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Translation of Reovirus Messenger RNAs Synthesized *In Vitro* into Reovirus Polypeptides by Several Mammalian Cell-Free Extracts

(mouse ascites tumor/rabbit reticulocyte/HeLa cells/L cells/hamster ovary)

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ABSTRACT **Single-stranded reovirus RNA, synthesized *in vitro* by reovirus cores, functioned as messenger RNA in cell-free extracts prepared from several mammalian cells: Krebs II mouse ascites cells, mouse L cells, Chinese hamster ovary cells, HeLa cells, and rabbit reticulocytes. As shown by acrylamide gel electrophoresis, all eight polypeptides known to be specified by reovirus were synthesized in the reticulocyte system. In the other extracts, from 5 to 7 complete virus proteins were made.**

Many studies on mammalian *in vitro* protein synthesis became possible only when homogeneous species of messenger RNA could be prepared. Recently, several messenger RNAs isolated from differentiated animal tissues (1–11), as well as from animal viruses (12–18), have been translated *in vitro* into their corresponding polypeptides.

Single-stranded reovirus RNA is particularly suitable for study of protein synthesis *in vitro*, since large amounts of it can be synthesized *in vitro* by virion cores (19–23). In infected cells there are 10 species of reovirus messenger RNA, each corresponding to one of the 10 double-stranded RNA species in the virion, and 9 virus-specific polypeptides (molecular weights in parentheses); [λ_1 (155,000), λ_2 (140,000), μ_0 (88,000), μ_1 (80,000), μ_2 (72,000), σ_1 (42,000), σ_2 (38,000), σ_{2a} (36,000), and σ_3 (34,000)]. Polypeptide μ_2 is not a primary gene product, but is derived from μ_1 by proteolytic cleavage (24, 25). Why only 8, rather than 10, primary polypeptides can be resolved by gel electrophoresis is not clear; presumably, some reovirus proteins have the same molecular weight. All ten mRNAs are synthesized *in vitro* (19–23).

In vitro work with a cell-free extract that synthesizes proteins prepared from mouse fibroblasts (L cells) infected with reovirus has shown that endogenous reovirus mRNA is translated into all 8 primary viral polypeptides (26). When added to extracts of uninfected mouse L cells, reovirus RNA stimulates the synthesis of low molecular weight polypeptides (27); very recently, this RNA has been shown to stimulate the synthesis of several complete viral polypeptides (28).

We have developed, using modifications of published procedures (3, 5, 13, 15), a general procedure for preparing preincubated cell-free protein-synthesizing extracts from mammals that are dependent upon exogenous messenger RNA. In the experiments described below, we have identified 5 viral polypeptides, that were synthesized in response to added reovirus messenger RNA in cell-free extracts of ascites and HeLa cells, and 7 that were synthesized in extracts of mouse L and CHO cells. By contrast, by use of extracts from rabbit reticulocytes that are not preincubated, and that synthesize large amounts of globin, all eight known reovirus proteins are made.

Abbreviation: CHO cells, Chinese Hamster Ovary cells.

MATERIALS AND METHODS

Cells. Krebs II ascites cells were cultured by injection into peritoneal cavities of female white mice (strain CD1 purchased from Charles River Breeding Labs., Wilmington, Mass.); they were harvested after 7 days. Mouse L fibroblasts, CHO cells, and HeLa cells were grown in suspension culture in Eagle's minimal essential medium (Joklik's Modification, Grand Island Biological Co.) containing 5% fetal-calf serum.

Reticulocytes were obtained from New Zealand white rabbits as described (2), except that the rabbits were made anaemic by subcutaneous injection of 1.2% acetylphenylhydrazine according to the following schedule: 2 ml on day 1, 1.6 ml on day 2, 1.2 ml on day 3, 1.6 ml on day 4, and 2 ml on day 5.

