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Reconstitution of Functional mRNA-Protein Complexes in a Rabbit Reticulocyte Cell-Free Translation System

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A variety of evidence suggests that the cytoplasmic mRNA-associated proteins of eucaryotic cells are derived from the cytoplasm and function there, most likely in protein synthesis or some related process. Furthermore, the evidence suggests that protein-free mRNA added to a cell-free translation system should become associated with a set of proteins similar to those associated with mRNA in native polyribosomes. To test this hypothesis, we added deproteinized rabbit reticulocyte mRNA to a homologous cell-free translation system made dependent on exogenous mRNA by treatment with micrococcal nuclease. The resulting reconstituted complexes were irradiated with UV light to cross-link the proteins to mRNA, and the proteins were analyzed by gel electrophoresis. The proteins associated with polyribosomal mRNA in the reconstituted complexes were indistinguishable from those associated with polyribosomal mRNA in intact reticulocytes. Furthermore, reticulocyte mRNA-associated proteins were very similar to those of cultured mammalian cells. The composition of the complexes varied with the translational state of the mRNA; that is, certain proteins present in polyribosomal mRNA-protein complexes were absent or reduced in amount in 40S to 80S complexes and in complexes formed in the absence of translation. However, other proteins, including a 78-kilodalton protein associated with polyadenylate, were present irrespective of translational state, or else they were preferentially associated with untranslated mRNA. These findings are in agreement with previous data suggesting that proteins associated with cytoplasmic mRNA are derived from the cytoplasm and that they function in translation or some other cytoplasmic process, rather than transcription, RNA processing, or transport from the nucleus to the cytoplasm.

It is becoming increasingly clear that the proteins associated with cytoplasmic mRNA in eucaryotic cells associate in the cytoplasm with already existing mRNA rather than coming out of the nucleus along with newly synthesized mRNA and that they function in translation or in some related cytoplasmic process such as determining mRNA stability. This laboratory and others showed that these proteins differ dramatically from those of nuclear RNA-protein complexes containing mRNA precursors (21, 24, 35). Even cytoplasmic polyadenylate [poly(A)]-protein complexes differ in structure and composition from their nuclear counterparts (1, 35, 39). The labeling kinetics of the cytoplasmic mRNA-associated proteins were consistent with cytoplasmic addition to mRNA (13). These findings make it unlikely that they are packaging or transport proteins.

The mRNA-associated proteins are similar in size, charge, and amino acid composition to soluble RNA-binding proteins which can serve as initiation and elongation factors in translation (26, 28, 36, 37, 40). Furthermore, we showed that the proteins of polyribosomal mRNA-protein complexes (mRNP) continue to be synthesized and to become associated with mRNA in the absence of mRNA synthesis (13). Therefore, polyribosomal mRNP are dynamic structures, and their proteins exchange in the cytoplasm with a pool of similar proteins not bound to mRNA. This behavior is consistent with a role in translation. Presumably, the proteins of this pool are the soluble translation factors already mentioned. If this is the case, then the mRNA-associated proteins themselves should be able to serve as translation factors; indeed, Schmid et al. reported that isolated mRNP stimulated translation in a factor-dependent cell-free translation system, whereas deproteinized mRNA did not (34). This finding is the most direct evidence to date for a translational role for the proteins, and it would be conclusive except for uncertainty as to the purity of the isolated mRNP. In agreement with the results of Schmid et al., Butcher and Arnstein found that the proteins of isolated mRNP are incorporated into polyribosomes in the course of cell-free translation (6).

If the mRNA-associated proteins are derived from the cytoplasm and function in translation, then it is to be expected that protein-free mRNA added to a cell-free translation system would become associated with a set of proteins similar to the mRNA-associated proteins of native polyribosomes. To test this hypothesis, we used a rabbit reticulocyte cell-free translation system together with the methodology this laboratory developed previously for investigating mRNA-associated proteins (11, 12). This methodology involves cross-linking the proteins to mRNA by irradiation with 254-nm UV light. We found that deproteinized reticulocyte mRNA translated in this system does indeed become associated with a set of proteins indistinguishable from those of freshly isolated reticulocyte polyribosomes. Furthermore, the proteins of reticulocyte polyribosomal mRNP are associated with mRNA in intact cells as well as in isolated polyribosomes, and they are similar but not identical to the proteins of L and HeLa cell mRNP which we investigated previously. Finally, we found that the protein composition of the complexes varies with the translational state of the mRNA; that is, polyribosomal mRNP differ slightly from 40S to 80S mRNP in their composition and more dramatically from mRNP formed in the absence of translation. These findings constitute further evidence to support the conclu-

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sion that the proteins of the complexes are involved in translation or a related cytoplasmic process.

MATERIALS AND METHODS

Preparation of rabbit reticulocyte lysate. Female New Zealand white rabbits (2.2 to 3 kg) were made anemic by injection with neutral 2.5% phenylhydrazine (0.2 ml/kg). They were injected daily for 5 or 6 days, and reticulocytes were harvested by cardiac puncture under pentobarbitol sodium (Nembutal) anesthesia on day 9. The extent of reticulocytosis was 50 to 70% as judged by staining with Cresyl brilliant blue. Packed cells were lysed by addition of 1 volume of water, and the cleared lysate was frozen in aliquots at −80°C after centrifuging for 20 min at 10,000 rpm in a Sorvall HB-4 rotor. In one experiment, commercial lysate obtained from Green Hectares (Oregon, Wis.) was used with equivalent results. In some experiments, fresh reticulocytes were irradiated with UV light as described below.

Cell culture, fractionation, and labeling. Mouse L cells and HeLa S3 cells were propagated in spinner cultures in Joklik-modified Eagle medium containing 6% calf serum and 1% fetal calf serum. In one experiment, L cells were labeled for 18 h with 2.5 μCi of [^{35}S]methionine (Amersham, 1,000 Ci/mmol) per ml in medium containing 1.9 μg of L-methionine per ml. The medium also contained nondialyzed serum. L cells were lysed in isotonic KCl buffer containing Triton X-100 as described previously (35). HeLa cells were lysed with 1% Tween 40 and 0.5% sodium deoxycholate in the hypertonic medium of Cervera et al. (7). After nuclei were pelleted by centrifuging for 2 min at 1,500 rpm in an International PR-J centrifuge, the cytoplasmic extracts were centrifuged for 5 min at 16,000 × *g* in a Sorvall SS34 rotor. Ribonucleoproteins were isolated by centrifuging in sucrose density gradients as described previously (35). In the case of micrococcal nuclease-treated reticulocyte lysate, the gradients also contained 0.002 M EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] which had been neutralized with NaOH. Fractions corresponding to polyribosomes, 40S to 80S particles, and smaller material were collected.

