DIRECT ASSOCIATION OF MESSENGER RNA LABELED IN THE PRESENCE OF FLUOROOROTATE WITH MEMBRANES OF THE ENDOPLASMIC RETICULUM IN RAT LIVER

JAMES CARDELLI, BYRON LONG, and HENRY C. PITOT

From the Departments of Oncology and Pathology, McArdle Laboratory for Cancer Research, The Medical School, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Liver rough endoplasmic reticulum (RER) membranes were isolated from rats given [^{3}H]orotic acid for 48 h (ribosomal RNA [rRNA] label) or for 3 h along with 5-fluoroorotate; this latter procedure permits the labeling of cytoplasmic messenger RNAs (mRNAs) in the absence of rRNA labeling. More than 50% of the labeled mRNA remained attached to membranes of the RER after complete removal of ribosomes with a buffer of high ionic strength in the presence of puromycin. Under similar conditions, membranes retained 40% of their polyadenylate as determined by a [^{3}H]-polyuridylate hybridization assay.

Treatment of mRNA-labeled endoplasmic reticulum membranes with pancreatic RNase indicates that the polyadenylate and possibly nonpolyadenylate-pyrimidine portions of the messenger are involved in the binding of mRNA to the membranes. The implication of these results in furthering our understanding of the mechanisms of the translational regulation of genetic expression is discussed.

Polyribosomes exist in eucaryotic cells either bound to intracellular membranes or unattached as free polysomes in the cytoplasm (27). Proteins destined for secretion from the cell appear to be synthesized almost exclusively on membrane-bound polysomes, whereas intracellular proteins do not appear to be synthesized exclusively on either class of polyribosome. Little is known of the mechanism by which polysomes or messenger RNA's (mRNA's) coding for secretory proteins are selected for interaction with the endoplasmic reticulum.

Sites on the surface of the endoplasmic reticulum recognize and allow the attachment of the large ribosomal subunits (19, 22) and polysomes (27). In rat hepatocytes the nascent peptide chain also appears to play a role in anchoring polysomes to the endoplasmic reticulum (1).

The possibility that mRNA plays a role in the binding of polysomes to intracellular membranes, although proposed theoretically by this laboratory (27), has not been thoroughly investigated, but evidence for direct mRNA-membrane interaction does exist. Membrane-bound polysomes can reform in vivo in the absence of further RNA synthesis after removal of agents that cause degranulation of the RER (4, 7, 18, 21). It appears that the endoplasmic reticulum may contain nontranslated messages (23). mRNA in the form of ribonucleoprotein particles may interact with intracellular membranes before entering the free polysome pool, the last step being dependent on initiation of translation (6, 25). Recent evidence for a direct association between mRNA and intracellular membranes has been obtained (2, 11, 13, 14).

In this paper, we will present evidence to sup-

port the concept of a direct mRNA-membrane interaction independent of ribosomes and polysomal structure in the rat hepatocyte. Furthermore, the evidence indicates that the site of interaction with membranes may involve regions of the mRNA molecule other than the poly(riboadenylic acid) (Poly A)[1] region.

MATERIALS AND METHODS

Animals, Chemicals, Labeling

Male Holtzman rats (Holtzman Co., Madison, Wisc.), weighing 300–400 g and maintained ad lib. on a chow diet, were used for all experiments. Rats were given intraperitoneal injections of 100 μCi of [^{3}H]orotic acid 48 h before being killed to obtain long-term labeled rRNA. For short-term labeling of mRNA, rats were given 100 μCi of [^{3}H]orotic acid by the same route together with 2.6 μmol of 5-fluoroorotic acid (FOA) 3 h before being killed.

Sucrose (RNase-free) was purchased from the Schwarz/Mann Division of Becton, Dickinson, & Co., Orangeburg, N. Y.; [^{3}H]poly(ribouridylic acid) (Poly U) and [^{3}H]Poly A, from Miles Laboratories, Inc., Miles Research Products, Elkhart, Indiana; puromycin dihydrochloride and pancreatic RNase, from Sigma Chemical Co., St. Louis, Mo.; [^{3}H]orotate (21 mCi/mmol), from Amersham/Searle Corp., Arlington Heights, Ill. All glassware was autoclaved before use. Homogenizing equipment was first washed with 30% H_2O_2 followed by copious amounts of double-distilled H_2O.

Isolation of Membranes and Polysomes

Rats were killed by decapitation, and the livers were excised, minced, and homogenized in 2 vol of 0.44 M STKM (sucrose molarity as designated, 50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.4) with eight strokes in a Potter-Elvehjem homogenizer. A postmitochondrial supernate (PMS) was prepared by spinning the homogenate for 10 min at 12,000 rpm in an SS-34 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). STKM (2 M) was added to the PMS to a final sucrose concentration of 1.35 M, and the PMS was layered over 10 ml of 2.0 M STKM. STKM (0.44 M) was then layered on top of the PMS, and the discontinuous gradient was spun for 3 h at 55,000 rpm, 4°C, in a 60 Ti rotor. The smooth membranes at the 0.44–1.35 M interphase were removed by clamping the tube with a hemostat above the RER membranes banding at the 1.35–2.0 M sucrose interphase and subsequently removing the liquid above the clamp with a pipet. After the walls of the tubes were rinsed with TKM, the RER was isolated by clamping the tubes below the band of membranes with a hemostat and gently pipetting out the membranes. Free polysomes, found as a pellet at the bottom of the tube, were rinsed on their surface with TKM and stored at −70°C until further use. The RER was adjusted to 0.44 M STKM with TKM and spun for 1 h at 28,000 rpm in a no. 30 rotor. The pellets were washed once by being gently resuspended in 50% S_3 (105,000 *g* supernate of rat liver prepared by homogenizing 1 g liver/ml STKM) with a rubber policeman and being homogenized by hand. The suspensions were then centrifuged for 30 min at 28,000 rpm in a no. 30 rotor. The membrane pellets were resuspended by hand to 0.66 g equivalents/ml (0.75 mg RNA/ml) in 0.25 M STKM.

