Structure and Function of Rat Liver Polysome Populations

I. Complexity, Frequency Distribution, and Degree of Uniqueness of Free and Membrane-bound Polysomal Polyadenylate-containing RNA Populations

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ABSTRACT

Free and membrane-bound polysomes were isolated from rat liver in high yields with minimal degradation, cross-contamination, or contamination by nuclear or nonpolysomal cytoplasmic ribonucleoprotein. Poly(A)⁺ RNA fractions isolated from free and bound polysomal RNA (poly(A)⁺ RNAfree and poly(A)⁺ RNAbound) by oligo(dT) cellulose chromatography exhibited number-average lengths of 1,600 and 1,200 nucleotides, respectively, on formamide sucrose gradients. Poly(A)⁺ RNAfree and poly(A)⁺ RNAbound contain 9.1 ± 0.55 and 10.7 ± 0.50% poly(A) as measured by hybridization to [³H]poly(U) and comprise 2.37 and 1.22% of their respective polysomal RNA populations.

Homologous poly(A)⁺ RNA-cDNA hybridizations revealed that greater than 95% of the mass of poly(A)⁺ RNAfree and poly(A)⁺ RNAbound contain nucleotide complexities of about 3.4 × 10⁷ and 6.0 × 10⁶, respectively. This represents about 20,000 and 5,000 poly(A)⁺ RNA species of average sizes. Heterologous hybridizations suggested that considerable overlap exists between poly(A)⁺ RNAfree and poly(A)⁺ RNAbound sequences that cannot be attributed to cross-contamination. This was confirmed by conducting heterologous reactions using kinetically enriched cDNA populations. Heterologous hybridizations involving poly(A)⁺ RNA derived from tightly bound polysomes and cDNAfree indicated that most of the overlapping sequences are not contributed by loosely bound (high-salt releasable) polysomes. The ramifications of these findings are discussed.

Early ultrastructural studies (39) and cell fractionation procedures (for review, see reference 31) revealed the existence of two morphologically distinct types of polyribosome structures in eucaryotic cells, those existing free in the cytoplasm and those bound to intracellular membranes. Subsequent work has been directed toward elucidating functional distinctions between free and membrane-bound polysome populations (for review, see reference 48). Most of this work has involved the use of immunochemical methods to identify the site of synthesis of specific proteins or types of proteins, and can be summarized as follows: (a) secretory proteins are synthesized preferentially on membrane-bound polysomes. (b) most proteins of the extravesicular cytoplasmic compartment are synthesized preferentially on free polysomes. (c) membrane proteins and proteins residing within membranous organelles may be synthesized preferentially on either polysome class.

These generalizations refer to quantitative rather than qualitative differences in the site of synthesis of a particular protein, and there are several examples of anomalous findings that cannot be readily explained by current theories concerning the genesis of polysome classes (e.g., see reference 6). For example, serine dehydratase (32) and globin (35), proteins of the extravesicular soluble cytoplasm of rat liver and mouse reticulocytes, respectively, are synthesized in significant quantities on membrane-bound polysomes. Neither is secreted or undergoes the types of processing or modification known to occur within
RER. While a primary function of membrane-polysome interaction is to allow transfer of polypeptides into or across membranes for subsequent processing, intracellular transport, or secretion (38), these examples, among others, raise the possibility of additional functions (for review, see reference 51).

Is the synthesis of most proteins confined to a single polysome class or does it occur on both classes? The answer to this question is crucial to a full understanding of the function(s) of polysome-membrane interaction. To further our knowledge in this area we have begun a detailed characterization of rat liver polysomal mRNA populations. Here we report our findings on the complexity, frequency distribution, and degree of uniqueness of free and membrane-bound polysomal poly(A) mRNA populations of rat liver.

MATERIALS AND METHODS

Tissue Fractionation and Isolation of Free and Membrane-bound Polysomes

Free and membrane-bound polysomes were prepared by a modification of the procedures of Ramsey and Steele (46). The modifications were used to preserve integrity of mRNA and increase recovery of polysomes from the livers of fed rats. Adult male rats of the Holtzman strain were given chow and water ad lib. 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, 0.5 mg/ml sodium heparin. The homogenate was centrifuged in a Beckman SW27 rotor (Beckman Instruments, Fullerton, Calif.) for 2 min at 74,000 g, and 12 min at 131,000 g. The supernatant fluid was decanted and adjusted to 10 mM MgCl2, 1–2 mg/ml sodium heparin. The pellet was homogenized in rat liver high-speed supernatant fluid (46) containing 1% Triton X-100, 20 μM MgCl2, 250 mM KCl, 3 mM GSH, and centrifuged in a Sorvall SS34 rotor (DuPont Instruments, Sorvall, DuPont Co., Newtown, Conn.) for 5 min at 1,470 g. The supernate was adjusted to 50 mM MgCl2, 2 mg/ml sodium heparin, 1.3% sodium deoxycholate and centrifuged at 15,000 g for 5 min in a Sorvall SS34 rotor (Sorvall, DuPont Co.) to pellet insoluble material. The final supernate or "microsomal fraction" and the initial 131,000 g supernatant or "free fraction" were layered over 2 M sucrose cushions prepared as described (46) with the addition of 1–2 mg/ml sodium heparin. Polysomes were pelletted at 303,000 g for 18–20 h in a Beckman 60Ti rotor (Beckman Instruments, Inc.).

Isolation of Poly(A) + RNA Fractions

Polysome pellets were extracted by the SDS-phenol-chloroform procedure of Perry et al. (44) or Palmiter (40) with the addition of lmg/ml bentonite in the extraction buffer. Ethanol-precipitated polysomal RNA was washed two to three times with 3 M NaOAc (pH 5.0) to remove glycogen, DNA, and low molecular weight RNA. In some instances polysomal RNA preparations were routinely sized by sucrose gradient centrifugation under partially denaturing conditions. A small aliquot of poly(A) + RNA was dissolved in 75% deionized formamide, incubated at 65°C for 3 min, and layered onto a 9–ml 5–15% linear sucrose gradient containing 75% formamide, 10 mM HEPS, pH 7.6, 3 mM EDTA underlayered with a 2.2–ml cushion of 25% sucrose in the same buffer. Gradients were centrifuged in a Beckman SW41 rotor at 22°C for 24 h at 38,000 rev/min. Fractions were collected using a Beckman fraction recovery system (Beckman Instrument, Inc.) and peristaltic pump. 0.1 ml fraction aliquots were added to 0.4 ml of 2.5 x SSC, pH 7.4 (SSC is 0.15 M NaCl in 0.015 M sodium citrate) and their poly(A) content determined by hybridization to [3H]poly(U) (46). Assuming that RNA molecules of different sizes have, on the average, phosphate tails of the same length (49), the number-average size of a heterogeneous population is given by (Cpm)1/(Ccpm) where Cpm is proportional to the number of RNA molecules of length 1. This same formula will yield a mass-average size if RNA molecules are labeled uniformly throughout their length. Mass-average sizes of free and bound poly(A) + RNA populations were determined after selective labeling of mRNA with [3H]-orotic acid in the presence of 5-fluoroorotic acid (56). In this case gradients fractions were collected directly into scintillation vials, 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and 0.5 ml water added, and the samples counted in an Isocap/300 liquid scintillation counter (Searle Radiographics, Inc., Des Plaines, Ill.) interfaced with a pc/3 computer.

