SYNTHESIS OF RAT LIVER MICROSOMAL CYTOCHROME b_5 BY FREE RIBOSOMES

R. A. RACHUBINSKI, D. P. S. VERMA, and J. J. M. BERGERON

From the Departments of Anatomy and Biology, McGill University, Montreal, Quebec, Canada H3A 2B2

ABSTRACT

Free and membrane-bound polyribosomes were separated from liver homogenates and characterized by electron microscopy. Using the wheat germ cell-free translation system, total translation products of poly A^+RNA extracted from free polyribosomes (poly A^+RNA_f) showed some correlation to total liver cytosol proteins. In contrast, translation products of poly A^+RNA from membrane-bound polyribosomes (poly A^+RNA_{mb}) showed some similarity to rat serum. Antibody to purified rat serum albumin immunoprecipitated from only the translation products of poly A^+RNA_{mb} a single polypeptide of mol wt 68,000., i.e., 3,000 greater than secreted serum albumin. In contrast, antibody to detergent-extracted cytochrome b_5 immunoprecipitated from only the translation products of poly A^+RNA_f a single polypeptide of mol wt 17,500, identical to that of microsomal cytochrome b_5. A consideration of the known properties of cytochrome b_5 is consistent with an exclusive site of synthesis on free ribosomes.

KEY WORDS   membrane biogenesis   ·   cell-free translation   ·   subcellular fractionation

Cytochrome b_5 is an integral membrane protein of the endoplasmic reticulum (ER)[1] of the hepatocyte (1, 41, 61, 66, 67, 70). The protein has been purified and sequenced (40, 42, 43, 46, 47, 61). The purified protein has been shown to bind to liposomes and microsomes in vitro with complete functional fidelity (56, 69). Both for the native protein *in situ* and for that bound to membrane in vitro, the membrane interaction has been documented to a hydrophobic sequence at the carboxy-terminal end of the molecule (20, 44, 56, 69), while the heme-containing amino-terminal portion protrudes into the cell sol (45, 61, 69). On the basis of these detailed findings, as well as reports documenting the ubiquitous distribution of cytochrome b_5 to several intracellular organelles other than the ER (i.e., Golgi apparatus [7, 12, 19, 26, 28, 38], plasmalemma [28], mitochondria [55, 60, 69], peroxisomes [14, 55], nuclear envelope [4, 21, 29, 59]), the synthesis of cytochrome b_5 might be assigned to the free polyribosome population of the hepatocyte rather than to membrane-bound ribosomes as has been suggested by the studies of Elhammer et al. (17) and Harano and Omura (24).

Using the heterologous wheat germ translation system, poly A^+RNA coding for cytochrome b_5 is herein reported to be associated preferentially with free polyribosomes of the hepatocyte.

MATERIALS AND METHODS

Chemicals

All routine laboratory chemicals were obtained from Sigma

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ER, endoplasmic reticulum; poly A^+RNA_f, free ribosomal polyadenylated RNA; poly A^+RNA_{mb}, membrane-bound ribosomal polyadenylated RNA; RSA, rat serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Montreal, Canada) and were of the highest purity available.

Separation of Free and Membrane-bound Ribosomes

The separation procedure was based on the method of Ramsey and Steele (51) but modified[2] to retain mRNA integrity. Thus, livers from Sherman rats fasted overnight were removed and homogenized (1:8 wt/vol) in 0.25 M sucrose in buffer A (0.2 M Tris-acetate (pH 8.5 at 4°C), 0.075 M potassium acetate, 0.01 M magnesium acetate, 0.006 M 2-mercaptoethanol, cycloheximide (5 µg/ml), heparin [150 U/ml]). Membrane-bound ribosome fractions were sedimented for 2 min at 740 g_{avg}, then for 12 min at 131,000 g_{avg} in a Beckman SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and RNA was extracted from the pellet (see below). Free ribosome fractions were isolated from the supernate by isopycnic centrifugation on discontinuous sucrose gradients according to the procedure of Ramsey and Steele (51), except that the specimens were centrifuged at 362,000 g_{max} for 7 h in a Beckman 60Ti rotor and all sucrose solutions were made up in buffer A. All results are based on six separate fractionations carried out as above.

Electron Microscopy

After resuspension of the membrane-bound and free ribosome fractions in 0.25 M sucrose in buffer A, small aliquots from each fraction were fixed in buffered 2% glutaraldehyde and sedimented at 50,000 g_{max} for 30 min. Pellets were postfixed in 1% OsO_4, block-stained with uranyl acetate (6), and embedded in Epon. Thin sections were cut through the depth of the oriented pellet as described previously (16). For quantitative analysis of ribosome distributions, fractions were processed by the modified (6) random sampling technique of Baudhuin et al. (3) and Wibo et al. (75) using Millipore filters (0.22 µm, type GS, Millipore Limited, Mississauga, Ontario), and scoring was carried out on electron micrographs printed to a final magnification of 50,000.

Isolation of RNA

The pelleted subcellular fractions were immediately resuspended (5 ml/g weight of liver) in 0.1 M Tris-acetate (pH 9.0 at 20°C), 0.1 M sodium acetate, 0.01 M EDTA, 1% SDS (58) containing Proteinase K (Beckman Instruments, Spinco Div.) at 200 µg/ml (76). RNA was extracted in phenol:chloroform:isoamyl alcohol and precipitated from the aqueous phase as described by Shore and Tata (58). DNA was removed by three washings in 3 M sodium acetate (pH 6.0 at 4°C) (73).

Poly A+RNA was isolated by oligo-(dT) cellulose chromatography (2) (type III, Collaborative Research Inc., Waltham, Mass.) and pelleted at 210,000 g_{max} for 12 h in an A321 rotor (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). RNA content was measured as described by Munro and Fleck (39) and Bergeron et al. (5).