UV cross-linking. For cross-linking in intact reticulocytes, 0.5-ml batches of packed cells were diluted to 20 ml with Tris-buffered isotonic saline and irradiated with stirring at 0 to 4°C in 10-cm petri dishes to a dose of 4×10^5 ergs/mm^2. The irradiated cells were pelleted by centrifugation and lysed as for unirradiated reticulocytes. For cross-linking of isolated ribonucleoproteins, sucrose gradient fractions containing polyribosomes, 40S to 80S particles, and smaller material were irradiated in 15- to 20-ml batches by the method used for intact cells except that the dose was 10^5 ergs/mm^2. In some experiments, whole reconstitution reactions were irradiated similarly, except that volumes of 3 to 3.8 ml were irradiated in 6-cm petri dishes and the dose was 8×10^5 ergs/mm^2. All irradiations were done with a fixture containing two 15-W germicidal tubes (Sylvania G15T8). The dose was determined with the aid of a DM254-N shortwave UV meter (Spectronics Corporation, Westbury, N.Y.).

mRNA isolation. For isolation of reticulocyte and L-cell mRNAs, a crude polyribosome preparation was made by layering lysate or L-cell extract over a sucrose step gradient consisting of layers of 0.5 and 2 M sucrose made up in sucrose gradient buffer and centrifuging for 2 h at 60,000 rpm in a Beckman 60 Ti rotor. The pellets were suspended, digested with proteinase K, and phenol-chloroform extracted as described previously (11). The RNA was ethanol precipitated and dissolved in buffer containing sodium dodecyl sulfate (SDS), and mRNA was selected by chromatography on oligodeoxythymidylate [oligo(dT)]-cellulose. It was ethanol precipitated again and dissolved in 10 mM Tris-hydrochloride (pH 7.6).

Cell-free translation and reconstitution. Translation was carried out as described by Pelham and Jackson (29). Reaction mixtures contained 65% reticulocyte lysate, 10 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 75 mM KCl, 1 mM $MgSO_4$, 1 mM ATP, 0.2 mM GTP, 10 mM Tris-hydrochloride (pH 7.6), 10 to 20 μM hemin, and 150 μM amino acids. For measuring protein synthesis, 100-μl reaction mixtures were supplemented with 25 μCi of [^{3}H]leucine per ml (50 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). At the times indicated, 5-μl samples were withdrawn, decolorized with alkaline hydrogen peroxide, and precipitated with trichloroacetic acid. The precipitates were collected on glass fiber filters and counted in a liquid scintillation counter. For analysis of the proteins synthesized, the reactions were supplemented with 300 μCi of [^{35}S]methionine per ml; the unlabeled methionine concentration was 75 μM. Reconstitution reaction mixtures were made up exactly as were translation reaction mixtures except that the final volume was 3.8 ml and labeled amino acid was omitted. Portions of the lysate were made dependent on exogenous mRNA by treatment with micrococcal nuclease (50 U/ml for 12.5 min at 20°C; P-L Biochemicals). Digestion was stopped by addition of 2 mM EGTA. Reticulocyte poly(A)-containing mRNA was added to some reactions at 40 μg/ml. The reactions were incubated at 30°C for the times indicated. In some experiments, protein synthesis was inhibited by addition of 40 μM aurintricarboxylic acid, 0.5 μM pactamycin, or 1 μM edeine or by omission of leucine, and in others, lysate was depleted of ribosomes by centrifuging for 90 min at 65,000 rpm in a Beckman 75 Ti rotor before reconstitution or translation reaction mixtures were made up. Pactamycin was obtained from P. F. Wiley of The Upjohn Co., Kalamazoo, Mich. Edeine was a gift from Gisela Kramer and Boyd Hardesty of the University of Texas at Austin.

Isolation and labeling of mRNA-protein complexes. Polyribosomes and 40S to 80S particles were concentrated from sucrose gradients by pelleting through sucrose cushions as described (35). When micrococcal nuclease was used, the cushions also contained 2 mM EGTA. Poly(A)-containing mRNP were isolated by chromatography on oligo(dT)-cellulose in the presence of SDS as described (12). In experiments in which material sedimenting slower than 40S was analyzed, the material was concentrated by ethanol precipitation before chromatography. Protein labeling by reductive methylation was based on the procedures of Rice and Means (31) and Benne et al. (2). Ethanol-precipitated and dried mRNP isolated by one cycle of chromatography on oligo(dT)-cellulose were dissolved in 0.08 ml of 10 M urea–0.014 M sodium borate (pH 9.0). [^{14}C]formaldehyde (10 μCi) was added (50 mCi/mmol; New England Nuclear Corp., Boston, Mass.), and then 10 1-μl portions of freshly prepared $NaBH_4$ solution (0.64 mg/ml) were added at 3-min intervals. The final volume was 0.1 ml. Urea was needed to dissolve precipitated mRNP. Also, it was found that the presence of urea increased labeling of a test protein (bovine serum albumin) by at least a factor of two compared with labeling done in the absence of urea. Labeled mRNP were freed of unincorporated radioactivity by dilution with 1 ml of oligo(dT)-cellulose-binding buffer followed by rechromatography. The mRNP were resolved into poly(A)-contain-

ing and non-poly(A)-containing regions by digestion with RNases A and T_1 followed by a third cycle of chromatography on oligo(dT)-cellulose (12).

Polyacrylamide gel electrophoresis. The proteins of mRNP were analyzed by electrophoresis in 10% SDS-polyacrylamide gels as described (12). Before electrophoresis, precipitated complexes were dissolved in buffer containing 0.5% sodium lauroyl sarcosinate, 10% glycerol, 1% 2-mercaptoethanol, and 0.01 M sodium acetate (pH 4.5). The samples were digested with 1.25 U of RNase T_2 for 1 h at 37°C. They were adjusted to a concentration of 0.0625 M in Tris-hydrochloride (pH 6.8) and 2% in SDS and then boiled before loading on gels. Proteins synthesized in the cell-free translation system were electrophoresed similarly, except that 12.5% gels were used and RNase digestion was omitted. After electrophoresis, the gels were impregnated with En^{3}Hance (New England Nuclear Corp.) and fluorographed with Kodak XAR-5 film.

RESULTS

Proteins associated with polyribosomal mRNA in rabbit reticulocytes. Before reconstitution of mRNP in the reticulocyte cell-free translation system was attempted, it was necessary to describe the reticulocyte mRNA-associated proteins and to compare them to the murine and human cultured cell mRNA-associated proteins described previously in this laboratory (12, 13, 35; unpublished data). For this purpose, polyribosomes were isolated by centrifugation in sucrose density gradients and irradiated with 254-nm UV light to cross-link proteins to RNA. Cross-linked poly(A)-containing mRNP were isolated by chromatography on oligo(dT)-cellulose under conditions preventing the binding of proteins not covalently attached to mRNA (12). The proteins cross-linked to mRNA were analyzed by electrophoresis in SDS-polyacrylamide gels after digestion with appropriate RNases.