Membrane Stripping

To strip membranes of ribosomes, we used a procedure similar to the one employed by Adelman et al. (1). A volume of membranes in 0.25 M STKM (0.75 mg RNA/ml) was mixed with an equal volume of a high salt buffer containing either 1.2 M or 2.0 M KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 7.4, in 0.25 M sucrose. After the addition of puromycin to a concentration of 10^{-3} M, the membranes were either incubated for 30 min at 25°C and analyzed on sucrose gradients or incubated for 30 min at 4°C and pelleted through 0.44 M STKM of the same salt composition.

Sucrose Density Gradient Analysis

To analyze membranes stripped of ribosomes, we layered 0.75 ml of the membrane sample onto an 11.5-ml 15–40% linear sucrose gradient of the same salt composition as the sample. The gradients were spun in an SW-41 rotor with an L2-65B Beckman ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 2 h at 38,000 rpm and 4°C. After the centrifugation tubes were punctured and their contents removed from the bottom, gradients were passed through a UV analyzer (Gilford 2400, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) by means of a peristaltic pump (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.), and 0.5 ml fractions were collected. Fractions were treated with cold 5% trichloroacetic acid (final concentration) after the addition of 0.4-mg of bovine serum albumin (BSA), collected on glass fiber filters, and the radioactivity was measured in the presence of toluene-2,5-diphenyloxazole (PPO), or they were collected directly in scintillation vials, and the radioactivity was determined after the addition of Scintisol (Isolab, Inc., Akron, Ohio).

RNA Extraction

Frozen polysome or membrane pellets were resuspended in 3–5 ml of NETS buffer (1% SDS, 100 mM

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; FOA, 5-fluoroorotate; PMS, postmitochondrial supernate (see Materials and Methods); Poly A, Poly U, poly(riboadenylic acid), poly(ribouridylic acid); RER, rough endoplasmic reticulum; and SDS, sodium dodecyl sulfate.

NaCl, 10 mM Tris, pH 7.4, 1 mM ethylenediaminetetraacetic acid [EDTA]) containing 1 mg/ml bentonite and 100 μg/ml sodium dextran sulfate. An equal volume of Tris-saturated (pH 7.4) phenol-chloroform (1:1 vol/vol) was added, and the mixture was shaken for 5 min at 25°C, then centrifuged at 5,000 rpm for 10 min to separate the phases. The aqueous phase was removed, and the organic phase extracted again with an equal volume of NETS-chloroform. The combined aqueous phase was extracted twice with chloroform-phenol (1:1 vol/vol). The RNA in the final aqueous phase was precipitated overnight with 2 vol of 95% ethanol after the addition of NaCl to a final concentration of 100 mM. 10 A_{260} U of yeast RNA were added as a carrier to the aqueous phase containing RNA extracted from stripped membranes before ethanol precipitation. The ethanol precipitate was washed twice with 95% ethanol, drained, and resuspended in a small volume of water.

Millipore Filter Analysis of Poly A-Containing RNA

The method of Lee et al. (12) was used to measure the amount of Poly A-containing RNA present. 50-μl aliquots of the isolated ^{3}H-labeled RNA was added to 1.0 ml of binding buffer (500 mM NaCl, 10 mM Tris, pH 7.6, 1 mM $MgCl_2$). This mixture was slowly passed under vacuum through 0.45-μm HAWP Millipore filters (Millipore Corp., Bedford, Mass.) that had been soaked for 30 min in binding buffer. The filters were then washed with 30 ml of binding buffer, dried, and the radioactivity was measured after the addition of toluene-PPO.

Analysis of Poly A with [^{3}H]Poly U

Poly A content was determined according to the technique of Rosbash and Ford (21). Samples of RNA were added to 0.5 ml of 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.4), followed by 5 μl of [^{3}H]Poly U (34 μg/ml, 3.3 × 10^5 dpm/μg). The mixture was incubated for 30 min at 45°C, cooled, digested with pancreatic RNase (20 μg/ml), precipitated with trichloroacetic acid (final concentration, 10%), filtered, and the radioactivity was measured after the addition of 5 ml of toluene-PPO. The assay was independent of [^{3}H]Poly U concentration or the time of incubation and was linear with respect to Poly A concentration. No hybridization of [^{3}H]Poly U to RNA that did not bind to Poly U-Sepharose was observed.

RESULTS

Selective Labeling of mRNA

In the past, this laboratory has reported the use of the analog, FOA, to label cytoplasmic mRNA selectively. Relatively low levels of FOA inhibit the maturation of hepatic 18S and 28S rRNA (28), but allow the synthesis of a cytoplasmic RNA characterized as mRNA by the studies employed (8). To examine the class of RNA labeled in the presence of FOA, we extracted RNA from free polysomes isolated from the livers of rats injected 3 h previously with [^{3}H]orotate in the presence of FOA. Fig. 1 A shows the distribution of this labeled RNA from SDS-treated free polysomes in 15–30% linear sucrose gradients. As can be seen, the labeled RNA sediments heterogeneously in the gradients with S values ranging from 7S to 28S. Phenol-chloroform-extracted RNA from free polysomes was also examined on acrylamide-agarose gels (Fig. 1 B). Again, the RNA labeled in the presence of FOA showed a large variation in S values, with a maximum around 20S. These results are in agreement with