Preparation of Cell-Free Extracts. Liquid tumors of ascites cells were harvested from five mice and filtered through cheesecloth into cold isotonic buffer (35 mM Tris·HCl, pH 7.5–146 mM NaCl–11 mM glucose). The cells were washed six times by differential centrifugation (80 × *g* for 5 min) in isotonic buffer to remove reticulocytes. Mouse L fibroblasts, HeLa cells, and CHO cells were each collected by centrifugation from 2 liters of suspension culture (1 to 2 × 10^9 cells), then washed three times with isotonic buffer. After washing, the procedure was identical for all four types of cells. One volume of packed cells was resuspended in 3 volumes of hypotonic buffer (10 mM Tris·HCl, pH 7.5–15 mM KCl–1.5 mM $MgAc_2$–6 mM 2-mercaptoethanol). After 10 min at 0°, the cells were disrupted in a Dounce homogenizer. 0.1 Volume of 10 × HEPES buffer (200 mM HEPES, pH 7.5–1200 mM KCl–50 mM $MgAc_2$–60 mM 2-mercaptoethanol) was then added, the homogenate was centrifuged at 30,000 × *g* for 20 min, and the pellet was discarded. ATP was added to a final concentration of 1 mM, GTP to 0.2 mM, creatine phosphate to 8 mM, and creatine kinase to 0.2 mg/ml, and the extract was incubated at 37° for 30 min. The extract was then passed at 4° through a Sephadex G-25 column (3 × 30 cm) that had been equilibrated with 1 × HEPES buffer. The opalescent fractions were pooled and stored at −70° in small aliquots.

Preparation of reticulocyte extract has been described (2).

Preparation of Reovirus Messenger RNA. The Dearing strain of reovirus type 3 was used. Procedures for the growth and purification of the virus have been described (29). Purified virus was treated with chymotrypsin, and reovirus cores

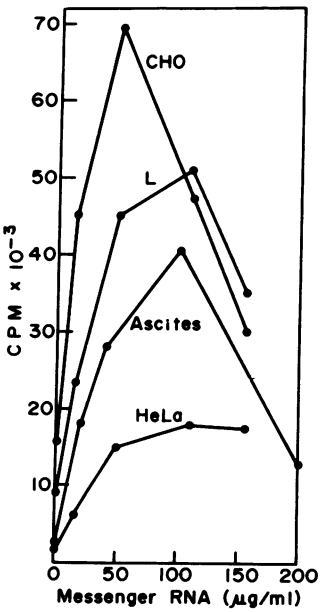

FIG. 1. Stimulation in the preincubated systems of *in vitro* protein synthesis by various concentrations of added reovirus messenger RNA. 50-μl Aliquots of reaction mixture were incubated for 100 min. The label was [^{3}H]leucine. Cell-free extracts were prepared from the indicated cell types.

were isolated from CsCl equilibrium density gradients (21). The 10 species of messenger RNA were synthesized *in vitro* from reovirus cores (21). After incubation, the reaction mixture was centrifuged to remove all cores, extracted with 1 volume of phenol, and mixed at −20° with 2 volumes of ethanol. The RNA precipitate was twice redissolved in distilled water and reprecipitated with 2 M LiCl at 4°, then twice redissolved in 0.2 M potassium acetate and reprecipitated with 2 volumes of ethanol at −20°. The final precipitate of purified messenger RNA was rinsed with ethanol, dissolved in distilled water, and stored at −20°. The incubation conditions used resulted

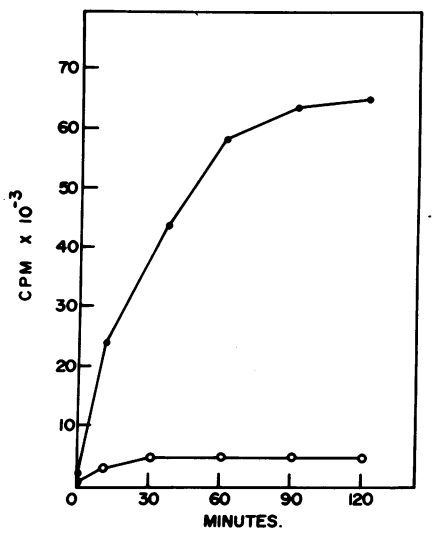

FIG. 2. Rate of protein synthesis in ascites cell-free extract. The label was [^{3}H]leucine. 20-μl Aliquots were taken at the indicated times. No message added (○——○). 110 μg/ml of reovirus message added (●——●).

