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)$^+$ RNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$) by oligo(dT) cellulose chromatography exhibited number-average lengths of 1,600 and 1,200 nucleotides, respectively, on formamide sucrose gradients. Poly(A)$^+$ RNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$ contain 9.1 ± 0.55 and 10.7 ± 0.50% poly(A) as measured by hybridization to [^{3}H]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)$^+$ RNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$ contain nucleotide complexities of about 3.4×10^7 and 6.0×10^6, 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)$^+$ RNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$ 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 cDNA$_{free}$ 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 prefer-

entially 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.[1] 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 procedure 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 $MgCl_2$ and 100 $\mu g/ml$ 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 $MgCl_2$, 75 mM KCl, 3 mM GSH, 0.5 mg/ml sodium heparin. The homogenate was centrifuged in a Beckman SW27 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 2 min at 740 g_{max} and 12 min at 131,000 g_{max}. The supernatant fluid was decanted and adjusted to 10 mM $MgCl_2$, 1-2 mg/ml sodium heparin. The pellet was homogenized in rat liver high-speed supernatant fluid (46) containing 1% Triton X-100, 20 mM $MgCl_2$, 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_{max} to pellet nuclei. The supernate was adjusted to 50 mM $MgCl_2$, 2 mg/ml sodium heparin, 1.3% sodium deoxycholate and centrifuged at 15,000 g_{max} 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_{max} supernate 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 pelleted at 303,000 g_{max} 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 1 mg/ml bentonite in the extraction buffer as an added ribonuclease inhibitor. In some instances polysome pellets were treated with proteinase K before extraction as described (50). 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 (40).

Poly(A)$^+$ RNA fractions were isolated by oligo(dT) cellulose chromatography essentially as described by Bantle et al. (3) except that RNA was denatured in 75% formamide rather than dimethylsulfoxide before the second cycle. Total polysomal and poly(A)$^+$ RNA fractions prepared by these procedures were judged to be intact by their ability to produce full-length pre-proalbumin and alpha-2$_u$-globulin (bound) or ornithine aminotransferase (free) when translated in a rabbit reticulocyte cell-free system (A. Bosch and M. Mueckler, unpublished experiments).

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[1] *Abbreviations used in this paper:* AMV, avian myeloblastosis virus; cDNA$_{free}$ or cDNA$_{bound}$, complementary DNA transcribed from polyadenylated free or membrane-bound polysomal RNA; dCTP, deoxycytidine triphosphate; dNTP, deoxynucleoside triphosphate; FOA, 5-fluoroorotic acid; poly(A)$^+$ RNA$_{free}$ or poly(A)$^+$ RNA$_{bound}$, polyadenylic acid-containing free or membrane-bound polysomal RNA that binds to oligo(dT) cellulose; RER, rough endoplasmic reticulum; RNP, ribonucleoprotein; rRNA, ribosomal RNA.

Sizing of Poly(A)$^+$ RNA Fractions

Poly(A)$^+$ 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 HEPES, pH 7.6, 3 mM EDTA underlayed with a 2.8-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 × 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 [^{3}H]poly(U) (49). Assuming that RNA molecules of different sizes have, on the average, poly(A) tails of the same length (49), the number-average size of a heterogeneous population is given by $(\sum cpm_i l_i)/(\sum cpm_i)$ where cpm_i is proportional to the number of RNA molecules of length l_i. 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 [^{3}H]-orotic acid in the presence of 5-fluoroorotic acid (56). In this case gradient 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 pds/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 $\mu g/ml$ template poly(A)$^+$ RNA, 75 $\mu g/ml$ oligo(dT)$_{12-18}$, 1.4 mM unlabeled deoxynucleoside triphosphate (dNTPs), 0.3 mM [^{3}H]-deoxycytidine triphosphate (dCTP), 300-400 U/ml AMV reverse transcriptase, 2.5% glycerol, 2 mM sodium pyrophosphate, 8 mM $MgCl_2$, 0.375 mg/ml dithiothreitol, 50 $\mu g/ml$ 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 Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). Void fractions containing cDNA were combined, ethanol precipitated, and resuspended in an appropriate volume of sterile, double-distilled H_2O. The specific activities of cDNA preparations used in these experiments ranged between 4 and 5 × 10^7 dpm/μg assuming a 25% dCTP residue content. Yields ranged from 20 to 30% by mass of input RNA.

