Isolation of Messenger-Like Ribonucleoproteins†

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ABSTRACT: Subribosomal and polyribosomal messenger ribonucleoproteins (mRNPs) were isolated from Ehrlich ascites tumor cells by a method involving sedimentation of polyribosomal and subribosomal particles, dissociation with EDTA, and rate-zonal sedimentation. The fractions containing mRNA protein particles were applied to glass fiber filters and extensively washed with buffer containing 0.5 *M* KCl. The eluted material was demonstrated to be an RNA–protein complex containing poly(A)-rich RNA, heterogeneous in size, and free of 18S or 28S rRNA. mRNA function for the RNA was suggested by its ability to direct protein synthesis in a cell-free protein-synthesizing system derived from wheat germ embryos. Analysis of the proteins associated with subribosomal and polyribosomal mRNPs by iodination and sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed at least seven similar proteins. The apparent molecular weights of the three most prominent proteins were 78,000, 52,000, and 34,000. Analysis of reticulocyte polyribosomal mRNPs revealed an increased prominence of the 78,000 and 52,000 molecular weight proteins relative to the other protein bands.

In eukaryotic cells, mRNA appears to be associated with specific proteins in the nucleus and the cytoplasm (Perry and Kelley, 1968; Henshaw, 1968; Cartouzou et al., 1969; Olsnes, 1970; Spohr et al., 1970; Lebleu et al., 1971; Schochetman and Perry, 1972; Blobel, 1972, 1973; Bryan and Hayashi, 1973). Although mRNA liberated from polysomes may interact nonspecifically with cytoplasmic proteins (Baltimore and Huang, 1970; Leytin et al., 1970), many studies suggest that specific mRNA-protein complexes (mRNPs) occur (Lebleu et al., 1971; Schochetman and Perry, 1972; Blobel, 1972, 1973; Bryan and Hayashi, 1973) and may be of functional significance (Ilan and Ilan, 1973; Cashion and Stanley, 1974). Lebleu et al. (1971) reported two major protein bands (mol wt 68,000 and 130,000) associated with reticulocyte polyribosomal mRNPs. Blobel (1972), using puromycin to dissociate reticulocyte polyribosomes, also demonstrated two protein bands which, however, migrated much faster on SDS[1]–polyacrylamide gel electrophoresis. Similar results were obtained with polyribosomal mRNPs purified from L cells, although application of this technique to liver polyribosome-associated mRNPs revealed at least 13 protein bands (Blobel, 1973). By using sedimentation in a zonal rotor to increase yield, Bryan and Hayashi (1973) demonstrated that brain polyribosomal mRNA was associated with two proteins of molecular weights 52,000 and 78,000, and, perhaps, one or more less prominent protein bands. It remains unclear whether more than two proteins are specifically associated with mRNA. The present study describes a method for the isolation of mRNA–protein complex. The observation that mRNA–protein complexes were specifically retained on glass fiber filters permitted their convenient isolation under conditions unfavorable to formation of nonspecific mRNA–protein complexes. This method also permitted isolation of subribosomal mRNA–protein complexes. Augmented sensitivity of analysis of associated proteins was accomplished by their iodination prior to gel analysis. At least seven mRNA-associated proteins were identified in mRNA–protein complexes isolated from Ehrlich ascites cells.

Experimental Procedures

Materials

[^{3}H]Uridine (25 Ci/mmol), [^{3}H]adenosine (15 Ci/mmol), and [^{3}H]leucine (55 Ci/mmol) were obtained from Schwartz/Mann. Minimal essential medium and synthetic poly(ribouridylic acid) and poly(riboadenylic acid) were purchased from Miles Laboratories, Inc. Globin mRNA was obtained from Searle Diagnostic; electrophoretically purified ribonuclease was purchased from Worthington Biochemical Corporation; phenylmethanesulfonyl fluoride was obtained from Sigma Chemical Company.

Methods

Preparation of mRNA Protein Particles. Ehrlich ascites tumor cells were harvested 7 days after inoculation of Swiss mice. The cells were collected by centrifugation at 1000*g* and suspended in F-10 medium with 15 m*M* Hepes (pH 7.4) at a concentration of 10^6 cells/ml. After incubation for 16 hr during which labeling of cellular RNA with [^{3}H]adenosine or [^{3}H]uridine was accomplished, cells were harvested by centrifugation and washed twice with 50 ml of 0.9% NaCl containing 1 m*M* adenosine and 1 m*M* uridine. For preferential labeling of mRNA, rRNA synthesis was inhibited by additon of actinomycin D (0.05 μg/ml) to the culture medium. Thirty minutes later, 1 mCi of [^{3}H]uridine (25 Ci/mmol) and 0.5 mCi of [^{3}H]adenosine (15 Ci/mmol) were added and incubation was continued for an additional 60 min. Total cellular RNA was labeled by incubation of cell suspensions for 16 hr at 37° in the presence of 0.2 mCi of [^{3}H]uridine (25 Ci/mmol). All procedures were performed at 4° using acid and alkali, diethyl pyrocarbonate-