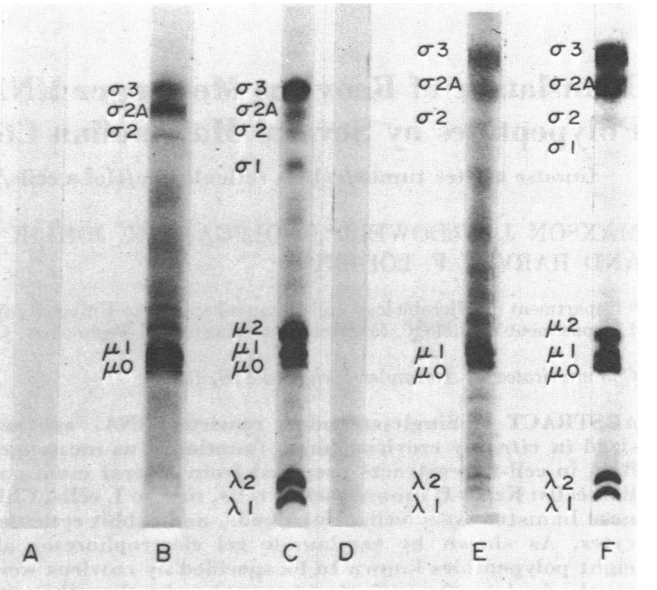

FIG. 3. Autoradiograms of reovirus polypeptides synthesized in L cell and ascites cell-free extracts, and separated by sodium dodecyl sulfate–acrylamide gel electrophoresis. Polypeptides, were labeled with [^{35}S]methionine, except for those shown in 1*C*, which were labeled with [^{14}C]protein hydrolyzate. Migration was from *bottom* to *top*. (*A*) ascites extract, endogenous synthesis; (*B*) ascites extract, reovirus message added; (*C*) viral polypeptides synthesized *in vivo;* (*D*) L cell extract, endogenous synthesis; (*E*) L cell extract, reovirus message added; (*F*) viral polypeptides synthesized *in vivo*.

in the synthesis of the large, medium, and small classes of messenger RNAs in approximate molar ratios of 1:2:3.

Conditions for In Vitro Protein Synthesis. For ascites, mouse L, HeLa, and CHO cells, the concentrations of components

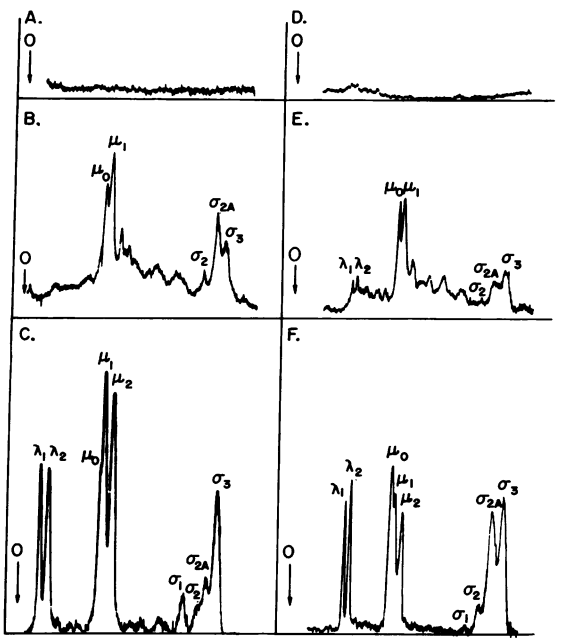

FIG. 4. Absorbance traces of the autoradiograms shown in Fig. 3. Migration was from *left* to *right*. (*A*) ascites extract, endogenous synthesis; (*B*) ascites extract, reovirus message added; (*C*) viral polypeptides; (*D*) L cell extract, endogenous synthesis; (*E*) L cell extract, reovirus message added; (*F*) viral polypeptides.