Synthesis of cDNA

DNA complementary to poly(A) + RNA populations was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase. Reaction conditions were optimized to produce long cDNA transcripts. The reaction mixture included 100 μg/ml template poly(A) + RNA, 75 μg/ml oligo(dT)12–18, 1.4 μM unlabeled deoxynucleoside triphosphate (dNTPs), 0.3 mM [3H]deoxycytidine triphosphate (dCTP), 300–400 μM AMV reverse transcriptase, 2.5% glycerol, 2 mM sodium pyrophosphate, 8 μM MgCl2, 0.375 mg/ml dithiothreitol, 50 μg/ml actinomycin D, 50 mM Tris-HCl (pH 8.0). No additional monovalent cation was included. The reaction mixture was incubated at 45°C for 20–30 min and the reaction terminated by the addition of EDTA and SDS to concentrations of 5 mM and 0.5%, respectively. RNA was hydrolyzed by adjusting the reaction mixture to 0.25 N NaOH and incubating overnight at room temperature. The mixture was neutralized by addition of HCl and HEPES to 0.25 N and 0.1 M, respectively. 100 μg yeast transfer RNA (tRNA) was added as carrier and the cDNA chromatographed on Sephadex G-50 (fine) overlaying a small pad of Celite-100 (Bio-Rad Laboratories, Richmond, Calif.). Void fractions containing cDNA were combined, ethanol precipitated, and resuspended in an appropriate volume of sterile, double-distilled H2O. The specific activities of cDNA preparations used in these experiments ranged between 4 and 5 x 10^6 cpm/μg assuming a 25% dCTP residue content. Yields ranged from 20 to 30% by mass of input RNA.

Kinetic Fractionation of cDNA

cDNAfree and cDNAbound were hybridized to an excess of the homologous poly(A) + RNA to rot values of 0.15 and 0.015, respectively, at which all of the abundant class cDNA should be a hybrid form according to computer fits of the homologous hybridization data (see Results). Double- and single-stranded nucleic acids were separated on 0.2 g hydroxyapatite (Bio-Gel HTP, DNA grade, Bio-Rad Laboratories, Richmond, Calif.) columns at 60°C (10). Samples were applied and single-stranded nucleic acids eluted in 0.12 M sodium phosphate buffer (pH 6.8). Hybrids were eluted in 0.5 M sodium phosphate buffer. Gentile positive air pressure was used to increase flow rate. Columns were preloaded with 500 μg yeast RNA and 0.4% SDS to reduce nonspecific and irreversable binding. The kinetically enriched cDNA populations were isolated as described above.

Hybridization Reactions

Hybridization reactions were carried out in torch-sealed, silanized microtubes at 68°C. The hybridization buffer contained 50 mM HEPES (pH 7.4) at 68°C, 0.5 M NaCl, 0.1% SDS, 1 mM EDTA. Rot values were obtained by varying the concentration of RNA and the length of incubation. The RNA/cDNA mass ratio ranged from about 10 to 105.5 x 10^6 dpm of cDNA was used per rot point. An SI nucleic assay was used to determine the percentage of cDNA remaining single-stranded at each rot point. Reactions were terminated by plunging the microtubes into a solid CO2-ethanol bath. The reaction mixtures were expelled into 2.2 ml of digestion buffer (0.3 M NaCl, 30 mM NaOAc (pH 4.5), 3 mM ZnSO4, 10 μg/ml denatured calf-thymus DNA). 1 μl aliquots were immediately adjusted to 10% trichloroacetic acid (TCA) and 100 μg/ml yeast DNA.
rRNA, and the precipitates collected onto glass-fiber filters. Filters were washed with 10 ml 5% TCA and 10 ml 100% EtOH, dried, and counted in 10 ml of OCS scintillator cocktail (Amersham Corp., Arlington Heights, Ill.). 1 ml aliquots were treated identically after digestion with S1 nuclease (54) for 1 h at 37°C. The amount of S1 nuclease used was sufficient to digest to acid-solubility greater than 95% of single-stranded DNA and less than 5% of double-stranded DNA under the conditions used in these experiments. Background values were determined on reaction mixtures lacking RNA and were subtracted from the datapoints. These generally ranged from 3 to 5%. We consistently observed terminal hybridization values of >90% (after background subtraction) with several different free and membrane-bound RNA and cDNA preparations.

**Data Analysis**

Hybridization data were analyzed by a nonlinear least squares method employing a computer program designed by Pearson et al. (41) and modified by Dr. W. W. Wai-nam Mak in our laboratory. The program analyzes the complex hybridization curves as the sum of two or more pseudo-first order reaction components according to the equation: 

$$C/Co = F + \sum_{i} f_i \exp(-K_i t)$$

where $C/Co$ is the fraction of cDNA remaining single stranded, $F$ is the fraction remaining unreacted at the termination of the reaction, $f_i$ is the fraction of cDNA in an individual component having a rate constant $K_i$, and $r$ is the number of components. Initial guesses are provided for the rate constants and the proportion of cDNA comprising each component, and the program is allowed to converge to a "best fit" by minimizing the sums of squares of deviations. This analysis is used as a means of describing, quantitating, and allowing statistical evaluation of hybridization data. The components or "abundance classes" defined by the analysis do not necessarily correspond to the actual frequency composition of the RNA populations.