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; MEM, minimal essential medium; poly(A), poly(riboadenylic acid); poly(U), poly(ribouridylic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

washed glassware heated to 180°, and RNase-free buffers. The cells were lysed in 10 m*M* $(EtOH)_3N$-HCl (pH 7.6), 10 m*M* KCl, and 1 m*M* $MgCl_2$ by gentle hand homogenization using a glass pestle. After lysis, sucrose was added to a final concentration of 0.25 *M* and polyribosomes and crude "subribosomal" mRNPs were prepared as described in Figure 1. Sedimentation through 15–35% linear sucrose density gradients revealed an intact polyribosomal profile (data not shown). The resuspended polyribosomes were dissociated with 50 m*M* EDTA as described in Figure 1 and 40–60 A_{260} units of EDTA-dissociated polysomes were applied to 38 ml of a 10–30% linear sucrose density containing 16 m*M* $(EtOH)_3N$ (pH 7.6) and centrifuged at 70,000*g* for 18 hr. The gradients were then collected from the top in 1.2-ml fractions, absorbancy was measured at 260 nm, and radioactivity was determined using an aliquot of each fraction. Sedimentation of pulse-labeled polyribosome-associated RNA (Figure 2) was used as a marker for sedimentation of mRNPs and the appropriate fractions were pooled.

Acid, base-washed glass fiber filters (GF/A, Whatman) or poly(U)-impregnated filters prepared by immobilizing 1.2 mg of synthetic poly(ribouridylic acid) to GF/A glass fiber filters (Sheldon et al., 1972) (60% retained) were used for additional purification. The filters were extensively prewashed with 10 m*M* $(EtOH)_3N$ (pH 7.6)–200 m*M* KCl. The pooled regions containing polyribosomal or subribosomal mRNPs were diluted in 20 volumes of 10 m*M* $(EtOH)_3N$ (pH 7.6)–200 m*M* KCl and slowly filtered (less than 0.5 ml/min) at 4° through glass fiber filters. To minimize retention of proteins nonspecifically associated with RNA, the filters were then rapidly washed with 300 ml of 10 m*M* $(EtOH)_3N$ (pH 7.6) and 500 m*M* KCl followed by 5 ml of 10 m*M* $(EtOH)_3N$ (pH 7.6) and 100 m*M* KCl, and dried by suction. mRNPs were eluted from the filters in 1 ml of sterile water or in 0.1% SDS by gentle agitation of the filter at 4° for 4–6 hr.

For the preparation of mRNPs from rabbit reticulocytes, 10-week old white male rabbits were given 25 mg of phenylhydrazine and 0.1 μmol of glutathione subcutaneously daily for 5 days; 2 days later, they were given heparin intravenously and blood was obtained via cardiac puncture. The red blood cells were washed in isotonic phosphate-buffered saline, and the buffy coat was completely removed by aspiration. The red blood cells were lysed in two volumes of sterile water containing 2 m*M* $MgCl_2$ and 1 m*M* dithiothreitol, and polyribosomal mRNPs were prepared as described above.

RNase Treatment of mRNPs. Affigel beads (Bio-Rad Laboratories), on which pancreatic RNase has been immobilized, were prepared by incubating RNase (50 mg/ml) with 0.1 g of Affigel beads in 0.1 *M* potassium phosphate (pH 7.0) for 120 min at 4°. The beads were washed successively with 0.1 *M* ethanolamine (pH 8.0), 0.1 *M* Tris-HCl at pH 4.0 and 9.0, and finally with distilled water. One-hundred microliters of beads with immobilized RNase rendered greater than 95% of radiolabeled rRNA (100 μg/ml) and less than 3% of radiolabeled poly(A) (100 μg/ml) Cl_3CCOOH soluble when incubated in 50 m*M* Tris-HCl (pH 7.6)–100 m*M* KCl for 20 min at 37°. When desired, mRNPs were incubated in 50 m*M* Tris-HCl (pH 7.6) and 100 m*M* KCl for 20 min at 37° with 100 μl of the Affigel beads to which pancreatic RNase had been immobilized and the beads were removed by centrifugation.

Cesium Chloride Gradients. mRNP solutions were adjusted to 2.5% glutaraldehyde and allowed to remain at 4° for 1 hr. The fixed mRNPs were then applied to a preformed cesium chloride gradient and centrifuged at 39,000 rpm for 18 hr at 22° in an SW50L rotor. Gradients were collected from the top and the density was determined on every fifth fraction. Ten micrograms of carrier rRNA was added to each fraction, 1 ml of 10% Cl_3CCOOH was added and, after 30 min, the samples were applied to nitrocellulose (Millipore, 0.45 M) filters, washed with 10 ml of ice-cold 5% Cl_3CCOOH and 5 ml of cold ethanol, and counted in 10 ml of Bray's solution (Bray, 1960).

Deproteinization of mRNPs and Translation of mRNA. mRNPs were adjusted to 1% SDS, and 20 min later were extracted three times with phenol–chloroform (v/v, 1:1) and three times with ether. For translation, the RNA was precipitated by the addition of 2.5 volumes of ethanol and stored for 18 hr at −20°. The RNA was pelleted by centrifugation at 20,000*g* for 20 min and resuspended in water. The RNA content was estimated from the original A_{260} absorbancy assuming 40 μg of RNA/A_{260} unit. A cell-free protein-synthesizing system derived from wheat germ was prepared as previously described (Roberts and Paterson, 1973). The protein synthesis assays contained, in a final volume of 60 μl, 20 μl of wheat germ S-30, 20 m*M* Hepes (pH 7.6), 2 m*M* dithiothreitol, 1 m*M* ATP, 0.08 m*M* GTP, 8 m*M* creatinine phosphate, 40 μg/ml of creatinine phosphokinase, 60 μ*M* unlabeled amino acids, 80 m*M* potassium chloride, 2 m*M* magnesium acetate, and 1.5 μ*M* of [^{3}H]leucine (55 Ci/mmol). Incubations were for 90 min at 28° and incorporation of [^{3}H]leucine in protein was determined by trichloroacetic acid precipitation as previously described (Roberts and Paterson, 1973). Addition of 10 μg of rRNA or tRNA produced no stimulation of protein synthesis in this system.