in the reaction mixtures were as follows: cell-free extracts, 0.6 ml/ml; 30 mM HEPES, pH 7.5, 86 mM KCl, 3 mM $MgAc_2$, 10 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 11 mM creatine phosphate, 1 mg/ml of creatine kinase, 0.2 mM (each) of nineteen unlabeled amino acids, 50 μCi/ml [^{3}H]leucine (40 Ci/mmol; New England Nuclear) or 250 μCi/ml [^{35}S]methionine (150 Ci/mmol; New England Nuclear), and 100 μg/ml (unless otherwise indicated) of reovirus messenger RNA. The reaction mixture was usually incubated for 100 min at 30°. Conditions for reticulocyte cell-free protein synthesis have been described in detail (2, 30). Reactions contained either [^{14}C]lysine (0.3 Ci/mmol, 5 μCi/ml) or [^{35}S]methionine (150 Ci/mmol, 250 μCi/ml) and reovirus messenger RNA, as indicated. Incubation was at 25°.

For all five systems the reaction was stopped by addition of cold 5% Cl_3CCOOH containing 3% casamino acids. When the total incorporation of radioactivity into polypeptides was to be measured, the mixture was heated at 100° for 5 min, filtered onto glass-fiber filters, washed with 5% Cl_3CCOOH containing 3% casamino acids, dried, and counted in a toluene-based POP–POPOP scintillation fluid. For analysis by polyacrylamide gel electrophoresis, the polypeptides synthesized *in vitro* were prepared as described (26).

Labeling of Viral Polypeptides In Vivo. Viral polypeptides were prepared from mouse L cells that had been infected with reovirus in the presence of actinomycin D, and subsequently pulse-labeled with ^{3}H- or ^{14}C-labeled reconstituted protein hydrolyzate (Schwarz/Mann), or with [^{35}S]methionine (26).

Conditions of Electrophoresis. 10% Acrylamide gels containing 0.1% sodium dodecyl sulfate–6 M urea were used as described (26). Electrophoresis was at 25 volts for 20 hr for low-resolution gels and for 40–60 hr for high-resolution gels. Radioactivity in 1-mm slices was counted after they were shaken overnight at 37° in a 3.5% solution of NCS tissue solubilizer (Amersham/Searle) in toluene-based scintillation fluid.

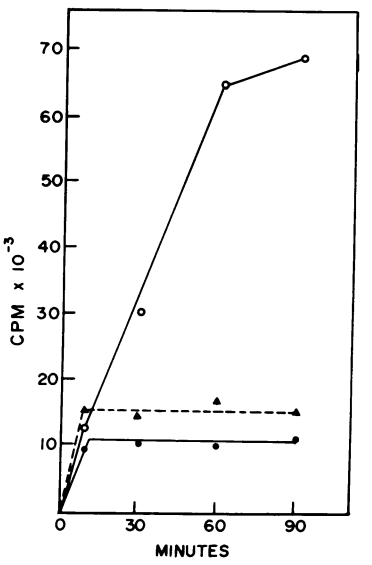

FIG. 5. The effect of addition of reovirus message on the rate of protein synthesis in a reticulocyte cell-free extract. The label was [^{14}C]lysine. 5-μl Aliquots were taken at the indicated times. No message added (O——O). 30 μg/ml of reovirus message added (▲- - -▲). 170 μg/nl of reovirus message added (●——●).