In the work with cultured cells, the mRNA-associated proteins were short-term metabolically labeled with [^{35}S]methionine and detected fluorographically after separation by gel electrophoresis. However, reticulocytes are not suitable for metabolic labeling, and it was necessary to use an in vitro protein-labeling procedure. We chose to use labeling by reductive methylation with [^{14}C]formaldehyde (2, 31). This procedure differs in two important respects from the short-term metabolic labeling used previously: it labels primarily lysine residues rather than methionine residues (31), and it labels the steady-state population of mRNA-associated proteins rather than the most rapidly synthesized ones. To distinguish real differences between reticulocyte and cultured cell mRNA-associated proteins from differences due to labeling conditions, we compared [^{14}C]formaldehyde-labeled reticulocyte proteins with similarly labeled mouse L- and human HeLa-cell proteins. We also compared the [^{14}C]formaldehyde labeling results with those obtained by long-term [^{35}S]methionine metabolic labeling in mouse L cells (Fig. 1). With long-term metabolic labeling, several bands could be seen which were faint or undetectable with short-term metabolic labeling (see references 12, 13, and 35). These included bands of 43, 38, 34, 29, and 15 kilodaltons (kDa) (Fig. 1, ^{35}S-Met, lane N). These bands could also be seen with [^{14}C]formaldehyde labeling, and they were present in HeLa-cell as well as L-cell mRNA-associated proteins (^{14}C-HCHO, lanes N). One band seen with short- or long-term metabolic labeling with [^{35}S]methionine was not seen with [^{14}C]formaldehyde labeling, presumably because it was relatively methionine rich and lysine poor. This was the 75-kDa band. Other bands could be detected by all labeling procedures. These included the 78-kDa protein associated with poly(A) (lanes B) and the 98-, 78-, 68-, 62-, and 52-kDa proteins associated with sequences other than poly(A) (lanes N). It has been shown previously that the 78-kDa protein associated with poly(A) differs in its partial peptide map from the protein of the same size not associated with poly(A). Therefore, these are different proteins (35).

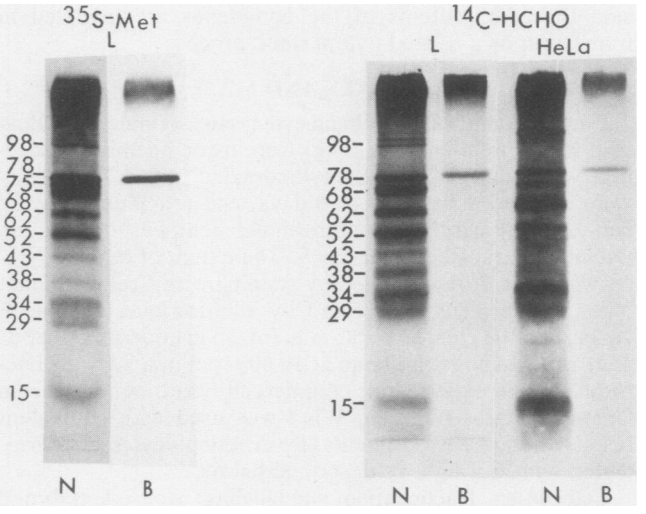


FIG. 1. Long-term metabolic labeling and in vitro labeling of L and HeLa cell polyribosomal mRNA-associated proteins. Isolation and labeling of mRNP and analysis of the proteins were carried out as described in the text. The numbers at the left of the lanes indicate the molecular weights of the proteins in thousands. ^{35}S-Met, [^{35}S] methionine-labeled proteins; ^{14}C-HCHO, [^{14}C]formaldehyde-labeled proteins; L, L-cell proteins; HeLa, HeLa cell proteins; N, not rebound to oligo(dT)-cellulose after digestion with RNases A and T_1, i.e., not associated with poly(A); and B, rebound to oligo(dT)-cellulose after digestion with RNases A and T_1, i.e., associated with poly(A).

For analysis of reticulocyte mRNA-associated proteins, intact cells were irradiated as well as isolated polyribosomes. This approach makes it possible to find out whether there is any change in the composition of the mRNA-associated proteins as a result of polyribosome isolation. Longer irradiation times were required with intact cells because of their higher nonspecific UV absorption. Otherwise, the analytical procedures were identical to those used with isolated polyribosomes. Figure 2A shows sucrose density gradient scans of polyribosomes from irradiated (bottom) and unirradiated (top) reticulocytes. Although there was some loss of optical density due to cell lysis during irradiation, the polyribosomes were not extensively degraded.

Figure 2B shows the polyribosomal mRNA-associated proteins from irradiated intact cells (lanes Cell) and irradiated isolated polyribosomes (lanes Poly). These two sets of proteins were nearly indistinguishable. Moreover, reticulocyte mRNA-associated proteins were strikingly similar to those of L and HeLa cells. There was a protein of 78 kDa cross-linked to poly(A) (Fig. 2B, B lanes) and proteins of 78, 68, 62, 52, 34, 29, and 15 kDa cross-linked to sequences other than poly(A) (N lanes). However, the 43- and 38-kDa proteins seen with L and HeLa cells were not seen with reticulocytes, and the 98-kDa doublet of L and HeLa cells