Iodination of Proteins. All samples were adjusted to 0.1% SDS. Iodination was accomplished using the solid-state lactoperoxidase method of David and Reisfeld (1974) using 250 μCi of carrier-free ^{125}I (New England Nuclear) and 10–20 μl of Sepharose-bound lactoperoxidase beads. The samples were dialyzed for 48 hr against large volumes of 1% SDS and 10^{-2} *M* sodium iodide at room temperature to remove the nonspecifically bound ^{125}I. The final dialysis was against 1% SDS, 10% glycerol, and 0.025% Phenol Red. Samples were adjusted to 100 m*M* dithiothreitol and boiled for 1 min prior to electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis. Samples were analyzed by slab polyacrylamide gel electrophoresis using the discontinuous pH 9.18 system described by Neville (1971) with the exception that the final acrylamide concentration was 10% and the methylenebisacrylamide concentration was 0.33%. When possible, an equal amount of Cl_3CCOOH precipitable counts was applied in a constant volume of 20 μl. The slabs were run at 10 mA and stained overnight in 0.025% Coomassie Brilliant Blue, 25% 2-propanol, and 10% acetic acid. The solution was changed to 0.0025% Coomassie Brilliant Blue, 10% 2-propanol, and 10% acetic acid. The final destaining was done in 10% acetic acid. The gels were dried overnight on a vacuum drying plate and radioautographed on Kodak RP Royal X-Omat RP/54 X-ray film following Kodak instructions for developing.

Results

mRNPs were prepared by sedimentation of the EDTA-dissociated polyribosomes (Figure 1) through linear sucrose density gradients. Sedimentation of pulse-labeled polyribo-

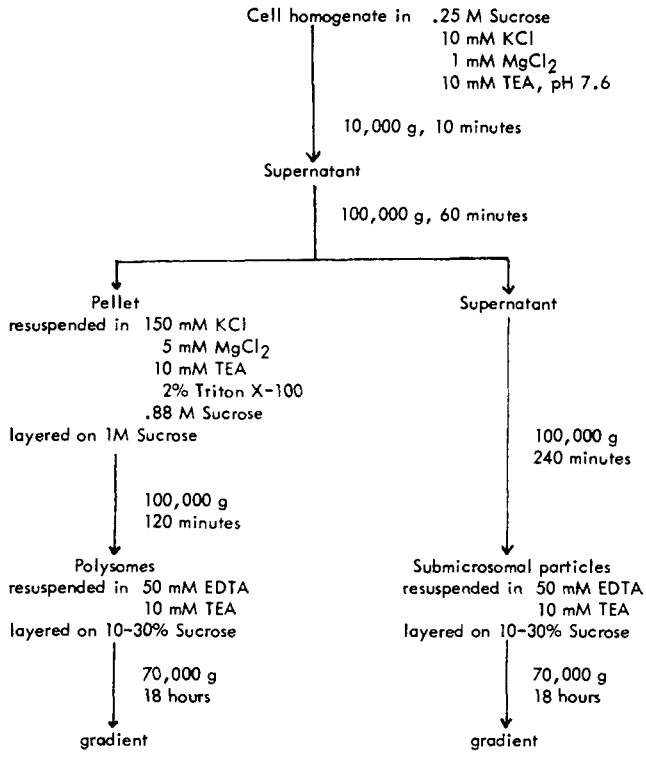

FIGURE 1: Technique for isolation of polysomes and crude cytoplasmic mRNPs.

Table I: Retention of mRNPs by GF/A and Poly(U) GF/A Filters.[a]

Sample	Filter	Poly(A) Added	cpm Added	cpm Retained	% Retained
Polysomal mRNPs	GF/A	None	5100	2416	47.6
	Poly(U) GF/A	None	5100	2072	40.6
	Poly(U) GF/A	6 mg	5100	1870	36.6

[a] Polyribosome-associated mRNPs were prepared through the sucrose gradient step from cells labeled for 16 hr in the presence of 0.2 mCi of [^{3}H]uridine (25 Ci/mmol) and the region indicated in Figure 1 was applied to filters as described in the Experimental Section. Filters were dried by suction and counted in Liquifluor–toluene. Synthetic poly(riboadenylic acid) was added prior to filtration. Similar results were obtained with polyribosomal and subribosomal mRNPs in two other experiments of similar design.

