

Structure and Function of Rat Liver Polysome Populations.

II. Characterization of Polyadenylate-containing mRNA Associated with Subpopulations of Membrane-bound Particles

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ABSTRACT Poly(A)⁺RNA fractions prepared from free and loosely and tightly membrane-bound polysome populations (poly(A)⁺RNA_{free}, poly(A)⁺RNA_{loose}, and poly(A)⁺RNA_{tight}) were used to drive cDNA in homologous and heterologous hybridization reactions. A large fraction by mass of sequences was shared among the three poly(A)⁺RNA populations, but shared sequences exhibited distinct frequency distributions within the different populations. 13–15 *in vitro* translation products of poly(A)⁺RNA_{free} and poly(A)⁺RNA_{loose} detected by gel electrophoresis were shared. Most of these were produced in different relative quantities by the two RNA populations. Five or six higher mol wt polypeptides were produced by poly(A)⁺RNA_{loose} that were not detected as products of either poly(A)⁺RNA_{free} or poly(A)⁺RNA_{tight}. We suggest that loosely bound polysomes may not be artifactually derived as reflected in their quantitatively distinct poly(A)⁺RNA population.

Two tightly membrane-bound RNP fractions were prepared from rat liver on the basis of their release from or retention on purified rough microsomes or a crude membrane fraction after *in vitro* disaggregation of polysomes with high-salt and puromycin. Homologous and heterologous hybridizations involving their poly(A)⁺RNA fractions revealed that a large portion by mass of sequences was shared but that these sequences exhibited distinct frequency distributions in the two fractions. The RNA fractions produced an identical set of *in vitro* translation products but individual polypeptides were produced in different relative quantities. This indicates that the two RNP fractions do not arise by any random artifactual process and suggests that they may represent functionally distinct populations.

Numerous investigations have revealed the existence of membrane-bound ribosomes or polyribosomes that apparently exhibit distinct interactions with rough microsomal or rough endoplasmic reticulum (RER) fractions (See references in 24, 42). Rosbash and Penman (38, 39) coined the terms “loose” and “tight” to describe ribosomes that are either susceptible or resistant to release from a HeLa cell membrane fraction by treatment with EDTA, puromycin, or ribonuclease. They interpreted their findings to suggest that two distinct classes of membrane-bound polyribosomes exist in HeLa cells. Subsequent work by Mechler and Vassalli (25, 26) with myeloma cells did not support this interpretation. Their findings suggested that “loose” and “tight” ribosomes are derived from different ends of the same polyribosome structure, the former

being only indirectly attached to the membrane by means of the mRNA molecule due to a shortage of 60s subunit binding sites on RER of cultured cells. Such “dangling” ribosomes are present in relatively small quantities in rough membrane fractions from liver (8). Instead, a significant fraction of liver membrane-bound polyribosomes are released by incubation of membrane fractions in buffers containing high monovalent cation concentrations (37). “Loosely bound” polyribosomes of this type have been identified in other systems (17, 45). In many cases these high-salt-releasable polyribosomes have been shown to exhibit similar characteristics (37) or synthesize the same polypeptides as free polyribosomes (15, 29, 45). This has led to the conclusion by some investigators (37, 45) that they represent artifactually adsorbed free polysomes. However,

much of the available data concerning this question is difficult to interpret since either (a) the integrity of mRNA was not shown to be preserved during polyribosome isolation or (b) the extent of free polysome contamination of rough membrane fractions was not determined. The latter is particularly significant since contaminating free polysomes will be included in the high-salt releasable fraction.

In addition, to these two polyribosome classes, two classes of membrane-bound mRNA or mRNP ribonucleoprotein have been defined in numerous systems on the basis of membrane release or retention after various treatments that cause polyribosome breakdown and release of most of the membrane-bound ribosomes (2, 3, 10, 14, 18, 20–23, 25–27, 31). In rat liver divergent results have been obtained. The membrane-associated fraction has been found by different investigators to account for 20–90% of the total, and there is controversy over whether the results indicate the existence of a direct interaction between mRNA and the membrane (10, 14, 18). In this case the available data are extremely limited since the two mRNA fractions have not been isolated in undegraded form and characterized.

We have addressed the question concerning the existence of functionally distinct subpopulations of membrane-bound polyribosomes and mRNP by using hybridization and in vitro translation technologies to characterize and compare their poly(A)⁺ mRNA populations. Our data indicate that, although the populations exhibit considerable sequence overlap, distinct quantitative differences exist among them. This suggests that they may be functionally distinct.

MATERIALS AND METHODS

Isolation of free and total membrane-bound polysomes, synthesis and kinetic fractionation of cDNA, and hybridization reactions were conducted exactly as described previously (28).

Tissue Fractionation and Isolation of Loosely and Tightly Membrane-bound Polysome Fractions

Adult male rats of the Holtzman strain were used. Rats were given chow and water *ad libitum* and were not starved before sacrifice. Rats were maintained on a 12 h light/dark cycle and were kept in the dark between 8 pm and 8 am. Sacrifice occurred between 2 and 4 pm. Loosely and tightly membrane-bound polysomes were prepared by the procedure of Ramsey and Steele (36, 37) with some modifications to preserve integrity of mRNA, increase recovery of polysomes from the livers of fed rats, and decrease the levels of cross-contamination.

ISOLATION OF LOOSELY MEMBRANE-BOUND POLYSOMES: Rats were decapitated and the livers perfused via the inferior vena cava with ice-cold 0.25 M sucrose containing 5 mM MgCl₂ and 100 μg/ml sodium heparin. Perfused livers were excised and homogenized in 3 vol of a solution containing 0.25 M sucrose, 50 mM HEPES, pH 7.6, 5 mM MgCl₂, 75 mM KCl, 3 mM GSH (reduced glutathione), and 250 μg/ml heparin. The homogenate was centrifuged in a Beckman SW 27 rotor (Beckman Instruments, Inc., Fullerton, CA) for 2 min at 740 g_{max} and 12 min at 131,000 g_{max}. The supernate was discarded and the pellet homogenized with three to four strokes in a loose-fitting Potter-Elvehjem homogenizer in 3 vol/g liver of the initial homogenization buffer containing 17% rat liver high-speed supernatant fluid (36). The homogenate was centrifuged as above and the supernate, containing residual free polysomes, discarded.

Loosely membrane-bound polysomes were extracted from the pellet by homogenizing with three to four strokes as above in 2 vol of the initial homogenization buffer containing 17% rat liver high-speed supernatant fluid and 0.25 M KCl rather than 0.075 M. The homogenate was incubated for 20 min on ice and centrifuged as described above. The supernate containing loosely membrane-bound polysomes was adjusted to 1 mg/ml heparin, layered over sucrose cushions prepared as described previously (28), and polysomes pelleted by centrifuging at 303,500 g_{max} in a Beckman 60 Ti rotor (Beckman Instruments, Inc.) for 20 h.

ISOLATION OF TIGHTLY MEMBRANE-BOUND POLYSOMES: A 131,000 g_{max} pellet was prepared as described above, except that the initial homogenization

buffer contained 0.25 M KCl rather than 0.075 M and 500 μg/ml sodium heparin rather than 250 μg/ml. The pellet was washed free of residual contaminating free and loosely membrane-bound polysomes by homogenizing in 3 vol/g liver of the initial homogenization buffer containing 15% rat liver high-speed supernatant fluid, and recentrifuging. The supernate was discarded and the 131,000 g_{max} pellet homogenized in 50% rat liver high-speed supernatant fluid containing 1% Triton X-100, 20 mM MgCl₂, 250 mM KCl, 3 mM GSH and centrifuged at 1,470 g_{max} in a Sorvall SS34 rotor (Du Pont Instruments-Sorvall Biomedical Div., Du Pont Co., Newtown, CT) for 5 min to pellet nuclei. The supernate was adjusted to 50 mM MgCl₂, 2 mg/ml heparin, 1.3% sodium deoxycholate and centrifuged at 15,000 g_{max} for 5 min to pellet insoluble material. Polysomes were pelleted from the supernate as described above for loosely membrane-bound polysomes.