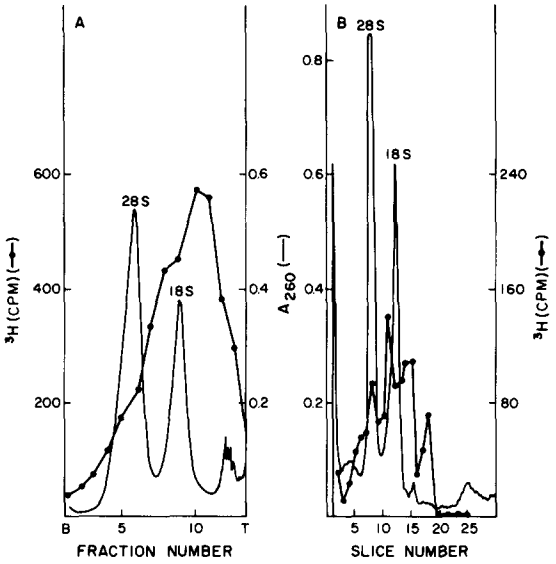

FIGURE 1 Size distribution of RNA labeled in the presence of FOA. Free polysomes were isolated as described in Materials and Methods, from livers of rats given injections 3 h previously of 100 μCi of [^{3}H]orotic acid and 2.6 μmol of FOA. The polysomes either were solubilized in NETS buffer and examined on 15–30% linear sucrose gradients of the same buffer composition without SDS (10 A_{260} U, SW-41, 38,000 rpm, 7 h, 4°C), or were extracted with phenol-chloroform as described in Materials and Methods and analyzed on acrylamide-agarose gels (1 A_{260} U, 2.1% acrylamide, 0.5% agarose, 6 mA/gel, 90 min). Fractions from the gradient (A) were counted in Scintisol, whereas slices of the gel (B) were heated at 55°C for 3 h in vials with 0.2 ml of concentrated perchloric acid and 0.4 ml of H_2O_2 and the radioactivity was measured after the addition of Scintisol.

previous data from this laboratory, which indicated that FOA, like actinomycin D (17), allows the selective labeling of a messenger-like class of RNA molecules.

To determine how effective FOA was in inhibiting the labeling of 18S and 28S microsomal RNA, we isolated the RER from livers of animals labeled with [^{3}H]orotate in the presence or absence of FOA. The membranes were treated with SDS and layered onto 15–30% sucrose gradients, which were centrifuged and analyzed for the distribution of radioactivity and A_{260} material. Fig. 2 B shows that essentially no label appeared in either the 18S or 28S peaks when the microsomal RNA was labeled in the presence of FOA. Fig. 2 A shows that incorporation of [^{3}H]orotate into rRNA peaks occurred when FOA was absent. Under the conditions used to treat the membranes with SDS, labeled mRNA was degraded. This finding accounts for the presence of the label at the top of the gradient in Fig. 2 B.

It was of importance to determine the percent of the labeled messenger-like RNAs on the endoplasmic reticulum membranes that were part of polysomal structures and thus functional in vivo. RER membranes containing mRNA selectively labeled in the presence of FOA were treated with sodium deoxycholate (DOC) in the presence of RNase inhibitors, layered over 2.0 M STKM, and centrifuged overnight as described in Materials and Methods. The centrifugation time should have been sufficient to sediment completely all polysomes, subunits, and any labeled nonfunctioning mRNA associated with the membranes as a ribonucleoprotein particle. Fig. 3 A shows the distribution of the membrane-bound polysomes and labeled RNA on 10–40% sucrose gradients. Approximately 90% of the labeled mRNA sediments

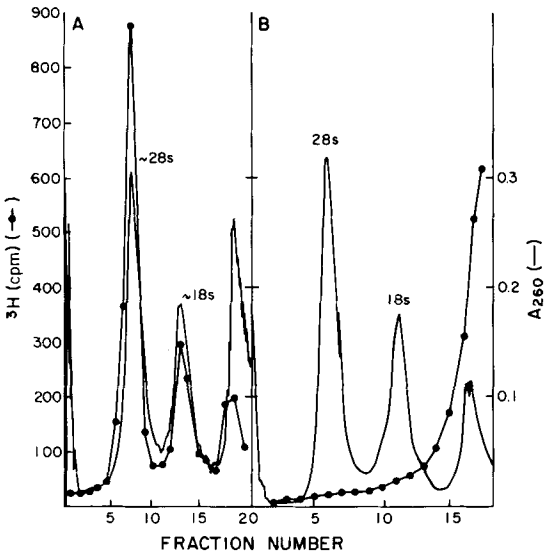

FIGURE 2 Inhibition of rRNA maturation by FOA. RER was isolated as described in Materials and Methods from livers of animals labeled with [^{3}H]orotic acid for 48 h (rRNA label) or for 3 h in the presence of 2.6 μmol of FOA (mRNA label). Membranes were solubilized in NETS buffer and analyzed on 15–30% sucrose gradients of the same composition minus SDS (0.11 mg RNA, SW-41, 38,000 rpm, 7 h, 4°C). Fractions were collected and counted in Scintisol. (A) RNA profiles from liver membranes of animals given [^{3}H]orotic acid alone or (B) [^{3}H]orotic acid with FOA.