**Polycrylamide Gel Electrophoresis in 98% Formamide**

Estimation of tRNA contamination of poly(A)+ RNA preparations and sizing of cDNA were performed by electrophoresis in 3.5% polycrylamide tube gels in the presence of 98% formamide as described by Duesberg and Vogt (17). Gels were cast in 0.6 × 15-cm quartz tubes and electrophoresed at 2 mA/gel for 2 h. Samples in 75% formamide were electrophoresed for 5 min at 2 mA/gel and 13 h at 1 mA/gel. Gels containing [3H]tDNA were fractionated by an automated gel crusher-fractionator (Gibson Medical Electronics, Inc., Middleton, Wis.) into 2 mm portions and collected directly into scintillation vials. 2.5 ml of Nuclear-Chicago Solubilizer (NCS) tissue solubilizer (Amersham Corp.) were added and 1 h later the samples were counted in 10 ml of toluene-polyphenoloxidase. For estimation of tRNA contamination of poly(A)+ RNA preparations gels were scanned at 260 nm in a Gilford 2400 spectrophotometer using a linear transport device (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

**Density Gradient Analyses**

The buoyant density of polysome fractions labeled under various conditions (see Results) was determined on preformed, linear, 4.4 ml 35 to 55% wt/wt CsCl gradients. Samples dissolved in 10 mM triethanolamine (pH 7.6), 100 mM NaCl, 2 mM MgCl2, were fixed for 24 h at 4°C in 3.7% formaldehyde-10 mM triethanolamine. Gradients were centrifuged in a Beckman SW60 Ti rotor for 18-20 h at 300,000g.

**RNA and DNA Concentrations**

RNA content of subcellular fractions and polysome pellets was determined by the method of Fleck and Munro (19) as described by Biobel and Potter (7). DNA content was determined by the method of Burton (11). The concentration of poly(A)+ RNA was determined by assuming that a concentration of 40 µg/ml gives an absorbance reading of 1.0 at 260 nm.

**Determination of Poly(A) Content**

The poly(A) content of total polysomal and poly(A)+ RNA populations was determined relative to poly(A) standards by hybridization to [3H]poly(U) (49). RNase-resistant [3H]scurves were plotted as a function of 5 different concentra-

**Materials**

Materials were obtained from the following sources: [H]-orotic acid, [H]-dCTP, New England Nuclear, Boston, Mass.; oligo(dT) cellulose type 3, Collaborative Res., Inc., Waltham, Mass.; unlabeled dTTP, oligo(dT)25-32, PL Biochemicals, Inc., Milwaukee, Wis.; reagent grade formamide, Fisher Scientific Co., Itasca, Ill., which was deionized before use with AG 501-X8 mixed bed resin; Bio-Rad Laboratories, Richmond, Calif.; poly(A), poly(A)+, H-poly(U), polyadenylate-K+ salt, Miles Biochemicals, Elkhart, Ind.; optical grade CsCl, Bethesda Research Lab., Inc., Rockville, Md.; "Aristar" guanidine-Cl, BDH Chemicals, Poole, England; AMV reverse transcriptase was kindly supplied by Dr. Joseph Beard, Life Sciences, Inc., St. Petersburg, Fla.; routine laboratory chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

**RESULTS**

Isolation and Purity of Free and Membrane-bound Polysomes

To obtain a meaningful comparison of the complexity and frequency distribution of rat liver free and membrane-bound polysomal poly(A)+ RNA populations it was essential to isolate undegraded polysomes in high yield with minimal levels of cross-contamination. We used the fractionation scheme of Ramsey and Steele (46) with minor modifications to achieve this end. Because we wished to characterize RNA populations from the livers of rats that had not been subjected to starvation, it was necessary to confirm that the fractionation protocol would be effective for liver from fed rats. Table I demonstrates that RNA distributions in subcellular fractions and recoveries in polysome pellets are comparable to those obtained by Ramsey and Steele (46) using starved-rat liver and to the results of others who used different fractionation schemes (8, 16).

Sucrose gradient centrifugation of pelleted "free" and "microsomal" fractions revealed the presence of large polyribosome structures (Fig. 1), indicating that minimal degradation of mRNA occurred during the fractionation procedure. To determine the extent of cross-contamination of free and membrane-bound polysomes we repeated the experiments of Ramsey and Steele (46). These involve adding labeled free polysomes or purified rough microsomes to a liver homogenate and following the distribution of label during the fractionation
procedure. For the amount of membrane-bound polysome contamination of free polysome fractions we obtained the same result for livers from starved and from fed rats, a result identical to that obtained by Ramsey and Steele (46). We estimated a <1% contamination of free polysomes by membrane-bound (data not shown). Table II shows that free polysome contamination of microsomal fractions was lower with fed-rat liver than with starved-rat liver. This may be attributed to the slower sedimentation rate of polysomes in the presence of high glycogen concentrations (30). Taking into account the 3:1 ratio of membrane-bound to free polysomes (Table I), the former are contaminated with <2% free polysomes using livers of fed rats.

We believe this to be the most direct method for determining polysome cross-contamination levels, but an important consideration was whether the endogenous free polysomes inhibited the pelleting of the labeled exogenous polysomes with the microsomal fraction, thus giving an artifactualy low level of cross-contamination. To determine whether this is the case, a 131,000 g_{max} pellet was prepared in the presence of 250 mM KCl to remove most of the free and loosely bound (high-salt extractable) polysomes (47). An amount of labeled free polysomes from an equivalent portion of liver was homogenized with the pellet in 75 mM KCl buffer and the homogenate recentrifuged. Table II indicates that the apparent cross-contamination level increases from 1.9 to 5.2%.

In the isolation of polysomes we have avoided the use of sucrose-gradient selection procedures (15) that may result in the loss of specific message sequences. However, polysomes purified from subcellular fractions by pelleting through sucrose cushions may be contaminated with significant amounts of nonpolysomal cytoplasmic or nuclear ribonucleoprotein particles (43). Because this would make interpretation of hybridization data difficult, it was necessary to estimate the extent of such contamination in our polysome preparations.

