RELEASE OF POLY A(+) MESSENGER RNA FROM RAT LIVER ROUGH MICROSOMES UPON DISASSEMBLY OF BOUND POLYSOMES

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ABSTRACT

Several procedures were used to disassemble rat liver rough microsomes (RM) into ribosomal subunits, mRNA, and ribosome-stripped membrane vesicles in order to examine the nature of the association between the mRNA of bound polysomes and the microsomal membranes. The fate of the mRNA molecules after ribosome release was determined by measuring the amount of pulse-labeled microsomal RNA in each fraction which was retained by oligo-dT cellulose or by measuring the poly A content by hybridization to radioactive poly U. It was found that ribosomal subunits and mRNA were simultaneously released from the microsomal membranes when the ribosomes were detached by: (*a*) treatment with puromycin in a high salt medium containing Mg^{++}, (*b*) resuspension in a high salt medium lacking Mg^{++}, and (*c*) chelation of Mg^{++} by EDTA or pyrophosphate.

Poly A-containing mRNA fragments were extensively released from RM subjected to a mild treatment with pancreatic RNase in a medium of low ionic strength. This indicates that the 3′ end of the mRNA is exposed on the outer microsomal surface and is not directly bound to the membranes. Poly A segments of bound mRNA were also accessible to [^{3}H]poly U for *in situ* hybridization in glutaraldehyde-fixed RM.

Rats were treated with drugs which inhibit translation after formation of the first peptide bonds or interfere with the initiation of protein synthesis. After these treatments inactive monomeric ribosomes, as well as ribosomes bearing mRNA, remained associated with their binding sites in microsomes prepared in media of low ionic strength. However, because there were no linkages provided by nascent chains, ribosomes, and mRNA, molecules were released from the microsomal membranes without the need of puromycin, by treatment with a high salt buffer containing Mg^{++}.

Thus, both in vivo and in vitro observations are consistent with a model in which mRNA does not contribute significantly to the maintenance of the interaction between bound polysomes and endoplasmic reticulum membranes in rat liver hepatocytes.

Polysomes bound to endoplasmic reticulum (ER) membranes are thought to be engaged in the translation of selected classes of messenger RNA (mRNA), coding for proteins which are segregated into the ER lumen for eventual discharge from the cell or for inclusion in other membrane-bounded organelles. Proteins destined to the ER membranes are also thought to be synthesized in membrane-bound polysomes (cf. references 29 and 33 for reviews). Individual ribosomes within the bound polysomes are known to interact with specific sites in the ER membranes, directly through their large subunits (34) and through their nascent polypeptide chains (31, 2). Although the molecular interactions which stabilize the association between ribosomes and membranes have been studied in detail, especially in rat liver microsomes (cf. reference 33), the process of assembly of bound polysomes and the mechanism leading to the selection of specific classes of mRNA's are less well understood.

It has been proposed that a segment of the nascent polypeptide chain located at its amino terminal end acts as a signal which directs ribosomes translating specific messengers to the ER membranes (7, 32, 28). Such a mechanism has received support from recent studies carried out with the primary translation products of messengers from pancreas (9, 10, 14), parathyroid (21), and myeloma cell microsomes (36, 28). There have also been several reports (27, 23, 26) suggesting that mRNA's of bound polysomes in tissue culture cells are associated with the membranes independently of the ribosomes and the nascent polypeptide chains. These messengers have been reported to be bound to the membranes through a segment located near the poly A region at the 3′ end of the mRNA. Although the association between an mRNA molecule and the ER membrane would not be sufficient to determine the fate of the protein for which it codes, this association could facilitate binding of ribosomes to neighboring membrane sites, as well as ensure reutilization of certain messengers in membrane-associated polysomes.

In this paper we present a study of the relationship of mRNA and microsomal membranes carried out with purified rat liver RM in which we determined the fate of mRNA after polysome disassembly in vitro by various procedures. We found that in contrast with the situation reported by another group of investigators using the same system (12), and the results found with cultured human diploid fibroblasts (23) and HeLa cells (27), the mRNA of rat liver bound polysomes is released together with the ribosomal subunits when the polysomes are disassembled in vitro. A similar behavior has been reported for the light chain mRNA in myeloma cells (19). A preliminary communication of this work has been published (22).

MATERIALS AND METHODS

Tissue Fractionation

Rat liver RM and membrane-bound polysomes were prepared by a modification of the procedure of Adelman et al. (1). After removal of nuclei from the homogenate (1) a postmitochondrial supernate (PMS) was obtained by centrifugation for 20 min at 17,000 rpm. The PMS was adjusted to 1.35 M sucrose by addition of a 2.5 M sucrose solution and layered over a step gradient consisting of 5 ml of 2.0 M sucrose-TKM and 5 ml of 1.55 M sucrose-TKM both containing 0.2 vol of rat liver high-speed supernate as an inhibitor of RNase (6). After centrifugation for 8–10 h at 44,000 rpm in a Ti60 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the RM was collected with a syringe from the 1.55 to 2.0 M interface. After 0.2 vol of rat liver high-speed supernate was added, the RM suspension was diluted 1:1 with 2 × HSB and sedimented (20 min at 35,000 rpm in a Ti60 rotor) to obtain the final RM preparation which was used immediately. Rat liver high-speed supernate (2 h × 105,000 *g*) prepared in 0.25 M sucrose was added (2.5 ml per 10 ml) to the RM suspension (RM derived from 20 g of tissue in 50 ml of 1.55 sucrose-TKM) as a source of RNase inhibitor (6). The ionic composition of the mixture was adjusted to that of high salt buffer (HSB: 0.5 M KCl, 50 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$), by adding appropriate amounts of 2 M KCl, 1 M Tris-HCl, pH 7.4, and 1 M $MgCl_2$, before sedimenting the RM by centrifugation (20 min at 35,000 rpm in the Ti-60 Beckman rotor) to remove inactive ribosomes and artefactually adsorbed free polysomes. This eliminates the so called "loosely bound" polysomes. The functional significance of these is obscure, and their mRNA would certainly be released by the in vitro procedures we used to disassemble the polysomes. The high salt wash released approx. 25–30% of the RNA from the RM.

We observed during the course of this work that in order to obtain undegraded mRNA from RM it was absolutely necessary to do the treatments in the presence of RNase inhibitors. Since we failed to recover undegraded mRNA molecules after incubating at room temperature, we decided to carry out all release experiments at 4°C and were thus able to control the residual RNase activity of the HSB-washed RM.

Disassembly of RM into Ribosomal Subunits, mRNA, and Stripped Vesicles

Freshly prepared, high salt-washed RM were resuspended in rat liver high-speed supernate (RM from 20 g of tissue in 25 ml). Heparin was added to the RM suspension to a final concn of 0.5–1.0 mg/ml before polysome disassembly and ribosome detachment by one of the following procedures:

(*a*) RM suspensions received an equal volume of a chelating solution to obtain a final concn of either 40 mM EDTA in 50 mM triethanolamine (TEA) HCl, pH 7.4 (34) or 30 mM sodium pyrophosphate, pH 8.2 (35) and were then incubated for 30 min at 4°C.