some-associated RNA was used as a marker for sedimentation of mRNPs, and the fractions were pooled as indicated in Figure 2 for additional purification. Analogous regions were pooled from gradients of the subribosomal particles obtained from the post-microsomal supernatant (Figure 1). The pooled fractions were then applied to glass fiber filters as described in the Experimental Section to permit extensive washing in a high salt buffer. Thirty to forty percent of the applied material was retained on the filters after thorough washing (Table I). Less than 1% of applied protein-free transfer or ribosomal RNA (100 μg) and less than 3% of synthetic poly(riboadenylic acid) (100 μg) were retained on GF/A filters; greater than 95% synthetic poly(riboadenylic acid) and greater than 90% globin mRNA were specifically retained on glass fiber filters on which poly(ribouridylic acid) was immobilized. The poly(U) filters did not retain a greater percentage of labeled mRNPs than GF/A filters nor did addition of excess poly(riboadenylic acid) alter retention on poly(U) filters (Table I). Hybridization was,

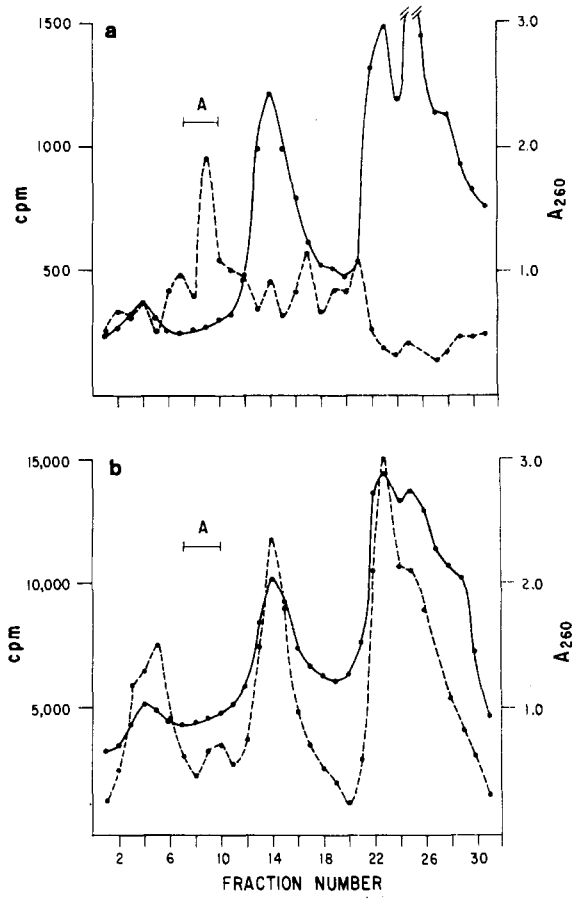

FIGURE 2: Preparation of crude polysomal mRNPs. EDTA-dissociated polyribosomes were sedimented through 10–30% linear sucrose density gradients as described in the Experimental Section. In panel a, mRNA was preferentially labeled; in panel b, total cellular RNA was labeled (see Methods). The area indicated (A) was pooled and used to prepare polysomal RNPs. (●—●) A_{260}; (●- - -●) cpm.

Table II: Retention of mRNPs on Filters.[a]

RNA Applied		Total RNA Retained		RNA Eluted	
A_{260} units	cpm	cpm	%	cpm	%
0.56	20,160	7,480	38	6,400	85
2.7	151,000	57,990	39	55,400	96
5.4	302,400	113,240	38	96,500	85

[a] [^{3}H]Adenylate polyribosomal mRNPs were prepared in the usual manner; the fractions indicated as A in Figure 2 were pooled and aliquots of various sizes were applied to a poly(U) GF/A filter as described in the Experimental Section. Half of the filter was counted in Liquifluor–toluene; the other half was eluted in 1 ml of sterile water and adjusted to 10% Cl_3CCOOH. The precipitate was collected on GF/C filters, washed with 5% Cl_3CCOOH and ethanol, and counted in Liquifluor–toluene.

therefore, apparently not required for the retention of mRNPs by these filters, although it was required for the retention of deproteinized poly(A)-rich RNA. The percentage of RNA retained remained constant over a wide range of RNP concentrations (0.5–6 A_{260} unit) and greater than 95% of the retained RNP was eluted with either ribonuclease-free distilled water or 0.1% SDS (Table II). A similar percentage of RNA obtained by deproteinization of mRNPs was retained on poly(U), GF/A filters (Table III); less than 3% of this RNA was retained on a GF/A filter on which poly(U) had not been immobilized.

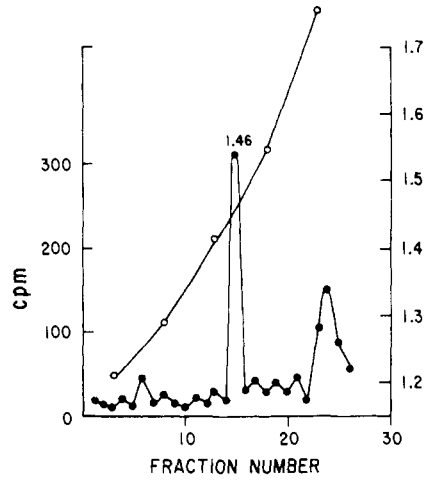

FIGURE 3: Cesium chloride gradients of mRNPs. [^{3}H]Adenylate-labeled mRNPs were fixed with glutaraldehyde, applied to preformed cesium chloride gradients, and centrifuged as described in the Experimental Section. Fractions were collected and buoyant density (right ordinate) and Cl_3CCOOH precipitable counts (left ordinate) were determined as described in the Experimental Section. (●—●) cpm; (○—○) buoyant density (g/cm^3).