Nuclear RNA contamination of polysome preparations was evaluated by pulsing a rat for 10 min with [3H]-orotic acid and determining the distribution of labeled RNA in subcellular fractions and polysome pellets. After a 10-min pulse, label will be primarily confined to nuclear RNA species (42) although some may be present in cytoplasmic species as well (43). Table III shows that leakage and/or lysis of nuclei does occur during homogenization and detergent treatment, but <0.3 and 1.2% of the pulse-labeled RNA is sedimented with free and membrane-bound polysomes, respectively. To determine the nature of the labeled RNA species that pellet, polysome fractions were subjected to CsCl-density gradient analysis in the presence and absence of EDTA. Fig. 2A and C show that most of the label in the free polysome fraction is present as a peak at 1.57 g/cm^3 with a shoulder at 1.53 g/cm^3, and that EDTA causes a shift in the mean density of the prominent peak to about 1.54 g/cm^3. This indicates that most of the label is in newly synthesized mRNP associated with ribosomes. The relatively high buoyant density of the free polysomal mRNP is due to exposure to 250 mM KCl as described previously (13). The shoulder at 1.53 g/cm^3 may represent newly synthesized cytoplasmic 40S struc-

![Figure 1: Polysome profiles. Free and membrane-bound polysomes were isolated as described in Materials and Methods. Polysome pellets were resuspended by homogenization in 50 mM HEPES pH 7.6, 25 mM KCl, 5 mM MgCl_2 with (—) or without (—) 50 mM EDTA. Approximately 6 A_{260} U of free (A) and 18 A_{260} U of membrane-bound (B) polysomes were layered onto 10-50% linear sucrose gradients containing 50 mM HEPES pH 7.6, 75 mM KCl, 5 mM MgCl_2. The quantities layered are from equivalent amounts of liver. Gradients were centrifuged in a Beckman SW41 rotor at 38,000 rpm for 75 min. Profiles were recorded by pumping gradient contents through a flow cell in a Gilford 2400 spectrophotometer, using a peristaltic pump and Beckman fraction recovery system.](image)

### Table II

| Experiment | 
|------------|
| Total DPM added | 8.14 x 10^6 (100) | 7.77 x 10^6 (100) | 7.01 x 10^6 (100) |
| DPM recovered in free fraction | 6.87 x 10^6 (84.4) | 7.16 x 10^6 (92.7) | 5.78 x 10^6 (82.4) |
| DPM recovered in microsomal fraction | 9.21 x 10^6 (11.3) | 4.53 x 10^6 (5.8) | 1.09 x 10^6 (15.5) |
| Total DPM recovered, % | 95.7 | 98.0 | 98.0 |

Approximately 10 mg (isolated from 10 g liver) of [3H]-orotic acid-labeled free polysomes was added to 10 g liver, a 1:3 wt/vol homogenate prepared and centrifuged at 740 g_{max} for 2 min and at 131,000 g_{max} for 12 min as described in Materials and Methods. Aliquots of the supernate or “free fraction” and pellet or “microsomal fraction” resuspended in H_2O were digested in NCS tissue solubilizer and radioactivity determined after addition of 10 ml of OCS scintillator cocktail. The percentage of total DPM recovered in the respective fractions are given in parentheses.

* Liver obtained from a rat starved for 18 h before sacrifice.

† Liver obtained from a fed rat.

§ Liver from a fed rat was homogenized in 3 vol of 0.25 M KCl buffer and a 131,000 g_{max} pellet prepared. The pellet was homogenized in 0.075 M KCl buffer containing 10 mg labeled free polysomes and the homogenate recentrifuged as above. Distribution of radioactivity was determined as described above.
Extent of Nuclear Leakage and Contamination of Polysome Pellets

| Fraction*       | DNA  | Total DPM | Total |
|-----------------|------|-----------|-------|
|                 | mg   | %         | %     |
| Total homogenate| 13.4 | 100       | 4.50 x 10^6 | 100  |
| Free            | 0.97 | 7.2       | 8.60 x 10^5 | 19.1 |
| Microsomal      | 1.66 | 12.4      | 6.75 x 10^6 | 15.0 |
| Nuclear         | 12.9 | 96.1      | 2.22 x 10^7 | 49.3 |
| Free polysome pellets | 0.025 | 0.37 | 1.22 x 10^6 | 0.27  |
| Bound polysome pellets | 0.035 | 0.52 | 5.26 x 10^6 | 1.17  |

A male rat, fed ad lib., was injected intraperitoneally with 1.0 mCi of [3H]orotic acid and sacrificed 10 min later. Subcellular fractions and polysome pellets were prepared from 5 grams of liver as described in Materials and Methods. Aliquots of the total homogenate, subcellular fractions and polysome pellets (resuspended in 1.0 ml H2O) were processed as described in Table I for RNA determinations. Nuclear RNA-enriched 3H-labeled counts were determined on aliquots of the NaOH hydrolysates. DNA contents were determined on the final perchloric acid (PCA) precipitates by the method of Burton (11). Recoveries of DNA and DPM in the subcellular fractions were 115 and 83.4%, respectively.

* Fractions are as described in Table I.

FIGURE 2 Estimation of nuclear RNP contamination of polysome pellets: CsCl density gradients of pelleted fractions. Free and membrane-bound polysome pellets were prepared from the liver of a rat, pulse-labeled for 10 min with [3H]-orotic acid as described in Table III. Pellets were rinsed with 10 mM triethanolamine pH 7.6, 2 mM MgCl2, 100 mM NaCl and resuspended by homogenizing in 0.6 ml of the same buffer. 0.3-ml (free) or 0.4-ml (membrane-bound) aliquots were adjusted to 50 mM EDTA. EDTA-treated (C and D) and untreated (A and B) aliquots of resuspended free (A and C) and membrane-bound (B and D) polysome pellets were fixed in 3.7% formaldehyde–10 mM triethanolamine pH 7.2 and layered onto 4.4 ml, 35-55% (wt/vol) linear CsCl gradients. Gradients were centrifuged in a Beckman SW60 Ti rotor at 40,000 rpm, 22°C, for 24 h. Gradient fractions were collected into scintillation vials and the absorbance at 260 nm monitored as described in the legend to Fig. 1. Arrows indicate the positions of ribosomes in the gradient as indicated by absorbance peaks at 260 nm.

FIGURE 3 Examination of free polysomes for contamination by nonpolysomal cytoplasmic RNP: profiles of pelleted free fraction selectively labeled in mRNA. Free polysomes were prepared from the liver of a rat injected intraperitoneally with 300 μCi of [3H]-orotic acid + 1 mg FOA 3 h before sacrifice. Profiles were conducted on EDTA-treated (---) and untreated (-----) resuspended free polysome pellets as described in Fig. 1. Fractions were collected directly into scintillation vials and radioactivity determined by counting in 10 ml of Aquasol; (●) EDTA, (○) no EDTA.
absence of EDTA no peak of radioactivity is discernible in the density range characteristic of rat liver free cytoplasmic RNP particles, i.e., 1.35–1.45 g/cm³ (22). For this experiment poly
somes were pelleted through 2 M sucrose cushions containing 25 mM KCl rather than 250 mM KCl to avoid removing proteins from RNP. These data suggest that cytoplasmic RNP contamination of free polysome pellets is very low. The same experiments with membrane-bound polysome pellets gave similar results (data not shown).