(*b*) The puromycin-KCl procedure (2) was used to disassemble the polysomes. In this case, a suspension of RM was brought to 500 mM KCl, 50 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$ by adding an equal volume of a compensating salt solution and incubated with 10^{-3} M puromycin for 30 min at 0°C.

(*c*) RM were brought to a solution of high ionic strength in the absence of Mg^{++} ions (32) as follows: 4 ml of RM resuspended in high-speed supernate received 1.25 ml of 4 M KCl and 250 μl of 1 M TEA-HCl, pH 7.4 before incubation at 4°C for 45 min, after which the samples were diluted with 5 ml of a cold solution containing 1 M KCl, 50 mM TEA-HCl, pH 7.4.

In all cases, membrane vesicles stripped of ribosomes were recovered by differential centrifugation (20 min at 35,000 rpm in a Ti-60 Beckman rotor). Supernate, containing the released ribosomal subunits, and pellets were saved for subsequent RNA extraction by phenol.

RM treated for ribosome detachment were also analyzed by sucrose density gradient centrifugation. In these cases, small amounts of high salt-washed RM (20 OD_{260} units measured after addition of sodium dodecyl sulfate (SDS) to a final concn of 0.5%) were resuspended in 0.5 ml of a solution containing partially purified RNase inhibitor (DEAE-cellulose fraction, reference 37) and then brought to 1 ml of the corresponding incubation buffer. After incubation, the RM suspensions were layered on sucrose density gradients of the composition indicated in the figure legends and centrifuged at 40,000 rpm for 1 h, at 4°C in the SW41 Beckman rotor.

RNase Treatment of RM

Aliquots (8 ml) of a suspension of freshly prepared RM, which were not washed in HSB, were diluted with 1 vol of TKM (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 10 mM $MgCl_2$) and received 0.5 ml of a ribonuclease A (Worthington Biochemical Corp., Freehold, N. J.) solution (500 μg/ml). Samples were then incubated at 30°C for 10 min. After this time, the microsomal vesicles were recovered by centrifugation (20 min, 35,000 rpm–Ti60). Pellets and supernate were saved for RNA extraction.

Extraction of RNA

SUPERNATES: Half a volume of extraction buffer (7 M urea, 50 mM Na acetate, pH 5.5, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) was added to 1 vol of supernate. The suspension was brought to 7 M with crystalline urea and adjusted to pH 5.5 with 1 N acetic acid. The RNA was extracted by shaking in a vortex with 1 vol of phenol:chloroform:isoamyl alcohol (25:24:1; vol:vol:vol) for 5 min.

The phases were separated by centrifugation in an IEC centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 2,500 rpm for 20 min, and the water phase was re-extracted with 1 vol of the chloroform-isoamyl alcohol (24:1; vol:vol). After removal of the water phase the combined organic phases were re-extracted with a small volume of the extraction buffer. The pooled water phases were extracted again with 1 vol of chloroform-isoamyl alcohol, and the RNA was precipitated by adding 2.5 vol of ethanol and storing overnight at −20°C. The RNA precipitate was recovered by sedimentation in a Sorvall HB4 rotor (DuPont Instruments, Sorvall Operations, Newtown, Conn.) at 10,000 rpm for 15 min at 3°C. The pellets were dissolved in water, and the amount of RNA as well as the poly A content was determined.

PELLETS: Microsomes and ribosome-stripped fractions were resuspended in 3 ml of extraction buffer containing 7 M urea and heparin (0.5–1 mg/ml) by homogenization in a Dounce homogenizer (Kontes Co., Vineland, N. J.). The RNA was extracted as described above.

SUCROSE DENSITY GRADIENT FRACTIONS: After 1 ml of extraction buffer and approx. 200 μg of *Escherichia coli* tRNA were added to each 0.6-ml gradient fraction, the RNA was extracted with 1 vol of the phenol-chloroform-isoamyl alcohol mixture. The organic layer was re-extracted with 0.5 ml of extraction buffer without urea, and the combined aqueous phases were precipitated with cold ethanol as above. The RNA was collected by sedimentation in an IEC centrifuge at 2,500 rpm for 20 min. The supernate was decanted, and the walls of the tubes were rinsed with 1 ml of 70% ethanol containing 100 mM NaCl. The washing solution was aspirated after centrifugation, and the pellets were dissolved in 0.5 ml of 2 × Standard Saline Citrate (SSC) (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 6.8) for determination of the poly A content by the method of Rosbash and Ford (30).

Poly U Hybridization

The poly A content of the extracted RNA fractions was measured by hybridization to radioactive poly U according to either Gillespie et al. (18) or Rosbash and Ford (30). With the former procedure, shorter incubations (2–3 h) were carried out at 35°C in 50-μl mixtures which contained 10–20 μg of RNA and an excess of [^{3}H]poly U (Miles Laboratories, Miles Research Prod-

ucts, Elkhart, Ind., 10^5 cpm/μg). The ratio of added poly U to the expected amount of poly A was at least 5:1.

Isolation of Poly A-Containing mRNA by Affinity Chromatography

mRNA was purified from phenol-extracted polysomal RNA by adsorption either to poly U-Sepharose or to oligo-dT cellulose.

(*a*) Poly U-Sepharose was prepared after cyanogen bromide activation of Sepharose as described by Cuatrecasas and Anfinsen (13), and poly U was coupled as described by Wagner et al. (40). Remaining active groups were inactivated by incubation with 0.1 N ethanolamine, pH 8.0, at 20°C for 2 h, and the final product was washed with three alternating cycles of acetate buffer (1 M NaCl, 0.1 M Na acetate pH 4.0) and borate buffer (1 M NaCl, 0.1 M borate, pH 8.0). The material was suspended in 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% Sarkosyl and stored in a cold room. The capacity of the poly U-Sepharose was tested with [^{3}H]poly A. The radioactivity in aliquots of the starting buffer and elution buffer was determined in Bray's solution (11). Polysomal RNA was applied to the poly U-Sepharose columns in binding buffer (300 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% SDS). Nonadsorbed material was removed by washing with binding buffer and with NETS solution (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% SDS). The bound poly A-containing mRNA was eluted with 90% formamide containing 10 mM Tris-HCl pH 7.4, 10 mM EDTA, and 0.2% SDS (24).

(*b*) Oligo-dT cellulose chromatography (5, 38) was performed as follows: 1 g of oligo-dT cellulose was resuspended in water, poured into a glass column, and washed with 6–10 ml of 0.5 N KOH solution (16). A similar washing was used to remove residual adsorbed material after each chromatography run. The column was then washed with 20 ml of 0.05% diethylpyrocarbonate or until the effluent was neutral and then equilibrated with the loading buffer (0.4 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% SDS). The phenol-extracted polysomal RNA was dissolved in a small volume of loading buffer and applied to the column. The column was washed with loading buffer and then with buffer of lower ionic strength (0.1 M NaCl) before eluting the poly A-containing mRNA either with 10 mM Tris-HCl, pH 7.4 or with 20 mM HEPES, pH 7.5. The mRNA fraction was precipitated with 2.5 vol of cold ethanol after the addition of 3 N sodium acetate, pH 6.0 to a final concn of 0.1 N. If necessary, tRNA was added as a carrier. RNA fractions not adsorbed to the column, containing rRNA and tRNA, were immediately precipitated with 2.5 vol of cold ethanol. The size distribution of the RNA fractions was determined by centrifugation in 15–30% sucrose gradients containing NETS buffer.