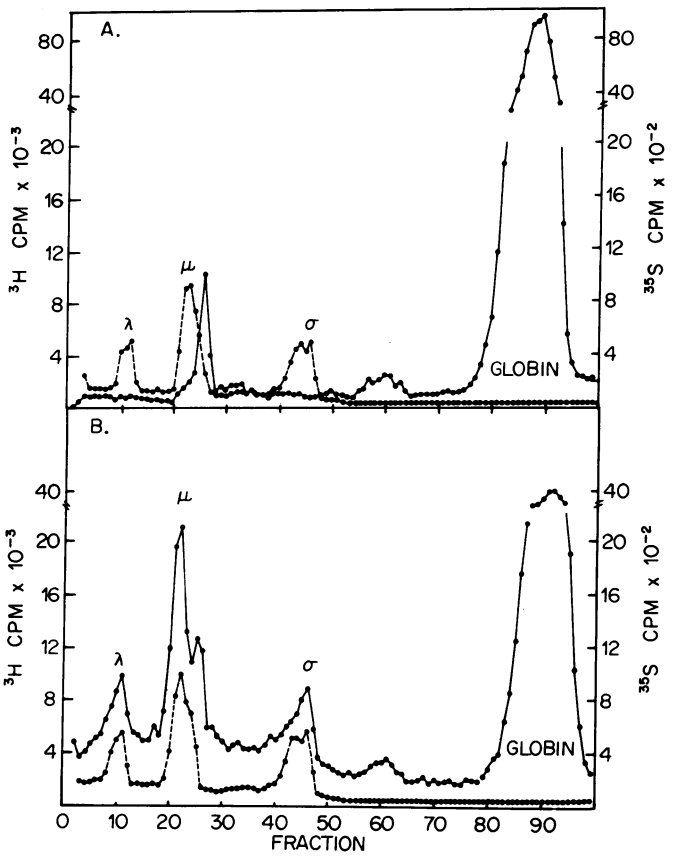

FIG. 6. Low-resolution sodium dodecyl sulfate–gel electrophoretic analysis of total polypeptides synthesized in a reticulocyte cell-free extract. Incubation was for 100 min. Polypeptides were labeled *in vitro* with [^{35}S]methionine (●——●) and coelectrophoresed with viral polypeptides labeled *in vivo* with [^{3}H]protein hydrolyzate (O——O). Migration was from *left* to *right*. (*A*) polypeptides synthesized due to endogenous message. (*B*) polypeptides synthesized in the presence of 200 μg/liter of reovirus message. The volume of reaction mixture analyzed on gel *B* was 3.5 times that in gel *A*.

RESULTS

Cell-free extracts from ascites, mouse L, HeLa, and Chinese hamster ovary cells

In each of these extracts, the addition of reovirus messenger RNA stimulated protein synthesis 5- to 10-fold (Fig. 1). Maximum stimulation occurred when the concentration of added message was between 50 and 120 μg/ml. In the stimulated extracts, protein synthesis continued for about 60 min at 30° (Fig. 2). In the ascites cell-free extract, reovirus-directed synthesis was abolished over 90% by inhibitors of cytoplasmic ribosomes: cycloheximide, emetine, and anisomycin (all at 100 μM) but unaffected by chloromphenicol, an inhibitor of mitochondrial protein synthesis.

The polypeptides that were synthesized in each extract were identified by electrophoresis in parallel with viral polypeptides synthesized in infected cells, in 10% polyacrylamide–sodium dodecyl sulfate gels. The gels were analyzed by autoradiography. Figs. 3 and 4 show the autoradiograms, and the absorbance traces derived from them, for the ascites and L-cell extracts. When reovirus message was added, polypeptides μ_0, μ_1, σ_2, σ_{2a}, and σ_3 were clearly synthesized to completion. σ_1 could not be resolved from the background. Varia-