Isolation and Characterization of Poly(A) + RNA

Perhaps the greatest source of inaccuracy in evaluating kinetic hybridization data results from inaccurate determination of driver RNA concentrations. We found it necessary to include a denaturation step in the presence of formamide during oligo(dT)-cellulose chromatography to obtain low levels of rRNA contamination of poly(A)+ RNA fractions. Without a denaturation step rRNA contamination varied from 20 to 60% in different preparations. Through the addition of a denaturation step, rRNA contamination of both free and membrane-bound polysomal poly(A)+ RNA preparations was consistently less than 5 to 10% as measured by electrophoresis on formamide polyacrylamide gels (not shown). Yields of poly(A)+ RNAfree and poly(A)+ RNAbound were 5.9 ± 1.1 and 9.8 ± 2.0 μg/g liver (four preparations), or 0.5% and 0.3% of free and membrane-bound polysomal RNA. The maximum theoretical yields can be estimated from the poly(A) content of polysomal and poly(A)+ RNA fractions. From Table IV it can be calculated that poly(A)+ RNA comprises 0.217/9.15 or 2.37% of free polysomal RNA and 0.131/10.7 or 1.22% of membrane-bound polysomal RNA. There are several possible explanations for the lower poly(A) content of membrane-bound polysomal RNA. It may indicate that a larger proportion of poly(A)-lacking mRNA (20) is translated in association with RER membranes than free in the cytoplasm. A trivial explanation is that a selective loss of poly(A) tails from membrane-bound polysomal RNA occurs during tissue fractionation or phenol-chloroform extraction. Both of these phenomena have been described (4, 44). If loss of poly(A) sequences is occurring at some point it must be an all or none phenomenon since the number-average length of poly(A) tails from poly(A)+ RNAbound is actually greater than that from poly(A)+ RNAfree (80 vs. 60 nucleotides) as determined by the method of Kaufman and Gross (23).

Complexity and Frequency Distribution of Poly(A) + RNAfree and Poly(A) + RNAbound

DNA complementary to poly(A)+ RNAfree and poly(A)+ RNAbound was prepared using AMV reverse transcriptase. cDNA was sized on formamide polyacrylamide gels (not shown). Mass-average sizes were determined by the method of Ordahl et al. (37) that takes into account the unequal length-ranges of cDNA that are contained in linear segments from different regions of the gel. Mass-average sizes were found to
Copies/cell = (grams driverRNA/cell X 6 X 10^23 molecules/mol)/(RNA nucleotide complexity X 330 g/mol nucleotide). There are 9.6 pg DNA/average liver cell and a RNA/DNA mass ratio of 2.82 (8). This gives 27.1 pg total RNA/cell, 60% of which is membrane-bound polysomal RNA and 20% free polysomal RNA.

The cDNAs were hybridized to an excess of the homologous (C and D) poly(A)^+ RNA either unlabeled (B and D) or labeled (A and C) with ^3H-orotic acid + FOA as described in Materials and Methods. The RNase A-digested hybrid mixtures were TCA precipitated onto glass-fiber filters, washed with 5% TCA and 95% ethanol, and dried, and radioactivity was determined in toluene-PPO. 28S, 18S, and 5S rRNAs were run in a parallel gradient as size markers.

**TABLE V**

Complexity and Frequency Distribution of Rat Liver Free and Membrane-Bound Polysomal Poly(A)^+ RNA Populations

| Polysome class | Abundance class | cDNA hybridized* | Rate Constant | Nucleotide complexity† | Number mRNA species‡ | Copies/cell** |
|----------------|-----------------|------------------|---------------|------------------------|---------------------|--------------|
|                | Abundant        |                  |               |                        |                     |              |
| Free           | 24.2 ± 11.4     | 15.0 ± 12.5      | (7.74 ± 6.44) X 10^6 | 48 ± 40                | 739 ± 615           |
| Intermediate   | 36.8 ± 11.1     | 1.31 ± 0.81      | (1.34 ± 0.83) X 10^6 | 837 ± 517              | 65 ± 40             |
| Rare           | 38.9 ± 6.9      | 0.059 ± 0.024    | (3.15 ± 1.23) X 10^6 | 19,700 ± 8,060         | 3 ± 1              |
| Bound          | 37.9 ± 2.6      | 170 ± 30.6       | (1.08 ± 0.19) X 10^6 | 9 ± 2                  | 12,800 ± 2,300     |
| Abundant       | 27.2 ± 5.3      | 3.94 ± 1.52      | (3.35 ± 1.29) X 10^6 | 279 ± 108              | 294 ± 113          |
| Intermediate   | 27.2 ± 5.3      | 0.299 ± 0.097    | (5.66 ± 1.83) X 10^6 | 4,710 ± 1,540          | 22 ± 7             |
| Rare           | 34.9 ± 5.6      | 0.31 ± 0.22      | (5.66 ± 1.83) X 10^6 | 4,710 ± 1,540          | 22 ± 7             |

* Normalized to a terminal hybridization value of 100%. Observed terminal values were 94.2 and 95.2% for poly(A)^+ RNA free and poly(A)^+ RNA bound, respectively.

† Rate constant expected for an RNA population consisting of a single abundance class.

§ Calculated relative to the rate constant observed for the reaction of rabbit alpha + beta globin mRNA with its cDNA under our hybridization conditions. The observed value (2,827 liter/mol-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 Wemur and Davidson (35) and Chamberlin et al. (14), one obtains: KT = K_1 (LT/L_1) (\sqrt{L_2} / \sqrt{L_0}) where K_1 and K_2 are the rate constants observed for a reaction with driver, tracer lengths, L_1, L_2, or L_0, respectively. The driver and tracer lengths for the globin reaction were 650 and 400 nucleotides. The corresponding mass-average lengths for the poly(A)^+ RNA free reaction were 2,250 and 1,064 nucleotides. We assume globin mRNA has a complexity of 1,200 nucleotides. The corrected K-values corresponding to a complexity of 1,200 nucleotides are thus 3,990 and 4,042 liter/mol-s for poly(A)^+ RNA free and poly(A)^+ RNA bound, respectively.

¶ Number of unique mRNA species 1,200 (membrane-bound) or 1,600 (free) nucleotides in length.

** Copies/cell = (grams driver RNA/cell X 6 X 10^23 molecules/mol)/(RNA nucleotide complexity X 330 g/mol nucleotide). There are 9.6 pg DNA/average liver cell and a RNA/DNA mass ratio of 2.82 (8). This gives 27.1 pg total RNA/cell, 60% of which is membrane-bound polysomal RNA and 20% free polysomal RNA.