Treatment of RM with Glutaraldehyde

Microsomes resuspended in 50 mM triethanolamine, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$ (TEA-KM) were washed once by centrifugation and resuspended in the same buffer. Glutaraldehyde was added to 0.1% final concentration, and the samples were incubated at 4°C for 30 min. Free aldehyde groups were inactivated with 10 ml of TKM buffer, and the microsomes were recovered by sedimentation (20 min, 35,000 rpm—Ti60).

RNase Digestion of Poly A-Containing mRNA Fractions

Poly U-Sepharose purified mRNA fractions labeled in vivo with [^{32}P]orthophosphate were dissolved in 0.5 ml of 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA and incubated at 37°C for 30 min with a mixture of pancreatic RNase (2 μg/ml) and T1 RNase (5 U/ml) as previously described (23). The residual RNase-resistant material containing the poly A segments was extracted with the phenol-chloroform mixture. Poly A radioactivity in an aliquot of the final aqueous phase was analyzed by polyacrylamide gel electrophoresis. All of the acid-precipitable radioactivity in the fraction was retained by poly U-Sepharose and hence represented poly A.

Polyacrylamide Gel Electrophoresis of Poly A

For determination of the size of poly A sequences, samples containing [^{32}P]poly A fragments obtained by RNase digestion and phenol extraction were lyophilized and dissolved in electrophoresis buffer containing 15% glycerol and bromphenol blue as a marker. Electrophoresis was carried out in 3-mm slab gels of 10% acrylamide and 0.25% bisacrylamide in the buffer system of Dingman and Peacock (15). ^{32}P-labeled 5S RNA and tRNA were used as markers. The distribution of ^{32}P-radioactivity in gels was determined in 2-mm slices by measurement of Cerenkov radiation in a Beckman scintillation counter.

Sucrose Density Gradient Analysis

Sedimentation analysis of RM and membrane-bound polysomes (usually equivalent amounts of ribosomes in 1 ml) was carried out in 12.5-ml linear sucrose gradients which were centrifuged for 1 h at 40,000 rpm and 4°C in the Beckman SW41 rotor.

After centrifugation, the optical density distribution was monitored at 254 nm by withdrawing the gradients from the top of the tubes with a Buchler Auto Densiflow probe (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.). The effluents were collected in about 20 fractions. Radioactivity in each fraction was measured by liquid scintillation counting on glass fiber filter disks after fractions were precipitated with 5% cold trichloroacetic

acid (TCA), using 200 μg of bovine serum albumin as a carrier. Alternatively, aliquots were spotted on Whatman filter paper disks and processed for counting as described by Mans and Novelli (25).

Sources

Enzyme grade sucrose was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); Tris-base (Trizma), PIPES, HEPES, and ethionine from Sigma Chemical Co. (St. Louis, Mo.); acrylamide, bisacrylamide, CNBr, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) from Eastman Kodak Co. (Rochester, N. Y.); puromycin from ICN Pharmaceuticals, Inc., Life Sciences Group (Cleveland, Ohio); heparin from Fisher Scientific Co. (Pittsburgh, Pa.); glutaraldehyde from Polysciences, Inc. (Warrington, Pa.); sodium desoxycholate from Matheson, Coleman and Bell (Norwood, Ohio); Triton X-100 from Rohm and Haas Co. (Philadelphia, Pa.); Sepharose 6B from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc. (Piscataway, N. J.); DEAE-cellulose from Gallard-Schlesinger Chemical MfG. Corp. (Carle Place, N. Y.); oligo-dT cellulose (T3) Collaborative Research, Inc. (Waltham, Mass.); trypsin, ribonuclease A from Worthington Biochemical Corp. (Freehold, N. J.); Tl RNase from Calbiochem (San Diego, Calif.); *E. coli* tRNA from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); Poly U, [5-^{3}H]poly U, (sp act 54.2 μCi/mol P), [^{3}H]poly A, (sp act 30 μCi/mol P) from Miles Laboratories, Inc., Miles Research Products (Elkhart, Ind.); [5-^{3}H]orotic acid, New England Nuclear (Boston, Mass.); [^{32}P]orthophosphate (sp act 56 μCi/mg P) Amersham/Searle Corp. (Arlington Heights, Ill.).

Table I

Recovery of rRNA and mRNA in Bound Polysomes from Rat Liver Microsomes

	Rough microsomes	Bound polysomes
Total [^{3}H]RNA (cpm)	84,850	66,850 (78%)*
Poly A (ng)	9,300	8,450 (91%)
[^{3}H]mRNA (cpm)	6,430‡	6,500 (100%)§
rRNA (cpm)	55,940	45,580 (81%)
Recovery of total [^{3}H]RNA (%)	73.5	78

RM were isolated from [^{3}H]orotic acid-labeled rats (2 h). Half of the high salt-washed RM fraction was extracted immediately with phenol as described in Materials and Methods. From the other half, bound polysomes were prepared after detergent treatment by the addition of 0.1% Triton and 0.5% DOC and sedimentation through a 2 M sucrose cushion in TKM (Ti60 rotor, 44,000 rpm for 8 h). The RNA of the polysomal pellet and the cushion were extracted together. In separate experiments, we have shown that under these conditions of centrifugation not all the monosomes have sedimented through the cushion which contains approx. 20% of the OD_{260} and 15-20% of the poly A. Although it cannot be determined whether this small amount of poly A + mRNA is ribosome associated, even if this is not the case, our interpretation of the results will not be significantly affected. The RNA was recovered by ethanol precipitation, dissolved in the extraction buffer without 7 M urea, reextracted with phenol, and reprecipitated. This RNA was resuspended in 3 M sodium acetate, pH 6.0, to solubilize 5S RNA and tRNA and resedimented. The high molecular RNA fractions were dissolved in 100 mM sodium acetate, pH 7.0; the amount of total RNA was determined by acid precipitation (line 1), and the content of poly A was measured by [^{3}H]poly U hybridization (line 2). The RNA fractions were separated into rRNA (line 4) and mRNA (line 3) by affinity chromatography on oligo-dT cellulose. The acid-precipitable radioactivity was measured in each column fraction. The total recovery of [^{3}H]RNA before column chromatography is indicated in line 5.

* The numbers in parentheses indicate the percentage of each component of RM which is recovered in the bound polysome fraction.

‡ 11% of the sum of the radioactivity in [^{3}H]mRNA and [^{3}H]rRNA recovered from RM.