Table III: Retention of mRNAs and mRNPs by Poly(U) Filter.[a]

Sample	Extracted	Total cpm Applied	Total cpm Retained		RNase Resistance of cpm Retained
			cpm	%	
Polysomal	–	15,320	3,680	24	32
RNP	+	11,120	3,100	28	31
Cytoplasmic	–	19,240	5,200	27	30
RNP	+	6,500	1,700	26	33

[a] [^{3}H]Adenylate polyribosomal and subribosomal mRNPs were divided into aliquots. The first aliquot was directly applied to the poly(U) GF/A filter while the second aliquot was SDS–phenol extracted before it was applied to the filter (see Methods). The poly(U) filters were cut in half after washing and drying; half the filter was counted in Liquifluor–toluene. The other half of the poly(U) filter was eluted in water for 6 hr at 4°; 83–86% of total retained counts were eluted. The eluted material for each sample was adjusted to 50 m*M* Tris-HCl (pH 7.6)–150 m*M* KCl, and divided in two fractions. One fraction was incubated with pancreatic RNase (3 μg/ml) at 37° for 20 min prior to Cl_3CCOOH precipitation. Under these conditions, greater than 95% of tRNA (10 μg/ml) or rRNA (10 μg/ml) was rendered Cl_3CCOOH soluble. The Cl_3CCOOH precipitable material was collected and counted as described in the Experimental Section. When the identical experiment was performed using RNA derived from [^{3}H]uridylate-labeled RNPs, greater than 98% of the radioactivity was rendered Cl_3CCOOH soluble by incubation with pancreatic RNase.

The eluted material was found to contain complexes comprised of messenger-like RNA and protein by several criteria. Radiolabeled polyribosomal RNPs eluted from the filters were fixed with glutaraldehyde and sedimented through preformed CsCl gradients. A major discrete peak of radioactive material with a buoyant density of 1.46 g/cm^3 was obtained (Figure 3), a density for RNPs similar to that reported by others (Perry and Kelley, 1968). This peak disappeared with incubation of the RNP for 15 min at 37° with Pronase (20 μg/ml) or pancreatic RNase (3 μg/ml) prior to glutaraldehyde fixation. In addition, a less prominent peak of radioactivity was observed at a buoyant density of 1.68–1.72 (Figure 3). The digestion of [^{3}H]adenylate-labeled mRNPs with immobilized pancreatic ribonuclease

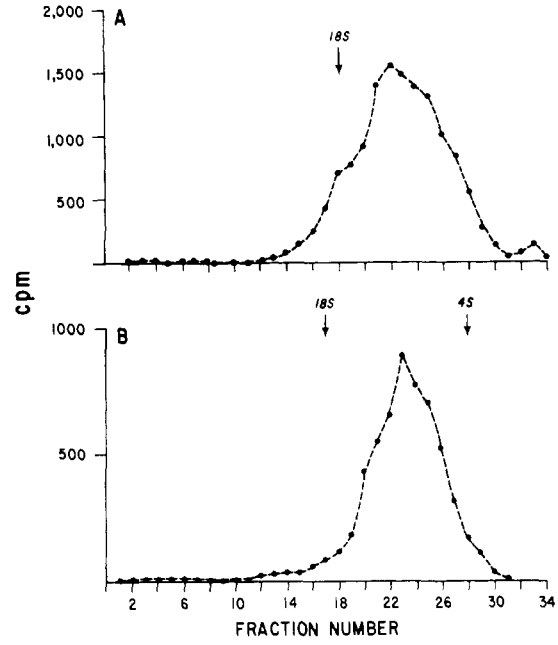

FIGURE 4: Sedimentation profile of RNA from purified polyribosomal and subribosomal mRNPs. mRNPs were prepared from cells incubated for 18 hr in the presence of [^{3}H]uridine (0.2 mCi/ml; 25 Ci/mmol). After elution from poly(U) GF/A filters, mRNPs were deproteinized as described in the Experimental Section, adjusted to 0.5% SDS, and applied to 5–20% linear sucrose density gradients containing 10 m*M* Tris-HCl (pH 7.6), 10 m*M* NaCl, and 0.5% SDS. Centrifugation was at 50,000 rpm for 240 min using an SW56 rotor. Fractions were collected from the bottom and counted in Bray's solution (Bray, 1960). The profile of RNA derived from polysomal mRNPs is shown in panel A and from cytoplasmic mRNPs in panel B.

prior to glutaraldehyde fixation was associated with disappearance of the 1.46 peak and an increase in the 1.68–1.72 peak suggesting that this second peak represented isolated poly(A) tracts with associated protein.

RNA present in polyribosomal and subribosomal RNPs was analyzed by sedimentation through linear SDS–sucrose density gradients (Figure 4). The heterogeneous sedimentation profile of the RNA associated with purified mRNPs was similar for both polyribosomal and subribosomal mRNAs. Radiolabeled peaks of RNA sedimenting at 18 S or 28 S were consistently absent in the gradient profile. When [^{3}H]adenylate-labeled RNA was prepared by deproteinization of RNPs, 30–35% of the counts remained Cl_3CCOOH precipitable after extensive digestion with pancreatic RNase (Table III). More than 95% of the RNase-resistant RNA was retained on a poly(U) filter, further supporting its identity as poly(A) tracts.