Cell-free Protein Synthesis and Characterization of Translation Products

Translation of poly A+RNA was carried out using a modified wheat germ system (72) (General Mills, Chemicals, Inc., Min-

[2] Attempts to use Triton X-100 to remove nuclei and ribosomes (51) resulted in a loss of mRNA integrity as assessed by cell-free protein synthesis.

neapolis, Minn.) prepared according to Marcu and Dudock (37) and treated with micrococcal nuclease (150 U/ml) for 10 min at 20°C (48). The translation was carried out as described by Verma et al. (72) containing in 100 µl of reaction mixture the following: 15 µl of micrococcal nuclease-treated wheat germ S-23, 1 mM ATP, 8 mM creatine phosphate, 4 µg of creatine phosphokinase, 25 µM GTP, 2 mM dithiothreitol, 90 mM potassium acetate, 2.5 mM magnesium acetate, 2 µg of wheat germ tRNA, 40 µM spermidine tetrahydrochloride (neutralized), 10 µCi L-[3,4,5-^{3}H(N)]leucine (115 Ci/mmol, New England Nuclear, Montreal, Canada), 50 µM each of 19 unlabeled amino acids (less leucine), and 20 mM HEPES buffer (pH 7.6 at 20°C). The reaction mixture was incubated for 90 min at 25°C, and the reaction was terminated by rapidly cooling the mixture on ice. The reproducibility of each batch of wheat germ S-23 was assessed with globin mRNA which had an activity of 6×10^5 cpm/µg RNA for the experiments described throughout the article.

The cell-free translation products were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as follows: Discontinuous SDS-PAGE was carried out using an apparatus based on that described by Studier (71). The resolving gel was either of a single phase (7.5 or 12.5% acrylamide) or of a linear gradient (7-20% acrylamide) essentially as described by Blobel and Dobberstein (9). The gels were stained in Coomassie Brilliant Blue. Quantification of Coomassie Brilliant Blue-stained proteins was done by the method of Fenner et al. (18). Gels were processed for liquid scintillation counting by slicing into 2-mm slices. Each slice was dissolved by the addition of 0.5 ml of 30% H_2O_2 maintained overnight at 37°C as modified from Bergeron et al. (5) and Strauss et al. (62). Counts per minute were converted to disintegrations per minute by the use of the channels ratio and external standard quench curves as outlined by Wang and Willis (74). Fluorography was according to Laskey and Mills (31).

Isolation of Serum and Cytosol

Normal rat serum was prepared as described by Bergeron et al. (6). Total liver cytosol proteins were isolated following intracardiac perfusion of the entire animal with lactated Ringer's buffer (8) to remove blood. The cell sol fraction was isolated from liver homogenates (1:3 wt/vol in 0.25 M sucrose, Tris-HCl (0.05 M, pH 7.4), 0.025 M KCl, 0.005 M $MgCl_2$) and differentially centrifuged at 10,000 g_{max} for 10 min, then the supernate centrifuged for 1 h at 100,000 g_{avg}. This final supernate was operationally defined as cell sol. SDS-PAGE of the fraction indicated little or no Coomassie Brilliant Blue-stained bands corresponding to albumin or other serum polypeptides.

Isolation and Characterization of Cytochrome b_5, Rat Serum Albumin, and Corresponding Antibodies

Cytochrome b_5 was partially purified from rat liver microsomes by the detergent extraction procedure of Spatz and Strittmatter (61) and monitored by the characteristic difference spectrum between the reduced and oxidized states at 409 and 424 nm (61). Reduction of oxidized cytochrome b_5 was achieved by the addition of sodium dithionite (61). Difference spectra were determined on an Aminco DW-2, UV-VIS spectrophotometer (American Instrument Co., Silver Spring, Md.) very generously made available by Dr. J. G. Joly, St. Luke's Hospital, Montreal. The isolation procedure was carried out seven times with similar results.

Cytochrome b_5 was purified to homogeneity by SDS-PAGE

on preparative 12.5% acrylamide gels. The prominent band of 17,500 mol wt was excised, and the protein was eluted from the gel as described by Lazarides (32). Antibodies were raised in rabbits essentially as described by Lazarides and Weber (33) except that the initial antigen injection was in 50% complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), 50% normal saline, and that booster injections were in 50% incomplete Freund's adjuvant, 50% normal saline. Five injections were given at 2-wk intervals.

Commercially prepared rat serum albumin (RSA) was purified to homogeneity by SDS-PAGE on preparative 7.5% acrylamide gels. The prominent band of 65,000 mol wt was excised, and the protein was eluted from the gel and the antibodies were obtained as for cytochrome b_5.

Protein Estimations

Protein concentration was determined by the procedure of Lowry et al. (36) with bovine serum albumin (BSA) as standard. Protein estimations on samples containing Triton X-100 detergent were carried out using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Mississauga, Canada) with bovine gamma globulin containing an equal percentage of Triton X-100 as standard.

Immunoelectrophoresis and Immunoprecipitation

Immunoelectrophoresis was performed in 1% agarose (Millipore Limited). Normal rabbit serum or antisera to cytochrome b_5 or to RSA were allowed to diffuse into the agarose for 24 h at room temperature against either total rat serum or solubilized, salt-washed rat liver microsomes. After washing for 48–72 h in 0.15 M NaCl, 0.1% sodium azide and for 1 h in glass doubly distilled, de-ionized water, the agarose was dried and stained in Coomassie Brilliant Blue. Immunoprecipitations of radiolabeled cytochrome b_5 and RSA synthesized in vitro were performed using antisera followed by incubation with protein A attached to inactivated Staphylococcus aureus (Enzyme Center, Boston, Mass.) according to Lingappa et al. (35). Immunoprecipitation was also carried out using goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, Ind.) instead of immobilized protein A as described above. The results were similar but background was reduced with the immobilized protein A method.

RESULTS

Separation of Free and Membrane-bound Ribosome Fractions and Characterization of Products Synthesized by Poly A^+RNA_f and Poly A^+RNA_{mb}

The amount of cross-contamination in the free and membrane-bound ribosome fractions was assessed by electron microscopy (Figs. 1 and 2). Through the depth pellet analysis of the membrane-bound fraction indicated significant contamination by free ribosomes which was most evident at the top of the pellet (Fig. 1 a) but progressively reduced through the depth of the pellet (Fig. 1 b and c). Quantitative analysis of

randomly prepared samples (see Materials and Methods) indicated that, of 996 ribosomes counted, 81.4% were judged as being clearly associated with membranous elements. The remaining 18.6% were ascribed to contamination by free ribosomes.