§ 12% of the sum of the radioactivity in [^{3}H]mRNA and [^{3}H]rRNA recovered from bound polysomes.

RESULTS

Association between mRNA of RM and Bound Polysomes

Table I shows that a major fraction (81%) of the labeled rRNA present in RM labeled in vivo for 2 h with [^{3}H]orotic acid was recovered in bound polysomes obtained by sedimentation after the microsomal membranes were dissolved by detergent treatment. It should be noted that almost all the poly A (determined by hybridization to [^{3}H]poly U) (Table I, second line) and the labeled microsomal poly A-containing mRNA assayed by oligo-dT cellulose chromatography (third line; 11% for RM and 12% for bound polysomes) were also recovered with these polysomes.

Release of mRNA from RM during Disassembly of Bound Polysomes

We examined the fate of mRNA molecules present in membrane-bound polysomes when nascent polypeptides were discharged by puromycin in media containing 250 or 500 mM KCl. At ionic strengths higher than 250 mM, the puromycin reaction has been shown to be accompanied by a release of ribosomes from the microsomal membranes (2). After treatment with puromycin, RM samples which contained [^{3}H]RNA labeled in vivo for 2 h with [^{3}H]orotic acid were sedimented through sucrose density gradients of the same ionic composition to separate the released material from the faster sedimenting microsomal membranes. The distribution of total [^{3}H]RNA after these treatments was determined by TCA precipitation. As was shown in Table I, after 2 h of labeling, 10% of radioactivity in the microsomal RNA corresponded to poly A-containing mRNA which hybridized to oligo-dT cellulose. Therefore, profiles of the acid-insoluble [^{3}H]RNA radioactivity distribution in sucrose density gradients mainly represent the distribution of ribosomal [^{3}H]RNA. In the same experiments the distribution of poly

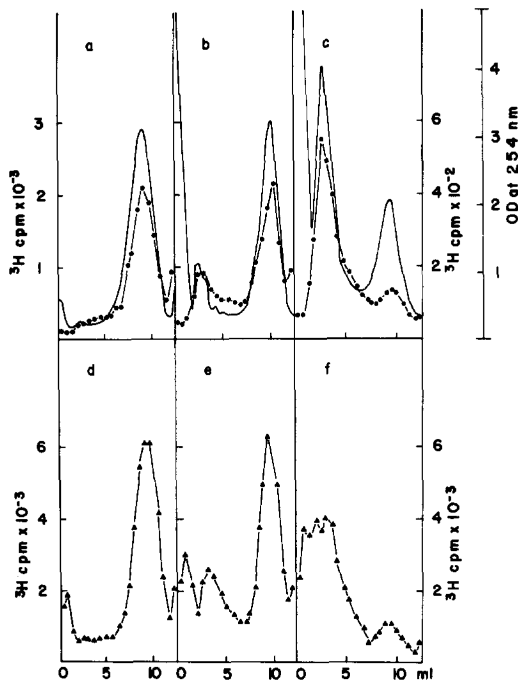

FIGURE 1 Release of mRNA after incubation of RM with puromycin in HSB. Rats were injected intraperitoneally with 250 μCi [^{3}H]orotic acid (sp act 17.7 Ci/mmol) 3 h before sacrifice. Livers were excised and RM prepared (as described in Materials and Methods), washed in HSB-containing media and resuspended in a partially purified RNase inhibitor solution (37) in HSB (*a* and *d*), in 250 mM KCl, 2.5 mM Mg^{++} (*b* and *e*), or in 500 mM KCl, 2.5 mM Mg^{++} (*c* and *d*). Samples *b* and *c* were treated with 1 mM puromycin for 30 min at 0°C and analyzed by sucrose density gradient centrifugation. The distribution of total [^{3}H]RNA was determined in small aliquots from each gradient fraction by TCA precipitation, and the profile of poly A-containing mRNA was assessed after phenol extraction of the residual gradient fraction by hybridization to [^{3}H]poly U as described in Materials and Methods. Under the above-specified labeling conditions, about 10% of the radioactivity from polysomes was poly A-containing mRNA as measured by affinity chromatography to oligo-dT cellulose. For reason of simplicity, we consider in the analytical gradients the radioactivity profile reflecting only the distribution of ribosomal RNA and measure separately the poly A-containing mRNA by annealing to [^{3}H]poly U. Since only the poly A/poly U hybrid is resistant to RNase digestion under the experimental conditions, the labeled part of the mRNA molecules does not interfere with this method of quantitation. Panels *a* and *d*: Control RM analyzed in 15-55% sucrose gradients in HSB. Panels *b* and *e*: RM analyzed after puromycin treatment in 10-50% sucrose gradients containing 250 mM KCl, 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.4. Panels *c* and *f*: RM analyzed after puromycin treatment in 10-40% sucrose gradients containing 500 mM KCl, 5 mM $MgCl_2$ in Tris buffer. (—) Absorbance at 254 nm; (●—●) ^{3}H-radioactivity in total RNA; and (▲—▲) ^{3}H-radioactivity in hybridized poly U.

In all figures, direction of sedimentation is from left to right. Membranes band at an isopycnic position in the bottom third of the gradients, and poorly resolved ribosomal subunits are represented by the absorbance peak at approx. fraction no. 5.

A-containing mRNA was determined by hybridizing [^{3}H]poly U to RNA prepared by phenol extraction from each fraction of the gradient. Labeled ribosomal RNA and mRNA did not interfere with these measurements because only small amounts of these RNA's with relatively low specific radioactivity were used in the assays and more than 90% of the labeled RNA's were degraded by the ribonuclease treatment used in scoring [^{3}H]poly U-poly A hybrids. As shown in Fig. 1 there was almost no release of ribosomes (Fig. 1 *a*) or mRNA (Fig. 1 *d*) from RM incubated in a high salt buffer alone for 45 min at 4°C. As expected from the fact that direct bonds between ribosomal subunits and membranes are rapidly disrupted only at ionic strengths greater than 0.5 M, incubation with puromycin in a medium containing 250 mM KCl led to the release of only a small fraction of ribosomes (Fig. 1 *b*). Under these conditions, a proportional amount of mRNA (Fig. 1 *e*) was released from the RM. On the other hand, when an extensive puromycin-dependent release of ribosomal subunits was effected by treatment in 0.5 M KCl (Fig. 1 *c*), there was also a dramatic rise in the release of poly A-containing mRNA (Fig. 1 *f*). Because mRNA and ribosomes were released concomitantly as a result of puromycin treatment at different salt concentrations, these observations provide no evidence for an independent attachment of mRNA to the microsomal membranes. Instead, they indicate that if mRNA is independently bound to the rough microsomal membranes, its affinity for the membranes could not be stronger than that of the bound ribosomes. The failure to produce a more extensive release of mRNA by puromycin treatment in vitro at 250 mM KCl is therefore likely to result from its association with inactive ribosomes. Pseudopolysome structures containing mRNA have been shown to be produced when free polysomes are treated with puromycin in media of low ionic strength (8).