Evidence that retained RNPs contained mRNA was provided by the ability of the RNA derived from mRNPs to direct the incorporation of radiolabeled precursor into proteins using a cell-free protein-synthesizing system derived from wheat germ. The incorporation was a linear function of the amount of RNA added. Intact mRNPs failed to direct incorporation of radiolabeled precursor into proteins in this system. Intact mRNPs isolated from reticulocyte polyribosomes by the same method, however, efficiently directed protein synthesis in this system; RNPs containing approximately 1 μg of RNA directed incorporation of more than 100 pmol of leucine into protein, a 50-fold stimulation above the endogenous activity of the translation system.

The proteins associated with mRNPs isolated from poly(U) filters were analyzed by SDS–polyacrylamide gel

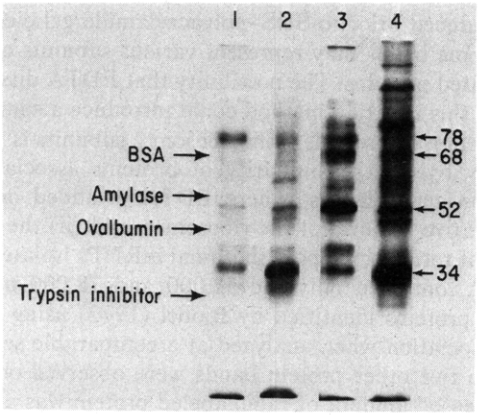

FIGURE 5: SDS-polyacrylamide gel electrophoresis of iodinated mRNP proteins. Polyribosomal mRNPs (samples 1 and 2) and subribosomal mRNPs (samples 3 and 4) were eluted from the poly(U) GF/A filters using sterile water. Samples 1 and 3 were treated with immobilized RNase as described in the Experimental Section. The samples were then iodinated and electrophoresed (see Methods). Migration of marker proteins was identified by staining. Molecular weights are bovine serum, 68,500; α-amylase, 56,000; ovalbumin, 43,000; and soy bean trypsin inhibitor, 21,500.

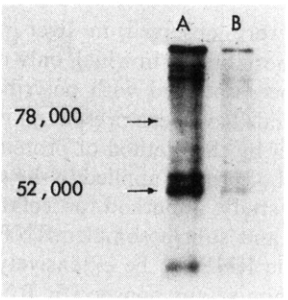

FIGURE 6: Effect of protease inhibitors in preparation of mRNPs. Subribosomal mRNPs were prepared as described in the Experimental Section with 1 m*M* phenylmethanesulfonyl fluoride, 0.5 m*M* L-1-tosylamido-2-phenylethyl chloromethyl ketone, and 0.5 m*M* *p*-tosyl-L arginine methyl ester present (panel A) or absent (panel B) in every buffer during the procedure. Following elution from the GF/A filters with 0.1% SDS at 4° for 60 min, proteins were iodinated and subjected to SDS-polyacrylamide gel electrophoresis (see Methods). Similar results were obtained with polyribosomal and subribosomal mRNPs in two other experiments using buffers containing these proteolytic enzyme inhibitors or 1 m*M* phenylmethanesulfonyl fluoride, 10 m*M* iodoacetamide, and 10 m*M* ϵ-aminocaproic acid.

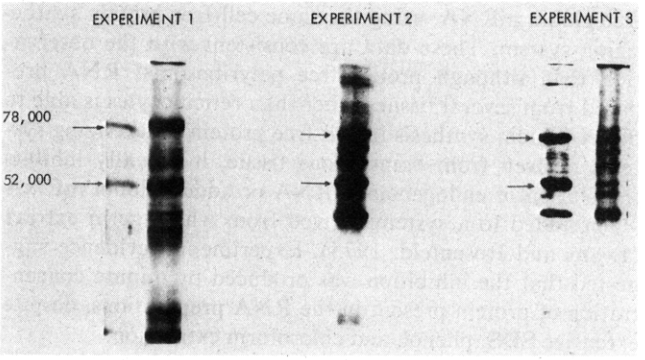

FIGURE 7: SDS-polyacrylamide gel electrophoresis of reticulocyte mRNPs. Reticulocyte polyribosomal and subribosomal mRNPs and Ehrlich ascites tumor cell subribosomal mRNPs were prepared, iodinated, and subjected to SDS-polyacrylamide gel electrophoresis as described in the Experimental Section. Molecular weights were determined from the migration of four standard proteins as described in Figure 6; the position of the 52,000 and 78,000 molecular weight proteins is indicated by arrows. Experiment 1: Reticulocyte polyribosomal (left) and subribosomal (right) mRNPs were prepared in parallel and analyzed on the same slab electrophoresis; developed for 7 days. Experiment 2: Ehrlich ascites tumor cell subribosomal nRNP; developed for 14 days. Experiment 3: Separate preparations of reticulocyte polyribosomal (left) and Ehrlich ascites tumor cell subribosomal mRNPs (right) run on separate slab gels. The counts of reticulocyte mRNP applied were threefold that applied in experiment 1 and the film was developed for 28 days.

electrophoresis after iodination. The iodinated material was degraded by digestion with Pronase and entirely resistant to digestion by pancreatic RNase. Seven protein bands were consistently observed on SDS-polyacrylamide gel analysis of the iodinated proteins (Figure 5). The most intense bands had apparent molecular weights of 78,000 ± 1500, 52,000 ± 1000, and 34,000 ± 2500, as calculated from ten separate determinations using six different preparations of mRNPs. Their electrophoretic migration was unaffected by exhaustive digestion with immobilized pancreatic RNase prior to iodination and electrophoresis (Figure 5). Several additional protein bands of high molecular weights were observed in the subribosomal RNP preparation (Figure 5).