Poly(A)^+ mRNA constitutes 2.37 and 1.22% of free and membrane-bound polysomal RNA, respectively. Thus, there are ~0.13 pg and 0.20 pg of poly(A)^+ RNA free and poly(A)^+ RNA bound per cell.

**FIGURE 5** Sizing of poly(A)^+ RNA fractions. Free (A and B) and membrane-bound (C and D) poly(A)^+ RNA either unlabeled (B and D) or labeled (A and C) with ^3H-orotic acid + FOA as described in Materials and Methods. Methods was dissolved in 75% formamide, 10 mM HEPEs pH 7.6, 3 mM EDTA and layered on formamide-sucrose gradients. Gradients were centrifuged in a Beckman SW41 rotor at 38,000 rpm, 22°C for 24-28 h. Gradient fractions of labeled poly(A)^+ RNA were collected directly into scintillation vials and counted in toluene-PPO. 28S, 18S, and 5S rRNAs were run in a parallel gradient as size markers.

**FIGURE 6** Homologous and heterologous hybridization reactions. cDNAbound (A) and cDNAfree (B) were hybridized to an excess of the homologous (C) or heterologous (D) poly(A)^+ RNA, and the extent of reaction was determined as described in Materials and Methods. RNA concentrations ranged from ~1 to 2,000 µg/ml. The homologous hybridization data are pooled from several independent experiments, using two different RNA and cDNA preparations for each curve. The curves were drawn with the aid of a computer as described in Materials and Methods.

that the driver RNA molecules exist in widely varying concentrations, because a single first-order reaction component occupies about 1.5 to 2.0 log rot. It can be seen that almost 50% of the cDNAbound (Fig. 6A) has hybridized by a log rot of ~1 whereas only ~20% of cDNAfree (Fig. 6B) has hybridized by...
this rot value. The homologous "bound" reaction is complete by a log rot of 1, but the homologous "free" reaction does not terminate until a log rot value of 2. These observations suggest that there are RNA species present at higher frequency in poly(A)\(^+\) RNA\(_{\text{bound}}\) than in poly(A)\(^+\) RNA\(_{\text{free}}\) and that poly(A)\(^+\) RNA\(_{\text{free}}\) is comprised of a greater number of different RNA species than is poly(A)\(^+\) RNA\(_{\text{bound}}\). Table V summarizes a quantitative description of both homologous hybridizations when each is analyzed as the sum of three kinetic components or "abundance classes" (5). By this analysis poly(A)\(^+\) RNA\(_{\text{bound}}\) is composed of about 5,000 species and poly(A)\(^+\) RNA\(_{\text{free}}\) of about 20,000 species. A large portion of the mass of poly(A)\(^+\) RNA\(_{\text{bound}}\) is comprised of nine mRNA species present at about 13,000 copies per cell. mRNA species present at this high frequency are absent from poly(A)\(^+\) RNA\(_{\text{free}}\), which has a large portion of its mass consisting of 20,000 species present at about three copies per cell.

**Are Poly(A)\(^+\) RNA\(_{\text{free}}\) and Poly(A)\(^+\) RNA\(_{\text{bound}}\) Qualitatively Unique?**

The data in Table V would suggest that poly(A)\(^+\) RNA\(_{\text{bound}}\) should be lacking RNA species present in poly(A)\(^+\) RNA\(_{\text{free}}\) because the latter apparently has a fivefold greater nucleotide complexity. The heterologous hybridizations in Fig. 6 demonstrate that this is not so. Within the limits of the technique, both poly(A)\(^+\) RNA\(_{\text{free}}\) and poly(A)\(^+\) RNA\(_{\text{free}}\) are able to hybridize to completion with the heterologous cDNA populations. Evidently the more rare mRNA species comprise too small a fraction (~5% or less) of the mass of poly(A)\(^+\) RNA\(_{\text{bound}}\) to be detected in the homologous reaction. This clearly demonstrates the limitations of this technique for complexity determinations. Table V can be said to represent data describing only ~95% of poly(A)\(^+\) RNA\(_{\text{free}}\) and poly(A)\(^+\) RNA\(_{\text{bound}}\) populations.

This finding is not unexpected because a finite level of cross-contamination is unavoidable (see above). The important question is whether all or part of the "heterologous" hybridization can be attributed to cross-contamination. By combining the data of Tables II and IV the following upper limits can be set for cross-contamination: poly(A)\(^+\) RNA\(_{\text{free}}\) in poly(A)\(^+\) RNA\(_{\text{bound}}\) 15.5/3 \times 2.37/1.22 = 10.0%; poly(A)\(^+\) RNA\(_{\text{free}}\) in poly(A)\(^+\) RNA\(_{\text{free}}\) 1 \times 1.22/2.37 = 0.51%. Thus, if heterologous hybridizations are due solely to cross-contaminating sequences, these should be displaced from the homologous curves by 1.0 and 2.3 log rot. Fig. 6 shows that this is not the case for either curve. In both cases the heterologous curves are displaced by less than the required amount throughout most of their length.

To obtain more quantitative information it is necessary to conduct heterologous hybridizations with kinetically fractionated cDNA populations. cDNA was fractionated into abundant and less-abundant species by limited hybridization to homologous RNA and separation of hybridized and unhybridized molecules by hydroxyapatite chromatography (21). Fig. 7A and B show the homologous hybridizations with kinetically fractionated cDNA populations. Although none of the fractionated cDNAs is kinetically pure (hybridizes within 2 log rot), the procedure was effective in isolating kinetically enriched populations.

Fig. 7C demonstrates that all of the poly(A)\(^+\) RNA\(_{\text{bound}}\) sequences are present in poly(A)\(^+\) RNA\(_{\text{bound}}\) at significant levels above cross-contamination since both heterologous curves are displaced by <2.3 log rot throughout their lengths. In contrast Fig. 7D demonstrates that most of the abundant and at least 60% of less abundant poly(A)\(^+\) RNA\(_{\text{free}}\) sequences are present in poly(A)\(^+\) RNA\(_{\text{free}}\) at significant levels (the curves are displaced by <1 log rot) but the most rare poly(A)\(^+\) RNA\(_{\text{free}}\) sequences which constitute most of the RNA complexity in free polysomes are present only at the level expected for cross-contaminating species.
Heterologous Hybridizations Involving Tightly Bound Polysomal Poly(A) + RNA Populations

To determine what role poly(A) + RNA derived from loosely bound polysomes might play in the results obtained, membrane-bound polysomes were isolated in the presence of 250 or 500 mM KCl by the method of Ramsey and Steele (47). These polysomes are designated tightly bound. In the case of the 500 mM KCl isolation procedure the initial 131,000 g_max pellet was washed twice to reduce free or loosely bound polysome contamination to ~0.1% by the criteria described in an earlier section.