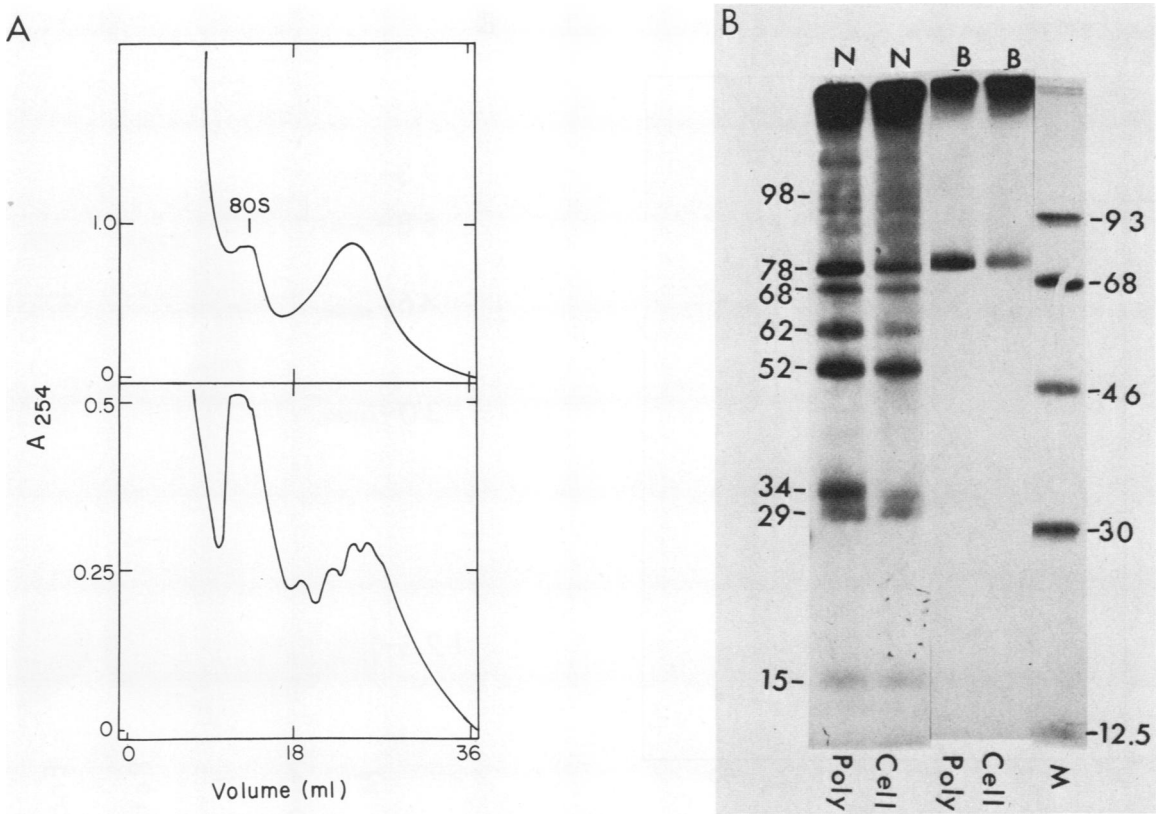


FIG. 2. Isolation of polyribosomes from irradiated and unirradiated reticulocytes and analysis of the mRNA-associated proteins. (A) Lysates were centrifuged in sucrose density gradients which were collected through the flow cell of a UV monitor. The direction of centrifugation was left to right. Top, unirradiated cells; bottom, irradiated cells. (B) Proteins cross-linked to polyribosomal mRNA by irradiation of intact cells and isolated polyribosomes. The polyribosomes used were those shown in (A). The proteins were labeled in vitro with [^{14}C]formaldehyde. The numbers at the left are the molecular weights in thousands of the mRNA-associated proteins, and the numbers at the right are those of the marker proteins, which were, from top to bottom, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*. Proteins were cross-linked to mRNA by irradiation of isolated polyribosomes (lanes Poly) or by irradiation of intact cells (lanes Cell). Gels were exposed for 5 days (N lanes) or for 21 days (B lanes). M, Marker proteins.

was less prominent with reticulocytes than with L and HeLa cells.

Protein synthesis in the reticulocyte lysate. To establish that our reticulocyte lysates could carry out protein synthesis, we performed conventional translation reactions with [^{3}H]leucine as a radioactive precursor. Portions of the lysate were made dependent on exogenous mRNA by treatment with micrococcal nuclease, and protein synthesis was reconstituted by adding back deproteinized reticulocyte mRNA (29). Figure 3A shows that the kinetics of trichloroacetic acid-precipitable [^{3}H]leucine incorporation were approximately linear for 60 min. The endogenous and reconstituted reactions synthesized similar amounts of protein, and maximal incorporation corresponded to 180 pmol of globin synthesized per 100 μl of reaction mixture. The nuclease-treated reaction without added mRNA incorporated about 1/12 as much [^{3}H]leucine.

The proteins synthesized in these reactions were analyzed by electrophoresis in a 12.5% SDS–polyacrylamide gel after labeling with [^{35}S]methionine (Fig. 3B). In the reconstituted and endogenous reactions, the major protein product synthesized had the mobility expected for globin (Fig. 3B, lanes Ret. and End.). In the nuclease-treated reaction without added mRNA, much less of this globin-like protein was synthesized (lane Nuc.). The lysate was capable of translating mRNAs for proteins of larger size, as shown by analysis of a reaction containing L-cell mRNA (lane L).

Reconstitution of mRNP. The mRNA-associated proteins of nuclease-treated, endogenous, and reconstituted protein synthesis reactions were analyzed by carrying out relatively large-scale reactions without labeled amino acid. After 45 min of translation, the reactions were centrifuged to isolate polyribosomes and more slowly sedimenting mRNP. Optical scans of these gradients are shown in Fig. 4A. The nuclease-treated reaction without added mRNA showed no detectable polyribosomes, whereas the endogenous and reconstituted protein synthesis reactions contained similar levels of polyribosomes which were comparable to those of freshly lysed cells. The polyribosomes and postpolyribosomal ribonucleoprotein were collected and irradiated, and the mRNA-associated proteins were labeled and analyzed as for those of irradiated intact cells and freshly isolated polyribosomes (Fig. 4B). Virtually no labeled mRNA-associated proteins were recovered from the nuclease-treated reaction without added mRNA (Fig. 4B, Nuc. lanes). The endogenous (End. lanes) and reconstituted (Rec. lanes) reactions yielded amounts of labeled mRNA-associated proteins similar to those recovered from freshly lysed cells. Roughly equal amounts of mRNA-associated proteins were recovered from the polyribosomes (Fig. 4B, Poly lanes) and from the 40S to 80S particles (Post lanes). The proteins of the endogenous and reconstituted polyribosomal mRNP were indistinguishable from one another and from those of the polyribosomal mRNP of intact or freshly lysed cells. In particular, there