Staining of the same gels with Coomassie Brilliant Blue generally revealed no more than one or two protein bands. The 78,000 molecular weight protein was the protein usually visualized by this technique, and the 34,000 molecular weight protein was never visualized by protein staining.

When proteolytic enzyme inhibitors were used during the entire preparative procedure for preparation of subribosomal and polyribosomal mRNPs, identical gel patterns of iodinated proteins were obtained in subribosomal or polyribosomal mRNPs (Figure 6).

Reticulocyte polyribosomal and subribosomal mRNPs were prepared and analyzed using identical procedures. Reticulocyte polyribosomal mRNPs were found to be associated with two proteins (Figure 7, experiment 1) of apparent molecular weights 52,000 and 78,000, as previously reported (Blobel, 1972), with only trace amounts of other protein bands present. In other preparations, application of larger quantities of radiolabeled material and development for long periods of time enabled visualization of several minor bands (Figure 7, experiment 3). Subribosomal mRNPs from reticulocyte exhibited multiple minor bands, many similar to those observed in subribosomal mRNPs prepared from Ehrlich ascites tumor cells (Figure 7).

Discussion

The proposal that mRNA is associated with specific proteins (Henshaw, 1968; Cartouzou et al., 1969; Schochetman and Perry, 1972) has been given support by demonstration of the apparent constancy of proteins associated with mRNPs from several tissues (Blobel, 1972, 1973; Bryan and Hayashi, 1973). It has been suggested that the proteins may have functional importance in the process of mRNA translation (Cashion and Stanley, 1974; Ilan and Ilan, 1973).

Polyribosomal RNPs isolated from the reticulocyte by EDTA dissociation and sedimentation techniques were reported to contain two major proteins (mol wt 68,000 and 130,000) and several minor proteins (Lebleu et al., 1971), while Blobel (1972), using a puromycin dissociation technique, reported two proteins (mol wt 52,000 and 78,000). Two similar proteins were reported to be associated with polyribosomal mRNA in L cells and brain cells (Blobel, 1973; Bryan and Hayashi, 1973), although numerous addi-

tional proteins were observed in liver mRNPs (Blobel, 1973). Even in those tissues in which only two proteins have been found to be associated with polyribosomal mRNPs, minor protein bands have been present on gels or could have escaped detection by the method of protein staining due to the small amount of protein applied to the gels.

In the current study, a method for isolation of both polysome-associated and subribosomal mRNPs has been used which enables the RNPs to be extensively washed in high salt buffer to minimize any nonspecific RNA–protein interaction. This procedure is based on the retention of mRNPs on glass fibers filters. The material eluted from the filters was demonstrated to be an RNA–protein complex. Although RNA derived from this complex by SDS, phenol, and chloroform extraction directed incorporation of radiolabeled precursor into protein using a cell-free protein synthesizing system derived from wheat germ, the intact mRNPs failed to direct protein synthesis using this system. Similar results were obtained with mRNPs isolated from porcine parotid gland (unpublished observation). In contrast, intact mRNPs isolated from reticulocytes by this method directed protein synthesis as effectively as protein-free globin mRNA, using the same cell-free protein synthesizing system. These data are consistent with the observation that although protein-free polyribosomal RNA prepared from several tissues other than reticulocytes is able to direct protein synthesis in cell-free protein synthesizing systems derived from homologous tissue, it actually inhibits translation of endogenous mRNA or added globin mRNA when added to a system derived from wheat germ extract (Evans and Rosenfeld, 1975). Experimental evidence suggested that the inhibition was produced by minute concentration of protein present in the RNA preparations, despite extensive SDS, phenol, and chloroform extraction.

An increased sensitivity for detection of proteins was achieved by iodination prior to SDS–polyacrylamide gel electrophoresis. By this method at least seven similar proteins were found to be associated with polyribosomal and subribosomal mRNPs. Since iodination by the method used (David and Reisfeld, 1974) requires the presence of exposed tyrosine residues, the density of the bands reflects the degree of iodination as well as the protein concentration. Furthermore, protein devoid of tyrosine residues would not be detected. The level of sensitivity for detection of proteins afforded by this method is much greater than that achieved by protein staining which generally revealed no visible bands or only one or two bands, particularly the 78,000 molecular weight protein. The protein with an apparent molecular weight of 34,000, often very prominent by radioautography, was never seen by protein staining, indicating that it contains more exposed tyrosine residues. It probably represents the protein associated with trace amounts of coisolated 5S RNA–protein particles, which have been isolated from several tissues (Petermann et al., 1972; Blobel, 1971; Lebleu et al., 1971). A 5S RNA–protein particle was also isolated from Ehrlich ascites cells and a single protein of apparent molecular weight 34,000 was found associated with 5S RNA on SDS–polyacrylamide gel electrophoresis after iodination (unpublished data).