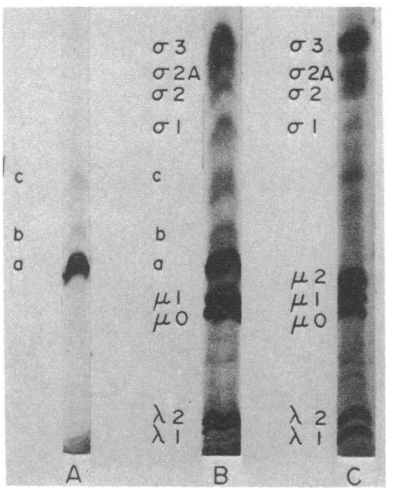

FIG. 7. Autoradiograms of high molecular weight polypeptides synthesized in a reticulocyte cell-free extract and separated by sodium dodecyl sulfate–gel electrophoresis. Polypeptides were labeled *in vitro* with [^{35}S]methionine and *in vivo* with [^{14}C]protein hydrolyzate. Migration was from *bottom* to *top*. (*A*) polypeptides synthesized *in vitro* due to endogenous message. (*B*) polypeptides synthesized *in vitro* in the presence of 200 μg/ml of reovirus message. (*C*) viral polypeptides synthesized *in vivo*.

tions in the relative intensities of the bands were due to the use of different radioactive amino acids for labeling (see legends). Polypeptides λ_1 and λ_2 were not synthesized to completion in significant amounts in the mouse ascites-cell extract, but small amounts were synthesized in the mouse L-cell extract. Reovirus message also stimulated the synthesis of large heterogeneous polypeptides, which were seen as a diffuse background on the autoradiograms. The viral polypeptide pattern obtained with the HeLa cell extract was similar to that obtained with the ascites cell extract, while the CHO cell pattern was similar to that obtained for the L cell extract.

Reticulocyte cell-free extract

The reticulocyte extract was not preincubated. Without added RNA, endogenous protein synthesis continued for 60 min at 25°, but in the presence of reovirus mRNA, synthesis ceased by 10 min (Fig. 5).

The polypeptides synthesized in the presence and in the absence of reovirus message were analyzed at low resolution by coelectrophoresis with viral polypeptides labeled *in vivo* (Fig. 6). Reovirus messenger RNA inhibited the synthesis of globin, but stimulated the synthesis of higher molecular weight polypeptides that coelectrophoresed with reovirus polypeptides. The high molecular weight polypeptides were more clearly identified by electrophoresis in parallel with viral polypeptides labeled *in vivo*, by use of high-resolution polyacrylamide–sodium dodecyl sulfate gels. Figs. 7 and 8 show autoradiograms of the gels and the absorbance traces derived from them. Under the conditions of electrophoresis used, globin migrated out of the gel. Polypeptides λ_1, λ_2, μ_0, μ_1, σ_1, σ_2, σ_{2a}, and σ_3 were all clearly synthesized to completion. Bands *a*, *b*, and *c* were coded for by endogenous messenger RNA, since their synthesis was independent of reovirus message. Globin has migrated from these gels.

DISCUSSION

In the work reported here, two different types of cell-free protein-synthesizing extracts were used to translate reovirus messenger RNA into complete reovirus polypeptides. Those derived from Krebs II ascites cells, mouse L cells, CHO cells, and HeLa cells were preincubated so as to reduce endogenous protein synthesis; the extract prepared from rabbit reticulocytes was not preincubated.

When reovirus messenger RNA was added to the preincubated extracts, total protein synthesis was stimulated 5- to 10-fold, and continued for 60 min at 30°. This long duration of protein synthesis implies that initiation of polypeptide chains continued as the incubation proceeded. In these ex-