Kinetic Fractionation of cDNA

cDNA$_{free}$ and cDNA$_{bound}$ 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 in 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. Gentle positive air pressure was used to increase flow rate. Columns were preloaded with 500 μg yeast tRNA and 0.4% SDS to reduce nonspecific and irreversible binding. The kinetically enriched cDNA populations were isolated as described above.

Hybridization Reactions

Hybridization reactions were carried out in torch-sealed, silanized micropipettes 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 10^2 to 10^5. 5-10 × 10^3 dpm of cDNA was included per rot point.

An S1 nuclease assay was used to determine the percentage of cDNA remaining single-stranded at each rot point. Reactions were terminated by plunging the micropipettes into a solid CO_2-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 $ZnSO_4$, 10 $\mu g/ml$ denatured calf-thymus DNA). 1-ml aliquots were immediately adjusted to 10% trichloroacetic acid (TCA) and 100 $\mu g/ml$ yeast

tRNA, 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 data points. 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. 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:

$$C/Co = F + \sum_{i=1}^{r} f_i \exp(-K_i Rot),$$

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.

Polyacrylamide Gel Electrophoresis in 98% Formamide

Estimation of rRNA contamination of poly(A)⁺ RNA preparations and sizing of cDNA were performed by electrophoresis in 3.5% polyacrylamide 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 preelectrophoresed 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]cDNA were fractionated by an automated gel crusher-fractionator (Gilson 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 rRNA 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 24 h at 40,000 rev/min at 22°C. Gradients were pumped through a flow cell in a Gilford 2400 spectrophotometer and fractions collected into scintillation vials. Density calibration was achieved by weighing 25-μl aliquots of several fractions per gradient. Radioactivity was determined by counting fractions in 10 ml of Aquasol.

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 Blobel 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]-counts were plotted as a function of 5 different concentra-

tions for each experiment and a line regressed through the data points. Poly(A) content is given by the ratio of the slope of this line to that of the poly(A) standard curve.

Materials

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

TABLE I

Distribution of RNA in Subcellular Fractions and Polysome Pellets

Fraction	mg RNA/g liver	Total
		%
Total homogenate	6.80 ± 0.34 (9)	100
Free*	2.46 ± 0.27 (9)	36.1 ± 3.0
Microsomal‡	4.05 ± 0.22 (9)	56.5 ± 3.4
Nuclear§	0.50 ± 0.07 (9)	7.4 ± 0.98
Free polysome pellet¶	1.08 ± 0.48 (4)	15.2 ± 8.1
Bound polysome pellet¶	3.29 ± 0.48 (4)	47.0 ± 7.1

Subcellular fractions and polysome pellets were prepared by the method of Ramsey and Steele (46) as described in Materials and Methods. RNA content was determined by the procedure of Fleck and Munro (19) as described by Blobel and Potter (7). Values given are mean ± SE. The number of determinations is given in parentheses and represents data from independent experiments.
* 131,000 g_{max} supernate of 1:3 wt/vol liver homogenate.
‡ 1,470 g_{max} supernate of resuspended, detergent-treated 131,000 g_{max} pellet.
§ 1,470 g_{max} pellet.
¶ Free and detergent-treated microsomal fractions were pelleted through 2 M sucrose cushions for 18–20 h at 303,500 g_{max}.

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
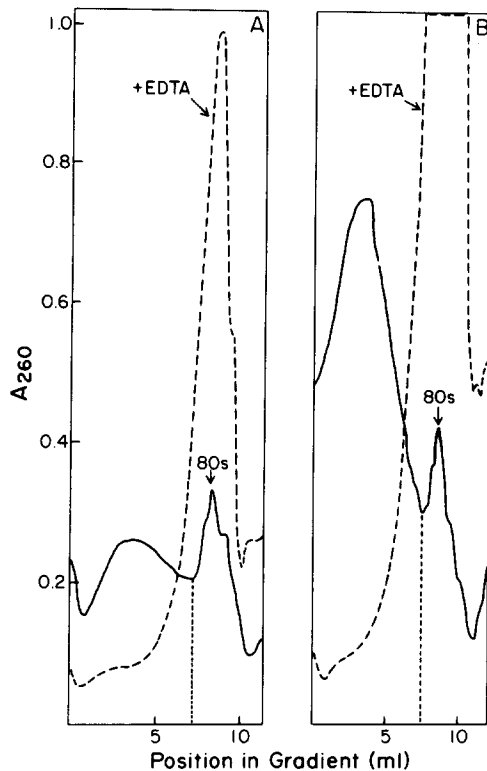

FIGURE 1 Polysome profiles. Free and membrane-bound polysomes were isolated as described in Materials and Methods. Polysome pellets were resuspended by homogenization in 50 mM HEPES pH 7.6, 25 mM KCl, 5 mM MgCl₂ with (- - -) or without (———) 50 mM EDTA. Approximately 6 A_{260} U of free (A) and 18 A_{260} 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 MgCl₂. 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.