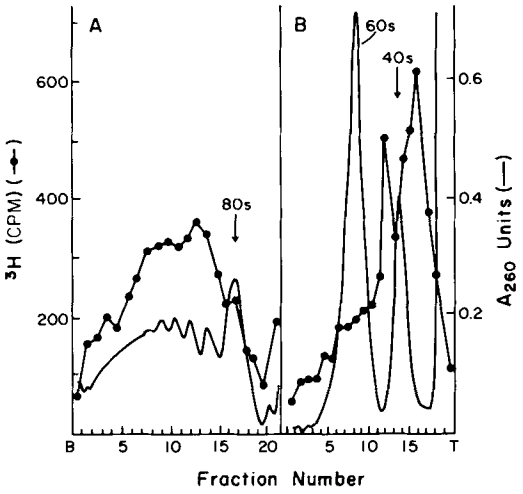

FIGURE 3 The association of 3 h labeled mRNA with membrane-bound ribosomes. An S2 was prepared from livers of animals labeled for 3 h with [^{3}H]orotic acid in the presence of 3 μmol FOA. The S2 preparation was fractionated on a discontinuous sucrose gradient as described in Materials and Methods, and the band corresponding to the RER membranes was isolated. A $^1/_{10}$ vol of 15% DOC was added to 1 vol of the membranes after the addition of heparin to a final concentration of 500 μg/ml. The membrane preparation was layered on top of 4 ml of 2.0 M STKM + heparin (500 μg/ml), centrifuged at 45,000 rpm, 16 h, 4°C, and the pellet resuspended in TKM (A); or 50 mM Tris, pH 7.4, 70 mM KCl, 10 mM EDTA (B). After removal of insoluble material, an aliquot of the resuspended polysome pellet was layered on 10–40% sucrose gradients of the same buffer composition as the applied polysomes. After centrifugation (SW-41, 40,000 rpm, 4°C, 1 h (A); 4 h (B)), the gradients were processed as described in Materials and Methods.

faster than the 80S monosome peak and is found mainly in the region of the gradient where the polysomes sediment. Fig. 3 B shows that the rapidly sedimenting, labeled RNA is sensitive to EDTA and displays a quantitative shift to the 30S–70S region of the gradient, suggesting that most of the labeled mRNA is associated with ribosomes in the configuration of membrane-bound polysomal structures.

Retention of mRNA by Membranes Stripped of Ribosomes

It appears that at least two forces operate to bind ribosomes to membranes of the endoplasmic reticulum (1). Both ionic interactions and nascent polypeptide chains appear to play a role in anchoring ribosomes to these membranes. The use of high ionic strength KCl buffers with puromycin allows one to remove most ribosomes from the intracellular membranes nondestructively (1). We have used this technique to follow the fate of labeled mRNA after disassembly of bound polysomes. Liver RER was isolated from rats preinjected either for 48 h with [^{3}H]orotate (rRNA label) or for 3 h with both [^{3}H]orotate and FOA (mRNA label). Membranes were then stripped with high salt buffer containing 5 mM Mg^{2+} and 1 mM puromycin (see Materials and Methods) and were analyzed by sedimentation through linear sucrose gradients. As can be seen, very little (less than 10%) of the labeled rRNA remained on membranes stripped with either 600 mM KCl plus 1 mM puromycin (Fig. 4 A) or 1.0 M KCl plus 1 mM puromycin (Fig. 4 B). On the contrary, more than 50% of the labeled mRNA remained attached to the endoplasmic reticulum after more than 90% of the ribosomes had been removed (Fig. 4 C and D).

To rule out the possibility that the small percentage of ribosomes left on stripped membranes act as points of attachment for the mRNA that remains, we performed the following experiment. Ribosomes can be completely removed from intracellular membranes by using a medium of high ionic strength lacking Mg^{2+} (1). Membranes were resuspended in high salt buffer *(vide supra)* with 1 mM puromycin and enough EDTA to chelate the Mg^{2+} present in the buffer. The membranes were analyzed on gradients containing 1.0 M KCl and no Mg^{2+}. Fig. 5 B shows that more than 50% of the labeled mRNA remained attached to the membranes stripped of all ribosomes. Fig. 5 A

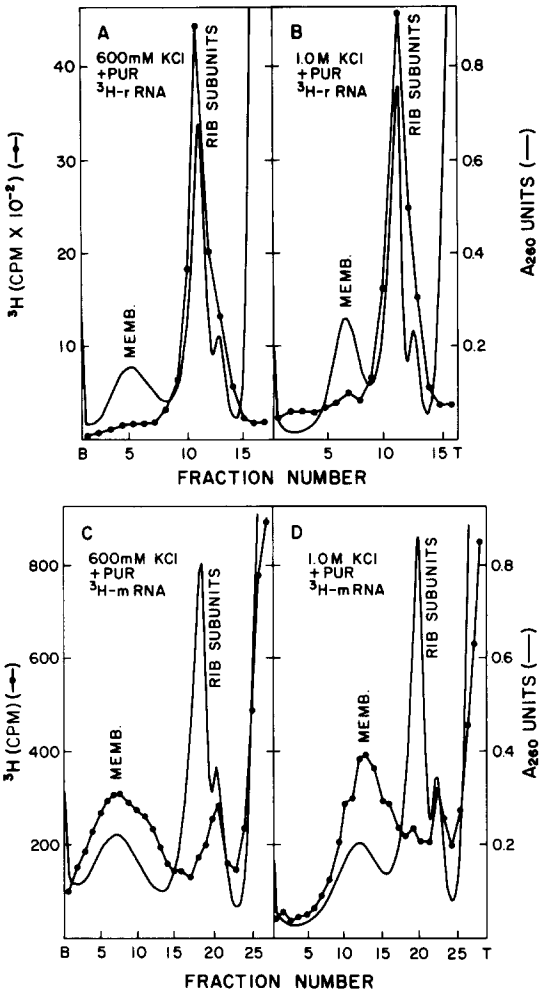

FIGURE 4 Retention of mRNA by membranes stripped of ribosomes. RER was isolated as described in Fig. 2 from livers of rats labeled for 48 h with 100 μCi of [^{3}H]orotic acid or 3 h with 100 μCi of [^{3}H]orotic acid in the presence of 2.6 μmol of FOA. 1 ml of membranes (0.70 mg RNA/ml in 0.25 M STKM) was mixed with 1 ml of either 1.2 M KCl or 2.0 M KCl (in 0.25 M STKM). Puromycin (PUR) was added to a final concentration of 1 mM. After incubation at 25°C for 30 min, 0.75 ml of the membrane suspension was layered onto 15–40% linear sucrose gradients of the same ionic strength followed by centrifugation (SW-41, 38,000 rpm, 2 h, 4°C). Fractions were collected and processed as described in Materials and Methods. (A) Membrane ribosomal RNA labeled, stripped in 600 mM KCl; (B) ribosomal RNA labeled, stripped in 1.0 M KCl; (C) mRNA labeled, stripped in 600 mM KCl; (D) mRNA labeled, stripped in 1.0 M KCl. Control membranes maintained in low ionic strength (0.25 M KCl) lose less than 10% and 20% of their labeled rRNA and mRNA under these conditions.