The free ribosome fractions, in contrast, was found to be highly purified. Although an occasional smooth-surfaced vesicle was found (Fig. 2), ribosomes were not attached to these vesicles.

Quantitative analysis of the proportion of RNA found in the two fractions indicated 56% associated with the membrane-found fraction (Table I) with similar proportions of poly A^+RNA from both the membrane-bound and free ribosome fractions. However, the efficiency of translation was much higher with free ribosomal polyadenylated RNA (poly A^+RNA_f) than with membrane-bound ribosomal polyadenylated RNA (poly A^+RNA_{mb}). The poly A^+RNA_{mb} was translated into a spectrum of polypeptides with some homology to serum proteins, whereas poly A^+RNA_f translation products showed some similarity to liver cytosol proteins (Fig. 3). Of note was the synthesis of a polypeptide identified as albumin (noted by arrow, Fig. 3, see also Fig. 7) by poly A^+RNA_{mb}. Only small amounts of this polypeptide were synthesized by poly A^+RNA_f (noted by double-headed arrow in Fig. 3). In contrast, a band with a mobility equivalent to 37,000 mol wt (noted by asterisk, Fig. 3) appeared to be synthesized primarily by poly A^+RNA_f.

Isolation of Cytochrome b_5 and Characterization of Antiserum to Cytochrome b_5

After purification of cytochrome b_5 by the method outlined by Spatz and Strittmatter (61), it was found that the final material was contaminated (Fig. 4). Assessment of four selected steps during the isolation procedure indicated a 37-fold purification over salt-washed microsomes (Table II). This corresponded to only 14.6% of