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 artifactually 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 shows that most of the label in the free polysome fraction is present as a peak at 1.57 g/cm³ with a shoulder at 1.53 g/cm³, and that EDTA causes a shift in the mean density of the prominent peak to about 1.54 g/cm³. This indicates that most of the label is in newly synthesized mRNP associated with ribosomes. The relatively high bouyant density of the free polysomal mRNP is due to exposure to 250 mM KCl as described previously (13). The shoulder at 1.53 g/cm³ may represent newly synthesized cytoplasmic 40S struc-

TABLE II

Extent of Free Polysome Contamination of Microsomal Fractions

	Experiment		
	1*	2‡	3§
Total DPM added	8.14 × 10⁶ (100)	7.77 × 10⁶ (100)	7.01 × 10⁶ (100)
DPM recovered in free fraction	6.87 × 10⁶ (84.4)	7.16 × 10⁶ (92.1)	5.78 × 10⁶ (82.4)
DPM recovered in microsomal fraction	9.21 × 10⁵ (11.3)	4.53 × 10⁵ (5.8)	1.09 × 10⁶ (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 H₂O 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.

TABLE III

Extent of Nuclear Leakage and Contamination of Polysome
Pellets

Fraction*	DNA	Total	DPM	Total
	mg	%		%
Total homogenate	13.4	100	4.50×10^6	100
Free	0.97	7.2	8.60×10^5	19.1
Microsomal	1.66	12.4	6.75×10^5	15.0
Nuclear	12.9	96.1	2.22×10^6	49.3
Free polysome pellets	0.025	0.37	1.22×10^4	0.27
Bound polysome pellets	0.035	0.52	5.26×10^4	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.
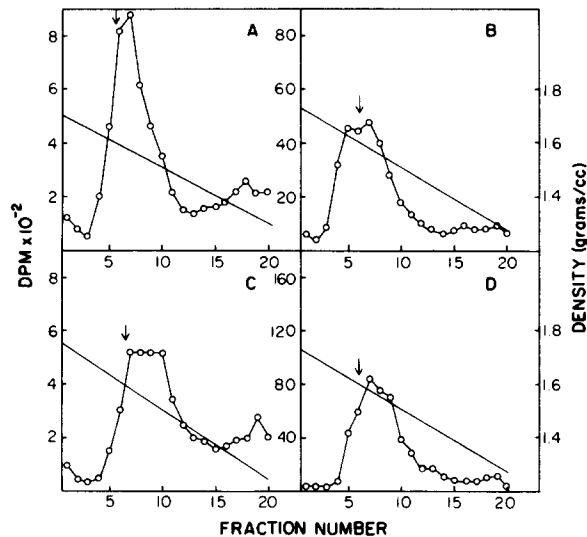
* Fractions are as described in Table I.

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.

tures that have been extensively characterized in rat liver (22, 52). Fig. 2B and D shows that most of the label in the membrane-bound polysome fraction is present as a broad peak coincident with ribosomes exhibiting a mean density of 1.60 g/cm³. EDTA shifts the peak of radioactivity to 1.56 g/cm³, indicating that the label is present in polysomal mRNP. The relatively high bouyant density of the membrane-bound poly-

somal mRNP is due to exposure to 250 mM KCl and sodium deoxycholate (13). In either case up to 20% of the pelleted label may be attributed to genuine nuclear RNP contamination. Taken as a whole these data suggest a negligible amount of nuclear RNP contamination of polysome pellets. Table III also shows that <1% of the total cellular DNA is found in free and membrane-bound polysome pellets. This represents <0.5 and 0.3% contamination by mass of free polysomal RNA and bound polysomal RNA, respectively, with DNA. DNA contamination of sodium acetate-washed, phenol-chloroform-extracted polysomal RNA was unmeasurable.