In the experiments just described, it was neces-

sary to carry out the incubation with puromycin at 4°C to minimize degradation of mRNA which occurs during the conventional incubation at 37°C (2). To determine whether the incomplete ribosome release which occurs at the lower temperature accounts for the retention of mRNA on the stripped membranes, RM were incubated at 4°C in a high salt medium (1 M KCl) containing no Mg^{++}. This treatment leads to the destructive disassembly and extensive detachment of bound polysomes, as a result of the dissociation and unfolding of the ribosomal subunits, while peptidyl-tRNA molecules remain associated with the microsomal membranes (32). An almost complete release of ribosomes in parallel with an extensive release of poly A occurred under these conditions (Fig. 2*a-d*).

An even more effective means of producing a concomitant release of ribosomes and undegraded mRNA from the microsomes at low temperatures involved the chelation of divalent cations with EDTA (Table II) or with 30 mM sodium pyrophosphate (not shown). This treatment does not lead to the extensive aggregation of microsomes which with the puromycin-KCl procedure prevents a clean separation of the released material from the sedimentable membranes by differential centrifugation. The aggregation of stripped membranes is responsible for the high proportion of ribosomes (39% in Table II) found in the sedimentable fraction of puromycin-KCl-treated samples. The release of mRNA from the membranes produced by EDTA did not result from the degradation of mRNA contained in the polysomes. As is shown in Fig. 3*a*, even after treatment for polysome disassembly, the released poly A-containing mRNA, although heterogeneous in size, remained of high molecular weight with an average sedimentation coefficient of approx. 17S. On the other hand, the sedimentation profile of the mRNA retained in the membranes indicated either partial degradation or a smaller size distribution.

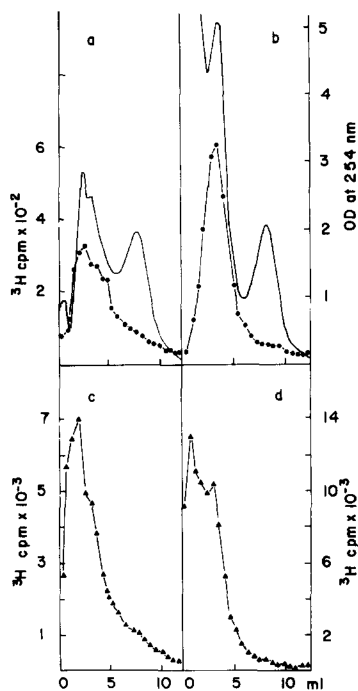

FIGURE 2 Release of bound mRNA after disassembly of RM in 1 M KCl solutions in the absence of Mg^{++} ions. About 25 A_{260} U of [^{3}H]orotic acid RM (same preparation as in Fig. 1) were incubated as described in Materials and Methods. In panels *a* and *c*, a partially purified RNase inhibitor fraction (37) and in panels *b* and *d*, a nonpurified high speed supernate from rat liver (6) were used to inhibit the endogenous RNase activity. Ribosomal subunits were separated from the stripped membranes by gradient centrifugation in 10–35% sucrose containing 1 M KCl and 50 mM Tris-HCl, pH 7.4. The [^{3}H]RNA distribution and the poly A-containing mRNA profile were determined as described in the legend to Fig. 1. (—) Absorbance at 254 nm; (●—●) ^{3}H-radioactivity in total RNA; and (▲—▲) ^{3}H-radioactivity in hybridized poly U.

Length of Poly A Segments in mRNA of Free and Membrane-Bound Polysomes

mRNA molecules and their poly A segments were labeled in vivo in rats injected intraperitoneally with 3 mCi [^{32}P]orthophosphate 7 h before sacrifice. With poly U-Sepharose chromatography, mRNA was isolated from RNA extracts prepared from: (*a*) free polysomes, (*b*) bound polysomes, (*c*) intact RM, and (*d*) the material released from the RM treated with 1 M KCl in the absence of Mg^{++}. The size distribution of the RNase-resistant ^{32}P-radioactivity corresponding to poly A segments was determined by electrophoresis in polyacrylamide gels. The results (Fig. 4) showed that there was no difference in the size distribution of the poly A segments in mRNA molecules from free or bound polysomes (Fig. 4*a* and *b*). The range in length of these segments was estimated to be between 140 and 180 nucleotides, using 5S and 4S RNA as reference standards. Poly A segments obtained from total microsomal RNA or from the released mRNA molecules were found to have the same size distribution as poly A

TABLE II
Distribution of mRNA and rRNA after Dissociation of RM

Treatment	Fraction	rRNA	mRNA	rRNA*	mRNA*
		cpm	*cpm*	%	%
None	Control	33,705	5,475		
1 mM Puromycin-HSB 30 min at 0°C	Supernate	42,038	7,275	61	79
	Pellet	27,000	2,000	39	21
40 mM EDTA	Supernate	61,278	9,680	86	90
	Pellet	9,765	1,000	14	10

RM were isolated from rats which were injected intraperitoneally with 250 μCi [^{3}H]orotic acid (sp act 15.5 Ci/mmol) 2 h before sacrifice. Membrane-bound polysomes were disassembled as indicated, and the stripped membranes were sedimented by centrifugation. The RNA was extracted with phenol from the supernates and pellets, and the poly A-containing mRNA fraction was isolated by affinity chromatography in oligo-dT cellulose. The amount of RM extracted in the control corresponds to half the material used in the release experiments. The reproducibility of the extraction procedure can be judged by the total RNA recovery which was for the control: $^1/_2 \times$ 79,560 cpm, for the puromycin experiment: 78,310 cpm, and for the EDTA experiment: 81,720 cpm.

* Relative percentages of rRNA and mRNA in each individual experiment.

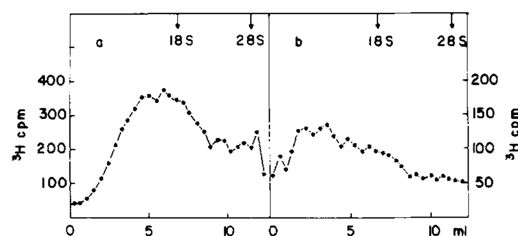

FIGURE 3 Size distribution of released and residual mRNA molecules. High salt-washed RM containing labeled RNA (2 h [^{3}H]orotic acid, sp act 15.5 Ci/mmol) were resuspended in rat liver high-speed supernate in 0.25 M sucrose containing heparin (500 μg/ml) as an additional RNase inhibitor. 40 mM EDTA and 50 mM Tris-HCl, pH 7.4 were added to disassemble the bound polysomes. RNA was extracted with phenol from the released material and from the stripped membranes (residual fraction) which were separated by differential centrifugation in a Beckman Ti 60 rotor at 35,000 rpm for 20 min. Released and residual mRNA fractions were isolated from the RNA samples by oligo-dT cellulose chromatography, and their size distribution was determined by centrifugation in a Beckman rotor SW 41 at 40,000 rpm and 22°C for 6 h in 15-30% sucrose gradients containing NETS buffer. The position of 18S and 28S rRNA's in similar gradients is indicated by arrows. (*a*) released poly A(+) mRNA; (*b*) residual poly A(+) RNA on the stripped membranes. (●—●) ^{3}H-Radioactivity in mRNA.

segments in the mRNA from bound polysomes (Fig. 4*c* and *d*). Because of the small amounts of radioactivity remaining in the membranes, it was not possible to make an independent measurement of the size of poly A segments in the small fraction of mRNA which remained associated with the membranes.