Poly(A) + RNA derived from tightly bound polysomes was used to drive heterologous hybridizations with cDNA_free. The results are shown in Fig. 8. Poly(A) + RNA from both tightly bound polysome fractions drove the reaction with very similar kinetics. At lower rot values the curve is displaced slightly to the right of the poly(A) + RNA bound driven reaction, whereas at higher rot values the reaction proceeds slightly more rapidly. Thus, removal of "loosely bound" polysomes appears to cause only a relatively minor shift in the overall frequency distribution of poly(A) + RNA_free sequences in membrane-bound populations. A decrease of two orders of magnitude in free polysome contamination had a minimal effect on the overall concentration of poly(A) + RNA_free sequences. Also shown in Fig. 8 is the heterologous reaction of cDNA_free driven by poly(A) + RNA derived from polysomes isolated from extensively washed, purified rough microsomes (12). This reaction proceeds more rapidly than any of the others and also goes to completion. This demonstrates that poly(A) + RNA obtained from membrane-bound polysomes isolated by an independent fractionation scheme also contains a significant amount of poly(A) + RNA_free sequences.

Tightly bound poly(A) + RNA was also used to drive heterologous reactions with kinetically fractionated cDNA_free populations (Fig. 9). Again, the data points representing hybridization to both abundant cDNA_free (Fig. 9A) and less abundant cDNA_free (Fig. 9B) are only minimally displaced from the analogous heterologous hybridizations driven by poly(A) + RNA_bound. At higher rot values the heterologous data points are displaced to the right relative to the homologous curves by 1 log rot. Because we estimate poly(A) + RNA_free contamination at about 0.1%, these sequences are present at 100-fold concentrations over the cross-contamination level. We conclude that a large portion of poly(A) + RNA_free and poly(A) + RNA_bound sequences are shared and that the shared sequences are present at different concentrations in the two populations.

Validity of the Assay for Duplex Formation

Because of the unexpected finding of an extensive overlap between poly(A) + RNA_free and poly(A) + RNA_bound sequences, it was necessary to ensure that we were actually measuring the formation of well-matched RNA-cDNA duplexes. To eliminate the possibility that S1 nuclease resistance was due to contaminating DNA-cDNA or cDNA-cDNA hybridization or aggregation, we measured the buoyant densities on CsCl-guanidinium chloride gradients (36) of cDNA_free and cDNA_bound hybridized to high rot values to an excess of the homologous and heterologous RNA. Most of the cDNA in each case had a density intermediate between that of pure RNA and DNA (not shown). To determine whether these RNA-cDNA structures were well-matched duplexes as opposed to aggregates or poorly matched duplexes in the case of the heterologous structures, we conducted thermal melting profiles. The sharp transitions and relatively high T_m values indicate the existence of well-matched duplexes (Fig. 10). The heterologous duplexes exhibit T_m values about 1.5°C lower than the corresponding homologous duplexes indicating that little, if any, mispairing has occurred (9).

DISCUSSION

Our results indicate that free and membrane-bound polysomes from rat liver contain overlapping poly(A) + RNA sequences.
contained all of these sequence complexity of the template RNA strand that cDNA transcribed from mouse liver poly(A)'RNA cDNA does accurately represent its template RNA (e.g., see arereasonably accurate. Several lines of evidence suggest that their template RNAs; (b) our cross-contamination estimates are an accurate qualitative and quantitative representation of rests mainly on two assumptions: (a) cDNA populations per cell.

RNArree contains 20,000 mRNA species present at three copies 20,000 species. The largest abundance class by mass in poly(A)'RNA rree is comprised of about 5,000 different mRNA species while the other abundant poly(A)'RNA sequences from loosely bound polysomes represent, at most, only a small mass-fraction of poly(A)'RNA rree.

This overlap involves a large fraction by mass of the poly(A)' RNA, and apparently cannot be attributed to cross-contamination. Poly(A)' RNA rree contains a complete complement of poly(A)' RNA bound sequences and poly(A)'RNA bound appears to contain most of the abundant and intermediate-abundant poly(A)'RNA rree sequences. Although poly(A)'RNA bound may lack a quantitatively significant level of rare poly(A)' RNA free sequences, poly(A)'RNA from tightly bound polysomes appears to contain a complete complement of poly(A)'RNA rree. The homologous hybridizations indicate that poly(A)' RNA rree and poly(A)'RNA bound are quantitatively distinct. About 95% or more of the mass of poly(A)'RNA bound is comprised of about 5,000 different mRNA species while the same mass-fraction of poly(A)'RNA rree is comprised of about 20,000 species. The largest abundance class by mass in poly(A)'RNA bound contains 9 mRNA species present at 13,000 copies per cell, while the largest abundance class by mass in poly(A)'RNA rree contains 20,000 mRNA species present at three copies per cell.

The interpretation of the hybridization data presented here rests principally on two assumptions: (a) cDNA populations are an accurate qualitative and quantitative representation of their template RNAs; (b) our cross-contamination estimates are reasonably accurate. Several lines of evidence suggest that cDNA does accurately represent its template RNA (e.g., see references 21, 27). Recently, Van Ness and Hahn (53) demonstrated that cDNA transcribed from mouse liver poly(A)'RNA contained all of the sequence complexity of the template RNA when both were used to drive reactions with single-copy DNA.

The estimation of cross-contamination levels is most crucial to the interpretation of the heterologous hybridizations. We believe the method we used is the most direct for estimating these levels, and that the values given are maximal ones. For example, we find that ~3% of rough microsomes fail to pellet at 131,000 g max for 12 min. When 3H-labeled purified rough microsomes are layered onto 2 M sucrose cushions and centrifuged under the conditions used in these experiments, ~10% of the label pellets. Assuming all of this is due to the release of membrane-bound polysomes caused by homogenization and/or centrifugal-shearing forces, this gives 3 x 0.1 x 3 or 0.9% contamination of free polysomes with membrane-bound. However, it is more likely that most of the pellet label is due to free polysomes contaminating the purified rough microsome preparation (our unpublished observations).

In the case of poly(A)+ RNA rree contaminating poly(A)+ RNA bound, we have assumed the poly(A)+ RNA rree concentration in the contaminating polysomes to be the same as that in free polysome pellets. If the lower poly(A) concentration of membrane-bound polysomal RNA is due to loss of poly(A) segments during isolation, then it is likely the poly(A) concentration of the contaminating free polysomal RNA would be reduced by the same extent. This would lower the calculated poly(A)+ RNA rree contamination by a factor of 2.37/1.22 to a value of 5.1%. Furthermore, we have assumed the higher free polysome contamination level is applicable (see Table II), whereas it may be an artifact caused by the initial pelleting step in 0.25 M KCl.