To determine whether free cytoplasmic RNP (57) contaminate free polysome pellets, we pulse labeled a rat for 3 h with [³H]-orotic acid in the presence of 5-fluoroorotic acid (FOA). This regimen allows the selective labeling of mRNA by inhibiting the maturation of rRNA species and their appearance in the cytoplasm without the unwanted side-effects of such drugs as actinomycin D (12, 56). Polysome profiles of resuspended free polysome pellets prepared from rats labeled by this procedure show that 90% of the label is present in the polysome (>100S) region of the gradient (Fig. 3). EDTA causes a shift of this label to the 0–80S region which indicates it is present in polysome structures (42, 43). When analyzed on CsCl-density gradients the peak of radioactivity is coincident with the ribosomal absorbance peak (Fig. 4). EDTA causes a shift in the density of the mRNA label to 1.47 g/cm³ characteristic of mRNP derived from free polysomes (13). In the
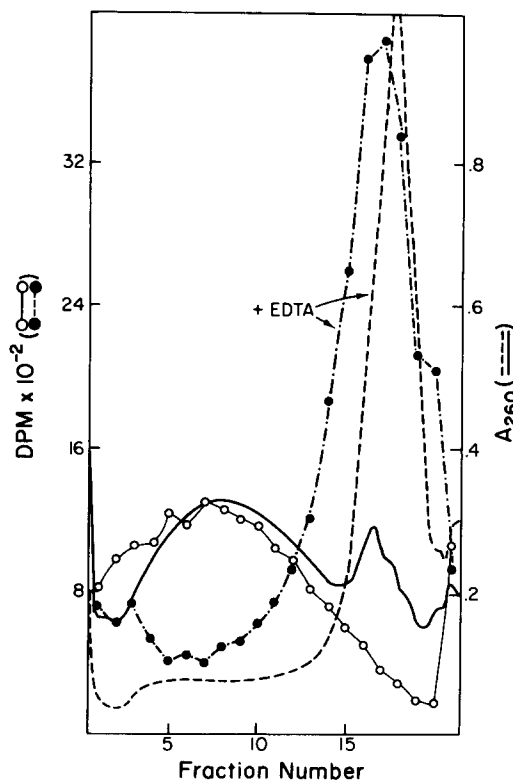

FIGURE 3 Examination of free polysomes for contamination by nonpolysomal cytoplasmic RNP: profiles of pelleted free fraction selectively labeled in mRNA. Free polysomes were prepared from the liver of a rat injected intraperitoneally with 300 μCi of ³H-orotic acid + 1 mg FOA 3 h before sacrifice. Profiles 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 polysomes 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$_{free}$ and Poly(A)⁺ RNA$_{bound}$

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)⁺ RNA$_{free}$ and poly(A)⁺ RNA$_{bound}$ 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)⁺ RNA$_{bound}$ is actually greater than that from poly(A)⁺ RNA$_{free}$ (80 vs. 60 nucleotides) as determined by the method of Kaufman and Gross (23).

Mass-average sizes of driver and tracer molecules must be known for accurate interpretation of hybridization data since they affect the kinetics of the reaction (14). The number-average size of RNA populations is useful in quantitating the number of different species present (see Table V). These values were obtained as described in Materials and Methods by sedimentation of labeled or unlabeled poly(A)⁺ RNA in formamide-sucrose gradients (Fig. 5). The mass-average sizes of poly(A)⁺ RNA$_{free}$ and poly(A)⁺ RNA$_{bound}$ were 2,500 and 2,250 nucleotides and the corresponding number-average sizes were 1,600 and 1,200 nucleotides.

Complexity and Frequency Distribution of Poly(A)⁺ RNA$_{free}$ and Poly(A)⁺ RNA$_{bound}$

DNA complementary to poly(A)⁺ RNA$_{free}$ and poly(A)⁺ RNA$_{bound}$ 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
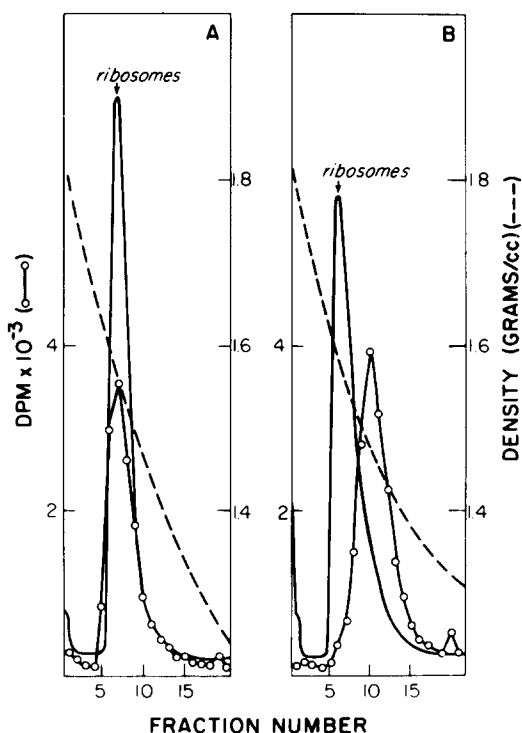