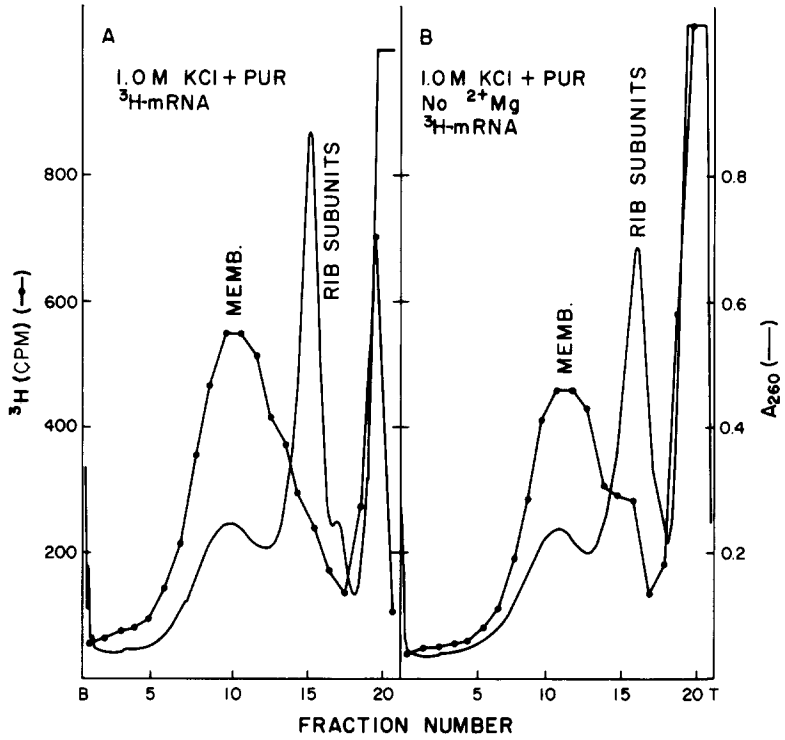

FIGURE 5 Effect of complete removal of ribosomes on mRNA-membrane association. Membranes, mRNA-labeled, were isolated as described in Fig. 4 and suspended in 0.25 M STKM. 1 ml of the membranes was mixed with 1 ml of high salt buffer (2.0 M KCl, 50 mM Tris, pH 7.4, 10 mM EDTA, 2 mM puromycin 0.25 M sucrose), and incubated for 30 min at 25°C; 0.75 ml was layered on 15–40% sucrose gradients (1.0 M KCl, 50 mM Tris, pH 7.4) without Mg^{2+}. After centrifugation (SW-41 rotor, 39,000 rpm, 7 h, 4°C), fractions were collected and processed as described in Fig. 4. (A) Membranes stripped (1.0 M KCl plus 1 mM puromycin) and analyzed in the presence of 5 mM $MgCl_2$; (B) same as (A) without Mg^{2+}.

shows membranes stripped as in Fig. 5 B but in the presence of Mg^{2+}. The fact that labeled mRNA remained on membranes in the absence of polysomes strongly indicates a direct mRNA-membrane association.

Up to this point, the stripping procedure used to examine the retention of labeled mRNA by degranulated endoplasmic reticulum involved incubation at 25°C in a high salt buffer *(vide supra)*. When the RNA was extracted from membranes stripped at this temperature, it was found that the mRNA on gradients appeared to be rather small in size (about 4S), presumably as a result of endogenous RNase activity. Therefore, we modified the stripping technique and removed more than 90% of the ribosomes from the endoplasmic reticulum but still left the membrane-associated mRNA relatively intact. Fig. 6 B shows that essentially all the ribosomes were removed from the membranes relative to the control (Fig. 6 A) when they were stripped under the conditions of that experiment. Fig. 7 shows the size distribution on gradients of the labeled mRNA from control and KCl-puromycin stripped membranes. The labeled mRNA remaining on stripped membranes (40% relative to control membranes) appears relatively intact though somewhat slower sedimenting than the labeled mRNA from control membranes. Whether this is due to partial degradation during the preparation or to some other factors is not clear at present.

To determine the percentage of the [^{3}H]Poly A-containing RNA remaining on membranes stripped by the above technique, we used the Millipore filter assay. With KCl concentrations above 500 mM, 90% of synthetic [^{3}H]Poly A and 40–45% of ^{3}H-labeled mRNA from extracted free polysomes bound to filters (results not shown).

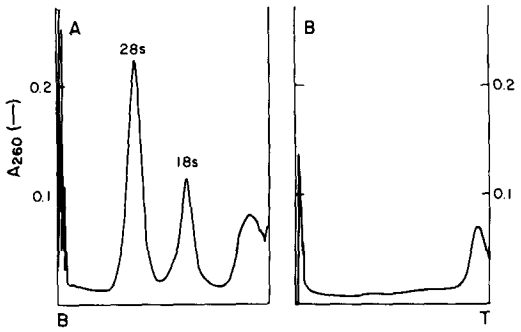

FIGURE 6 Removal of ribosomes from membranes under conditions less destructive to mRNA. RER membranes were isolated as described in Fig. 2. 1 ml of the membranes (0.70 mg RNA/ml in 0.25 M STKM) was mixed with 1 ml of high salt buffer (0.25 M sucrose, 2.0 M KCl, 50 mM Tris, pH 7.4, 2 mM puromycin, 10 mM EDTA), incubated for 30 min at 4°C, layered on top of 9 ml of 0.44 M sucrose (1.0 M KCl, 50 mM Tris, pH 7.4, no Mg^{2+}) in an SW-41 tube, and centrifuged (38,000 rpm, 1 h, 4°C). Membrane pellets were solubilized in 3 ml of NETS buffer in the presence of 1 mg/ml of bentonite centrifuged at 5,000 rpm for 5 min (to remove bentonite); 0.5 ml was layered onto 15–30% sucrose gradients in NETS buffer minus SDS and centrifuged (SW-41, 38,000 rpm, 7 h, 4°C). Tubes were punctured at the bottom, and the outflow was scanned at 260 nm with a peristaltic pump. (A) Control membranes; (B) stripped membranes.