Accessibility to Macromolecular Probes of Poly A Segments in mRNA of Bound Polysomes

We first determined whether the poly A sequences of mRNA's in bound polysomes are exposed on the surface of RM by measuring their accessibility to [^{3}H]poly U and to exogenous

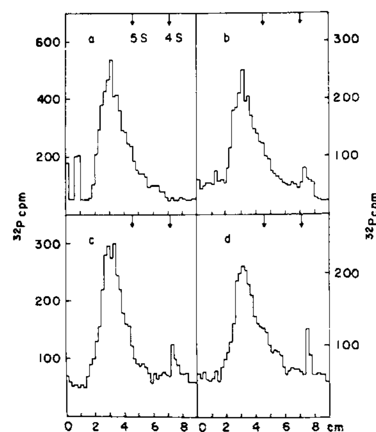

FIGURE 4 Size distribution of the poly A segment in free and bound mRNA fractions. Free and bound mRNA fractions were isolated from ^{32}P-labeled polysomes (2.5 mCi ^{32}P orthophosphate per 200 g rat, sp act 56 Ci/mg P for 7 h) by phenol extraction and affinity chromatography on poly U-sepharose. The poly A fragments were isolated after digestion with pancreatic and T1 RNase. The length distribution of poly A segments from free (*a*), bound (*b*), microsomal (*c*), and released (*d*) mRNA molecules was determined in 10% polyacrylamide gels with 5S and tRNA as marker substances. (—) ^{32}P-Radioactivity measured in 2-mm gel slices by Cerenkov counting.

RNase. [^{3}H]Poly U was hybridized to the poly A in RM which were prefixed in glutaraldehyde to prevent the release of ribosomes and mRNA which otherwise occurs under the conditions of hybridization. It was found that poly A sequences of mRNA's in bound polysomes are available to form RNase-stable hybrids with [^{3}H]poly U (Table III). It is possible, although unlikely, that by altering the conformation of the membranes, glutaraldehyde fixation rendered accessible to the poly U probe poly A segments otherwise not exposed on the surface of the microsomes.

The fact that poly A segments are exposed and not directly bound to the ER membranes was, however, demonstrated by their release when RM were treated with pancreatic RNase. A major fraction of the poly A (75%) was released by RNase even when RM not previously washed in high salt were used (Table IV). This observation excludes the possibility that proteins which could protect the messenger from RNase degradation were removed during washing in high salt wash and indicates that salt-sensitive linkages between poly A and the membranes were not being disrupted in the media of high salt concentration used in some of the ribosome-stripping procedures. Under the conditions of RNase treatment, ribosomes were not released from the membranes, and poly A is not degraded (20). Therefore, the poly A release indicates that, in addition to the poly A sequences, other regions of the messenger susceptible to RNase located between the poly A and the nearest membrane-bound ribosomes were also exposed on the surface of RM.

TABLE III
[^{3}H]Poly U Hybridization to Poly A mRNA in Glutaraldehyde-Fixed Microsomal Membranes

Fraction	Treatment	Poly A membrane	Phospholipids
		ng	*μg*
RNA	From unfixed RM	2,650	2,440
RM	Control	2,690	2,440
RM	40 mM EDTA	420	2,370
RM	15 μg/ml Trypsin	750	2,440
SM	Untreated	210	2,930

Rough microsome samples were treated as indicated before fixation with 0.1% glutaraldehyde for 30 min at 4°C. Samples were washed with TKM to inactivate residual unreacted aldehyde groups. Membranes were recovered by sedimentation and used for *in situ* hybridization with [^{3}H]poly U. Phospholipid phosphorus was measured according to Ames and Dubin (4).

TABLE IV
Release of Poly A by RNase Treatment of RM

Exp	Treatment	Incubation buffer	Fraction	Poly A
				ng %
1	None	–	Control	4,900 (100)
2	RNase	TKM	Supernate	3,000 (61)
			Pellet	690 (14)
3	RNase	HSB	Supernate	3,000 (61)
			Pellet	990 (20)
4	None	–	Control	8,100 (100)
5	RNase	TKM	Supernate	4,500 (56)
			Pellet	1,630 (20)

High salt-washed (exp 1-3) and unwashed RM (exp 4 and 5) were incubated for 10 min at 30°C with pancreatic RNase (15 μg/ml) under the specified ionic conditions. The released material and the microsomal vesicles were separated by differential centrifugation, and their content of poly A was measured by annealing to [^{3}H]poly U (18) after extraction of the RNA by phenol. The sum of poly A in supernates and pellets does not add to 100%. The imperfect recovery (~80%) is due to losses during fractionation, RM extraction, and precipitation.

mRNA Content of Smooth Microsomes

Although we found that in rat liver RM almost all the mRNA was contained in polysomes, the finding of a direct association between RNA and membranes in other systems led us to consider the possibility that mRNA not being engaged in translation could be found in association with smooth microsomal membranes. We therefore extracted RNA from rough and smooth microsomal fractions prepared from rats which received [^{32}P]orthophosphate 3 h before sacrifice. The [^{32}P]RNA radioactivity obtained from smooth microsomes (SM) represented, on a phospholipid basis, less than 5% of that found in RM. Moreover, the amount of poly A contained in SM mRNA as measured by oligo-dT chromatography was less than 1% of that in RM. As shown in Table III, glutaraldehyde-fixed SM also had a low capacity for *in situ* hybridization with [^{3}H]poly U, under conditions where the full poly A content of RM was hybridizable.

Effect of Inhibitors of Protein Synthesis on the Association of Ribosomes and mRNA with Microsomal Membranes

We examined the effect of inhibitors of protein synthesis which lead to polysome disassembly by blocking initiation or the early steps of translation on (*a*) the intracellular distribution of ribosomes and (*b*) the extent of association of mRNA with

the ER membranes and the sensitivity of this association to treatment with high salt.