FIGURE 4 Examination of free polysomes for contamination by nonpolysomal cytoplasmic RNP: CsCl density gradients of pelleted free fraction selectively labeled in mRNA. Free polysome pellets from the liver of a rat labeled as in Fig. 3 were rinsed and resuspended in 10 mM triethanolamine pH 7.6, 2 mM MgCl₂, 100 mM NaCl. Equal aliquots either treated with EDTA (B) or left untreated (A) were fixed in formaldehyde and run on CsCl gradients as in Fig. 2. (———) A₂₆₀; (○) ³H dpm.

TABLE V

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

Polysome class	Abundance class	cDNA hy-bridized*	Rate Constant		Nucleotide complexity¶	Number mRNA species‖	Copies/cell**
			Observed‡	Corrected§			
		%					
Free	Abundant	24.2 ± 11.4	15.0 ± 12.5	62.0 ± 51.6	(7.74 ± 6.44) × 10^4	48 ± 40	739 ± 615
	Intermediate	36.8 ± 11.1	1.31 ± 0.81	3.56 ± 2.20	(1.34 ± 0.83) × 10^6	837 ± 517	65 ± 40
	Rare	38.9 ± 6.9	0.059 ± 0.024	0.152 ± 0.062	(3.15 ± 1.29) × 10^7	19,700 ± 8,060	3 ± 1
Bound	Abundant	37.9 ± 2.6	170 ± 30.6	448 ± 80.7	(1.08 ± 0.19) × 10^4	9 ± 2	12,800 ± 2,300
	Intermediate	27.2 ± 5.3	3.94 ± 1.52	14.5 ± 5.6	(3.35 ± 1.29) × 10^5	279 ± 108	294 ± 113
	Rare	34.9 ± 5.6	0.299 ± 0.097	0.857 ± 0.280	(5.66 ± 1.85) × 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.

‡ 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 (10).

§ 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 hydribization 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 (55) and Chamberlin et al. (14), one obtains: $K_{T2} = K_{T1} (L_{T2}/L_{T1}) (\sqrt{L_{D1}} / \sqrt{L_{D2}})$ 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 lengths for the poly(A)$^+$ RNA$_{free}$ reaction were 2,486 and 1,104 nucleotides and for the poly(A)$^+$ RNA$_{bound}$ reaction 2,250 and 1,064 nucleotides. We assume globin mRNA has a complexity of 1200 nucleotides. The corrected K-values corresponding to a complexity of 1,200 nucleotides are thus 3990 and 4042 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 × 6 × 10^{23} molecules/mol)/(RNA nucleotide complexity × 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 (8, 46). 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.
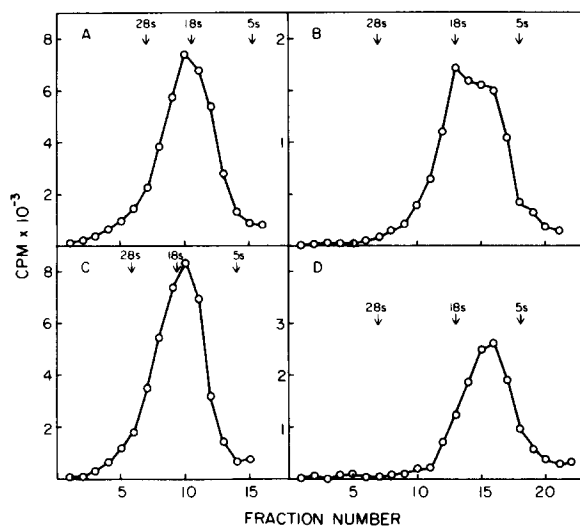

FIGURE 5 Sizing of poly(A)$^+$ RNA fractions. Free (A and B) and membrane-bound (C and D) poly(A)$^+$ RNA either unlabeled (B and D) or labeled (A and C) with ^{3}H–orotic acid + FOA as described in Materials and Methods was dissolved in 75% formamide, 10 mM HEPES pH 7.6, 3 mM EDTA and layered on formamide-sucrose 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 10 ml of Aquasol. 100-µl aliquots of unlabeled poly(A)$^+$ RNA gradient fractions were added to 400 µl of 2.5 × SSC buffer and hybridized to an excess of ^{3}H-poly(U) 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.