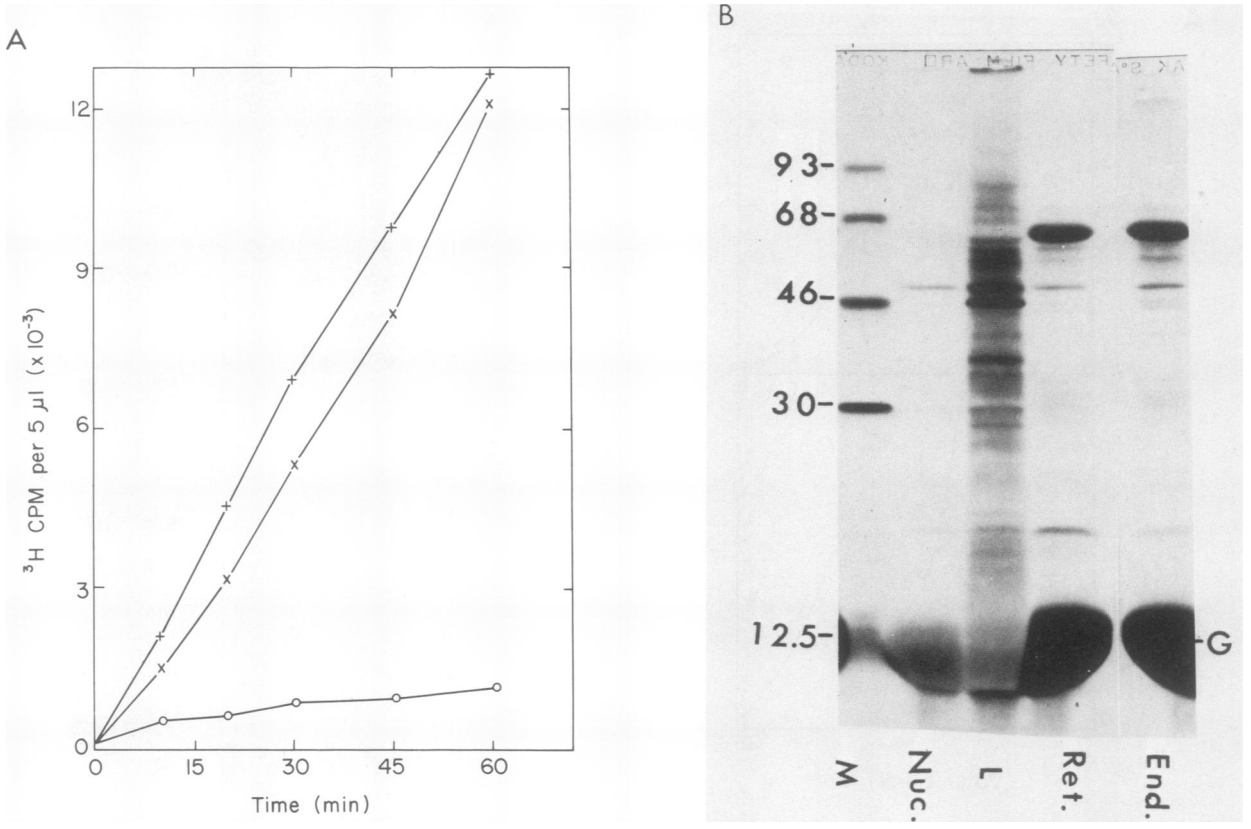


FIG. 3. Kinetics of protein synthesis in the reticulocyte lysate and analysis of the proteins synthesized. (A) Protein synthesis was carried out as described in the text, with [^{3}H]leucine as the labeled amino acid. Radioactivity precipitable by trichloroacetic acid is shown. Symbols: +, endogenous incorporation; ○, incorporation in a nuclease-treated reaction; and ×, incorporation in a nuclease-treated reaction supplemented with reticulocyte mRNA. (B) Protein synthesis was carried out as for (A) except that [^{35}S]methionine was used in place of [^{3}H]leucine. After 60 min of incubation, 5-μl samples were analyzed by electrophoresis in a 12.5% gel and by fluorography. The film was exposed for 3 days. Abbreviations: Nuc, proteins synthesized in a nuclease-treated reaction; L, proteins synthesized in a nuclease-treated reaction supplemented with L-cell mRNA; Ret, proteins synthesized in a nuclease-treated reaction supplemented with reticulocyte mRNA; End, proteins synthesized in an endogenous reaction without nuclease treatment; and G, Globin.

was a protein of 78 kDa cross-linked to poly(A) (Fig. 4B, lanes B) and proteins of 98, 78, 68, 62, 52, 34, 29, and 15 kDa cross-linked to sequences other than poly(A) (lanes N).

The protein composition of the mRNP varies with the translational state of the mRNA. Figure 4B shows the proteins of endogenous and reconstituted reticulocyte mRNP sedimenting at 40S to 80S (Post lanes) as well as those of polyribosomal mRNP (Poly lanes). Presumably, the former consisted of mRNA associated with monoribosomes and ribosomal subunits, since reticulocytes contain mainly globin mRNA and ribosome-free globin mRNP sediment at about 20S (10, 17). The 40S to 80S mRNP were qualitatively similar in protein composition to polyribosomal mRNP. However, some quantitative differences were apparent. In particular, some proteins of the polyribosomal mRNP were relatively scarce in the 40S to 80S mRNP. These included the proteins of 62, 34, 29, and 15 kDa. On the other hand, the 52-kDa protein was relatively more prominent in the 40S to 80S mRNP. These findings suggested that the protein composition of the mRNP varies with the translational state of the mRNA. Similar results have been obtained with L and HeLa cells (unpublished data).

To further investigate the dependence of protein composition on translational state, we reconstituted mRNP in the absence of protein synthesis. This was accomplished by adding deproteinized reticulocyte mRNA to translation reactions containing inhibitors of protein synthesis such as aurintricarboxylic acid, pactamycin, and edeine and also by using ribosome-depleted lysate or by omitting an amino acid. Figure 5A shows that protein synthesis was completely inhibited by aurintricarboxylic acid and edeine, whereas a control reaction showed the normal amount of protein synthesis. Aurintricarboxylic acid inhibits mRNA binding to ribosomes (38), and we anticipated that much of the mRNP formed under these conditions would sediment at less than 40S. Therefore, we recovered and analyzed the <40S material as well as the more rapidly sedimenting material (Fig. 5B). The control complexes (Fig. 5B, −ATA lanes) gave the expected results. In particular, bands of 62, 34, 29, and 15 kDa were conspicuous in the polyribosomal mRNP (Poly, lane N) but much reduced in the 40S to 80S material (40–80S, lane N). The material sedimenting more slowly than 40S (−ATA, <40S lanes) contained a significant amount of mRNP which were similar in composition to the 40S to 80S material, except that they contained at least one additional band at 82 kDa. The bands at 62, 34, 29, and 15 kd were present in very low amounts, if at all. Control experiments which are not shown established that no labeled proteins were bound to the oligo(dT)-cellulose column in the absence of irradiation. Therefore, the bands observed were not due to soluble proteins nonspecifically bound to the column. In the case of the aurintricarboxylic acid-treated reaction (Fig.