It was important to determine what effect removal of loosely bound polysomes had on the heterologous poly(A)+ RNA bound-cDNA free hybridization. We refer to loosely bound polysomes as those that are released from rough microsomes by high monovalent salt concentrations (47). These polysomes have been poorly characterized and their function is obscure. It has been suggested that they arise by artificial adsorption of free polysomes during cell fractionation (26, 47), but there is no direct evidence that supports this contention. Loosely bound polysomes constitute ~15% of the total membrane-bound polysome population (47). If they represent artificially adsorbed free polysomes, their removal along with a 100-fold reduction of residual free polysome contamination would cause a dramatic shift in the heterologous poly(A)+ RNA bound-cDNA free hybridization curves. Figs. 8 and 9 show that only minor shifts occur, which suggests that poly(A)+ RNA sequences from loosely bound polysomes represent, at most, only a small mass-fraction of poly(A)+ RNA rree. Furthermore, at high rot values poly(A)+ RNA from tightly bound polysomes drives cDNA free with faster kinetics than does poly(A)+ RNA bound. Thus, loosely bound polysomes are not a random sampling of artificially adsorbed free polysomes and may comprise a unique polysome population. We are currently conducting a more detailed characterization of this polysome class in rat liver.

In HeLa cells polysomes appear to be associated with a cytoskeletal structure (28). It is possible that some of the poly(A)+ RNA rree in the membrane-bound polysome fraction is due to an interaction of this type. However, since the cytoskeleton is reported to be disrupted in ionic strengths above 0.1 M NaCl (28), its presence may not be a factor in membrane-bound polysome fractions prepared in 0.25 M or 0.5 M KCl. Also, intermediate-abundant poly(A)+ RNA rree sequences would have to be preferentially associated with such a structure.
These findings must be interpreted in the light of current theories and evidence concerning the genesis and function of polysome classes. The results of the homologous hybridizations are in agreement with the general view that (a) membrane-bound polysomes are primarily engaged in the synthesis of a relatively small number of secretory and membrane proteins and (b) free polysomes synthesize a larger number of proteins destined for intracellular use, including those involved in various "housekeeping" functions. The presence of a large fraction of poly(A)\(^+\) RNA\(_{\text{bound}}\) sequences in the free polysome class is consistent with the theory of Blobel and Dobberstein (6) that initiation of synthesis of all proteins takes place free in the cytoplasm. From Fig. 7C it can be estimated that, on the average, abundant poly(A)\(^+\) RNA\(_{\text{bound}}\) sequences are present at a 30 to 150 fold reduced quantity and less abundant poly(A)\(^+\) RNA\(_{\text{bound}}\) sequences at a 6 to 30 fold reduced quantity in free polysomes. If all of the poly(A)\(^+\) RNA\(_{\text{bound}}\) sequences present in the free polysome class are newly initiated molecules that have not yet reached the membrane surface, this suggests that the rate of initiation on less abundant poly(A)\(^+\) RNA\(_{\text{bound}}\) is on the average fivefold lower than on abundant poly(A)\(^+\) RNA\(_{\text{bound}}\). This would indicate a correlation between the concentration of a mRNA species and the rate of protein synthesis initiation on that species. However, there is evidence suggesting that initiation of protein synthesis in vivo occurs on mRNA already bound to membranes (33).

The finding that a complete complement of poly(A)\(^+\) RNA\(_{\text{free}}\) is found in tightly bound polysome structures is difficult to reconcile with the hypothesis that signal peptide sequences are solely responsible for the segregation of free and membrane-bound polysomes (6). According to this hypothesis sequence overlap might result from: (a) newly synthesized and initiated putative membrane-bound messages that have not yet reached the membrane surface or (b) membrane-bound messages that are produced in excess of available ribosome binding sites. In cells of normal tissue under steady-state conditions the former would be expected to comprise a very small fraction of the total message. In the latter case we consider it unlikely that ~20% of free polysomes are engaged in the synthesis of secretory or membrane polypeptides that are consequently degraded in the cytoplasm due to the lack of ribosome binding sites. For example, assuming a poly(A)\(^+\) RNA\(_{\text{bound}}\)/poly(A)\(_{\text{free}}\) ratio of 1.54 it can be estimated from Figs. 7D and 9A that ~20% by mass of sequences that are abundant in free polysomes are present in 1.5-fold greater quantity in membrane-bound polysomes. If this distribution results from a limited number of binding sites on the membrane, then these sequences are produced in 40% excess of their capacity to be utilized for the synthesis and proper localization of polypeptides. This would constitute an enormous waste of cellular energy. An alternative explanation is that these sequences code for polypeptides that are not transferred into or across the RER and that membrane-polysome interaction serves some other function(s).

Because these mRNA species are present in polysome structures that are not released from the membrane in high salt, they must be attached to the membrane surface by some means other than or in addition to the direct interaction with the large ribosomal subunit (1). In some cases this additional interaction may involve the nascent peptidyl chains and be characteristic of proteins that are found both in the soluble cytoplasm or associated with the cytoplasmic face of membranes and within membranous organelles or associated with the noncytoplasmic face or lipid bilayer. An additional interaction may involve the mRNA molecule or mRNP proteins. There is evidence that such an interaction exists in rat liver (12, 18, but see 24) and several other systems (2, 25, 26, 29, 33, 34). An interaction of this type would be required for tight binding of polysomes synthesizing polypeptides which are not inserted into or across the membrane. We are currently characterizing a subpopulation of poly(A)\(^+\) RNA\(_{\text{bound}}\) that appears to exhibit such an interaction (12).

The results of these hybridization experiments are in agreement with those of numerous experiments localizing the site of synthesis of specific proteins between free and membrane-bound polysomes (31, 48, 51). They demonstrate that various proteins are synthesized on both free and membrane-bound polysomes but preferentially on one or the other. In many cases the lack of an exclusive site of synthesis has been ignored or attributed to cross-contamination without presenting appropriate data. In light of the results presented here a re-evaluation of such data seems appropriate. At present the reason for an extensive overlap between free and membrane-bound poly(A)\(^+\) RNA populations remains unknown. A possible artificial cause is not entirely ruled out but cannot be the result of random cross-contamination or adsorption of free polysomes, or any process that is random with respect to polysomal mRNA sequences.

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