FIGURE 6 Homologous and heterologous hybridization reactions. cDNA$_{bound}$ (A) and cDNA$_{free}$ (B) were hybridized to an excess of the homologous (●) or heterologous (○) 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.

be 1,064 and 1,104 nucleotides for cDNA$_{bound}$ and cDNA$_{free}$.

The cDNAs were hybridized to an excess of the homologous template RNA and curves fit to the data with the aid of a nonlinear least squares computer program (Fig. 6). Both reactions take place over a range of 4.5 to 5.0 log rot. This indicates
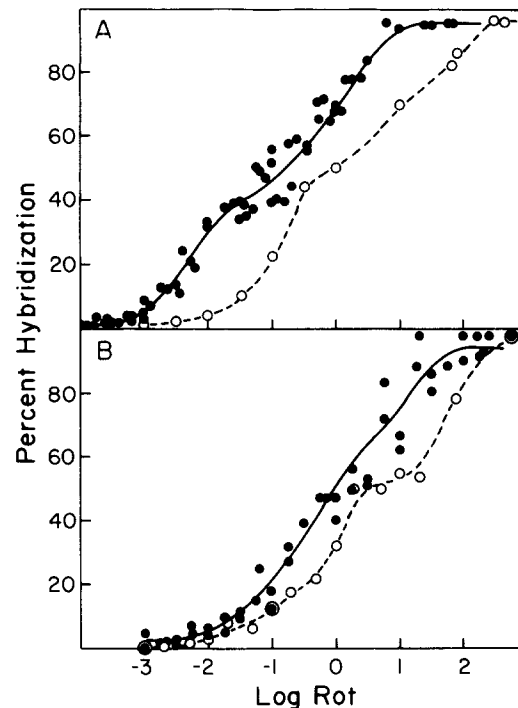
that the driver RNA molecules exist in widely varying concentrations, because a single first-order reaction component occupies about 1.5 to 2.0 log rot. It can be seen that almost 50% of the cDNA$_{bound}$ (Fig. 6A) has hybridized by a log rot of −1 whereas only ~20% of cDNA$_{free}$ (Fig. 6B) has hybridized 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_bound than in poly(A)⁺ RNA_free and that poly(A)⁺ RNA_free is comprised of a greater number of different RNA species than is poly(A)⁺ RNA_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_bound is composed of about 5,000 species and poly(A)⁺ RNA_free of about 20,000 species. A large portion of the mass of poly(A)⁺ RNA_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_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_free and Poly(A)⁺ RNA_bound Qualitatively Unique

The data in Table V would suggest that poly(A)⁺ RNA_bound should be lacking RNA species present in poly(A)⁺ RNA_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_free and poly(A)⁺ RNA_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_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_free and poly(A)⁺ RNA_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_free in poly(A)⁺ RNA_bound' 15.5/3 × 2.37/1.22 = 10.0%; poly(A)⁺ RNA_bound in poly(A)⁺ RNA_free' 1 × 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 poly(A)⁺ RNA_bound sequences have a much narrower frequency distribution in poly(A)⁺ RNA_free. The heterologous curves are displaced by a maximum of 1.2 log rot as opposed to 2.1 log rot for the homologous curves. The heterologous curve involving less abundant
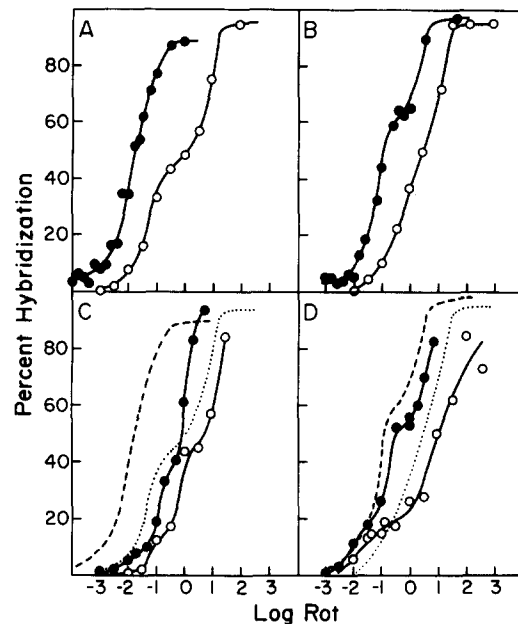