ISOLATION OF HIGH-SALT, PUROMYCIN-MEDIATED MEMBRANE-RELEASED AND MEMBRANE-ASSOCIATED RNP FRACTIONS: Two fractionation schemes were used for the isolation of these RNP fractions. In method A the two fractions were obtained from a high-salt washed 131,000 g_{max} nuclear-microsomal pellet. This method allowed isolation of the large quantities of poly(A)⁺RNA required in the hybridization reactions, and the two RNP fractions were obtained from a subcellular fraction that contained ~95% of the total tightly membrane-bound polysomes (36). However, the poly(A)⁺RNA fractions obtained by this procedure were partially degraded, exhibiting number-average sizes of 500–600 nucleotides on formamide-sucrose gradients and having low translational efficiencies in the rabbit reticulocyte lysate system. This occurred despite the liberal use of several ribonuclease inhibitors and presumably was due to the extensive manipulation and incubation periods involving the ribonuclease-rich 131,000 g_{max} pellet. In method B, the RNP fractions were prepared from high-salt-washed, purified, rough microsomes obtained from a postmitochondrial supernate. Poly(A)⁺RNA obtained by this procedure was intact (see Results), but was derived from a subcellular fraction containing only 5–10% of the total tightly membrane-bound polysomes.

Method A: A 131,000 g_{max} pellet was prepared and washed once as described for tightly membrane-bound polysomes. The resulting pellet was homogenized gently in 3 vol/g liver of the initial homogenization buffer containing 2 mM puromycin diHCl, 15% rat liver high-speed supernatant fluid, 0.1 mM EDTA, and 1.5 mM, rather than 5 mM MgCl₂. The homogenate was incubated at 4°C for 1 h and recentrifuged as above. The supernate containing the membrane-released tightly bound RNP fraction was decanted and stored on ice. The pellet was gently homogenized in the same buffer, incubated at 4°C for 15 min, and recentrifuged. The supernate was decanted and added to the membrane-released tightly bound RNP fraction. The pellet was washed once more in the same buffer lacking puromycin and the supernate discarded. Nuclei were removed from the pellet as described above for tightly membrane-bound polysomes using 1% Triton X-100 treatment, except that the buffer contained 1 mg/ml yeast tRNA as an additional ribonuclease inhibitor. Membrane-associated tightly bound RNP was liberated from RER by sodium deoxycholate treatment of the 1,470 g_{max} supernatant fluid as described above. Both tightly membrane-bound RNP fractions were pelleted through 1.8 M sucrose cushions prepared in the same buffer as for the isolation of polysomes.

Method B: Postmitochondrial rough microsomes were prepared essentially as described previously (10) except that all solutions contained 0.5 mg/ml sodium heparin and 3 mM GSH. The microsomes that banded at the 1.35 M-2.0 M sucrose interface were collected and diluted with 2½ vol of a solution containing 10% rat liver high-speed supernatant fluid, 0.75 M KCl, 5 mM MgCl₂, 50 mM HEPES, pH 7.6, 500 μg/ml sodium heparin, and 3 mM GSH. 50 ml of the rough microsome solution were layered over 10 ml of 1 M sucrose in the same buffer in 1 × 4½ inch polycarbonate bottles. Microsomes were washed free of contaminating free and loosely membrane-bound polysomes by centrifuging in a Beckman 45 Ti rotor (Beckman Instruments, Inc.) at 30,000 rpm for 35 min. The supernate was decanted and the walls of the bottles carefully swabbed. The pellet was gently hand-homogenized in 20 ml of a solution containing 25% rat liver high-speed supernatant fluid, 2 mM puromycin diHCl, 0.66 M KCl, 2 mM MgCl₂, 500 μg/ml sodium heparin, 50 mM HEPES, pH 7.6, and 3 mM GSH. After incubation for 1 h at 4°C, this mixture was layered over 30 ml of 0.5 M sucrose prepared in the same buffer lacking puromycin and pelleted as above. The supernate containing the membrane-released tightly bound RNP fraction was decanted into a fresh centrifuge bottle and spun in a Beckman 45 Ti rotor (Beckman Instruments, Inc.) at 40,000 rpm for 2½ h to pellet RNP. The high-salt, puromycin-stripped microsomal pellet containing the membrane-associated tightly bound RNP fraction was extracted directly in guanidinium thiocyanate as described below.

Isolation of Poly(A)⁺RNA fractions

Polysome pellets were extracted by the SDS-phenol-chloroform procedure of Palmiter (32) as described previously (28). RNP and microsomal pellets were extracted by the guanidinium thiocyanate-CsCl procedure of Chirgwin et al. (12). Poly(A)⁺RNA fractions were isolated by oligo(dT) cellulose chromatography using the procedure of Bantle et al. (4) as described previously (28).

In Vitro Translation and Analysis of Products

Poly(A)⁺RNA was translated in a nuclease-treated rabbit reticulocyte lysate system as described by Gonzalez and Kasper (15). Poly(A)⁺RNA was translated at a concentration of 5–15 µg/ml. [³⁵S]methionine (600–1400 Ci/mmol) was included at a concentration of 500 µCi/ml. Under these conditions incorporation of radioactivity into TCA-insoluble products varied from 10–50-fold over background with the various poly(A)⁺RNA fractions.

Translation was allowed to proceed for 45 min at 30°C and the reaction quenched by cooling on ice. 2 µl aliquots of the reaction mixture were spotted onto Whatman No. 1 filter paper (Whatman Inc., Paper Div., Clifton, NJ). Filters were washed by boiling for 10 min in 10% TCA and rinsed twice each in 5% TCA, methanol, and ether. Filters were dried and acid-precipitable radioactivity determined by scintillation spectrophotometry in 10 ml of OCS scintillator cocktail (Amersham Corp., Arlington Heights, IL). The translation products were analyzed by loading equal quantities of acid-precipitable counts onto 10% polyacrylamide, 1.5-mm slab gels using the system of Laemmli (19). The gels were stacked at 10 mA/slab for 4 h then run at 5 mA/slab for ~12 h. The gels were fixed and treated with Enhance (New England Nuclear, Boston, MA) as described by the manufacturer. Dried gels were fluorographed using Kodak XAR-5 film and fluorographs were subjected to microdensitometry.

Sizing of Poly(A)⁺RNA and cDNA Populations

Poly(A)⁺RNA populations were sized by hybridization of excess [³H]poly(U) to formamide-sucrose gradient fractions as described previously (28). Number-average sizes were estimated by using the formula: $\Sigma \text{cpm } l_i / \Sigma \text{cpm}$, and mass average sizes by the formula: $(\Sigma (\text{cpm}_i)^2 / \Sigma \text{cpm}_i) / l_i$, where cpm_i is proportional to the number of molecules of length l_i .

[³H]cDNA populations were run on 98% formamide–3.5% polyacrylamide gels as described previously (28), except that slab gels were used rather than cylindrical gels. Gels were treated with Enhance (New England Nuclear), dried, and fluorographed. Microdensitometric tracings were done and mass-average sizes determined by integrating areas under equal length-range segments as described by Ordahl et al. (30). pBR322 *Hinf*I restriction fragments were used as size markers.

RESULTS

Isolation and Characterization of Loosely and Tightly Membrane-Bound Polysomal Poly(A)⁺RNA Populations

Loosely and tightly membrane-bound polysomes were isolated and their respective poly(A)⁺RNA fractions obtained as described in Materials and Methods. Polysomes prepared by pelleting through sucrose cushions, as described, have previously been shown to be free of contaminating nuclear or cytoplasmic RNP particles. Cross-contamination levels were estimated to be <1% by the procedure used previously (28). Yields were 0.61 mg/g liver of loosely membrane-bound and 2.19 mg/g liver of tightly membrane-bound polysomal RNA (average of two preparations). Loosely membrane-bound polysomes thus comprise ~22% of the total membrane-bound polysome population. Polysome profiles have demonstrated the presence of large polyribosome structures with monomers comprising a relatively small proportion of each fraction (37). Yields of poly(A)⁺RNA were somewhat variable and ranged from 0.5–1.0% of the total polysomal RNA populations. Yield variability was due to aggregation rather than degradation of RNA during preparation. This aggregation is the result of the extremely high *g*-force necessary for maximum recovery of polysomes from subcellular fractions prepared from the livers of fed rats. All poly(A)⁺RNA preparations were sized on formamide sucrose gradients, as described in Materials and Methods. Number-average lengths of poly(A)⁺RNA_{loose} and poly(A)⁺RNA_{tight} were ~1,725 and 1,400 nucleotides, respectively.¹

