Association of Messenger Ribonucleic Acid with Mammalian Microsomal Membranes: Characterization by Analysis of Cell-Free Translation Products†

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ABSTRACT: Total rough microsomes, isolated from the dog pancreas, were stripped of membrane-bound polysomes by treatment with either EDTA or puromycin and 0.5 M KCl. The stripped microsomal membranes were isolated relatively free from contamination, by using buoyant density centrifugation, and mRNA was isolated from both the membrane fraction and the released material. Depending on the method used to strip the rough microsomes, we found a variable but small percentage (3–15%) of the cellular poly(A)-containing mRNA attached to the microsomal membranes. Reextraction of isolated microsomal membranes with puromycin and 0.5 M KCl reduced the content of membrane-associated mRNA by approximately 50%, resulting in less than 2% of the total membrane-bound polysomal mRNA remaining associated with the microsomal membranes. The membrane-associated mRNA was characterized by translation in the wheat germ cell-free protein synthesizing system, and the products were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The translation products of the membrane-associated mRNA were identical with those from the total pancreas mRNA and also with those obtained by using mRNA isolated from material released directly from the rough microsomes.

It is now well established that mammalian cells contain polyribosomes which are attached to the endoplasmic reticulum and which are free in the cytoplasm. Polysome attachment to the membrane of the endoplasmic reticulum has been shown to be mediated, in part, by the growing nascent polypeptide chain (Blobel & Dobberstein, 1975a,b). However, a number of reports have suggested that there could be direct interaction between bound polysomes and the membranes of the endoplasmic reticulum, via mRNA[1] (Milcarek & Penman, 1974; Mechler & Vassalli, 1975; Cardelli et al., 1976). To date the microsomal membrane-associated mRNA has been defined only by pulse–chase experiments (Lande et al., 1975) or by use of inhibitors that block either mRNA formation or the initiation of protein synthesis (Cardelli et al., 1976; Adesnik et al., 1976). Such experiments could not, however, determine if the mRNA associated with the microsomal membranes represented mRNA which was active in protein synthesis or merely partially degraded mRNA containing regions of poly(adenylic acid) [poly(A)]. Furthermore, it was unclear from these experiments how direct attachment of mRNA to microsomal membranes, by the 3′ poly(A) region of the mRNA molecule, was compatible with data on the cotranslational attachment of membrane-bound ribosomes via the NH_2-terminal signal sequence (Blobel & Dobberstein, 1975a,b; Blobel, 1977).

The object of this present study was to examine the interaction of mRNA associated with microsomal membranes by analyzing its translation products for specific or uniquely enriched polypeptides. The results presented here demonstrate that a variable but small amount of mRNA was present in association with dog pancreas microsomal membranes following treatment of total rough microsomes with either EDTA or puromycin plus high salt. Translation of this membrane-associated mRNA in the wheat germ cell-free protein synthesizing system, followed by product analysis, by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, demonstrated that the translation products were qualitatively identical with those obtained from dog pancreas total mRNA.

Materials

Oligo(dT)-cellulose was obtained from Collaborative Research, Inc., Waltham, MA; [^{35}S]methionine, specific activity 400–700 Ci/mmol, was purchased from Amersham/Searle Corp., Arlington Heights, IL; and puromycin was from Sigma Chemical Co., St. Louis, MO. Wheat germ was obtained from Pillsbury Co., Minneapolis, MN.

Methods

Preparation of Rough Microsomes from Dog Pancreas. Dog pancreas total rough microsomes were prepared as previously described (Shields & Blobel, 1978).

Preparation of "Stripped" Microsomal Membranes. Rough microsomes were stripped of ribosomes by treatment with either EDTA or puromycin and high salt (0.5 M KCl). The ribosome-depleted microsomal membranes were collected by flotation on sucrose gradients as described below.

(*1*) *EDTA Procedure.* Rough microsomes were thoroughly resuspended in 1 M sucrose containing 100 mM KCl and 20 mM Tris-HCl, pH 7.4, at 20 °C to a concentration of 100 A_{260} units/mL. Sufficient 0.2 M EDTA was added to the suspension to give a final concentration equivalent to 3 μmol of EDTA per 10 A_{260} units of rough microsomes. Following 10-min incubation at 2 °C, an equal volume of 2 M sucrose containing 100 mM KCl and 20 mM Tri-HCl, pH 7.4, at 20 °C was added. Aliquots (10 mL) were loaded in 12.5-mL centrifuge tubes and overlaid with 1 mL of 1.4 M sucrose containing the above ions and then 1 mL of 0.25 M sucrose containing 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, at 20 °C, and 50 mM KCl. The gradients were centrifuged for 5 h at 40000 rpm (190000g_{av}) in the SB 283 rotor of the IEC B-60 International ultracentrifuge at 2 °C. A turbid band comprising the EDTA-stripped microsomal membranes was obtained floating at the 1.4 M/0.25 M sucrose interface. The stripped membranes were frozen at −80 °C or used directly

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; mRNP, messenger ribonuclear protein.