the total protein of the purified material as assessed by the known extinction coefficient (163 cm^{-1} mM^{-1} [61]) of purified cytochrome b_5. The band of mobility corresponding to 17,500 mol wt (indicated by arrow in Fig. 4) was judged to be cytochrome b_5, as the band corresponded not only to 14.5% of the total protein (as assessed by the method of Fenner et al. [18]) but also to the known molecular weight of cytochrome b_5 (61). Thus, this band was excised from the gel, electrophoretically eluted,
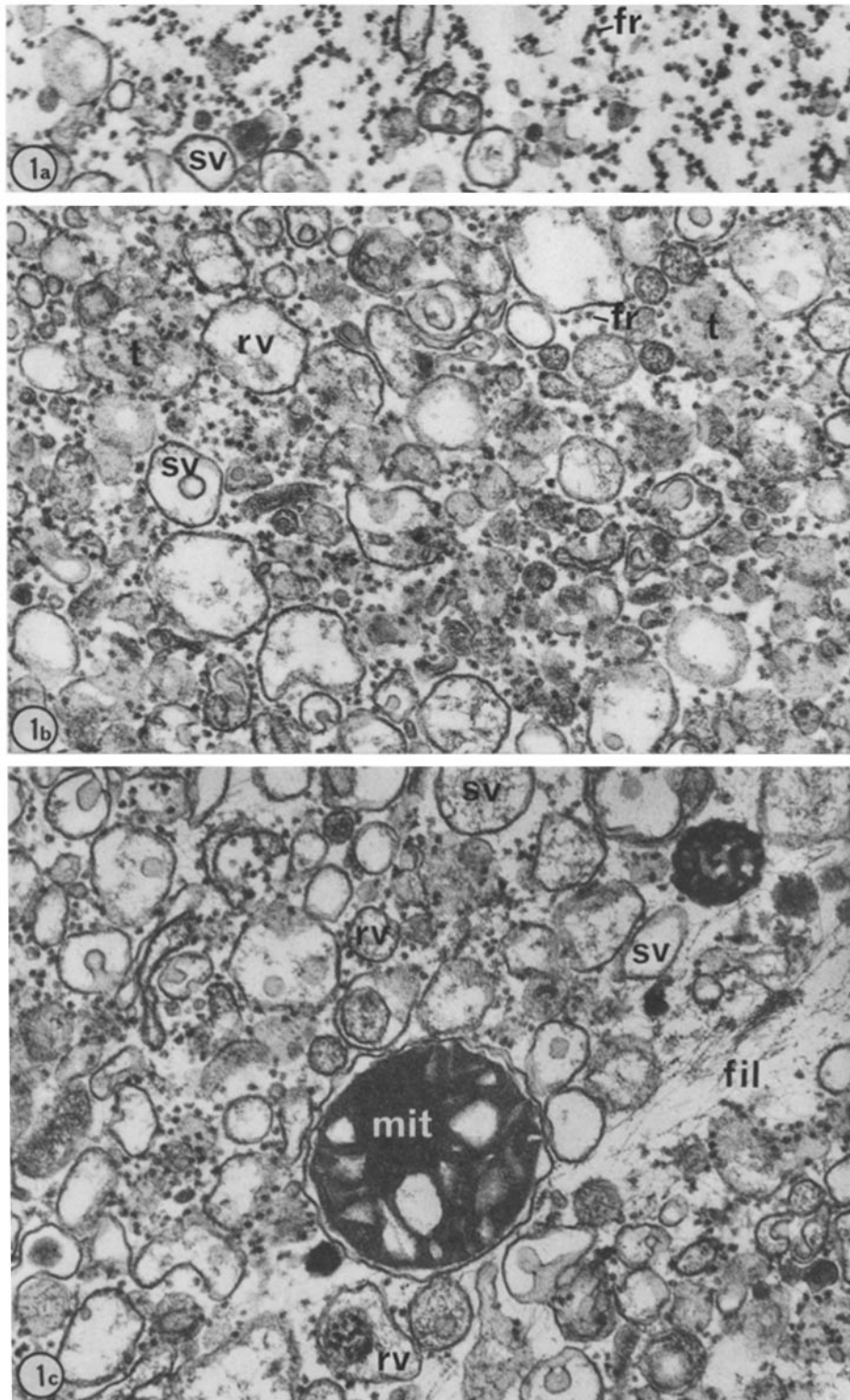
FIGURE 1 Section through a pellet of the total membrane-bound ribosome fraction showing the top, middle, and bottom of the pellet (a, b, and c, respectively). The preparation consists primarily of smooth vesicular elements (sv) or ribosome-studded vesicular elements (rv), which vary in size. Both tangential (t) and cross-sectional profiles of ribosome-studded vesicular elements are seen. There is contamination by free ribosomes (fr) at the top of the pellet (a), but this contamination is progressively reduced through the depth of the pellet (b and c). The bottom of the pellet (c) contains the larger elements such as mitochondria (mit) and filamentous structures (fil). Few intact nuclear profiles were seen; however, there was evidence for nuclear lysis. × 54,000.
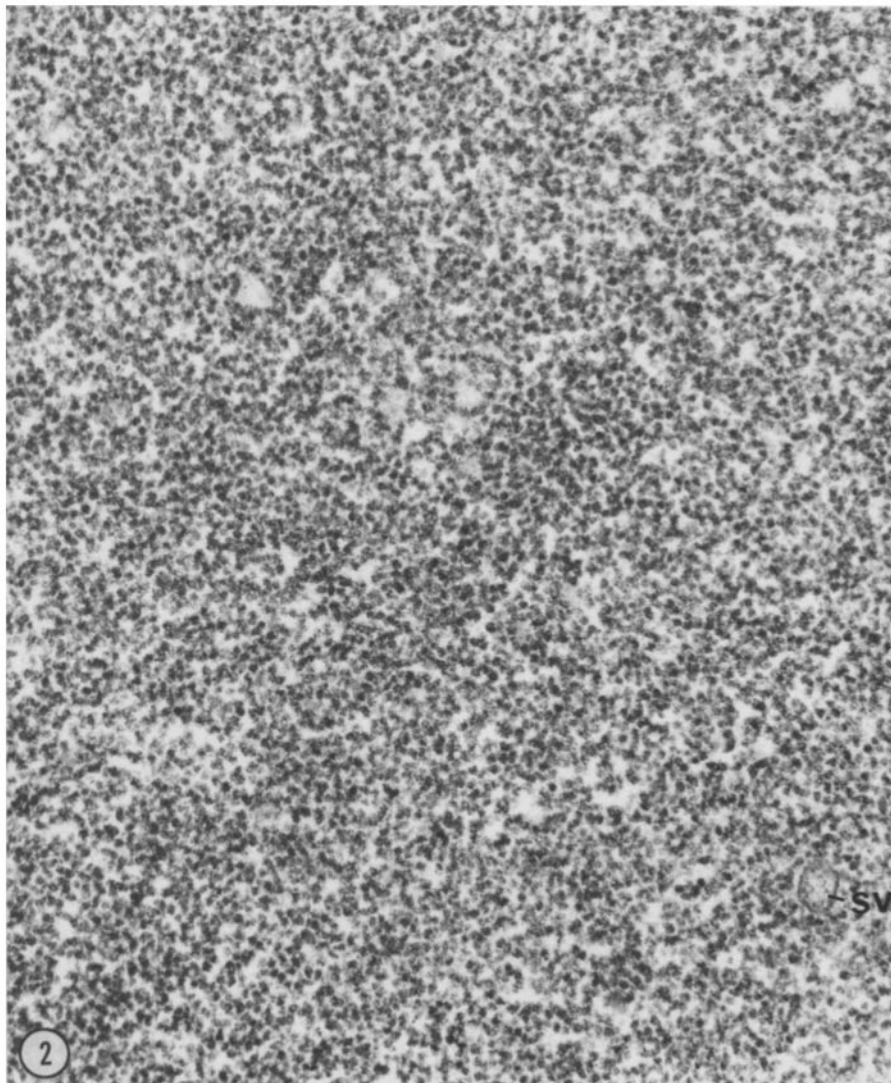
FIGURE 2 Free ribosome fraction, sectioned pellet showing a field at the middle of the pellet. Free ribosomes are seen with rare contamination by a smooth vesicle (sv). There is no contamination of the fraction by any vesicles with attached ribosomes. × 54,000.