The fact that a large proportion of isolated RNA labeled in the presence of FOA bound to Millipore filters under high salt conditions further supports the suggestion that FOA specifically labels mRNA. Under these salt conditions, less than 0.5% of labeled ribosomal RNA bound to the filters. Explanations for the lack of complete binding of [^{3}H]mRNA include the possibilities that a significant percentage of rat liver mRNA may not contain a Poly A region (15, 16) and/or that Millipore filters bind only RNAs that contain a Poly A segment of greater than a minimum length (9, 24). With this same procedure, RER containing RNA labeled in the presence of FOA (mRNA label) was stripped of ribosomes and pelleted; the RNA was extracted and filtered through HAWP Millipore filters under high salt conditions. Table I shows that 38% of the [^{3}H]Poly A-containing RNA remained on membranes stripped of their ribosomes compared to control membranes. This finding further supports the hypothesis of a direct mRNA-membrane interaction independent of ribosomes.

Quantitation of Poly A Remaining on Stripped Membranes

The possibility exists that the percentage of labeled mRNA left on membranes stripped of ribosomes is not truly representative of the steady-state mRNA left unlabeled in the 3-h labeling period. To examine this possibility, we stripped membranes with the 1 M KCl buffer and puromycin and determined the percentage of the Poly A remaining membrane-attached by a [^{3}H]poly U

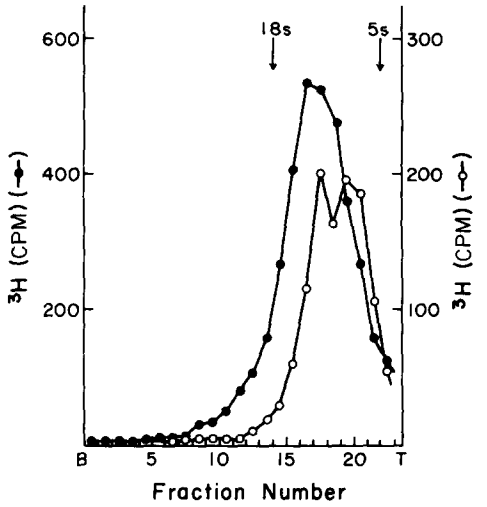

FIGURE 7 Size distribution of labeled mRNA extracted from membranes stripped of ribosomes. RER membranes, mRNA labeled, were isolated as described in Fig. 2 except that 500 μg/ml heparin was included during the washing. The membranes were stripped and pelleted as described in Fig. 6 except that heparin was present at 300 μg/ml. The pellets were phenol-chloroform extracted, and the isolated RNA was examined on 15–30% sucrose gradients as described in Fig. 1.

TABLE I

Binding of ^{3}H-mRNA to Millipore Filters

RNA isolated from	KCl	dpm applied	dpm bound	% bound*
	mM			
Control RER	0	3,904	9	–
Control RER	500	3,904	592	100
Stripped RER	0	1,060	20	–
Stripped RER	500	1,060	223	38

* Values are expressed relative to control value.

RER membranes, mRNA labeled (see Fig. 4), were isolated and stripped of ribosomes as described in Fig. 6, with 1.0 M KCl, 1 mM puromycin, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$. The RNA of the membrane pellet was extracted as described in Materials and Methods and the purified RNA filtered through Millipore filters. Filters were dried, and the radioactivity was measured in toluene-PPO.

hybridization assay (see Materials and Methods). As can be seen in Table II, a substantial percentage of Poly A remained on membranes stripped of all ribosomes with the high ionic strength buffer and puromycin. This is further support for the concept of a direct, ribosome-independent, mRNA-membrane interaction.

Sites of Membrane-mRNA Interaction

The question remains as to what segments of the mRNA may be involved in its binding to the endoplasmic reticulum. Thus far, data from systems in vitro have indicated that the Poly A segment is the major and perhaps only site of binding (11, 14). Table II shows that in liver, as in HeLa cells and fibroblasts, the Poly A segment appeared to remain bound to the endoplasmic reticulum after pancreatic ribonuclease treatment. To see whether other mRNA segments played a role in binding to the membrane, we treated isolated RER containing labeled mRNA with low levels of RNase and examined it on 15–40% sucrose gradients in TKM buffer. Under these conditions, 30% of the ribosomes were released as monosomes. Fig. 8 B shows that a significant portion (about 40%) of the labeled mRNA remained associated with the membranes under these conditions. As a control against nonspecific trapping of labeled RNA, nonlabeled membranes of the RER were mixed with [^{3}H]mRNA-labeled free polysomes and treated as described above with ribonu-

TABLE II

Poly A Content of RNA Measured by [^{3}H]Poly U Hybridization

Sample	[^{3}H]Poly U Hybridized	Hybridized
		%
Control Membranes	2,621	100
Membranes + KCl	1,979	75.5
Membranes + KCl + puromycin	1,041	39.7
Membranes + KCl + puromycin + EDTA	855	32.6
Membranes + RNase	1,218	46.5

RER membranes were isolated as described in Materials and Methods, resuspended in 0.25 M STKM (0.7 mg RNA/ml), treated as shown above and pelleted as described in Fig. 6. The pellets were resuspended in 3 ml of NETS buffer with 1 mg/ml bentonite, the bentonite was removed by centrifugation, and 40-μl aliquots were analyzed for Poly A with [^{3}H]Poly U as described in Materials and Methods. Final concentrations of various reagents shown in Table II and used to treat membranes were KCl, 1.0 M; puromycin, 1 mM; EDTA, 5 mM; and RNase, 3 μg/ml.