¹ Abbreviations used in this paper: cDNA^{assoc}, cDNA_{bound}, cDNA_{free},

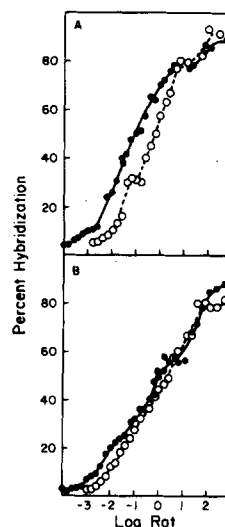


FIGURE 1 Homologous and heterologous hybridization reactions. cDNA_{tight} (A) and cDNA_{loose} (B) were hybridized to an excess of the homologous (●) or heterologous (○) poly(A)⁺RNA, and the extent of reaction was determined as described (28). RNA concentrations ranged from ~0.5 to 1,700 µg/ml. Most data points are averages of duplicates or triplicates. The curves were drawn with the aid of a computer (28).

cDNA was prepared from the poly(A)⁺RNA fractions using avian myeloblastosis virus reverse transcriptase as described previously (28). cDNA_{tight} and cDNA_{loose} were hybridized to their respective homologous poly(A)⁺RNA populations.¹ The homologous hybridization curves are shown in Fig. 1A and B. Both curves occupy ~5½ log rot (rot, product of RNA concentration and time of incubation in mol-s/l) which indicates that mRNA species within the populations are present at widely varying concentrations. We have analyzed the hybridization curves as if the poly(A)⁺RNA species are distributed into discrete abundance classes (5) using a nonlinear least squares computer program (33) as described previously (28). The results of this analysis are given in Table I. In both cases, best fits to the data were obtained by assuming the presence of four first-order reaction components. Assuming four rather than three components did not result in a significant increase, in either case, in the calculated complexity of the populations.

For both homologous curves, frequency classes are present that contain more abundant and more rare mRNA species than were resolved when the total membrane-bound polysomal poly(A)⁺RNA population was analyzed (28). We feel the present analysis is more accurate due to subfractionation and analysis of a greater number of data points, particularly at

cDNA_{loose}, cDNA_{tight} and cDNA^{rel}, complementary DNA fractions transcribed from poly(A)⁺RNA derived from membrane-associated RNP, total membrane-bound polysomes, free polysomes, loosely membrane-bound polysomes, tightly membrane-bound polysomes, and membrane-released RNP, respectively; FOA, 5-fluoroorotic acid; GSH, reduced glutathione; K_{loose}, rate constant for the most abundant component of the heterologous hybridization involving poly(A)⁺RNA_{tight} and cDNA_{loose}; K_{tight}, rate constant for the most abundant component of the heterologous hybridization involving poly(A)⁺RNA_{loose} and cDNA_{tight}; P_{loose}, fraction of hybridizable cDNA_{loose} comprising the most abundant component of the heterologous hybridization involving poly(A)⁺RNA_{loose} and cDNA_{tight}; P_{tight}, fraction of hybridizable cDNA_{tight} comprising the most abundant component of the heterologous hybridization involving poly(A)⁺RNA_{loose} and cDNA_{tight}; poly(A)⁺RNA^{assoc}, poly(A)⁺RNA_{bound}; poly(A)⁺RNA_{free}, poly(A)⁺RNA_{loose}, poly(A)⁺RNA_{tight} and poly(A)⁺RNA^{rel}, polyadenylic acid-containing RNA obtained from membrane-associated RNP, total membrane-bound polysomes, free polysomes, loosely membrane-bound polysomes, tightly membrane-bound polysomes, and membrane-released RNP by oligo(dT) cellulose chromatography, respectively; rRNA, ribosomal RNA; Rot, product of RNA concentration and time of incubation in mol-s/l.

TABLE I
Complexity and Frequency Distribution of Loosely Membrane-bound and Tightly Membrane-bound Polysomal Poly(A)⁺RNA Populations

Polysome class	Abundance class	Percent cDNA hybridized*	Rate Constant		Nucleotide complexity	No. of mRNA species [¶]	Copies/cell**
			Observed [‡]	Corrected [§]			
Loose	I	18.4	317	2420	2.05×10^3	1	39,000
	II	13.0	48.3	136	1.36×10^4	8	5880
	III	29.7	1.94	5.53	5.36×10^5	311	149
	IV	38.8	0.014	0.086	5.71×10^7	33,100	1
Tight	I	13.1	266	1450	1.38×10^3	1	205,000
	II	35.4	28.3	218	2.45×10^4	17	11,600
	III	35.1	1.64	5.52	6.04×10^5	431	470
	IV	16.3	0.020	0.052	3.86×10^7	27,600	7

* Normalized to a terminal hybridization value of 100%. Observed terminal values were 87.2 and 89.0% for loosely bound and tightly bound poly(A)⁺RNA, respectively.

[‡] Liter per mole-s. Values have not been adjusted to those that would be observed under standard salt conditions. Those values may be obtained by consulting the appropriate tables.

[§] Rate constant expected for an RNA abundance class reacting in isolation.

[¶] Calculated relative to the rate constant observed for the reaction of rabbit alpha + beta globin mRNA with its cDNA under our hybridization conditions using the relation: $K_1 C_1 = K_2 C_2$ where K and C represent the rate constants and complexities of the two RNA populations. The observed value for the globin reaction (2827 liter/mole-s) was corrected for the dependence of reaction rate on the square root of fragment length and the retardation effect of excess driver over tracer length. Combining the equations of Wetmur and Davidson (44) and Chamberlin et al. (11) one obtains: $K_{T2} = K_{T1} (L_{T2}/L_{T1}) (L_{D1}L_{D2})^{1/2}$ where K_{T1} and K_{T2} are the rate constants observed for a reaction with driver, tracer lengths $L_{D1}L_{T1}$, or $L_{D2}L_{T2}$. The driver and tracer lengths for the globin reaction were 650 and 400 nucleotides. The corresponding mass-average sizes for the loosely bound poly(A)⁺RNA reaction were 2324 and 743 nucleotides and for the tightly-bound poly(A)⁺RNA reaction 1451 and 662 nucleotides. We assume globin mRNA has a complexity of 1200 nucleotides. The corrected K-values corresponding to a complexity of 1200 nucleotides are thus 2468 and 2783 l/mol-s for loosely and tightly bound poly(A)⁺RNA.

^{¶¶} Number of unique mRNA species 1725 (loosely bound) or 1400 (tightly bound) nucleotides in length.

** Copies/cell = (grams driver RNA/cell $\times 6 \times 10^{23}$ molecules/mol)/(RNA nucleotide complexity $\times 330$ g/mol nucleotide). There is ~0.2 pg of total membrane-bound poly(A)⁺RNA/average liver cell (28), 21.8% of which is loosely-bound and 78.2% tightly-bound. This gives 0.044 pg loosely bound and 0.156 pg tightly bound poly(A)⁺RNA/cell.

higher rot values. The complexities of poly(A)⁺RNA_{loose} and poly(A)⁺RNA_{tight} are similar to each other, to total liver polysomal poly(A)⁺RNA (9, 40), as well as to free polysomal poly(A)⁺RNA (3, 13, 28). The complex component comprises ~39% of poly(A)⁺RNA_{loose} and only ~16% of poly(A)⁺RNA_{tight}. Both poly(A)⁺RNA fractions contain a very abundant component consisting of a single mRNA species present at several thousand copies per cell. Assuming that serum albumin mRNA comprises 10% of the total polysomal poly(A)⁺ mRNA population (34) and that it is synthesized exclusively on tightly membrane-bound polysomes, it can be estimated that it is present at about 100,000 copies per cell and comprises ~15% of poly(A)⁺RNA_{tight}. These values are reasonably similar to those determined for component I of poly(A)⁺RNA_{tight} of 200,000 copies per cell and 13%. A highly abundant polypeptide of ~39,000 daltons has been identified as the product of poly(A)⁺RNA_{loose} by in vitro translation (see below). This polypeptide accounts for ~15% of the total poly(A)⁺RNA_{loose} translation products detected under our conditions, which is close to the value of 18% for the percentage of poly(A)⁺RNA_{loose} in component I. It should be mentioned that it is not known what contribution, if any, mitochondrial poly(A)⁺RNA might make to the higher abundance classes of poly(A)⁺RNA_{tight}.