and antiserum was prepared. Immunoelectrophoresis (Fig. 5) of the antiserum showed the lack of cross-reaction to serum and the immunoprecipitation of one band from solubilized microsomes. In addition, the antiserum immunoprecipitated cytochrome b_5 from the Triton X-100 extract of salt-washed microsomes as judged by the decrease in b_5 spectrum of the supernatant fraction from the immunoprecipitates (Fig. 6).

Antiserum to purified RSA showed specificity to one band in serum as well as to one band in microsomes (Fig. 5). Ouchterlony immunodiffu-

sion experiments (not shown) revealed that this band corresponded to albumin.[3] Preimmune sera produced no immunoprecipitates against albumin, serum, or solubilized microsomes (Figs. 5 and 6).

In Vitro Translation of
Cytochrome b_5 and Albumin

Immunoprecipitation of in vitro synthesized cy-

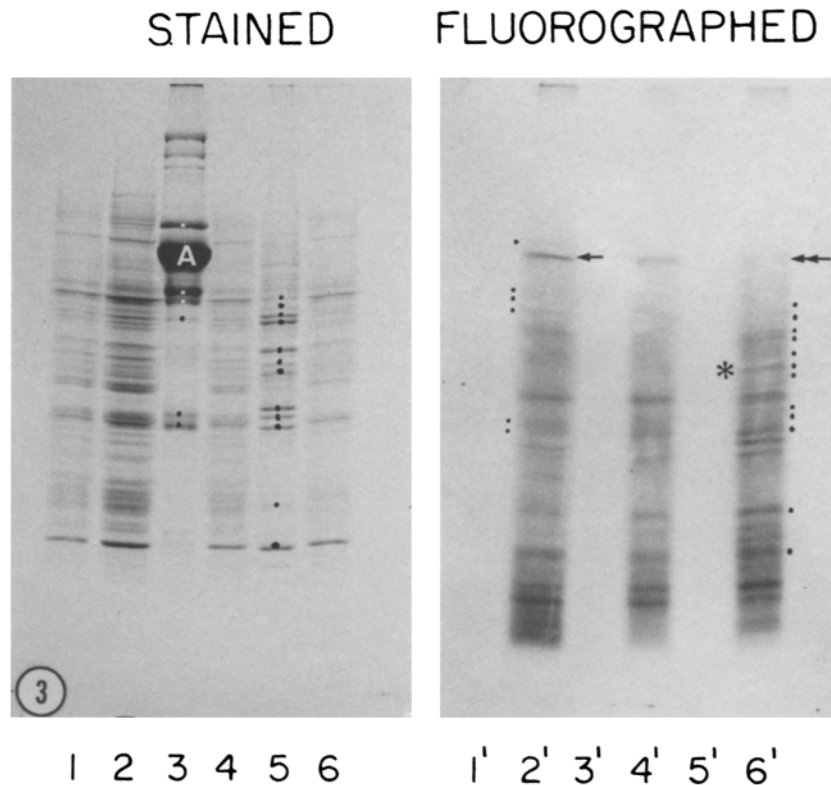[3] Presumably albumin in the content of microsomal vesicles (6, 15, 27, 49).

STAINED   FLUOROGRAPHED

1 2 3 4 5 6   1' 2' 3' 4' 5' 6'

FIGURE 3 Fluorography of labeled polypeptides synthesized in the wheat germ cell-free translation system by poly A^+RNA_{mb} and poly A^+RNA_f fractions. The Coomassie Brilliant Blue-stained gel is at left, while the corresponding fluorogram is at right. 10^5 cpm of radioactive product was applied to lane 2 (poly A^+RNA_{mb}), lane 4 (total liver homogenate polyadenylated RNA), lane 6 (poly A^+RNA_f). In addition, 20 μl of endogenous wheat germ translation product (a volume comparable to the volumes of the three heterologous translation products) were applied to lane 1. To lanes 3 and 5 were applied 100 μg of unlabeled rat serum proteins and unlabeled rat liver cytosol proteins, respectively. The major stained protein of rat serum (labeled A, lane 3) corresponds to the mobility of RSA. The radioactive band judged to correspond with albumin (and confirmed in Fig. 7) is noted by a single and double-headed arrow in lanes 2' and 6', respectively. Other bands in lanes 2' and 6' judged to bear a relationship to those in lanes 3 and 5, respectively, have been noted by dots and an asterisk. The asterisk refers to a polypeptide of 37,000 mol wt which is translated from poly A^+RNA_f but not poly A^+RNA_{mb} and corresponds in mobility to a protein found in the cytosol fraction (lane 5). The fluorograph shows noteworthy differences in the translation products of poly A^+RNA_{mb} (lane 2') and poly A^+RNA_f (lane 6') and no detectable endogenous synthesis of polypeptides by the wheat germ system (lane 1').

tochrome b_5 and albumin was carried out. Using as starting material equal counts of the primary translation products synthesized by poly A^+RNA_{mb} and poly A^+RNA_f (Fig. 3) and the antisera described above, single immunoprecipitates were produced (Figs. 7–10). The antiserum to albumin (Fig. 7) immunoprecipitated a single polypeptide of mobility corresponding to 68,000 mol wt (distinctly slower mobility compared to that of serum albumin) and synthesized only by poly A^+RNA_{mb}. By gel slicing (Fig. 8), it was

estimated that >90% of this protein was synthesized by poly A^+RNA_{mb} and only 10% by poly A^+RNA_f. In contrast, immunoprecipitation with anti-cytochrome b_5 antiserum produced only a single polypeptide of mobility corresponding to 17,500 mol wt. This corresponded exactly with the mobility of cytochrome b_5 as found in salt-washed microsomes (Fig. 9). This polypeptide was synthesized primarily by poly A^+RNA_f, and quantitation by gel slicing (Fig. 10) indicated 85% synthesized by poly A^+RNA_f and 15% by poly A^+RNA_{mb}.

TABLE I

Recovery and Activity of Poly A⁺RNA from Free and Membrane-bound Ribosomes

	Total RNA	Poly A⁺RNA	Activity
		%	
Free ribosome fraction	1,398*	2.58	6.2 × 10⁶‡
Membrane-bound ribosome fraction	1,792	2.30	1.3 × 10⁶

The distribution of poly A⁺RNA was estimated after chromatography on oligo-(dT) cellulose. The activity of poly A⁺RNA was determined from the total [³H]-leucine radioactivity incorporated by the in vitro wheat germ system into TCA-insoluble material.

* μg RNA/g liver wet weight.

‡ dpm [³H]leucine incorporated/μg poly A⁺RNA.

68,000 ►
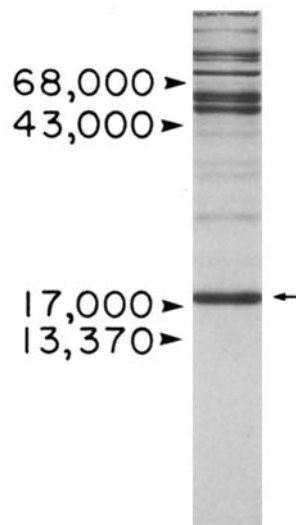43,000 ►

17,000 ► ◄
13,370 ►

FIGURE 4   12.5% SDS-PAGE gel of cytochrome b_5 purified according to the procedure of Spatz and Strittmatter (61). The arrow indicates a protein with electrophoretic mobility similar to that of cytochrome b_5. The remaining bands represent contaminating proteins of greater molecular weight. The numbers at left represent the molecular weights of standard protein markers; cytochrome c, 13,370; ribonuclease, 17,000; ovalbumin, 43,000; and BSA, 68,000.