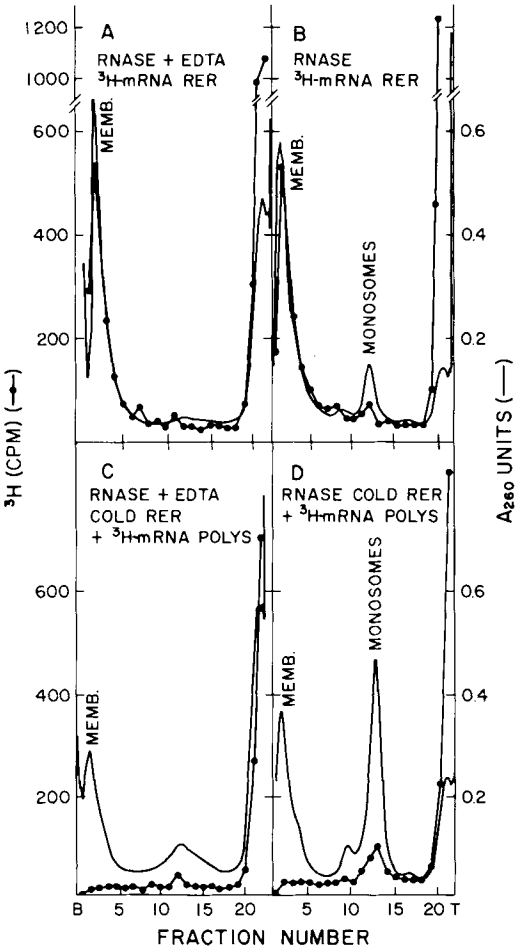

FIGURE 8 Effect of RNase treatment on mRNA-membrane association. RER membranes with associated mRNA labeled as described (Fig. 4) were isolated and resuspended in 0.25 M STKM (0.75 mg RNA/ml). Pancreatic RNase was added at a concentration of 3 μg/ml (B and D) or 15 μg/ml with 10 mM EDTA (5 mM effective concentration) (A and C). Membranes were treated for 30 min at 4°C; then 0.75 ml was layered onto 15–40% sucrose gradients in TKM buffer with 1.0 ml of a 2.0 M STKM cushion to "catch" the membranes and centrifuged (SW-41, 38,000 rpm, 2 h, 4°C). Fractions were processed as described in Materials and Methods. (A) Membranes treated with RNase (15 μg/ml) and 10 mM EDTA; (B) Membranes treated with RNase (3 μg/ml); (C) unlabeled membranes mixed with free polysomes (POLYS) labeled in mRNA (12.4 A_{260} U, 3,880 dpm) treated as in (A); (D) unlabeled membranes with mRNA-labeled free polysomes (12.4 A_{260} U, 3,880 dpm) treated as in (B).

clease. The results show that no radioactivity sedimented with the membranes (Fig. 8 D).

Even under more severe conditions (15 μg/ml RNase, 5 mM EDTA), a significant percentage of labeled mRNA sedimented with the membranes (Fig. 8 A). A mixing experiment similar in design to that discussed above also showed that the retained radioactivity was not the result of nonspecific association or trapping.

To eliminate the possibility that ribosomes were protecting the labeled mRNA from RNase attack, we stripped the membranes with high salt buffer and puromycin and treated them with low levels of RNase. Fig. 9 A shows that about 30% of the labeled mRNA still sedimented with the membranes after application on gradients. A mixing experiment again showed that the radioactivity sedimenting with the membranes was not artificially derived (Fig. 9 B). These experiments suggest that, in rat hepatocytes, mRNA may interact with intracellular membranes through its non-Poly A pyrimidine region as well as through its Poly A segments. Alternate explanations such as the secondary structure of the membrane-bound mRNA allowing partial resistance to exogenous ribonuclease cannot be excluded at the present time (10). Early experiments, though, suggest that the labeled mRNA in isolated polysomes is more sensitive to pancreatic ribonuclease than those mRNAs found as part of membrane-polysome complexes (our unpublished results).

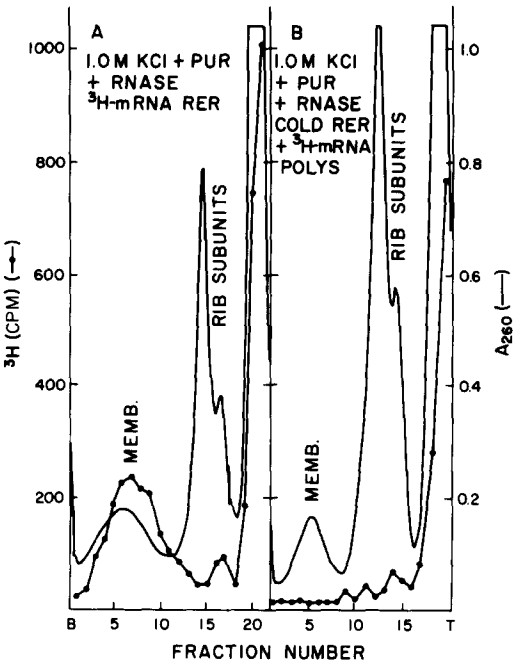

FIGURE 9 Effects of high salt-puromycin-RNase on mRNA-membrane association. Membranes with their mRNA labeled were isolated as described in Fig. 4 and resuspended in 0.25 M STKM to 0.70 mg RNA/ml. 1 ml of membranes was mixed with 1 ml of high salt buffer (1.2 M KCl, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.25 M sucrose), and pancreatic RNase was added to a final concentration of 3 μg/ml. After incubation (30 min, 25°C), 0.75 ml was layered onto 15–40% sucrose gradients in high salt buffer, centrifuged (SW-41, 38,000 rpm, 2 h, 4°C), and collected as described in Materials and Methods. (A) Labeled membranes treated as described above; (B) unlabeled membranes mixed with labeled free polysomes (POLYS) (12.4 A_{260} U, 3,880 dpm, see Fig. 9), treated as in (A).