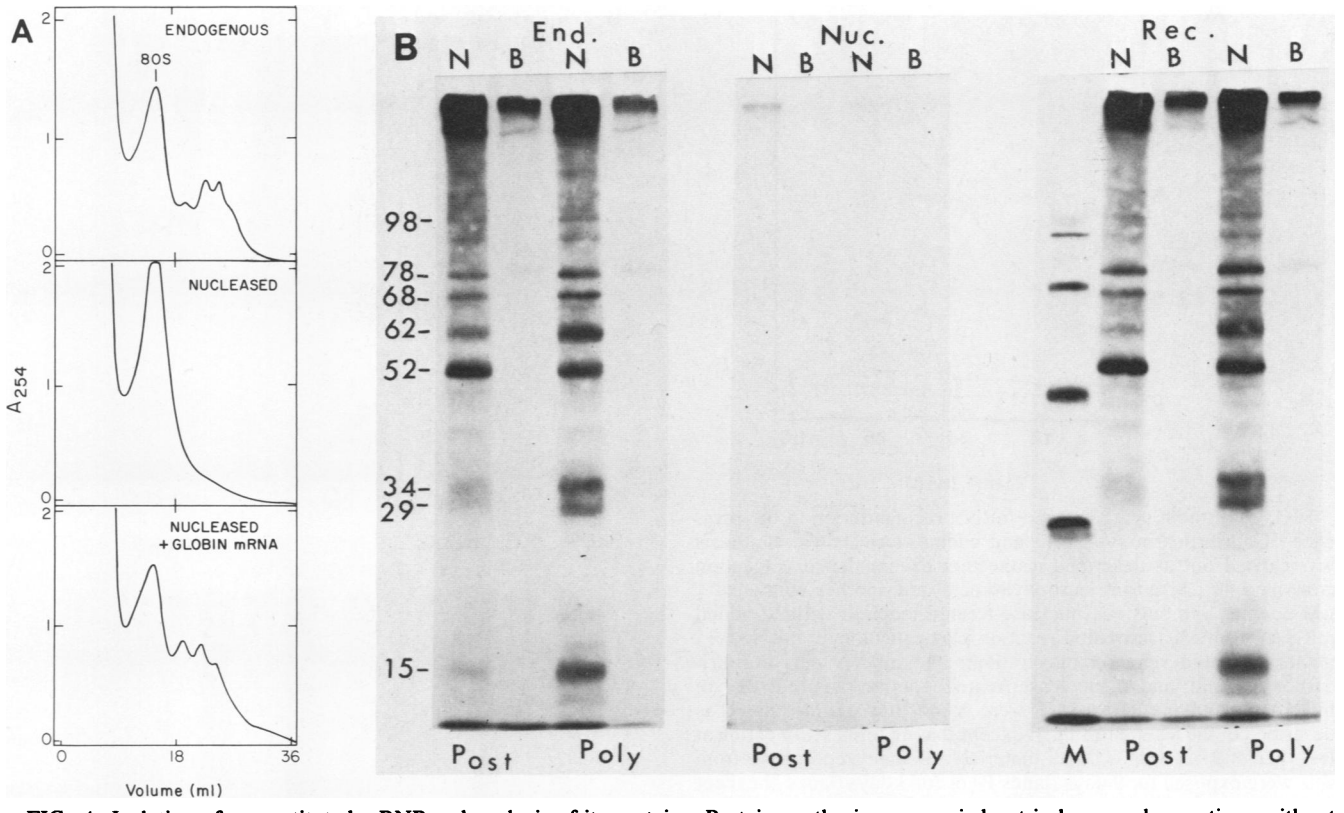
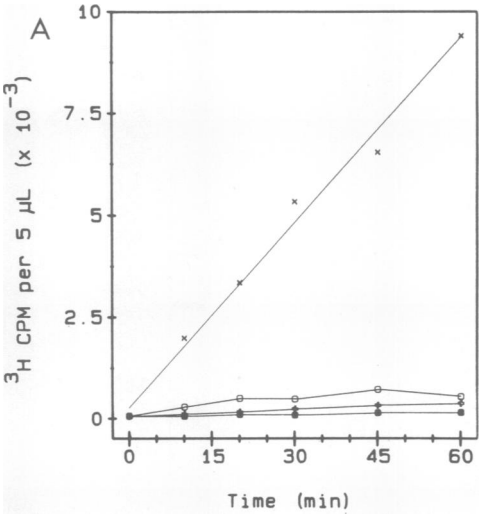


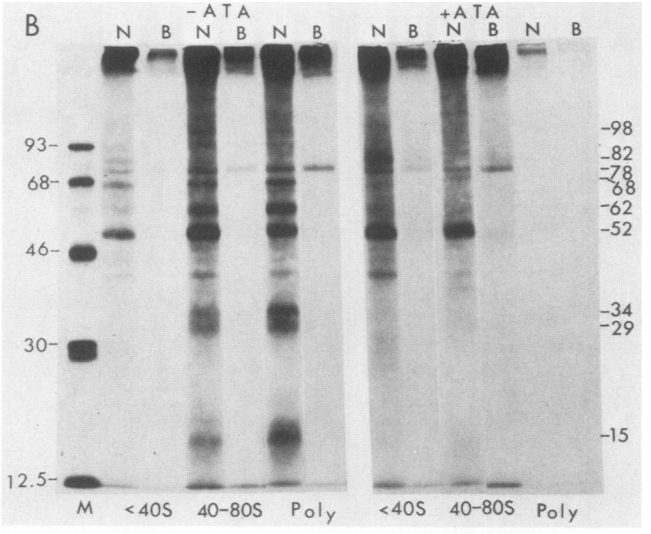
FIG. 4. Isolation of reconstituted mRNP and analysis of its proteins. Protein synthesis was carried out in large-scale reactions without labeled amino acid. After 45 min of incubation, the reaction mixtures were rapidly chilled, layered on sucrose gradients, and centrifuged. (A) UV scans of sucrose gradients. Reactions: ENDOGENOUS, reaction not treated with nuclease; NUCLEASED, reaction treated with nuclease; NUCLEASED + GLOBIN mRNA, reaction treated with nuclease and supplemented with reticulocyte mRNA. (B) Fluorograms of gels containing proteins cross-linked to mRNA. The proteins were labeled in vitro with [^{14}C]formaldehyde in this and subsequent figures. All lanes were exposed for 3 days. Proteins of polyribosomal mRNP (lanes Poly) or 40S to 80S (lanes Post) were cross-linked to mRNA from an endogenous reaction (lanes End.), to mRNA from a nuclease-treated reaction (lanes Nuc.), or to mRNA from a nuclease-treated reaction supplemented with reticulocyte mRNA (lanes Rec.).

5B, +ATA lanes) no bands were seen in the polyribosome region of the gradient (Poly lanes), and all of the mRNP sedimented at 80S or less (<40S and 40–80S lanes). In the 40S to 80S mRNP (+ATA, 40–80S lanes), the 52-kDa band was the most prominent. Bands at 78 and 68 kDa not associated with poly(A) were also seen, as was a band at 78 kDa associated with poly(A) (+ATA, 40–80S, lane B). However, bands at 62, 34, 29, and 15 kDa could not be detected. The material sedimenting at less than 40S (+ATA, <40S lanes) was similar in composition, except that it contained at least one additional band at 82 kDa. Thus, the mRNP formed in the presence of aurintricarboxylic acid were similar in composition to the control mRNP sedimenting at less than 40S. Similar results have been obtained in experiments in which mRNP were reconstituted in the absence of protein synthesis by using ribosome-depleted lysates; that is, the 62-, 34-, 29-, and 15-kDa bands were absent, and one additional band was present at 82 kDa.