DISCUSSION

The results indicate a near-exclusive location for the site of synthesis of cytochrome b_5—an integral membrane protein of the ER—to the free polyribosome population of the hepatocyte. The use of the Ramsey and Steele (51) procedure ensured that the total free and membrane-bound ribosomal

populations were assessed and not just a small and perhaps unrepresentative population of each as in other procedures (e.g., references 5, 10, and 58). However, it was necessary for us to determine by electron microscopy the degree of cross-contamination of the fractions, as such ultrastructural observations have not heretofore been carried out on these fractions. The estimate of a maximal 15% contamination of the membrane-bound ribosomal population by free ribosomes is greater than the estimate made by Ramsey and Steele (52) using biochemical criteria. However, our own biochemical criteria (Figs. 3 and 7–10) indicate minimal cross-contamination, in agreement with their findings.

To assess the possibly unrepresentative degradation of select RNA populations in each of the fractions, we attempted to correlate the synthesis of total polypeptides with the predited major functions of each of the ribosomal populations, namely, secretory proteins, serum (e.g., references 6, 22, 23, 49, and 54) by the membrane-bound ribosomes, and cytosol proteins by the free ribosomes. Within the limitations of the cell-free protein-synthesizing system used in the present study, such a correlation was indeed found to exist.

TABLE II

Purification of Cytochrome b_5

Purification* step	b_5‡	% b_5§ (Spectrophotometry)	Purification fold	% b_5‖ (SDS-PAGE)
A	0.24	0.39	1.0	0.40
B	0.77	1.3	3.3	ND¶
C	7.17	12.0	30.8	ND
D	8.71	14.6	37.4	14.5

* The letters refer to steps in the purification procedure of Spatz and Strittmatter (61) which were selected for spectrophotometric determinations of cytochrome b_5 content with the Aminco spectrophotometer. A, salt-washed microsomes; B, postmicrosomal supernate after detergent solubilization (1.5% Triton X-100 overnight at 4°C); C, eluant after G-75 Sephadex chromatography (in 0.4% deoxycholate); D, eluant after G-25 Sephadex chromatography (0.02 M Tris-acetate, pH 8.1, 0.2 mM EDTA).

‡ nmol Cytochrome b_5/mg protein.

§ % Total protein corresponding to cytochrome b_5 determined from consideration of the extinction coefficient (163 cm⁻¹ mM⁻¹ [61]) and mol wt (17,500) of cytochrome b_5 (61).

‖ Method of Fenner et al. (18), % of total Coomassie Brilliant Blue stained protein in band of 17,500 mol wt.
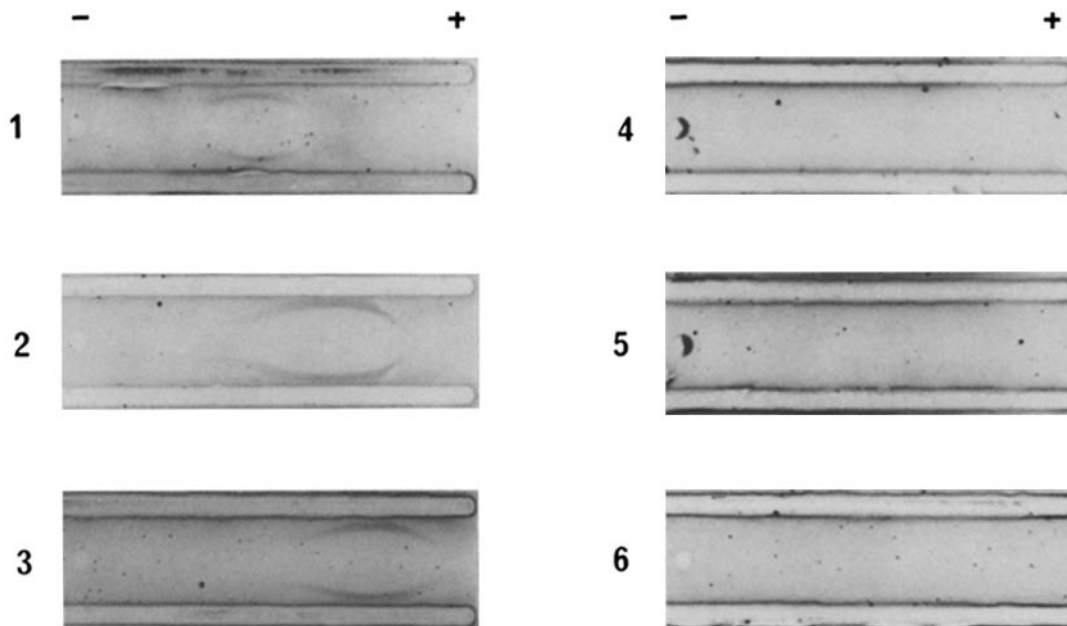
¶ Not determined.

FIGURE 5   Immunoelectrophoretic analysis of antisera to electrophoretically eluted cytochrome b_5 and RSA. Immunoelectrophoresis was performed as described in Materials and Methods. Lane *1*, 8.75 μg of solubilized liver microsomes against anti-cytochrome b_5; lane *2*, 1.8 μg of rat serum against anti-RSA; lane *3*, 17.5 μg of solubilized liver microsomes against anti-RSA; lane *4*, 6.0 μg of rat serum against anti-cytochrome b_5; lane *5*, 8.75 μg of total liver microsomes against normal rabbit serum; lane *6*, 1.8 μg of normal rat serum against normal rabbit serum.

It was necessary to characterize the purification procedure of Spatz and Strittmatter (61) for cytochrome b_5, as in our hands this procedure did not result in a single polypeptide on SDS-PAGE gels.[4] The 17,500 mol wt band (Fig. 4) on SDS-PAGE preparative gels was identified as cytochrome b_5 by two criteria. Firstly, it had a mol wt of 17,500, similar to that predicted for rabbit liver cytochrome b_5 (61). Secondly, by the technique of Fenner et al. (18), this band corresponded to 14.6% of the total protein, similar to the proportion independently assessed by spectrophotometry (Table II). Furthermore, the technique of Fenner et al. (18) showed a 37-fold purification of this polypeptide over that found in parent salt-washed microsomes, similar to that predicted by spectrophotometry of the total preparations (parent salt-washed microsomes and Sephadex G-25 eluant, Table II). The rabbit antiserum raised against this polypeptide was shown to interact with only one protein from microsomes as demonstrated by im-

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[4] The isolation procedure for cytochrome b_5 has undergone noteworthy changes (68) from the original report (61).

munoelectrophoresis. That the antiserum was specific for cytochrome b_5 was demonstrated by the dose-dependent loss of the characteristic difference spectrum of the cytochrome in supernates of immunoprecipitates.