These studies employed verrucarin A (41, 17) and ethionine (39) administered intraperitoneally to rats. The results concerning verrucarin are presented in Fig. 5. Within 45 min this drug produced an extensive in vivo disassembly of polysomes due to ribosome run-off. This was apparent in sucrose density gradient profiles obtained from the post-mitochondrial supernates analyzed in TKM (not shown) in which free polysomes were replaced by a prominent monomer peak. The amount of membrane-bound ribosomes in microsomes prepared in low salt medium was not significantly different from control animals. This should be expected since at low ionic strengths direct bonds between large subunits and ER membranes are known to be (2) capable of maintaining the ribosome-membrane association, even in the absence of nascent chains. When RM not washed in high salt buffer obtained from verrucarin-treated animals were analyzed in TKM-containing sucrose gradients, it was found that a normal proportion of mRNA, as assessed by [^{3}H]poly U hybridization, was also associated with the microsomal membranes (cf. Fig. 5*a* and *d* to Fig. 6*a* and *d*). An increase in ionic strength, however, led to the extensive removal of the membrane-bound ribosomes (Fig. 5*b*) and to the release of 80% of the poly A in mRNA molecules (Fig. 5*e*) without the requirement of puromycin treatment in vitro, which, if performed, had little additional effect (Fig. 5*c* and *f*). This is in marked contrast to the situation found with unwashed RM of untreated rats (Fig. 6). In this case, because of the presence of nascent polypeptide chains, high salt treatment alone was not sufficient to reduce the content of mRNA and ribosomes to the levels in microsomes of verrucarin-treated rats after high salt treatment. Similar results (not shown) were found with microsomes obtained from rats treated with ethionine. In this case, too, normal levels of mRNA and ribosomes were recovered with the microsomal membranes, but these were easily removed by increasing the ionic strength in vitro in the absence of puromycin.

DISCUSSION

We have attempted to define what role, if any, mRNA molecules play in the interaction between bound polysomes and endoplasmic reticulum membranes of rat liver cells. RM labeled in vivo

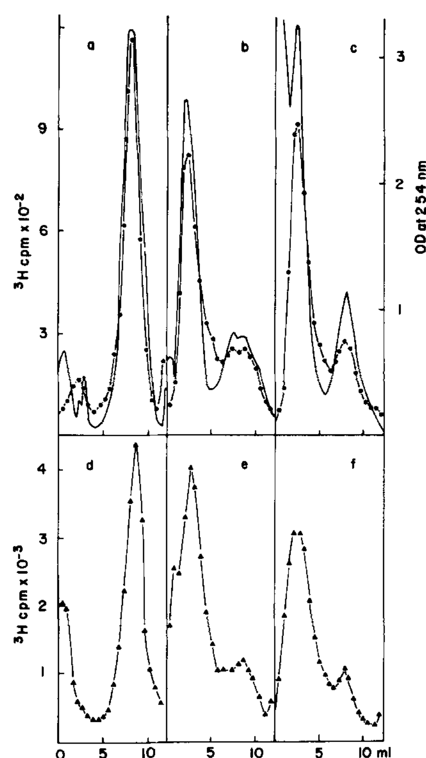

FIGURE 5 Release of ribosomes and mRNA from microsomal membranes by media of high ionic strength after polysome disassembly in vivo. Rats (150 g) were injected intraperitoneally with [^{3}H]orotic acid and 3 h later with 7.5 mg of verrucarin A dissolved in 0.3 ml of DMSO (50 μg/g body weight) in order to inhibit elongation of protein synthesis and induce polysome disaggregation in vivo. The animals were sacrificed 30 min later, and RM were prepared without washing in high salt. Aliquots resuspended in TKM were maintained in the same buffer (*a* and *d*) or adjusted to the high salt concentration of HSB with (*c* and *d*) or without (*b* and *e*) subsequent treatment with 1 mM puromycin for 30 min at 0°C. Samples were analyzed by sucrose density gradient sedimentation in media of the same salt concentration. The distribution of total [^{3}H]RNA and poly A-containing mRNA was determined as explained in the legend to Fig. 1. Panels *a* and *d*: RM resuspended in TKM and analyzed in 20–60% sucrose TKM gradients. Panels *b* and *e*: RM suspended in HSB and analyzed in 10–40% sucrose HSB gradients. Panels *c* and *f*: RM treated with puromycin in HSB and analyzed in 10–40% sucrose HSB gradients. (—) Absorbance at 254 nm. Panels *a, b,* and *c*. Distribution of ^{3}H-radioactivity in total RNA (●—●). Panels *d, e,* and *f*: Amount of [^{3}H]poly U radioactivity hybridized to total RNA from each fraction (▲—▲).

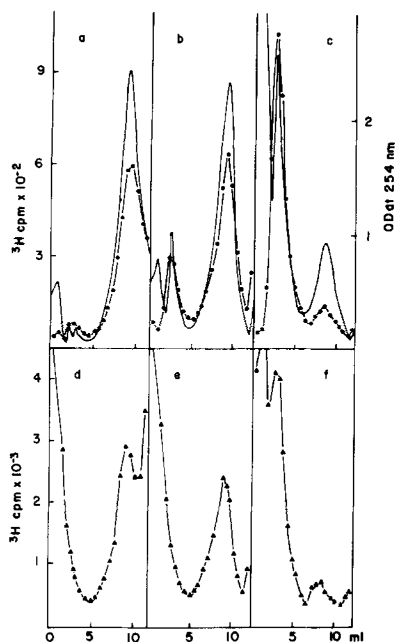

FIGURE 6 Distribution of rRNA and mRNA from low salt-washed RM of control rats. Radioactive labeling, preparation, and treatment of the RM fractions were identical to those for Fig. 5 except that the verrucarin A was omitted and the animals received 0.3 ml of DMSO. The separation was carried out in identical gradients which are drawn in the same order as in Fig. 5.

with [^{3}H]RNA precursors, containing largely undegraded mRNA molecules, were prepared and washed before use by centrifugation in a medium of high salt concentration to remove inactive ribosomes and unspecifically adsorbed polysomes. We showed that most, if not all, of the poly A-containing mRNA molecules associated with the microsomes are engaged in translational complexes involving one or several ribosomes. Using such high salt-washed liver RM we then demonstrated that various treatments which dissociate membrane-bound polysomes lead to the concomitant release of mRNA molecules and ribosomes from the membranes. In all cases, the extent of mRNA release from the RM paralleled the release of ribosomes. These results disagree with those of Cardelli et al. (12) who performed similar experiments with rat liver microsomes in which mRNA was specifically labeled with [^{3}H]orotic acid in the presence of fluoroorotate to inhibit ribosomal RNA synthesis. These authors reported that 40–50% of this labeled RNA remained associated with the microsomes after ribosome detachment. Cardelli et al. (12) also observed that, after treatment of RM prepared in low salt buffer with puromycin-KCl, 40% of the total poly A, monitored by poly U hybridization, was retained in the membranes. Although in these experiments a significant fraction of the mRNA was released upon polysome disassembly, the values for the retained mRNA were higher than those reported in this paper. Cardelli et al. (12) therefore concluded that in rat liver there is a direct association between mRNA and ER membranes, similar to that reported in tissue culture cells by Milcarek and Penman (27) and Lande et al. (23). The reason for the difference in the extent of mRNA release caused by polysome disassembly is not yet clear. It should be noted, however, that different microsomal fractions were used in the conflicting experiments. While the fraction used by us represents approx. 40–50% of the membrane-bound ribosomes (1), the one employed in the experiments by Cardelli et al. (12) contains a much lower proportion of the total microsomal RNA. Moreover, in our EDTA experiments, which gave the most complete release of mRNA, microsomes were resuspended in a solution of rat liver high-speed supernate containing 0.25 M sucrose with no added Mg^{++}. To this, EDTA was added to a final concn of 40 mM. On the other hand, in the experiments of Cardelli et al. (12) equal volumes of 10 mM EDTA were added to a suspension of RM in a buffer containing 5 mM Mg^{++}. It is possible that the higher EDTA-to-Mg^{++} ratio accounts for our more complete release.