We also reconstituted mRNP in the presence of pactamycin. This drug has been reported to inhibit initiation by preventing joining of the 60S ribosomal subunit with the initiation complex containing the 40S subunit and mRNA (19, 23). However, it has also been reported that in the presence of excess joining factor, initiation is not affected but pactamycin inhibits elongation (19). In our study, the minimum effective concentration for inhibiting [^{3}H]leucine incorporation (0.5 μM) resulted in an accumulation of nonfunctional polyribosomes. Thus, the major effect appeared to be on elongation rather than initiation. The pactamycin mRNP were indistinguishable from control mRNP (data not shown). Similarly, when elongation was inhibited by omitting leucine from the translation reaction, there was an accumulation of inactive polyribosomes which contained typical polyribosomal mRNP (data not shown).

The mRNP formed in the presence of edeine are shown in Fig. 5C. Edeine is reported to allow formation of 48S initiation complexes containing mRNA and the small ribosomal subunit, but to inhibit joining of the 60S subunit with this complex (20, 22, 33). In agreement with those data, we found that the majority of the mRNP formed in the presence of edeine sedimented at 40S to 80S (Fig. 5C, + Edeine, 40–80S lanes). There were no detectable mRNP recovered from polyribosomes (+ Edeine, Poly lanes) and only a small amount of mRNP sedimenting at less than 40S (+ Edeine, <40S lanes). The 40–80S mRNP formed in the presence of edeine differed substantially in composition from the polyribosomal mRNP formed in the absence of edeine. Most strikingly, there were prominent bands at ca. 98, 82, 65, and 47 kDa which were barely detectable in the control mRNP (− Edeine lanes). Also, the 68- and 62-kDa bands prominent in the control mRNP were barely detectable in the edeine mRNP. The 68-kDa band is probably present, although it is not resolved from the 65-kDa band in the gel shown, since a gel that had better resolution in this





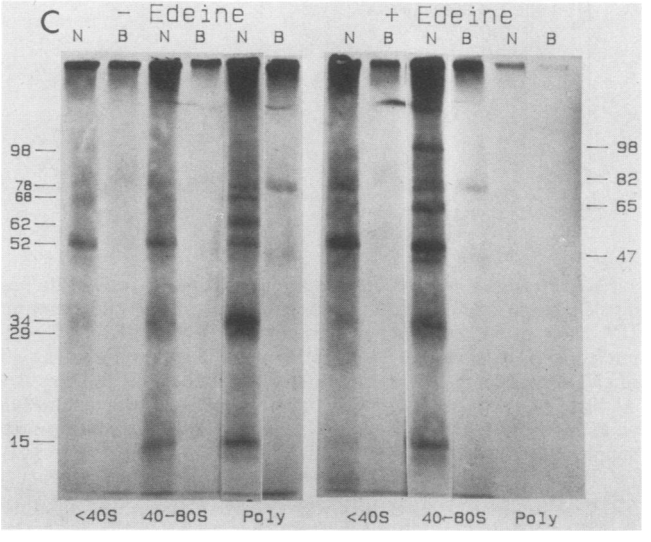


FIG. 5. Protein synthesis and mRNP reconstitution in the presence of aurintricarboxylic acid and edeine. (A) Protein synthesis was carried out as described in the text except that one reaction contained 40 µM aurintricarboxylic acid and another contained 1 µM edeine. Symbols: ○, nuclease-treated reaction without added mRNA; ×, nuclease-treated reaction plus reticulocyte mRNA; +, nuclease-treated reaction plus reticulocyte mRNA plus aurintricarboxylic acid; and ●, nuclease-treated reaction plus reticulocyte mRNA plus edeine. (B) mRNP were reconstituted and isolated as described in the legend to Fig. 4, except that material sedimenting at less than 40S, as well as larger material, was analyzed. mRNP from gels were exposed for 9 days (lanes B) or for 3 days (lanes N except the + ATA, <40S N lane, which was exposed for 9 days). − ATA, Absence of aurintricarboxylic acid; + ATA, presence of aurintricarboxylic acid. Proteins were cross-linked to mRNA sedimenting slower than 40S (<40S lanes), to mRNA sedimenting at 40S to 80S (40-80S lanes), or to polyribosomal mRNA (Poly lanes). (C) Same as (B) except that reactions took place in the presence (+ Edeine) or absence (− Edeine) of edeine instead of aurintricarboxylic acid. The poly N −Edeine lane and the 40–80S N + Edeine lane were exposed for 3 days. All other lanes were exposed for 9 days.

region revealed a distinct 68-kDa band (data not shown). The 34-, 29-, and 15-kDa bands which were very prominent in the control mRNP were less prominent in the edeine mRNP, whereas the 52kDa band was more prominent in the edeine mRNP than in the control. However, the 78-kDa band associated with poly(A) was equally prominent in the edeine and the control mRNP (− Edeine, Poly, lane B and + Edeine, 40–80S, lane B).

It is apparent from these results that the mRNP formed in the absence of translation may differ substantially from those formed under protein synthesis conditions. However, not surprisingly, different results are obtained with different inhibitors of translation. It is of interest that the 78-kDa protein associated with poly(A), as well as certain other bands, including the 52- and 78-kDa bands not associated with poly(A) always seem to be present in mRNP, whether or not translation occurs.

DISCUSSION

mRNA-associated proteins of reticulocytes and cultured cells. The overall pattern of proteins cross-linked to mRNA by irradiating rabbit reticulocyte polyribosomes was very similar to that obtained by irradiating L- and HeLa-cell polyribosomes. In particular, the 78-kDa poly(A)-associated protein and the 78-, 68-, 62-, 52-, 34-, 29-, and 15-kDa proteins not associated with poly(A) from rabbit reticulocytes could not be distinguished from the corresponding proteins of murine L cells and human HeLa cells on the basis of their mobilities in SDS-polyacrylamide gels. These findings suggest that these proteins are conserved in evolution, and that, like other conserved proteins, they may have an essential function in the cell. Similar conclusions about mRNA-associated proteins have been reached by other investigators, although they were based on a less extensive list of proteins and on older methodology (4, 5, 21, 24, 25). Some differences do exist between the mRNA-associated proteins of L and HeLa cells, and between these cells and rabbit reticulocytes. However, their significance is not clear.

The same proteins can be cross-linked to mRNA by irradiating intact reticulocytes or isolated polyribosomes. This observation suggests that these proteins are associated with mRNA in intact cells as well as in isolated polyribosomes and that there is no change in the composition of the mRNA-associated proteins as a result of polyribosome isolation, provided that appropriate conditions are used. In this respect, reticulocytes differ slightly from HeLa cells. When intact HeLa cells were irradiated, a few bands were seen

which were not seen when isolated polyribosomes were irradiated. This finding suggests that a few mRNA-associated proteins were lost when HeLa polyribosomes were isolated without prior cross-linking (unpublished data).