FIGURE 7 Homologous and heterologous hybridizations with kinetically enriched cDNA populations. Kinetically fractionated cDNA_bound (A and C) and cDNA_free (B and D) were hybridized to an excess of the homologous (A and B) or heterologous (C and D) poly(A)⁺ RNA populations and the extent of reaction determined as described in Materials and Methods. RNA concentrations ranged from 0.5 to 1,200 μg/ml. Curves were drawn with the aid of a computer. (●) Reactions involving abundant cDNA; (○) reactions involving less-abundant cDNA. The broken lines in C and D represent the corresponding homologous curves shown in A and B.

cDNA_bound is displaced from the corresponding homologous curve by 0.3 to 1.0 log rot. These sequences are present in poly(A)⁺ RNA_free on the average at about 20–100 fold greater concentration than can be accounted for by cross-contamination and are proportionately present in much greater amounts than are the abundant poly(A)⁺ RNA_bound sequences.

In contrast Fig. 7D shows that poly(A)⁺ RNA_free sequences are present at a greater frequency distribution in poly(A)⁺ RNA_bound. Most striking is the observation that ~20% by mass of less abundant poly(A)⁺ RNA_free is present at greater concentrations in poly(A)⁺ RNA_bound' and about an equal percentage by mass of abundant poly(A)⁺ RNA_free is present at equal concentrations in poly(A)⁺ RNA_bound. At high rot values the heterologous curve involving less abundant cDNA_free is displaced from the homologous curve by over 1.0 log rot which explains why these rare sequences were not detected in the homologous poly(A)⁺ RNA_bound hybridization. They would constitute a very small mass-fraction of poly(A)⁺ RNA_bound and be present at less than one copy per cell in membrane-bound polysomes.

Fig. 7C demonstrates that all of the poly(A)⁺ RNA_bound sequences are present in poly(A)⁺ RNA_free at significant levels above cross-contamination since both heterologous curves are displaced by <2.3 log rot throughout their lengths. In contrast Fig. 7D illustrates that most of the abundant and at least 60% of less abundant poly(A)⁺ RNA_free sequences are present in poly(A)⁺ RNA_bound at significant levels (the curves are displaced by <1 log rot) but the most rare poly(A)⁺ RNA_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 hybridi-
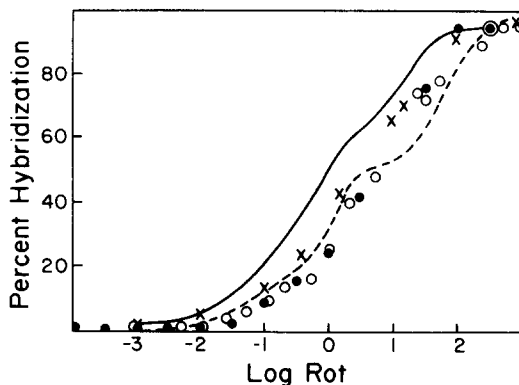
FIGURE 8 Heterologous hybridizations of free cDNA to tightly bound polysomal poly(A)$^+$ RNA fractions. cDNA$_{free}$ was hybridized to poly(A)$^+$ RNA prepared from membrane-bound polysomes isolated as described in Materials and Methods except that the initial homogenization buffer contained 250 mM (●) or 500 mM (○) KCl rather than 75 mM. In the latter case, the 131,000 g_{max} pellet was washed twice with 500 mM KCl buffer to reduce the level of contaminating free or loosely bound polysomes to ~0.1%. In addition, heterologous hybridizations were conducted between cDNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$ prepared from purified rough microsomes (12) washed free of contaminating free polysomes by repeated pelleting through 1 M sucrose (X). The solid line represents the homologous free hybridization curve and the broken line the heterologous cDNA$_{free}$-poly(A)$^+$ RNA$_{bound}$ hybridization curve depicted in Figure 6 B.
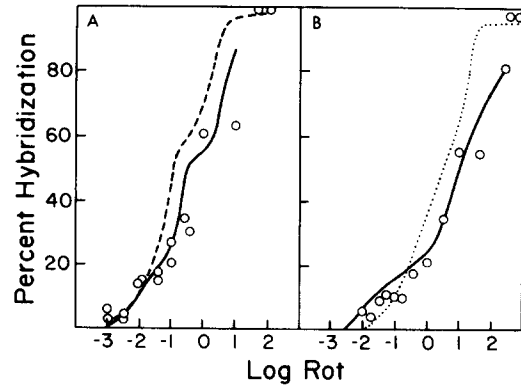
FIGURE 9 Heterologous hybridizations of kinetically enriched cDNA$_{free}$ populations with tightly bound polysomal poly(A)$^+$ RNA. Abundant cDNA$_{free}$ (A) and less-abundant cDNA$_{free}$ (B) were hybridized to an excess of poly(A)$^+$ RNA isolated from tightly bound polysomes prepared in the presence of 0.5 M KCl as described in the legend to Fig. 8. The extent of the reaction was determined by S1 nuclease assay as described in Materials and Methods. RNA concentration varied from 1 to 2,000 μg/ml. The broken lines represent homologous hybridization curves with kinetically fractionated cDNA$_{free}$ and the solid lines heterologous hybridization curves of kinetically fractionated cDNA$_{free}$ with poly(A)$^+$ RNA$_{bound}$ shown in Fig. 7 B and D.