The heterologous hybridizations of cDNA_{tight} to poly(A)⁺RNA_{loose} and cDNA_{loose} to poly(A)⁺RNA_{tight} are also shown in Fig. 1A and B. Comparing the plateau values of the homologous and heterologous reactions in Fig. 1A indicates that poly(A)⁺RNA_{loose} contains all of the sequences that are present in poly(A)⁺RNA_{tight}. Comparing the kinetics of the two reactions suggests that poly(A)⁺RNA_{loose} is relatively enriched in sequences that are rare in poly(A)⁺RNA_{tight}. Poly(A)⁺RNA_{loose} was also hybridized to kinetically fractionated cDNA_{bound} enriched for abundant and rare species (28).¹ The heterologous reaction curves are shown in Fig. 2A and B along

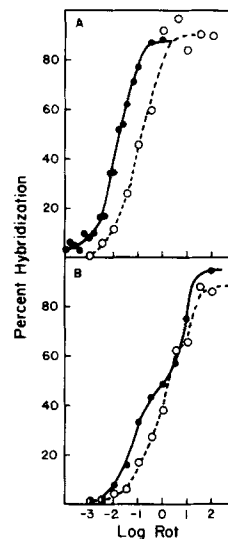


FIGURE 2 Homologous and heterologous hybridization reactions with kinetically-enriched cDNA_{bound}. cDNA_{bound} was kinetically fractionated into abundant and less-abundant fractions as described (28). Abundant cDNA_{bound} comprised 39% and less-abundant cDNA_{bound} 61% of the total. Abundant cDNA_{bound} (A) and less-abundant cDNA_{bound} (B) were hybridized to an excess of poly(A)⁺RNA_{bound} (●) or poly(A)⁺RNA_{loose} (○) and the extent of reaction determined as described (28). RNA concentrations ranged from ~4 to 400 μg/ml. Smooth curves were drawn without the aid of a computer.

with the respective homologous hybridizations involving poly(A)⁺RNA_{bound}.¹ This figure indicates that poly(A)⁺RNA_{loose} is relatively enriched for sequences that are rare in poly(A)⁺RNA_{bound} and is consistent with the finding from the homologous hybridization data that a large mass-fraction of poly(A)⁺RNA_{loose} is comprised of rare RNA species.

The hybridization curves in Fig. 1b indicate that poly(A)⁺RNA_{loose} may contain some sequences that are either lacking or present at very low levels in poly(A)⁺RNA_{tight}. The heterologous reaction plateaus at ~8% below the homologous reaction so that 8/0.88 or 9% by mass of poly(A)⁺RNA_{loose} sequences were not detected in poly(A)⁺RNA_{tight}. However, it should be realized that the difference of 9% observed here may not be significant, and is not strong evidence that the two populations are qualitatively unique. The kinetics of the reac-

tions indicate that on the average poly(A)⁺RNA_{loose} sequences are present in poly(A)⁺RNA_{tight} at only slightly reduced concentrations.

Since only sequences that are abundant in both populations will react at low rot values in the heterologous hybridizations, the first components of these hybridizations can be analyzed to reveal the extent to which abundant sequences are shared (13). Computer-best fits (28) revealed that the first component of the heterologous reaction involving cDNA_{tight} exhibits a rate constant (K_{tight})¹ of 36.4 liter/mole-s and contains 27.6% of the reacting cDNA_{tight} (P_{tight})¹. The values for the reciprocal heterologous reaction involving cDNA_{loose} were: $K_{\text{loose}} = 77.5$ and $P_{\text{loose}} = 15.7$.¹ If these values represent only abundant shared sequences, then $K_{\text{loose}}/P_{\text{tight}}$ should be equal to $K_{\text{tight}}/P_{\text{loose}}$ since the complexity of the reacting component is the same regardless of which population it is a part. These values are 281 and 232, respectively, which are reasonably close. Using an intermediate value of 256 l/mol-s, approximately four abundant species are shared. It can be estimated that these abundant shared sequences are present at ~2,000 copies/cell in poly(A)⁺RNA_{loose} and 9,000 copies/cell in poly(A)⁺RNA_{tight}. Thus, these sequences are represented in kinetic component II of poly(A)⁺RNA_{loose} and poly(A)⁺RNA_{tight}. It should be noted that, since there is about four times more poly(A)⁺RNA_{tight} than poly(A)⁺RNA_{loose} in the cell, these abundant species are present at about an equal relative concentration in the two RNA populations.

Are Loosely Membrane-bound Polysomes Artificially Adsorbed Free Polysomes?

Nonspecific adsorption of free polysomes to rough microsomes or RER, by means of the large ribosomal subunits as a result of cell disruption in a low-to-medium monovalent cation concentration buffer, would be expected to be a random event with respect to mRNA species. Therefore, if this accounts for the observation of loosely-membrane bound polysomes, poly(A)⁺RNA_{loose} should have characteristics identical or very similar to poly(A)⁺RNA_{free}.¹ A comparison of the homologous hybridization data of poly(A)⁺RNA_{free} presented earlier (28) with that of poly(A)⁺RNA_{loose} reveals a major difference. About 31% of poly(A)⁺RNA_{loose} is comprised of about nine abundant mRNA species present at several thousand copies/cell. Sequences of comparable abundance are completely absent from poly(A)⁺RNA_{free}.

To elucidate further distinctions between the two RNA populations, heterologous reactions were conducted. Hybridizations of poly(A)⁺RNA_{loose} to kinetically fractionated abundant and less abundant cDNA_{free} (28) are shown in Fig. 3 A and B. Fig. 3 A indicates that abundant poly(A)⁺RNA_{free} sequences are present on the average at about a 10-fold reduced concentration in poly(A)⁺RNA_{loose}. However, the most abundant species appear to be present at roughly equal frequency in the two populations. Fig 3 B indicates that the less abundant poly(A)⁺RNA_{free} sequences are present on the average at about a two fold reduced concentration in poly(A)⁺RNA_{loose}. Although clear plateaus were not achieved, the behavior of the curves at high rot values suggests that some sequences in poly(A)⁺RNA_{free} are either absent or present at greatly reduced concentration in poly(A)⁺RNA_{loose}. The heterologous reaction of cDNA_{loose} to poly(A)⁺RNA_{free} shown in Fig. 4 indicates that poly(A)⁺RNA_{loose} sequences are present at an overall reduced concentration in poly(A)⁺RNA_{free}. The heterologous curve pla-

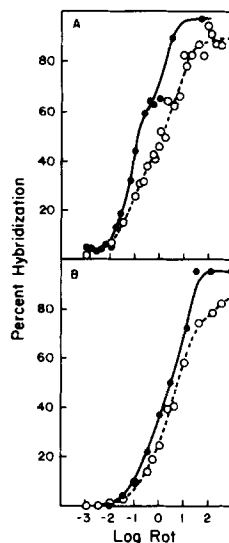


FIGURE 3 Homologous and heterologous hybridization reactions with kinetically-fractionated cDNA_{free}. cDNA_{free} was kinetically-fractionated into abundant and less-abundant fractions as described (28). Abundant cDNA_{free} and less-abundant cDNA_{free} comprised 32% and 68% of the total. Abundant cDNA_{free} (A) and less-abundant cDNA_{free} (B) were hybridized to an excess of poly(A)⁺RNA_{free} (●) or poly(A)⁺RNA_{loose} (○) and the extent of reaction assayed as described (28). RNA concentrations ranged from ~4 to 400 μg/ml. Smooth curves were drawn without the aid of a computer.

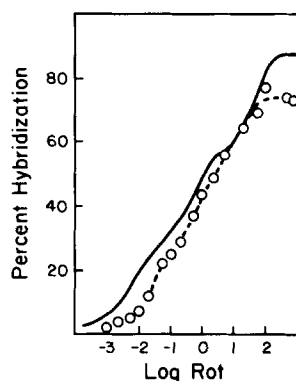


FIGURE 4 Heterologous hybridization reaction of cDNA_{loose} to poly(A)⁺RNA_{free}. cDNA_{loose} was hybridized to an excess of poly(A)⁺RNA_{free} and the extent of reaction assayed as described (28). The solid curve is the corresponding homologous reaction shown in Fig. 1 B. The broken curve representing the heterologous reaction was drawn with the aid of a computer (28). RNA concentrations ranged from 8 to 1,700 μg/ml.

teaus at ~13% lower than the homologous curve which indicates that 13/87 or 15% of poly(A)⁺RNA_{loose} sequences by mass may be either absent or present at greatly reduced concentrations in poly(A)⁺RNA_{free}.

