude RSW fraction. The specific activity (calculated as described in Table 1) of the purified σ 3 fraction for the factor activity was 50-fold higher than that of the RSW fraction. Analysis by gel electrophoresis of the translation products showed that the stimulation of the translation efficiency of the late viral mRNA corresponded to the increased synthesis of authentic viral proteins (data not shown). Furthermore, the addition of the purified σ 3 protein did not affect the translation efficiencies of TMV RNA and L-cell mRNA, indicating that the σ 3 protein does not directly inhibit the translation of other types of mRNA.

DISCUSSION

Previous work from our laboratory showed that, after infection with reovirus type 3, the translational machinery of the host cell is modified at the level of mRNA recognition (8, 15, 16). The exact nature of the modification(s) has remained unclear, but it results in the shutoff of host protein synthesis and in the efficient translation of late viral mRNA. The inhibition of host and early viral capped mRNA translation observed in late-infected cells and in the corresponding cell-free translation systems led us to postulate that an inactivation of the CBPs could be responsible for the mRNA discrimination observed. Indeed, a decrease in the amount of active CBPs has been observed in extracts prepared from late-infected cells (H. Zarbl, R. Lemieux, and S. Millward, unpublished observations). Although such a modification could be responsible for the shutoff of host and early viral capped mRNAs, it could not explain the efficient translation of the late viral uncapped mRNA, since this type of mRNA was not efficiently translated in extracts from uninfected cells even in the presence of antibodies against CBPs (19). The finding that the late viral mRNA can easily outcompete the cellular mRNA for translation in late-infected cell extracts (8) suggested that the hypothetical factor responsible for this effect plays a predominant role in the regulation of translation at late times postinfection.

In the present report, we showed that the viral capsid protein σ 3 is the factor responsible for the efficient translation of late viral mRNA in reovirus late-infected cells. This conclusion was derived from analysis of in vitro translation of late viral mRNA in extracts from uninfected cells supplemented with fractions prepared from infected cells. The results showed that the amount of σ 3 present in each fraction (S-200, ribosomes, and RSW) correlated with the amount of factor activity (Table 1 and Fig. 3B). In this system, the addition of σ 3-specific antibodies completely inhibited the stimulation of reovirus late mRNA translation (Fig. 4); furthermore, the addition of purified σ 3 protein stimulated the translation of late viral mRNA in the absence of any fractions prepared from infected cells (Table 2). An additional important finding is that the addition of crude RSW preparation from infected cells or purified σ 3 protein did not stimulate or inhibit the translation of several other types of mRNA, including reovirus capped mRNA and L-cell mRNA (Fig. 2 and Table 2). Thus, the viral protein σ 3 exerts positive control over the translation of late viral mRNA, possibly by assuming the role of a late-viral-mRNA-specific protein synthesis initiation factor.

These results strongly support our previous hypothesis (8) that two different mechanisms of translational control must be operative in reovirus late-infected cells to explain both the reduced efficiency of translation of capped mRNAs (host and early viral mRNA) and the efficient translation of the late (uncapped) viral mRNA. The finding that the viral protein σ 3 can greatly stimulate the translation of the late viral uncapped mRNA, apparently without affecting the translation of the host and early viral capped mRNA, suggests that the modification responsible for the arrest of capped mRNA translation (perhaps the inactivation of the CBPs) plays a predominant role in the transition of the early to the late stages of the reovirus multiplication cycle, rather than in the takeover of the host protein synthesis at late times postin-

TABLE 2. Effect of purified σ 3 protein on mRNA translation in nuclease-treated extracts from uninfected cells

Expt no.	Fraction added	[^{35}S]methionine incorporated (cpm, 10^3) with supplement			
		None	Reovirus late-infected-cell polyribosomal RNA (200 μg/ml)	TMV RNA (20 μg/ml)	L-cell polyribosomal RNA (100 μg/ml)
1	None	3.9	70.6	607.6	
	RSW fraction of infected cells[a]	5.1	354.9	630.8	
	Poly(I)-poly(C) chromatography				
	Unbound material[b]	5.3	95.6	616.8	
	1.0 M NaCl eluate[c,d]	5.8	287.5	628.8	
2	None	5.4	20.0		24.9
	Poly(I)-poly(C) chromatography				
	1.0 M NaCl eluate[d]	5.7	40.3		23.0