zation 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 bouyant 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.
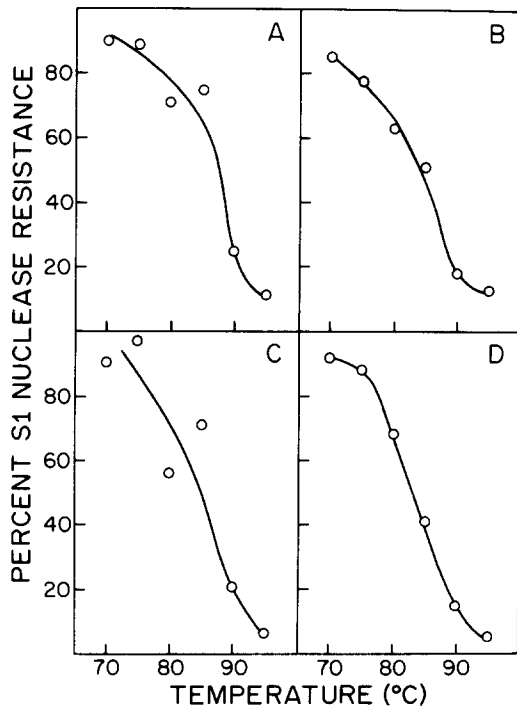
FIGURE 10 Validity of the assay for duplex formation: melting curves of homologous and heterologous hybrids. Aliquots of the homologous and heterologous duplexes of cDNA$_{free}$ and cDNA$_{bound}$ with poly(A)$^+$ RNA$_{free}$ and poly(A)$^+$ RNA$_{bound}$ were incubated in 0.18 M NaCl for 20 min at 70°–95°C. Reaction mixtures were frozen in liquid N$_2$, expelled into S1 nuclease digestion buffer and assayed as described in Materials and Methods. (A) Homologous cDNA$_{free}$–poly(A)$^+$ RNA$_{free}$, melting temperature (T_m) = 84.5°C; (B) Homologous cDNA$_{bound}$–poly(A)$^+$ RNA$_{bound}$, T_m = 86.8°C; (C) Heterologous cDNA$_{free}$–poly(A)$^+$ RNA$_{bound}$, T_m = 83.0°C; (D) Heterologous cDNA$_{bound}$–poly(A)$^+$ RNA$_{free}$, T_m = 85.3°C.

This overlap involves a large fraction by mass of the poly(A)$^+$ RNA, and apparently cannot be attributed to cross-contamination. Poly(A)$^+$ RNA$_{free}$ 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$_{free}$ 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$_{free}$. The homologous hybridizations indicate that poly(A)$^+$ RNA$_{free}$ 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$_{free}$ 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$_{free}$ 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 ^{3}H-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 × 0.1 × 3 or 0.9% contamination of free polysomes with membrane-bound. However, it is more likely that most of the pelleted label is due to free polysomes contaminating the purified rough microsome preparation (our unpublished observations).

In the case of poly(A)$^+$ RNA$_{free}$ contaminating poly(A)$^+$ RNA$_{bound}$, we have assumed the poly(A)$^+$ RNA$_{free}$ 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$_{free}$ 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 artifactual 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 artifactually 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$_{free}$. 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 artifactually 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$_{free}$ 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$_{free}$ 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$_{bound}$ 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$_{bound}$ sequences are present at a 30 to 150 fold reduced quantity and less abundant poly(A)$^+$ RNA$_{bound}$ sequences at a 6 to 30 fold reduced quantity in free polysomes. If all of the poly(A)$^+$ RNA$_{bound}$ 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$_{bound}$ is on the average fivefold lower than on abundant poly(A)$^+$ RNA$_{bound}$. 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$_{free}$ 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$_{bound}$/poly(A)$^+_{free}$ 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 peptide 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$_{bound}$ 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 polysomal poly(A)$^+$ RNA populations remains unknown. A possible artifactual 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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