In an attempt to determine whether there exist poly(A)⁺RNA_{loose} sequences that are completely absent from poly(A)⁺RNA_{free}, we performed the following experiment. A 10³ mass-excess of poly(A)⁺RNA_{free} was hybridized to cDNA_{loose} to a rot value of 320 mol-s/l at which the heterologous cDNA_{loose}-poly(A)⁺RNA_{free} hybridization had reached saturation. The hybridized and unhybridized molecules were separated by hydroxyapatite chromatography and the two cDNA fractions isolated as described previously (28). The cDNA that did not hybridize should be enriched in those sequences that are absent or greatly reduced in poly(A)⁺RNA_{free}. Hybridizations of poly(A)⁺RNA_{free} and poly(A)⁺RNA_{loose} to this cDNA_{loose} fraction and to the hybridized cDNA_{loose} fraction are shown in Fig. 5 B and A, respectively. As expected, both poly(A)⁺RNA populations reacted with the hybridized cDNA_{loose} fraction to about the same extent (Fig. 5 A). Clear plateaus could not be achieved at practical rot values when the unhybridized cDNA_{loose} fraction was reacted with either poly(A)⁺RNA population (Fig. 5 B). However, the difference in the apparent saturation levels was only ~10%. Since the unhybridized cDNA_{loose} fraction represented 20% of the total cDNA_{loose}, this indicates that only 0.2 × 0.2 or 4% of the total poly(A)⁺RNA_{loose} sequences by mass could not be detected in poly(A)⁺RNA_{free}. Thus, the apparent difference in plateau levels observed in Fig. 4 is not accurate, and at most only a very small fraction by mass of the two RNA populations

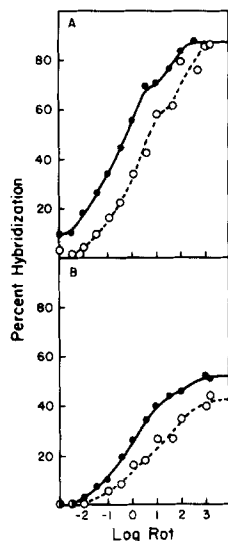


FIGURE 5 Homologous and heterologous hybridizations with $cDNA_{100se}$ enriched or deleted for poly(A)⁺RNA_{free} sequences. $cDNA_{100se}$ was hybridized with poly(A)⁺RNA_{free} to a rot value of 320 mol-s/liter. The hybridized and unhybridized molecules were fractionated on a hydroxyapatite column and the poly(A)⁺RNA_{free}-enriched and deleted $cDNA_{100se}$ fractions isolated as described (28). The hybridized cDNA represented 80% and the unhybridized cDNA 20% of the total. Poly(A)⁺RNA-enriched $cDNA_{100se}$ (A) and poly(A)⁺RNA-deleted $cDNA_{100se}$ (B) were hybridized to excess poly(A)⁺RNA_{100se} (●) or poly(A)⁺RNA_{free} (○) and the extent of reaction assayed as described (28). RNA concentrations ranged from 9 to 1,800 μg/ml. Curves were drawn with the aid of a computer (28).

do not overlap. The kinetics of the hybridization curves in Fig. 5B indicate that some of the sequences contained in this 20% that are present in poly(A)⁺RNA_{free} are reduced in relative concentration by about 100-fold.

Isolation and Characterization of High Salt, Puromycin-mediated Membrane-released and Membrane-associated Tightly-bound Poly(A)⁺RNA Fractions

Previous experiments in our laboratory (10) and others (14, 18) have demonstrated that a fraction of liver poly(A)⁺RNA remains associated with rough microsomes when various treatments are applied *in vitro* or *in vivo* to cause breakdown of polysome structures and release of most of the ribosomes from the surface of the membrane. These experiments have been interpreted by some (10, 14) but not others (18) to indicate the existence of a direct interaction between mRNP and the membrane. If such an interaction exists for some membrane-bound mRNA molecules but not others, a functional distinction might exist between the two populations that is reflected in their complexity and frequency distribution, as well as in their degree of uniqueness with respect to each other. The finding that the two populations are distinct in some manner would eliminate the possibility that the two fractions are observed as the result of a random incomplete release of ribosomes and mRNP from the membrane.

Earlier experiments demonstrated that after removal of >90% of the ribosomes from rough microsomes by treatment with 0.5 M KCl in the presence of 1 mM puromycin, ~40% of 3-h pulse-labeled mRNA and 40% of steady state poly(A)⁺RNA remained associated with microsomes. However, under the conditions used mRNA was degraded. Since we wished to characterize the translational activity of the membrane-associated RNA (poly(A)⁺RNA^{assoc})¹ and the released RNA (poly(A)⁺RNA^{rel})¹, we developed the fractionation scheme referred to as method B in Materials and Methods. Poly(A)⁺RNA^{assoc} and poly(A)⁺RNA^{rel} isolated using this method exhibited number-average lengths of ~1,460 and 1,500 nucleotides, respectively, on formamide-sucrose gradients (Fig. 6) and are translationally active in the rabbit reticulocyte lysate system (see below). Nine independent analytical experiments were conducted to determine the distribution of mRNA be-

tween the membrane-associated and membrane-released RNP fractions using this fractionation procedure (Fig. 7). 52.3 ± 17.3% of 3-h pulse-labeled mRNA and 62.1 ± 4.0% of steady state poly(A)⁺ mRNA remained associated with membranes after removal of 85.4 ± 2.5% of large ribosomal subunits.

To obtain poly(A)⁺RNA^{rel} and poly(A)⁺RNA^{assoc} that are representative of the total poly(A)⁺RNA_{tight} population for use in hybridization experiments, we used fractionation method A described in Materials and Methods. This was desirable since recent evidence suggests that postmitochondrial membrane-bound polysomes may be functionally distinct from rapidly sedimenting RER bound polysomes (16, 35, 43). Using method A, a lower percentage of mRNA was recovered in the membrane-associated fraction. This is probably the result of partial degradation of mRNA which occurred using this procedure.

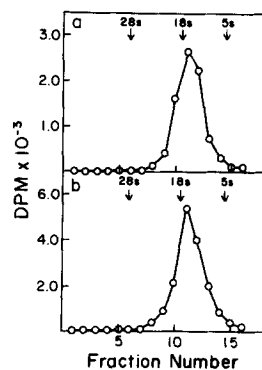


FIGURE 6 Sizing of poly(A)⁺RNA^{rel} and poly(A)⁺RNA^{assoc}. Poly(A)⁺RNA^{rel} (a) and poly(A)⁺RNA^{assoc} (b) were isolated using method B as described in Materials and Methods. Small aliquots were dissolved in 75% formamide, 10 mM HEPES, pH 7.6, 3 mM EDTA and layered on 75% formamide, 5–15% sucrose gradients. Gradients were centrifuged in a Beckman SW41 rotor at 38,000 rpm, 22°C, for 28 h. 100-μl aliquots of gradient fractions were added to 400 μl of 2.5 × SSC buffer and hybridized to an excess of [³H]poly(U) as described (28). The RNase A-digested hybrid mixtures were TCA precipitated onto glass fiber filters, washed with 5% TCA and 95% ethanol, dried, and radioactivity determined in OSC scintillator cocktail. 28S, 18S, and 5S rRNAs were run in a parallel gradient as size markers.

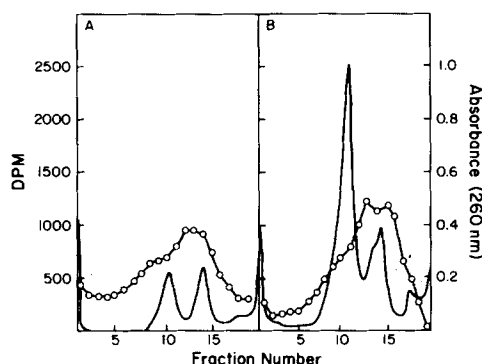


FIGURE 7 KCl-Puromycin stripping of rough microsomal membranes. A high-salt washed rough microsomal membrane fraction was prepared from the liver of a rat labeled for 3 h with 300 μCi [³H]-orotic acid + 1 mg FOA. Membrane-released and membrane-associated RNP fractions were isolated after treatment with 0.66 M KCl-2 mM puromycin by method B. RNA was obtained by phenol-chloroform extraction and ethanol precipitation, dissolved in 100 μl 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 100 mM NaCl buffer, and layered onto 15–30% aqueous sucrose gradients prepared in the same buffer. Gradients were centrifuged in a Beckman SW41 rotor, at 38,000 rpm at 4°C for 8 h. Fractions were collected by pumping gradient contents through a flow cell in a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) using a peristaltic pump and Beckman fraction recovery system. Radioactivity in gradient fractions was determined by counting in 10 ml Aquassure scintillator cocktail. (A) membrane-associated RNA (B) membrane-released RNA. (—), Absorbance (260 nm); (○) ³H-DPM.