The constancy of proteins in different preparations, after extensive washing with 0.5 *M* KCl, provides suggestive evidence for specificity of the association. Sedimentation of unfixed mRNPs through cesium chloride gradients failed to remove the associated proteins (unpublished data), suggesting a high affinity of these proteins for RNA. Since proteins were reduced prior to SDS–polyacrylamide gel electrophoresis, some bands may represent various subunits of larger, dissociated proteins. The possibility that EDTA dissociation used in this isolation method could introduce a spurious copurification of proteins from ribosomal subunits is rendered unlikely by (i) the similarity of proteins associated with subribosomal mRNPs, where EDTA is added only after quantitative removal of the ribosomes; and (ii) the observation that reticulocyte polyribosomal mRNPs isolated by this method contained only the 52,000 and 78,000 molecular weight proteins identified by Blobel (1972) using puromycin dissociation when analyzed at a comparable sensitivity. Four to five other protein bands were observed only when an increased amount of radiolabeled protein was applied to the gels or an increased time of development was allowed to augment the sensitivity of analysis. It is significant that proteins with various apparent molecular weights reported by different authors (Blobel, 1972, 1973; Bryan and Hayashi, 1973; Lebleu et al., 1971) were all detected in the same preparation by this method. Some of the lighter bands could represent proteolytic digestion products of the heavier proteins; however, the same gel patterns were observed when various proteolytic enzyme inhibitors were added during the entire isolation procedure.

The marked variation in the intensity of the protein bands suggests that not all of the proteins are associated with every mRNA. One possibility is that there are subpopulations of mRNAs, most of which are associated with the two more prominent proteins, but some of which are associated with additional or different proteins. In the reticulocyte mRNPs, where more than 90% of the mRNA represents globin mRNA, the minor bands are much less prominent than in Ehrlich ascites tumors where a greater heterogeneity of mRNA is present.

Absolute proof of the physiologic importance of these proteins will require demonstration of their function. The 78,000 molecular weight protein has been suggested to be associated with the poly(A) tracts of mRNA (Blobel, 1973). It has been observed in this laboratory that the isolated 78,000 molecular weight protein binds specifically to synthetic poly(riboadenylic acid) and is not removed by washing with 2 *M* salt or sedimentation through cesium chloride gradients (unpublished data). The ability to isolate intact subribosomal, as well as polyribosomal, mRNPs permits evaluation of possible regulatory function of the proteins as has been demonstrated for other protein–nucleic acid interactions (Gilbert and Muller-Hill, 1970).

Acknowledgments

We would like to thank Dr. Gary David for his advice and assistance with iodination techniques.

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Different Cyclic Adenosine 3′,5′-Monophosphate Requirements for Induction of β-Galactosidase and Tryptophanase. Effect of Osmotic Pressure on Intracellular Cyclic Adenosine 3′,5′-Monophosphate Concentrations†

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ABSTRACT: In this study we have tried to answer the following questions: (1) is it possible for different catabolite-repressible genes, although submitted to the same control, to be expressed selectively depending upon the growth conditions, and (2) what is the effect of increasing the osmolarity of the medium on the intracellular level of cAMP? Two conditions were found to cause a continuous variation of intracellular cAMP levels during growth. With different strains, higher cAMP levels are required for induction of the tryptophanase gene than are required for induction of the lactose operon. cAMP has also been provided externally in adenyl cyclase minus cells of a mutant that has been made permeable by EDTA treatment. Although external cAMP concentrations, 10 times higher than the usual intracellular levels, are required for induction of β-galactosidase and tryptophanase, the difference of requirements of cAMP is maintained. An increase in the osmolarity of the medium by sucrose addition causes a fourfold decrease in the intracellular cAMP level. As a consequence this prevents the induction of tryptophanase whereas β-galactosidase is still inducible. After pulse induction, a difference in the kinetics of expression of the tryptophanase and β-galactosidase genes was found. Its relationship with the previous results is discussed.

Many lines of evidence suggest that the major regulation of catabolite repression in *Escherichia coli* occurs through the interaction of cyclic adenosine 3′,5′-monophosphate (cAMP)[1] with a specific receptor protein (CRP) (Emmer et al., 1970; Riggs et al., 1971).

This complex binds to the promoters of catabolite-repressible genes and allows the initiation of transcription by the RNA polymerase (De Combrugghe et al., 1971; Beckwith et al., 1972). This functioning might be expected to be identical for all the genes submitted to this control. However, at least in one case, it has been shown that this is not the true situation. The levels of cAMP that are required to induce the positively controlled L-arabinose operon are higher than those needed to induce the negatively controlled lactose operon (Lis and Schleif, 1973). It is of interest to know if this is a unique case, or if other examples exist. In this report we describe a similar difference between the lactose operon and the tryptophanase gene. Four different systems showed that higher cAMP levels are required for induction of the tryptophanase gene than are required for induction of the lactose operon.

The effects of increasing the osmolarity of the medium on the intracellular cAMP level have been investigated. We have shown that a decrease of this level occurs and, as a consequence, this prevents the induction of tryptophanase whereas β-galactosidase induction is still possible.

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[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; CRP, cAMP receptor protein; IPTG, isopropyl β-D-thiogalactoside; ONPG, *o*-nitrophenyl β-D-galactoside.