The final control consisted of the use of anti-RSA antiserum. Immunoprecipitates of total in vitro translation products showed synthesis of a band of 68,000 mol wt only by RNA extracted from the membrane-bound ribosome fraction. The near-exclusive site of synthesis of albumin by this fraction has long been known (e.g., references 5, 25, 30, and 53). In addition, the decreased mobility of the product (3,000 daltons greater than the secreted form of serum albumin) would correspond to the "prepro" leader sequence (24 amino acids [63–65]) which would not be expected to be processed by the wheat germ system (13, 35, 57, 64, 72).

Previous work on the site of synthesis of cytochrome b_5 using less direct techniques has ascribed the site of synthesis to the membrane-bound ribosomes (17, 24). However, the present findings (deduced from the subcellular localization of poly A^+RNA coding for cytochrome b_5) are consistent
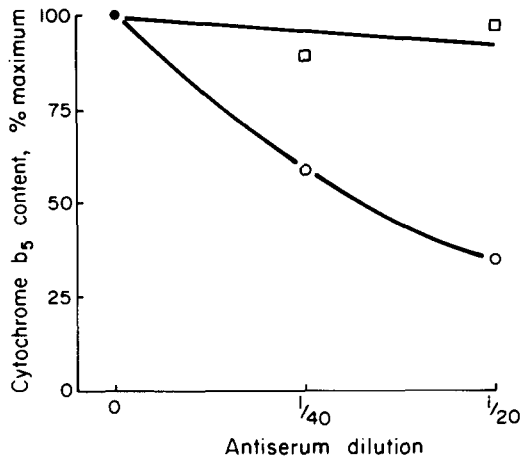
FIGURE 6 Specificity of antiserum to cytochrome b_5. Immunoprecipitation of the Triton X-100 extract of salt-washed microsomes was carried out with increasing concentrations of antiserum directed against the polypeptide of mol wt 17,500 of Fig. 4 (○) and compared to that of normal rabbit serum (□). After immunoprecipitation, the supernate was assessed for cytochrome b_5 content by spectrophotometry. Only the cytochrome b_5 antiserum results in a dose-dependent loss of b_5 spectrum. Maximum cytochrome b_5 content of the supernate (100%) was determined by the addition of H_2O instead of antiserum (●).
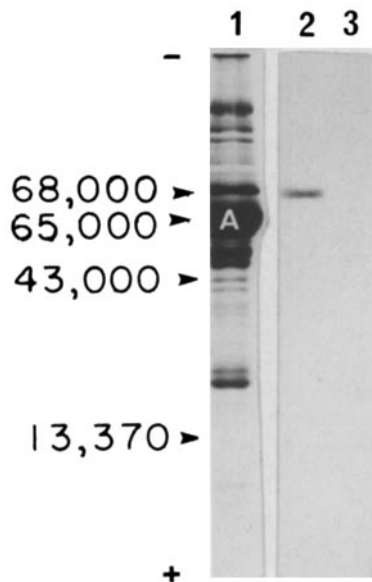
FIGURES 7-10 SDS-PAGE analysis of radiolabeled immunoprecipitates. Identical amounts in radioactivity units (2 × 10^5 cpm) of total translation products from poly A^+RNA_{mb} or poly A^+RNA_f were incubated with anti-RSA (Figs. 7 and 8) or anticytochrome b_5 (Figs. 9
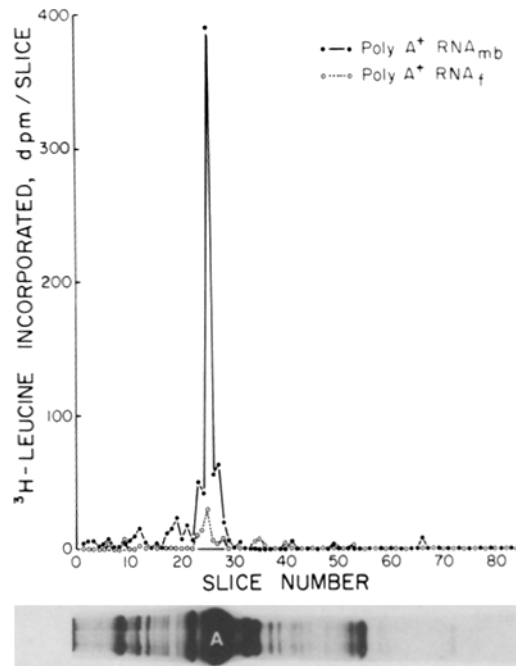

FIGURE 8 Radioactive content of gel slices of anti-RSA immunoprecipitate. Only one major peak of radioactivity from the in vitro membrane-bound ribosomal translation products is seen, and only a minor corresponding peak from the in vitro free ribosomal translation products is observed. 90% of the radioactivity identified as albumin is derived from the membrane-bound ribosomal translation products, and 10% from the free ribosomal translation products. The photograph shows a polyacrylamide gel of unlabeled rat serum proteins. The protein band designated A corresponds in electrophoretic mobility to RSA.

and 10) and immunoprecipitates electrophoresed on 7-20% polyacrylamide gradients.