DISCUSSION

The results on rat hepatocytes presented in this paper support the concept of a direct association of mRNA with endoplasmic reticulum membranes independent of ribosomal attachment. More than 50% of the mRNA labeled in the presence of FOA remained associated with membranes stripped of essentially all ribosomes by means of a buffer of high ionic strength with puromycin in the absence of Mg^{2+}. The finding that 40% of the RNA labeled in the presence of FOA and isolated from stripped membranes bound to Millipore filters relative to the amount bound from control membranes further supports the idea that the RNA retained on degranulated membranes is indeed mRNA. The fact that the amount of labeled mRNA released when membranes are KCl-puromycin treated is only slightly greater than the amount released by KCl treatment alone (our unpublished results) implies that those messengers not directly attached to the membranes are mainly associated with the loose class of polysomes (those released by high salt alone). Small doses of FOA effectively inhibited the maturation of 28S and 18S rRNA while labeling a messenger-like RNA species that appeared heterogeneous in size on sucrose gradients and polyacrylamide gels and that bound (45%) to Millipore filters under conditions where less than 1% of the labeled rRNA was bound. Fluoroorotic acid does not suffer from some of the drawbacks of actinomycin D, such as toxicity and inhibition of protein synthesis; its use would seem desirable in whole animal experiments with liver where a selective label of mRNA is needed.

In confirmation of other studies (11, 14), our

data demonstrate that the Poly A segment of mRNA represents a site for binding the mRNA to intracellular membranes because almost half of the Poly A remains on membranes treated with pancreatic ribonuclease. We found that other sites may exist on mRNAs for membrane binding, unlike the results of earlier work. This was demonstrated by the experiments that showed that a significant amount of pyrimidine-containing portion of membrane-bound mRNA was resistant to RNase even when ribosomes were removed from the membranes. Furthermore, unpublished results in our laboratory (Cardelli, J., unpublished observations) have shown that mRNA present as a component of the membrane-polysome complex is less sensitive to exogenous ribonuclease digestion than mRNA in "free" polysomes. Thus, the membrane must play a role in determining the resistance of membrane-bound mRNA to exogenous nuclease. On the other hand, our data cannot distinguish an interaction of the membrane directly with pyrimidine-containing portions of the mRNA molecule or a membrane-induced conformational change in the mRNA as the effector(s) of RNase resistance. Artifactual binding was ruled out by mixing experiments. It also appears that the RNase-resistant class of mRNA may represent a subset of the class of mRNA that is left on membranes disassembled from ribosomes by high salt-puromycin solutions (cf. Fig. 4 D with Fig. 9 A). Our data extend previous work that indicated that the mRNA that was part of membrane-bound polysomes was less sensitive to RNase than the mRNA in free polysomes (3, 5); this may indicate that the membrane offered some protection of mRNA to nuclease action.

Other investigators have reported that the non-Poly A portions of the mRNA associated with membranes are sensitive to the action of ribonuclease (11, 14). Although the systems they used were cultured cells in which the polysome-membrane interaction may be quite different from that in liver, the conditions utilized in those studies may not have allowed the non-poly A portions of mRNA to remain intact. The lower retention of labeled polyadenylated mRNA on the surface of the endoplasmic reticulum by high salt-puromycin stripped membranes in the hepatocyte growing in vivo as compared with the cell culture systems reported earlier may be the result of one or more factors. *(a)* In the studies reported in this paper, stripped membranes were compared with control membranes not previously washed in high ionic strength buffers. In Table II, it is evident that membranes treated with the high salt buffer alone lose 25% of their polyadenylated mRNA relative to the low salt-washed control. The percentage of polyadenylated mRNA remaining on the surface of high salt-puromycin- or ribonuclease-treated membranes would appear considerably greater if these membranes had been compared with the high salt-wash membrane control. *(b)* Membranes of the RER isolated from liver both contain large amounts of endogenous ribonuclease activity and are contaminated by lysosomes. Therefore, the amount of labeled mRNA released from membranes that have been degranulated in the high salt buffer-puromycin solution may reflect the effects of both high ionic strength and ribonuclease present in the preparation. *(c)* The differences in the percentage of mRNA tightly bound to membranes of the endoplasmic reticulum in cultured cells as compared with hepatocytes may be a real phenomenon.

Our results, which indicate a direct mRNA-membrane association of the RER in rat hepatocytes involving Poly A and possibly non-Poly A-pyrimidine regions of the mRNA, are in accord with the predictions of the Membron model (27). This model predicts that the structure of the endoplasmic reticulum is involved in selecting mRNAs for translation, in stabilizing the mRNA, in allowing and possibly modifying the translation of this mRNA, and subsequently in conserving the mRNA for further rounds of translation. For the membrane to select specific mRNAs for translation, regions other than the Poly A segment, which is found on free as well as bound mRNAs, would have to be recognized in polysome-membrane interaction. Our data indicate that regions of the mRNA other than the Poly A segment may interact with and be protected by intracellular membranes. Whether specific sequences of the mRNA are recognized by membrane sites or whether proteins attached to the mRNA play a role in binding is not known at the present time, although the recent report of Shires et al. (26) does demonstrate selectivity of liver membranes in their interaction in vitro with polysomes from liver as compared with those from a mouse myeloma. Work is in progress to determine what role ribonucleoproteins and/or specific mRNA sequences play in the binding of mRNAs to membranes of the endoplasmic reticulum.

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