Since mRNP that is released from the membrane due to nucleolytic degradation will contaminate the released fraction, any differences observed between the two RNA fractions must be considered minimal. Using method A, ~31% of 3-h pulse-labeled mRNA and 23% of poly(A)⁺RNA were recovered in the membrane-associated fraction when 85% of total ribosomes were removed.

Poly(A)⁺RNA^{rel} and poly(A)⁺RNA^{assoc} were isolated using method A and cDNA prepared as described in Materials and Methods. The homologous hybridization curves are presented in Fig. 8A. Note that the slight differences in the sizes of the driver and tracer populations for the two reactions can only account for a 10% difference in hybridization kinetics, which would not be discernible and would be in the opposite direction from the difference in kinetics that is apparent. The bulk of poly(A)⁺RNA^{assoc} hybridizes considerably faster than does poly(A)⁺RNA^{rel}, although a highly complex component is discernible in poly(A)⁺RNA^{assoc} that is absent from poly(A)⁺RNA^{rel}. An analysis of the homologous hybridizations is given in Table II. The best fits to the data were obtained by assuming four discrete components for poly(A)⁺RNA^{assoc} and three discrete components for poly(A)⁺RNA^{rel}. 54% of poly(A)⁺RNA^{rel} is comprised of 42 abundant RNA species

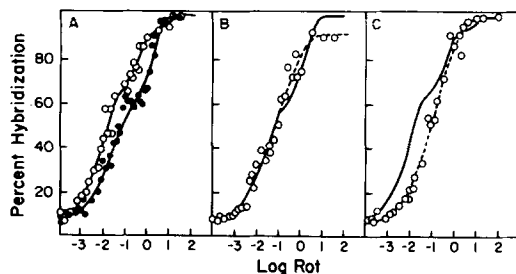


FIGURE 8 Homologous and heterologous hybridizations with cDNA^{rel} and cDNA^{assoc}. cDNA was prepared to poly(A)⁺RNA^{rel} and poly(A)⁺RNA^{assoc} isolated by method B as described in Materials and Methods. Homologous and heterologous reactions were conducted and assayed as described previously (28). RNA concentrations ranged from 0.5 to 500 µg/ml. Most data points represent averages of duplicates or triplicates. Curves were drawn with the aid of a computer (28). (A) cDNA^{assoc} (○) and cDNA^{rel} (●) hybridized to their homologous poly(A)⁺RNA populations. (B) Heterologous cDNA^{rel}-poly(A)⁺RNA^{assoc} hybridization. The solid curve is the homologous cDNA^{rel} reaction shown in (A). (C) Heterologous cDNA^{assoc}-poly(A)⁺RNA^{rel} hybridization. The solid curve is the homologous cDNA^{assoc} reaction shown in (A).

including one highly abundant species. 11% of the mass of poly(A)⁺RNA^{assoc} consists of a component containing ~19,000 RNA species. In contrast, poly(A)⁺RNA^{rel} has components which are neither as abundant nor as rare as those in poly(A)⁺RNA^{assoc}. The combined complexity of the two populations of 29,500 RNA species is very close to that of poly(A)⁺RNA_{tight} of 28,000 species. Although this suggests that they may be nonoverlapping populations, the heterologous curves in Fig. 8B and C indicate that this is not so. The heterologous cDNA^{assoc}-poly(A)⁺RNA^{rel} curve (Fig. 8C) plateaus at the same level as the corresponding homologous curve, which indicates that poly(A)⁺RNA^{rel} contains all of the sequences of poly(A)⁺RNA^{assoc}. The heterologous cDNA^{rel}-poly(A)⁺RNA^{assoc} curve (Fig. 8B) plateaus at ~10% below the level of the homologous curve, which indicates that 10% by mass of poly(A)⁺RNA^{rel} sequences may be either absent or present at greatly reduced concentrations in poly(A)⁺RNA^{assoc}. If all of these sequences were derived from the rare abundance class of poly(A)⁺RNA^{rel}, they would constitute ~2,000 RNA species. Again, however, differences in plateau levels of this magnitude may not be significant. Therefore, we cannot conclude that the populations are qualitatively unique.

Comparison of In Vitro Translation Products

To obtain further evidence that distinct poly(A)⁺ mRNA populations are associated with the various subpopulations of membrane-bound polysomes, we analyzed their in vitro translation characteristics in a rabbit reticulocyte lysate system. The translational efficiencies of poly(A)⁺RNA_{free}, poly(A)⁺RNA_{loose}, poly(A)⁺RNA_{tight}, poly(A)⁺RNA^{rel}, and poly(A)⁺RNA^{assoc} are presented in Fig. 9. The translational efficiencies of poly(A)⁺RNA_{free} and poly(A)⁺RNA_{loose} are comparable and are about threefold greater than that of poly(A)⁺RNA_{tight}. As would be expected, the translational efficiency of poly(A)⁺RNA_{tight} is intermediate between that of poly(A)⁺RNA^{rel} and poly(A)⁺RNA^{assoc}. The latter fraction exhibits the lowest efficiency, about one-half that of poly(A)⁺RNA_{tight} and one-third that of poly(A)⁺RNA^{rel}.

Translation products were analyzed by one-dimensional gel electrophoresis. A fluorograph of a gel containing the [³⁵S]-methionine-labeled polypeptides produced by all five poly(A)⁺RNA populations is shown in Fig. 10. The gel patterns can be divided into two groups on the basis of qualitative differences. poly(A)⁺RNA_{free} (Fig. 10, lane c) and poly(A)⁺RNA_{loose} (Fig. 10, lane b) produced a qualitatively

TABLE II

Complexity and Frequency Distribution of High-salt, Puromycin-mediated Membrane-associated and Membrane-released Tightly Bound Polysomal Poly(A)⁺RNA Populations

Polysome class	Abundance class	Percent cDNA hybridized*	Rate Constant		Nucleotide complexity	No. of mRNA species†	Copies/cell§§
			Observed‡	Corrected§			
Membrane-associated	I	8.80	1,220	13,900	3.74 × 10 ²	1	600,000
	II	46.4	98.3	212	2.45 × 10 ⁴	41	9,200
	III	33.4	2.59	7.75	6.70 × 10 ⁵	1,130	336
	IV	11.4	0.053	0.467	1.11 × 10 ⁷	18,800	20
Membrane-released	I	10.2	307	3,010	1.55 × 10 ³	3	89,100
	II	41.1	30.5	74.2	6.30 × 10 ⁴	124	2,190
	III	47.8	0.478	0.981	4.77 × 10 ⁶	9,890	29

See Table I footnotes.

‡ Calculated as described in Table I footnote (||). The mass-average sizes of the driver and tracer populations for the membrane-associated RNA reaction were 604 and 590 nucleotides and for the released RNA fraction 562 and 513 nucleotides.

§§ Calculated as described in Table I footnote (**). The membrane-associated RNA fraction represents ~62% of the total tightly membrane-bound polysomal poly(A)⁺RNA or 0.62 × 0.156 = 0.124 pg RNA/cell. The membrane-released RNA fraction makes up the remaining 38% or 0.38 × 0.156 = 0.044 pg/cell.

similar polypeptide pattern distinct from that of the three tightly membrane-bound poly(A)⁺RNA fractions (Fig. 10, lanes *d-f*). Within the two groups there are clear quantitative differences in the abundance of specific polypeptides as well as some qualitative differences.