Reticulocytes contain predominantly globin mRNA (8), whereas cultured cells such as L and HeLa cells contain thousands of other kinds of mRNA (3, 14, 30, 32) but virtually no globin mRNA (16). Therefore, the same proteins must be able to bind to different kinds of mRNA. mRNA molecules vary widely in sequence and in secondary and tertiary structure, and they contain only a few common features which might serve as universal protein-binding sites. These include poly(A), caps, and the AAUAAA polyadenylation signal. Only one of the mRNA-associated proteins mentioned in this paper appears to be poly(A) associated. Whether or not any of the other proteins bind to caps or to AAUAAA remains to be seen. Other possibilities are that the mRNA-associated proteins do not have fixed binding sites but, rather, that they are relatively nonspecific RNA binding proteins or else that they translocate along mRNA molecules.

Reconstitution of mRNP: implications for protein function. We have shown that addition of mRNA to nuclease-treated reticulocyte lysate under protein synthesis conditions permits the reconstitution of mRNP whose protein composition is indistinguishable from that of mRNP present in intact cells. Since no bands were detected without added mRNA, the bands observed after mRNA addition could not have been due to residual mRNP in the lysate. The mRNA was very rigorously deproteinized before addition to the lysate. It was digested with proteinase K, extracted with phenol-chloroform, and chromatographed on oligo(dT)-cellulose in the presence of high salt and SDS. Any one of these procedures by itself should have been sufficient to eliminate most of the mRNA-associated protein, and the combination makes it very unlikely that the bands observed in the reconstituted complexes were due to residual protein contaminating the mRNA. The fact that no bands were observed in the absence of irradiation virtually rules out this possibility.

A related issue is whether or not any of the proteins we see bound to oligo(dT)-cellulose after UV irradiation are associated with RNAs other than mRNAs. As far as we know, there are no polyadenylated RNAs other than mRNA present in the cytoplasm of reticulocytes, and it seems unlikely that nonpolyadenylated RNAs would survive the isolation procedure. Before the final RNase digestion and gel electrophoresis step, the mRNA of the reconstituted mRNP was subjected to no fewer than four cycles of chromatography on oligo(dT)-cellulose, including one step of denaturation in 10 M urea and three heating steps. This rigorous protocol makes it very unlikely that any non-mRNA molecules could remain attached to the mRNA by hydrogen or other noncovalent bonds. Nevertheless, as an additional check on the authenticity of our mRNA-associated proteins we did reconstitution experiments with L-cell mRNA and vesicular stomatitis virus mRNA metabolically labeled with $^{32}P_i$. The vesicular stomatitis virus mRNA was from cells labeled in the presence of actinomycin D, so that no nonviral RNAs should have been labeled. Rather than labeling the mRNP proteins with [^{14}C]formaldehyde, we relied on the ^{32}P-nucleotides attached to the proteins by cross-linking to detect them. The reconstituted vesicular stomatitis virus and L-cell mRNP gave the same major bands as did the reticulocyte mRNP. These results will be published separately.

Since reticulocytes are anucleate cells, the proteins of the reconstituted complexes are necessarily derived from the cytoplasm. The reticulocyte data alone do not rule out the possibility that the mRNP proteins may have been associated with intranuclear mRNA precursors before enucleation. However, in previous experiments with nucleated cultured cells, we did not detect cytoplasmic mRNP proteins in association with polyadenylated nuclear RNA (35). Also, our metabolic labeling and inhibitor studies on cultured cells showed that the mRNP proteins associate in the cytoplasm with already existing mRNA. Those findings, together with the present results, make it rather unlikely that the cytoplasmic mRNP proteins function in transcription, RNA processing, or transport of mRNA from the nucleus to the cytoplasm. Rather, it is much more likely that the mRNP proteins function in the cytoplasm. Since the major, perhaps the only, process in which mRNA is involved in the cytoplasm is protein synthesis, these proteins may function in some aspect of it. The presence in reticulocyte lysates of proteins capable of reconstituting mRNP from added mRNA may explain why some investigators have been unable to find any difference between the translation efficiency of mRNP and that of protein-free mRNA (15, 18, 27).

Our results showed that the protein composition of the mRNP varied with the translational state of the mRNA. In particular, certain proteins were most abundant in polyribosomes. These included the 62-, 34-, 29-, and 15-kDa proteins not associated with poly(A). They were less abundant in 40S to 80S mRNP which presumably consisted of mRNA attached to monoribosomes and ribosomal subunits, and they were virtually absent from mRNP not associated with ribosomes, such as the mRNP formed in the presence of aurintricarboxylic acid or in ribosome-depleted lysate. They were present in relatively small amounts in the 40S to 80S region under protein synthesis conditions and also in the presence of edeine, which permits formation of 48S initiation complexes but not subsequent steps in translation. They were also present in the polyribosomal mRNP of the inactive polyribosomes formed in the presence of pactamycin or in the absence of leucine. The data suggest that these proteins become associated with mRNA in the course of polyribosome formation, and that they may be required for translation. On the other hand, some mRNA-associated proteins were found preferentially in the blocked 48S initiation complexes formed in the presence of edeine. These included the bands at 98, 65, and 47 kDa. Their behavior is consistent with a transient association with mRNA during initiation, as might be expected for some initiation factors. Still others were associated with mRNA irrespective of its translational state. These included the 78-kDa poly(A)-associated protein and the 52- and 78-kDa bands not associated with poly(A). The data do not permit any conclusion as to whether or not they are required for translation.

Thus, the data suggest that there are three classes of mRNP proteins: those associated with mRNA irrespective of its translational state, those that become associated with mRNA in the course of translation and are found predominantly in polyribosomes, and those that associate with mRNA transiently at some step before polyribosome formation. More work will be required to identify the proteins and to determine precisely at which step in translation they become associated with mRNA. However, it is clear that the protein composition of mRNP varies with the translational state of the mRNA, and it seems likely that at least some of the proteins are involved in translation.

The question arises as to whether or not the proteins associated with mRNA preferentially during translation are

ribosomal proteins. The 62- and 34-kDa proteins are synthesized and become associated with mRNA in L cells treated with actinomycin D. Indeed, the synthesis of the 34-kDa protein seems to be enhanced by actinomycin D (13). The 29-kDa protein also appears to be synthesized and to become associated with mRNA in actinomycin-treated cells, although this band is relatively faint under these conditions (unpublished data). Actinomycin D inhibits de novo formation of ribosomes. Therefore, by this criterion they are not ribosomal proteins (9, 41). However, it has not been ruled out that they are proteins which associate with already existing ribosomes in the cytoplasm. The 15-kDa protein was not detected in short-term metabolic labeling. Therefore, it is not clear whether it is a ribosomal protein.

It remains to be seen to what extent the mRNA-associated proteins correspond to known translation factors and to what extent they do not. Studies along these lines are in progress. Indeed, on the basis of antibody binding and mobility in two-dimensional gels, it appears that eEF-Tu can be cross-linked to mRNA by irradiating polyribosomes with UV light (unpublished data of L. Slobin and J. Greenberg).

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