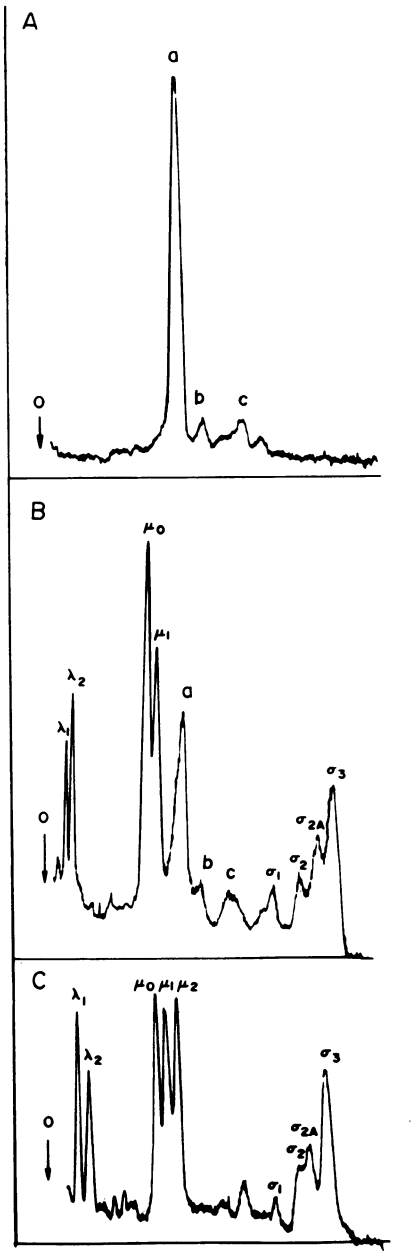

FIG. 8. Absorbance traces of the autoradiograms shown in Fig. 7. Migration was from *left* to *right*. (*A*) polypeptides synthesized *in vitro* due to endogenous message. (*B*) polypeptides synthesized *in vitro* in the presence of 200 μg/ml of reovirus message. (*C*) viral polypeptides synthesized *in vivo*.

tracts polypeptides of the μ and σ classes were synthesized in easily detectable amounts; polypeptides of the largest (λ) class were formed only in small amounts, but could be detected in the mouse-L and CHO cell extracts. It seems unlikely that poor production of λ_1 and λ_2 (and σ_1) proteins is due to inefficient initiation of translation by the appropriate mRNAs, since L cells infected with reovirus do make these proteins. Absence of synthesis of the larger proteins may be due to trace amounts of endoribonucleases in the extracts, which would affect predominantly the longer mRNAs. It is possible that the considerable background apparent in the region of the gel between λ and μ polypeptides was due to incomplete λ polypeptides.

In the reticulocyte extract, which was not preincubated, the situation was quite different. When no exogenous message was added, the translation of globin mRNA proceeded for at least 60 min at 25°, but in the presence of added reovirus message, all translation ceased within 10 min. Possibly, this result is due to traces of reovirus double-stranded RNA, which inhibits initiation of reticulocyte protein synthesis (30); alternatively, initiation of translation of both globin and reovirus mRNA could have been prevented in this extract by the exogenous single-stranded RNA (14, 31, 32). However, during the brief time in which protein synthesis persisted, globin, as well as all 8 known complete viral polypeptides, was synthesized. The relative efficiency with which the largest class of viral mRNA was translated was considerably higher than in the four preincubated extracts.

The synthesis of the largest proteins could be due to the relative absence of ribonucleases in reticulocyte extracts. Since, as mentioned above, it is possible that two or more reovirus proteins are not separable by sodium dodecyl sulfate–acrylamide gel electrophoresis, further analysis by peptide mapping will be essential to determine whether, in fact, *all* reovirus proteins are made in these lysates.

The experiments reported here have two general implications. First, they prove that the single-stranded reovirus RNA that is transcribed *in vitro* by reovirus cores is functional mRNA. It should now be possible, by translation of individual species of mRNA, to determine which mRNA species code for which polypeptides and to determine why only 8, rather than the expected 10, polypeptides have so far been detected. Second, the procedure used to prepare the preincubated cell-free extracts promises to be of general use for preparation of cell-free protein-synthesizing extracts from many types of eukaryotic cells.

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