Densitometric tracings of lanes *b* and *c* (Fig. 10) representing the translation products of poly(A)⁺RNA_{free} and poly(A)⁺RNA_{loose} are compared in Fig. 11 *a*. The two low mol wt polypeptides labeled 1 and 3 in the poly(A)⁺RNA_{free} tracing have not been detected as products of poly(A)⁺RNA_{loose}. The abundant polypeptides labeled 2, 16, and 17 synthesized by poly(A)⁺RNA_{loose} are barely detectable as products of poly(A)⁺RNA_{free}. Approximately six to eight polypeptides >70,000 daltons have been detected as products of poly(A)⁺RNA_{loose} and these were distinct from the high molecular weight polypeptides synthesized by the tightly membrane-

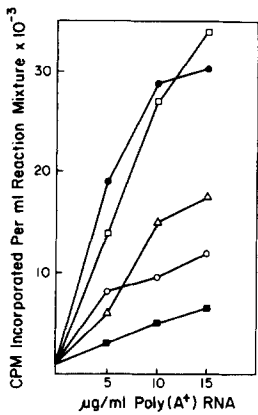


FIGURE 9 Translational efficiencies of poly(A)⁺RNA populations. Poly(A)⁺RNA_{free} (□), poly(A)⁺RNA_{loose} (●), poly(A)⁺RNA^{rel} (Δ), poly(A)⁺RNA_{light} (○), and poly(A)⁺RNA^{assoc} (■) were translated at 5–15 µg/ml final concentration in the rabbit reticulocyte lysate system as described in Materials and Methods. 2-µl aliquots of the reaction mixtures were spotted onto filter paper, boiled in 10% TCA, washed in 5% TCA, ethanol, and ether, dried, and radioactivity determined in 10 ml OCS scintillator cocktail.

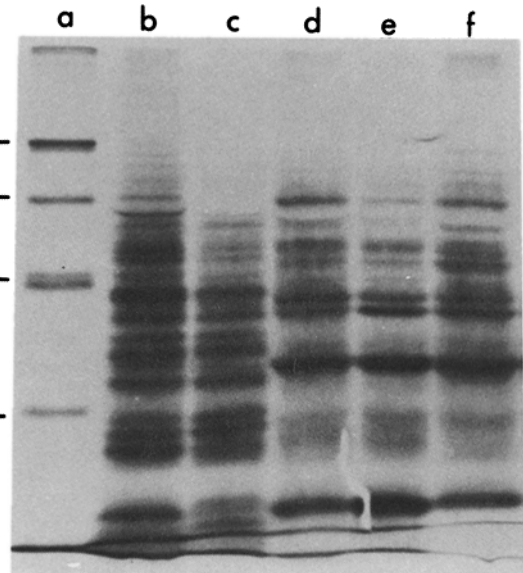


FIGURE 10 Comparison of in vitro translation products of poly(A)⁺RNA fractions. The five poly(A)⁺RNA fractions in Fig. 9 were translated in the rabbit reticulocyte lysate system as described in Materials and Methods. RNA concentrations were within the linear range for each fraction. A volume of lysate corresponding to 1.70×10^5 dpm of acid insoluble radioactivity from each RNA fraction was subjected to SDS PAGE by the method of Laemmli (19) as described in Materials and Methods. The gel was fixed, treated with Enhance, dried, and fluorographed. Kodak XAR-5 film was exposed for 20 h at -70°C . (Lane *a*) *M*, standards, phosphorylase B, 92,500; albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000. (Lane *b*) poly(A)⁺RNA_{loose} translation products; (lane *c*) poly(A)⁺RNA_{free} translation products; (lane *d*) poly(A)⁺RNA_{light} translation products; (lane *e*) poly(A)⁺RNA^{rel} translation products; (lane *f*) poly(A)⁺RNA^{assoc} translation products.

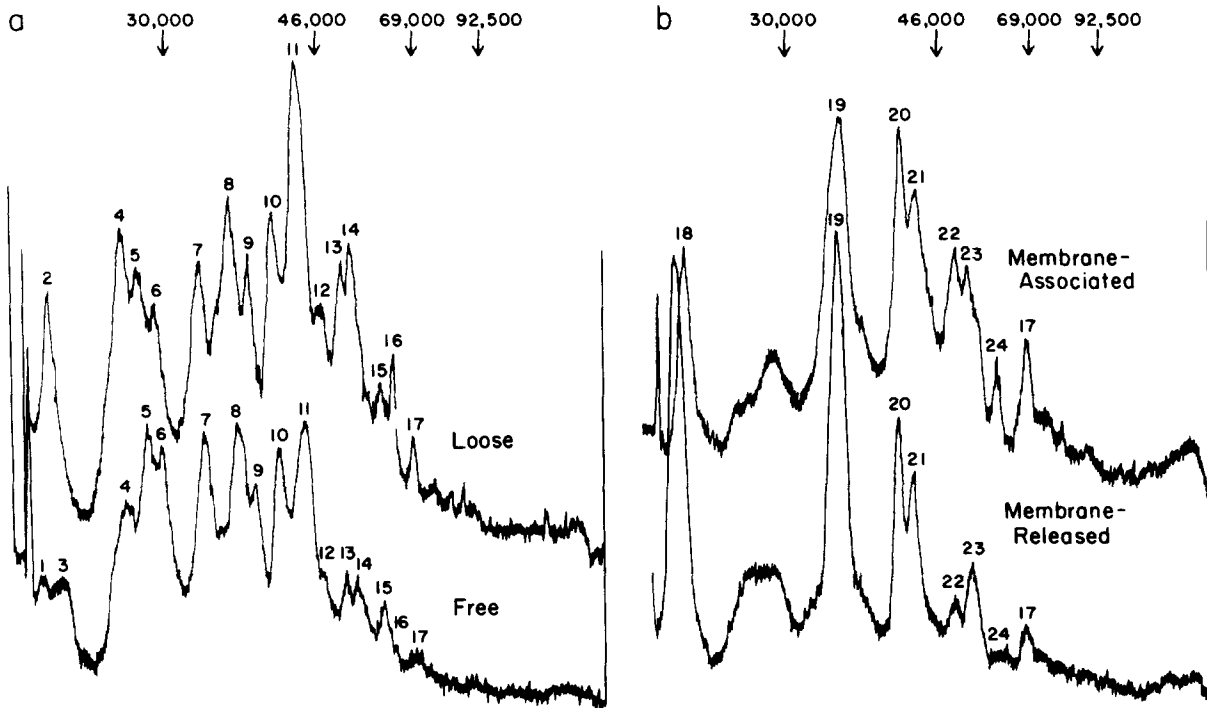


FIGURE 11 Comparison of in vitro translation products of poly(A)⁺RNA fractions. Densitometric tracings of the translation products shown in lanes *b*, *c*, *e*, and *f* of Fig. 10 were obtained using a Joyce-Loebel microdensitometer. (a) A comparison of the translation products of poly(A)⁺RNA_{loose} and poly(A)⁺RNA_{free} shown in Fig. 10, lanes *b* and *c*. (b) A comparison of the translation products of poly(A)⁺RNA^{assoc} and poly(A)⁺RNA^{rel} shown in Fig. 10, lanes *e* and *f*.

bound poly(A)⁺RNA fractions. Polypeptides 4 and 8–17 are all synthesized in greater quantities by poly(A)⁺RNA_{loose}, whereas polypeptides 5 and 6 are synthesized in greater quantities by poly(A)⁺RNA_{free}.

Densitometric tracings of the poly(A)⁺RNA^{assoc} and poly(A)⁺RNA^{rel} translation products are shown in Fig. 11 B. These were qualitatively very similar, but the same quantitative differences were consistently observed. The polypeptide-labeled 18 is 2.5-fold enriched in poly(A)⁺RNA^{rel}, polypeptides 19–21 are produced in similar quantities by the two fractions, and polypeptides 22–24 and 17 are enriched in the products of poly(A)⁺RNA^{assoc}. Polypeptide 17 has been identified as prealbumin by immunoprecipitation.

DISCUSSION

Our results demonstrate that poly(A)⁺RNA fractions derived from free polysomes, loosely and tightly membrane-bound polysomes, and membrane-released and membrane-associated RNP have unique characteristics. From this we can eliminate the possibilities that (a) loosely membrane-bound polysomes are the result of random artifactual adsorption of free polysomes to membrane fractions and (b) membrane-associated mRNP is the result of random entrapment of released mRNP or random incomplete release from membranes. Our results do not eliminate artifactual origins that are nonrandom in nature. It is logical to assume, however, that any nonrandom processes that might occur during cell fractionation would themselves be indicative of structural differences among RNP or polysomes.