[a] Starting material. Contains 2 μg of protein.
[b] Flowthrough. Contains 3.2 μg of protein.
[c] Contains 0.032 μg of protein.
[d] Prepared from different RSW preparations.

fection. The resulting inhibition of capped reovirus early mRNA translation would free this type of mRNA to serve as a template for minus-strand synthesis and the subsequent formation of progeny subviral particles. The correlation between a slower multiplication rate and the absence of inhibition of host protein synthesis in reovirus type 1 infection (9) supports this hypothesis. We have also recently observed that extracts from type 1-infected cells, despite the lack of host protein synthesis inhibition, also have an increased capacity to translate late viral mRNA compared with uninfected-cell extracts (unpublished observations). These last observations are also consistent with the presence of two types of translation control during type 3 infection. The recent finding that the expression of the cloned reovirus S4 gene (coding for the σ 3 protein) in transfected cell lines does not have any apparent harmful effect on the cells (7) while allowing efficient translation of the late viral mRNA (unpublished observations) also clearly supports the hypothesis that the σ 3 protein is not directly involved in the shutoff of host and early viral capped mRNA at intermediate times postinfection.

The mechanism by which the σ 3 protein can specifically stimulate the translation of late viral mRNA remains unclear and will require further investigation. However, it is reasonable to postulate that the uncapped 5′-end structure of the late viral mRNA (compared with the capped 5′-end structure of the early viral mRNA) (16, 22) is important for this effect. The most obvious mechanism would be that the σ 3 protein binds to a common structure present at the 5′ end of all 10 reovirus uncapped mRNAs and that the resulting structures facilitate recognition by the ribosomes. The previous observation that the σ 3 protein binds strongly to double-stranded RNA (3) suggests that the protein interacts with a secondary structure on reovirus uncapped mRNA; however, there is no evidence that σ 3 binds on mRNA. In fact, we have recently observed that the binding of σ 3 to the polysomal structure is not sensitive to degradation of mRNA by micrococcal nuclease (unpublished observations); furthermore, the σ 3 protein present in early-infected cells, as well as the σ 3 protein expressed in cell lines transfected with the reovirus S4 gene, is also bound to polysomes in the absence of viral uncapped mRNA (7). These results suggest that the σ 3 protein might instead interact with another protein synthesis initiation factor(s) and alter its specificity, so that late viral mRNA is translated efficiently without a normal cap structure. Additional experiments will be necessary to clarify this point and are in progress in our laboratory.

Recent results obtained in other systems suggest that the direct participation of viral protein(s) in the mechanisms of translational control might be a strategy used by several viruses either to confer upon the viral mRNA a translational advantage over host mRNA or to regulate the virus multiplication cycle. For Semliki Forest virus, it has been shown that a viral capsid protein can selectively inhibit the translation of cellular mRNA and early viral mRNA without affecting the translation of late viral mRNA (21). For vaccinia virus, a viral capsid protein can inhibit protein synthesis and cause a rapid shutoff of host protein synthesis at the early stage of infection (10). In these two cases, in contrast to the σ 3 protein effect reported here, the viral proteins appear to exert negative control over the translation of mRNAs. However, it has also been recently shown that two human T-cell lymphotropic virus type III and lymphadenopathy associated virus proteins (coded for by the *tat*III and *art* genes), apparently exerting positive control on the expression of viral mRNA, probably act at the level of translation (12, 18). It has been postulated that these viral proteins might control the latent and lytic phases of the virus (18). A positive effect of a viral (or virus-induced) initiation factor has also been shown to be responsible for the translation of late viral mRNA of frog virus 3 (11), but the exact nature of this factor is unknown.

In the case of reovirus, genetic recombination experiments have also shown that the S4 gene coding for the σ 3 protein is responsible for the inhibition of host protein synthesis after infection with type 2 reovirus (13). This is additional evidence that the σ 3 protein has a central role in the control of protein synthesis during reovirus infection; the exact situation occuring in type 2 infection is still incompletely known. Also, it has been shown that high-passage reovirus stocks contain a mutated S4 gene (1) and that this gene is apparently involved in the establishment of persistent reovirus infections (2). The finding that the σ 3 protein is essential for the efficient translation of viral mRNA in late-infected cells provides new perspectives concerning the mechanism by which the σ 3 protein might be involved in these phenomena and suggests that its role is crucial for the normal virus multiplication cycle.

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