FIGURE 7 Fluorographic analysis of RSA immunoprecipitate. Lane *1*, unlabeled rat serum proteins stained with Coomassie Brilliant Blue. The protein band designated by A corresponds in electrophoretic mobility to RSA. The mobility of the molecular weight markers BSA (68,000); RSA (65,000); ovalbumin (43,000); and cytochrome c (13,370) are indicated. Lane *2*, fluorograph of immunoprecipitate from [³H]leucine-labeled poly A^+RNA_{mb} translation products; lane *3*, fluorograph of immunoprecipitate from poly A^+RNA_f translation products. A single band with decreased electrophoretic mobility as compared to native RSA is seen in the fluorograph of the immunoprecipitate of poly A^+RNA_{mb} translation products. The fluorographically detected band has a mol wt of 3,000 greater than native RSA. This band is not visible in the fluorograph of the free ribosomal immunoprecipitate.

with the known chemical properties of cytochrome b_5. Thus, the hydrophobic membrane-insertion sequence of cytochrome b_5 is at the carboxy-terminal end of the molecule (20, 44, 56, 69) and consists of ~40 amino acid residues. During translation, this hydrophobic sequence would be buried within the 39 amino acid long tunnel (11) in the large ribosomal subunit even up to termination and discharge. Thus, the hydrophobic sequence would be unlikely to participate in the linking of the ribosome to the membrane during protein synthesis. Moreover, the known ubiquitous distribution of cytochrome b_5 to the ER, nuclear envelope (4, 21, 29, 59), Golgi apparatus (7, 12, 19, 26, 28, 38), plasmalemma (28), mitochondrial (55, 60, 69) and peroxisomal (14, 55) membranes of the hepatocyte is in accord with the site of synthesis documented here. In addition, the orientation of the protein to the cytoplasmic face of the ER (20, 44, 45, 56, 61, 69) is in accord with synthesis on free ribosomes, as is the demonstration by Rogers and Strittmatter
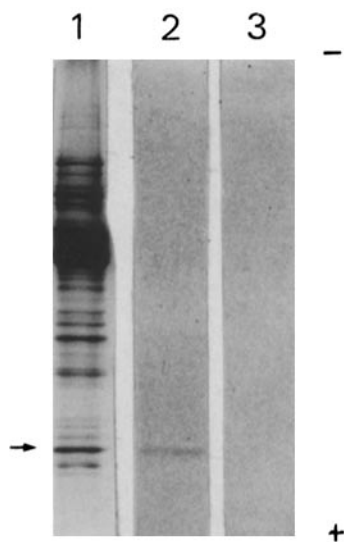

FIGURE 9 Fluorographic analysis of rat liver microsomal cytochrome b_5 immunoprecipitate. Lane 1, unlabeled rat liver microsomal proteins stained with Coomassie Brilliant Blue. The band designated by an arrow corresponds in electrophoretic mobility and protein content (see Table II) to rat liver microsomal cytochrome b_5. Lane 2, fluorograph of immunoprecipitate from translation products of poly A^+RNA_f. Lane 3, fluorograph of immunoprecipitate from translation products of poly A^+RNA_{mb}. A single band with identical electrophoretic mobility to rat liver microsomal cytochrome b_5 is seen in the fluorograph of the free ribosomal immunoprecipitate. This band is not visible in the fluorograph of the membrane-bound ribosomal immunoprecipitate.
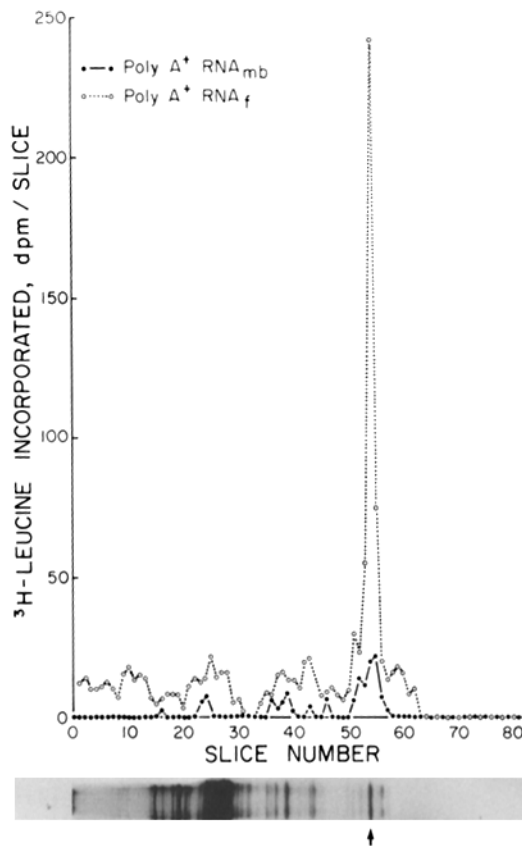

FIGURE 10 Radioactive content of gel slices of anti-cytochrome b_5 immunoprecipitate. Only one major peak of radioactivity from poly A^+RNA_f translation products is seen. This peak co-migrates with native rat liver microsomal cytochrome b_5. No substantial corresponding peak from poly A^+RNA_{mb} translation products is seen. 85% of the radioactivity identified as cytochrome b_5 is derived from the free ribosomal translation products, and 15% from the membrane-bound ribosomal translation products. The photograph shows a polyacrylamide gel of unlabeled rat liver microsomal proteins stained with Coomassie Brilliant Blue (step A, Table II). The protein band designated by the arrow corresponds in electrophoretic mobility (17,500 mol wt) to rat liver microsomal cytochrome b_5 and represents 0.40% of the total Coomassie Brilliant Blue-stained material on the gel as determined by the method of Fenner et al. (18). This corresponds to the amount of cytochrome b_5 in the preparation determined spectrophotometrically (Table II).

(56) and Strittmatter et al. (69) of complete functional fidelity of integration of the purified molecule into natural or synthetic membranes.

In conclusion, rat liver microsomal cytochrome b_5 has been shown to be synthesized on free ribo-

somes and probably not in precursor form. It is proposed that the protein is simply synthesized as a soluble protein in the cell sol. After ejection from the tunnel of the large ribosomal subunit, the 40 amino acid hydrophobic "tail" is unable to bury itself within the protein, and the polypeptide rapidly migrates to the nearest cytoplasmic membrane to reduce its free energy (see reference 34 for possibly relevant mechanisms).

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