Our observations with liver microsomes also contrast sharply with those on membrane fractions from tissue culture cells (27, 23), in which similar treatments produced a selective release of ribosomes while allowing retention of mRNA. In addition, we found that in rat liver microsomes all the mRNA was contained in polysomes. On the other hand, up to 40% of the mRNA radioactivity in membrane fractions from confluent human fibroblasts labeled in the presence of actinomycin D was not recovered with the polysomes after dissolving the membranes and was therefore attributed to membrane-associated mRNA molecules not involved in translation (23).

Lande et al. (23) and Milcarek and Penman (27) also showed that poly A segments could not be released from the membranes of tissue culture cells by incubating the fractions with RNase under conditions where mRNA molecules were extensively degraded. It was therefore concluded (23) that a direct linkage between mRNA and ER

membranes exists in the cultured fibroblasts which involves a region of the messenger adjacent to the poly A segments that is resistant and/or inaccessible to RNase. We found that, in rat liver RM not only are the poly A segments of mRNA molecules exposed on the surface of the membranes where they are accessible to [^{3}H]poly U for "*in situ*" hybridization, but they are easily released from the membranes by mild RNase digestion.

Because the experiments in which polysomes were dissociated in vitro provided no evidence for an independent attachment of mRNA to the microsomal membranes, we attempted to produce the release of mRNA from ER membranes in vivo. Verrucarin A (41) and ethionine (39) were administered to rats to selectively inhibit initiation of protein synthesis while permitting elongation and termination of nascent polypeptide chains to occur. The resulting ribosome run-off might have been expected to produce release of unattached mRNA from the microsomes. This, however, was not the case. Although the drugs led to extensive disassembly of both free and bound polysomes, the inactive bound ribosomes, as well as the mRNA molecules, remained associated with the membranes when the microsomes were prepared in media of low ionic strength. A similar observation has been made with human diploid fibroblasts (3). However, while we found that the mRNA was simultaneously released with the ribosomes from RM of verrucarin-treated rats during incubation in medium of high ionic strength, mRNA under similar conditions, was largely retained in membrane fractions of verrucarin-treated fibroblasts (3). If, as suggested by our observations, the majority of mRNA in microsomes of liver cells is not directly associated with the ER membranes, its retention on the microsomes after polysome disaggregation in vivo is likely to be accounted for by its involvement with both ribosomal subunits in the formation of a small number of functional 80S complexes which are blocked by verrucarin. This inhibitor prevents polysome formation by blocking 80S initiation complexes or translation after the first peptide bonds are synthesized (17). The blocked ribosomal complexes bearing mRNA and short peptides should therefore be resistant to dissociation into subunits by high salt treatment. Our observations indicate that the linkage between the blocked ribosomes and the membranes is, on the other hand, salt sensitive. This would be expected in light of the fact that linkages of normal ribosomes containing short polypeptides or ribosomes containing no nascent chains are disrupted in high salt (2). If, indeed, the association between mRNA and microsomal membranes was due to the binding of blocked 80S ribosomes, it should be of great interest to determine whether the association was selective and restricted to only specific classes of blocked ribosomes bearing messengers normally translated in bound polysomes. This would imply a recognition mechanism not involving an exposed segment of the nascent chain to direct the 80S complexes bearing specific messengers to the membranes. The alternative would be that, under the low-ionic-strength conditions, blocked ribosomes, as well as inactive ribosomes, were adsorbed nonspecifically onto available binding sites on the membranes.

Although it may appear difficult to reconcile in a single hypothesis the observations reported in this paper with those concerning the mRNA-membrane association in tissue culture cells, several explanations may be proposed. It could be argued that the difference between tissue culture cells and liver cells may be one of degree or strength of the binding of mRNA with the membranes. Thus, if in liver microsomes the binding sites are weaker than in tissue culture cells, messenger-membrane links could be disrupted under the same conditions which lead to ribosome dissociation. Several observations, however, indicate that if such weak or salt-sensitive binding sites exist in liver RM they are not likely to involve a region of the messenger adjacent to the poly A segments. Thus, poly A segmetns were easily removed by mild RNase treatment from RM which had not been previously washed in high salt medium, and were incubated under conditions which did not produce extensive ribosome release.

Another possible explanation for the divergent results with the different systems is that there may be more than one class of mRNA associated with ER membranes. One class predominating in fibroblasts and other tissue culture cells may be directly associated with the membranes. Another class of messenger may be only weakly bound, or not bound directly to the membranes, and depend on its association with ribosomes for retention on the ER membranes. Such a class would be the major one in liver cells and may be represented in myeloma cells by the light chain mRNA which has been shown to be released upon polysome disassembly (19). It should be noted that in the same myeloma cells a large fraction of poly A-containing mRNA was not released from the membranes

upon polysome disassembly. Although the unreleased mRNA was assumed to be mitochondrial (19), it represented such a large fraction of the total RNA that we believe it was probably microsomal in origin. That a considerable fraction of microsomal mRNA in myeloma cells is independently associated with the membranes has been concluded in other experiments by Mechler and Vassalli (26). These authors demonstrated that small ribosomal subunits, containing initiator tRNA but not yet bound to large subunits, remained associated with the microsomal membranes, presumably through the mRNA, after treatment of the cells with pactamycin to inhibit protein synthesis.

Alternatively, a direct binding of mRNA to the membrane may be greatly dependent on the physiological condition of the cells. For example, membrane-binding sites for messenger may be exposed only under conditions such as those found during growth in culture. An obvious third alternative is the possibility of an artefactual association between released mRNA and the membranes of tissue culture cells. Such an artefactual binding may be more likely to occur in membrane fractions from tissue culture cells since these fractions are impure and contain, in addition to the ER elements, membranous remains of other subcellular organelles which are absent in purified rat liver RM. The possibility of artefactual adsorption of free mRNA to membranes of fibroblasts under the conditions of dissociation was examined and ruled out by the results of in vitro mixing experiments (23).

The resolution of the apparent contradiction between results concerning the relationship of mRNA and ER membranes in some tissue culture cells and rat liver cells may have to await the characterization of specific mRNA's in both systems by in vitro translation and hybridization methodology. This may reveal whether the different behavior of these mRNA's is related to their functional properties, to the state of cell growth or to the conditions of in vitro cell fractionation.

We thank Dr. T. E. Martin for supplying us with verrucarin A and instructing us in its use. We are also grateful to Dr. M. Adesnik for many discussions during the work and the preparation of the manuscript.

This work was supported by a grant from the National Institute of Health GM 20277. J. Kruppa had a travelling fellowship from the Deutsche Forschungsgemeinschaft.

Received for publication 24 June 1976, and in revised form 14 April 1977.

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