Recently, Adesnik and Maschio (3) characterized poly(A)⁺RNA fractions derived from rat liver rough microsomal loosely and tightly bound polysome populations by analysis of their hybridization characteristics. From their data they concluded that loosely bound polysomes are artifactually adsorbed free polysomes. Any or all of the following may account for the different results obtained by these investigators and ourselves: (a) Adesnik and Maschio (3) isolated polysomes from a rough microsomal fraction representing only a small (7) and perhaps functionally distinct (16, 35) portion of the total RER. In this study we isolated polysomes from a crude membrane fraction containing at least 95% of the total RER. (b) Adesnik and Maschio (3) isolated poly(A)⁺RNA populations that were partially degraded while our poly(A)⁺RNA populations were essentially intact. (c) In no case did Adesnik and Maschio demonstrate that their free and loosely membrane-bound polysomal poly(A)⁺RNA populations were either qualitatively or quantitatively identical, since hybridizations were only carried out to relatively low rot values and plateaus were not observed. In most of our experiments clear plateaus were observed, which allowed us to conclude that the two fractions are at least quantitatively unique. (d) We have observed that rough microsomal fractions isolated by procedures similar to those used by Adesnik and Maschio (3) may be extensively contaminated with trapped free polysomes, i.e., polysomes that can be extracted by low salt washes (Mueckler and Pitot, unpublished data, see also reference 24). Since these contaminating free polysomes were not removed from microsomal fractions before extraction of high-salt releasable polysomes, they would be included in the latter fraction. Thus it is reasonable that Adesnik and Maschio (3) observed similar hybridization characteristics between these fractions, as a significant portion of their loosely bound polysome fraction may actually have consisted of trapped free polysomes. We have determined that low-salt-extractable polysomes comprise <1%

of our loosely membrane-bound polysome fraction, which comprises 22% of total membrane-bound polysomes. Adesnik and Maschio (3) found that their loosely membrane-bound polysome fraction comprised 33% of total microsomal-bound polysomes. Thus, about one-third of their loosely membrane-bound polysomes may, in fact, have been trapped free polysomes.

Adesnik and Maschio (3) also conducted hybridization experiments with microsomal-associated and nonassociated RNA after *in vivo* disaggregation of polysome structures with ethionine. Their data suggested that this treatment resulted in a random release of mRNA from microsomal membranes. They concluded that the retention of mRNA on microsomal membranes observed by us (10) after *in vitro* treatment with 0.5 M KCl-puromycin is not functionally significant. However, it is clear that the mRNA fractions examined in each case are not equivalent. After disaggregation of polysomes *in vivo*, they did not wash microsomal membranes with high-salt buffer to fractionate mRNA into membrane-associated and membrane-released components. Since it had been reported earlier (18) that ribosomes and mRNA are not released from microsomal membranes after ethionine treatment *in vivo* unless these are washed with high-salt buffer, the significance of the RNA fractions examined by Adesnik and Maschio (3) is questionable. The release they observed may have been due to random nucleolytic degradation of mRNA *in vitro* after mRNP had been denuded of ribosomes *in vivo*.

The *in vitro* translation experiments and homologous and heterologous hybridizations involving poly(A)⁺RNA^{assoc} and poly(A)⁺RNA^{rel} indicate that (a) these two RNA fractions contain a qualitatively identical set of abundant mRNAs that are differentially distributed between them, (b) poly(A)⁺RNA^{assoc} is enriched in species that are very abundant and very rare and (c) poly(A)⁺RNA^{rel} may contain some species that are absent from poly(A)⁺RNA^{assoc}, but the former contains all of the species present in the latter. Although these results suggest the possibility of a functional distinction between these RNA fractions, they do not suggest what this distinction is. To shed light on this question we are currently conducting hybridization experiments with several specific cloned cDNA probes to determine the relative distribution of specific mRNAs between these RNA populations.

Shields (41) used *in vitro* translation to characterize membrane-associated and membrane-released mRNA populations of dog pancreas. He found a much smaller percentage of mRNA in the membrane-associated fraction (3–15%) and the *in vitro* translation products of the two fractions were quantitatively and qualitatively very similar. These data are not necessarily in conflict with the results presented here, since there is no reason to suspect that the processes examined need be identical in liver and pancreas. Pancreatic acinar cells are highly specialized for the synthesis and secretion of a relatively small number of polypeptides. Hepatocytes, however, are functionally much more diverse. Thus, it is reasonable that these two cell types might exhibit unique characteristics.

The results of polysome disaggregation experiments have usually (10, 14), but not always (18), been interpreted to indicate the existence of a direct association between mRNA or mRNP and rough microsomal membranes, but there is no direct structural evidence to support this. Therefore, we refer to these fractions as “membrane-released” and “membrane-associated” in a purely operational sense. In particular, it cannot be ruled out that the membrane-bound ribosomes that

are resistant to release by high-salt and puromycin treatment are involved in the binding of mRNP. If so, this interaction probably doesn't involve nascent chains since the two RNA fractions encode for an identical set of abundant polypeptides, and puromycin is present at a large molar excess during fractionation. The nature of membrane interaction involving the resistant ribosomes is obscure (1). In the case of ethionine disaggregation of liver polysomes *in vivo*, ribosomes are apparently not involved in the binding of mRNA to microsomal membranes (14). Under conditions where the integrity of mRNA was preserved, Endo and Natori (14) also found a much larger proportion of mRNA associated with microsomal membranes than was reported by Adesnik and Maschio (3), and mRNA coding for serum albumin was found to be preferentially retained. These results again suggest that the randomization of mRNA sequences observed by Adesnik and Maschio (3) was the result of release caused by random nucleolytic degradation. If an interaction exists between mRNP and rough membranes *in vivo* as well as *in vitro*, it is not likely to be involved in the selection of messages for translation on membrane-bound polysomes, as experiments with myeloma cells *in vivo* (25) have demonstrated that this process is dependent on translation, and probably involves nascent chains (6).

Loosely membrane-bound polysomes have been shown to be active in the synthesis of cytochrome b_5 (15), ribosomal structural proteins (29), and histones (45). All of these are also synthesized on free but not on tightly membrane-bound polysomes at significant levels. Unfortunately, cross-contamination levels were only determined in the case of the ribosomal structural proteins (29). More work is needed to determine whether the results presented here are indicative of a functionally distinct subpopulation of membrane bound polysomes or simply of a nonrandom adsorption of free polysomes to membranes during cell fractionation. The necessity of working with polysome preparations that exhibit minimal cross-contamination between low and high-salt extractable fractions cannot be over-emphasized.

The finding that classes of proteins that must transverse the nuclear envelope are synthesized on loosely bound polysomes suggests a possible function for the loose interaction. RER is known to be intimately associated with the nuclear envelope. It is possible that the loose interaction functions to establish a concentration gradient of polysomes synthesizing nuclear proteins in the vicinity of the nucleus. In this regard Lönn (23) has demonstrated that Balbiani ring 75S RNA, which is associated with RER in a high-salt, puromycin-resistant linkage (22), is concentrated in the cytoplasm proximal to the nucleus. This message apparently codes for secretory polypeptides.

Similarly, in the case of integral membrane proteins such as cytochrome b_5 , whose synthesis occurs on loosely bound polysomes and whose integration is not cotranslational, the loose interaction may function to allow the proper topographical and spacial orientation of newly completed polypeptides for post-translational integration. The loose interaction may conceivably involve any of the component structures of polyribosomes. Specificity for the interaction could reside within the mRNA molecule itself or the polypeptide chain. In analogy with the signal hypothesis (6), the loose interaction could be initiated by transient ionic interactions between the growing nascent chain and specific membrane proteins exposed on the cytoplasmic face of the membrane. This would allow the large ribosomal subunits to bind to the receptor sites on the mem-

brane surface (1), establishing the high-salt sensitive binding that is observed *in vitro*. This, in turn, would allow disposal of newly completed polypeptides at the surface of the membrane, facilitating subsequent integration.

It should be mentioned that, while the free monovalent cation concentration of our initial homogenization buffer was close to physiological, nonphysiological concentrations are required to release loosely membrane-bound polysomes. This is consistent with the idea that this interaction exists *in vivo* as well as *in vitro*.

In conclusion, we have provided considerable evidence that the poly(A)⁺RNA fractions associated with free and loosely membrane bound polysomes, and membrane-associated and membrane released mRNP, are discrete in the sense that they are not identical. However, the data indicates that there are substantial qualitative similarities between these populations, much of which cannot be attributed to cross-contamination. Further experiments are required to determine whether the differences observed are of functional significance.

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