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Table I: Recovery of Microsomal Membranes by Buoyant Density Centrifugation[a]

treatment	expt	A_{260} units	RNA (mg)	phospholipid (mg)	microsomal membranes: RNA (mg) released	RNA (mg) Mb fraction	phospholipid (mg) released	phospholipid (mg) Mb fraction
EDTA	A	2257	44.8	83.5	35.6 (79)	11.2 (25)	23.3 (28)	62.3 (75)
	B	740	14.7	29.7	9.6 (65)	3.3 (22)	10.2 (34)	18.2 (61)
puromycin + 0.5 M KCl	A	2257	44.8	83.5	36.7 (82)	6.1 (13.6)	12.6 (15)	74.7 (89)
	B	887	13.0	32.4	10.4 (80)	0.6 (4.6)	3.9 (12)	26.6 (82)

[a] Dog pancreas rough microsomes resuspended to a concentration of 100 A_{260} units/mL were treated with either EDTA or puromycin and 0.5 M KCl, followed by buoyant density gradient centrifugation (see Methods). Both the microsomal membranes and material released into the gradients were analyzed for RNA and phospholipid content (see Methods). Numbers in parentheses represent the percentage recovery of each sample.

for RNA extraction. Material not banding by this procedure (referred to as released material) and which therefore remained in the gradient was treated similarly.

(*2*) *Puromycin and High-Salt Procedure.* This was based on the method of Adelman et al. (1973). Pellets of rough microsomes were resuspended in a solution of 1 M sucrose containing 500 mM KCl, 50 mM Tris-HCl, pH 7.4, at 20 °C, 2 mM $MgCl_2$, and 1 mM puromycin to a concentration of approximately 100 A_{260} units/mL. Samples were incubated for 10 min at room temperature, followed by 10 min at 37 °C. An equal volume of 2 M sucrose containing 500 mM KCl and 50 mM Tris-HCl, pH 7.4, at 20 °C was then added together with sufficient 1 M $MgCl_2$ to give a final Mg concentration of 5 mM. Samples (10 mL) were transferred to centrifuge tubes and overlaid with 1 mL of 1.4 M sucrose containing 500 mM KCl, 50 mM Tris-HCl, pH 7.4, at 20 °C, 5 mM $MgCl_2$, and 1 mL of 0.25 M sucrose containing 50 mM KCl, 50 mM Tris-HCl, pH 7.4, at 20 °C, and 5 mM $MgCl_2$. Samples were centrifuged as described for the EDTA procedure, and the band of puromycin–KCl stripped microsomal membranes was obtained at the 1.4 M/0.25 M interface.

(*3*) *mRNA Preparation.* Total RNA was extracted from microsomal membranes or from material released from the rough microsomes as previously described (Blobel & Dobberstein, 1975a; Shields & Blobel, 1977). Poly(A)-containing mRNA was isolated from total RNA by affinity chromatography on oligo(dT)-cellulose by using the procedure of Aviv & Leder (1972).

(*4*) *Cell-Free Protein Synthesis.* A wheat germ S-23 supernatant was prepared as described by Dobberstein & Blobel (1977), and mRNA activity (final concentration 1 A_{260} unit/mL) was assayed in the various mRNA preparations isolated from either stripped microsomal membranes or released material by using assay conditions identical with those previously described (Dobberstein & Blobel, 1977).

(*5*) *Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as previously described (Blobel & Dobberstein, 1975a).

(*6*) *Chemical Determinations.* (a) RNA was determined by using the procedure of Fleck & Munro (1962), 1 mg of hydrolyzed RNA being equivalent to 32.2 A_{260} units; (b) protein was determined by the method of Lowry et al. (1951); (c) phospholipids were determined by a combination of the procedures of Ames (1967) and Folch et al. (1957).

Results

Initial experiments were concerned with the isolation of microsomal membranes free from contamination by material released from the rough microsomes following treatment with either EDTA or puromycin plus 0.5 M KCl. A flotation gradient was used which separated the stripped microsomal membranes from disassembled polysomes and mRNP on the basis of the relative differences in buoyant density of these components. Ribosomal subunits and mRNP which were released from the rough microsomes, following treatment with EDTA or puromycin and high salt, remained in the gradient, whereas the microsomal membranes floated to the top (see Methods). Analysis of the various fractions for phospholipid content and total RNA (Table I) showed that relatively uncontaminated microsomal membranes could be obtained with up to 90% of the total rough microsomal phospholipid recovered in the membrane fraction. Consistent with this result, approximately 80% of the total RNA from the rough microsomes was released following either treatment and remained in the gradient, i.e., in association with material discharged directly from the microsomal membranes. In one experiment, when puromycin and 0.5 M KCl were used to strip the rough microsomes, as little as 4% of the total RNA was recovered with the microsomal membrane fraction (Table I). It is also apparent, from Table I, that EDTA is less efficient than puromycin and high salt for the removal of membrane-bound polysomes from rough microsomal membranes. Approximately 65% of the total phospholipid from the rough microsomes was recovered in the membrane fraction when EDTA treatment was used to strip rough microsomes, whereas up to 90% of the membrane phospholipid was recovered by using puromycin and 0.5 M KCl. Increasing the duration of centrifugation did not improve the recovery of microsomal membranes, indicating that recovery of the microsomal membranes reflected the efficiency of ribosome removal rather than some other factor related to the gradient conditions. Furthermore, analysis of the fractions for RNA content (Table I) showed that significantly less total RNA remained attached to the microsomal membranes following treatment with puromycin and high salt than after EDTA was used, results that further indicated the former procedure was more efficient than the latter for removal of membrane-bound polysomes from rough microsomes.

The RNA which remained associated with the microsomal membranes as well as that released from the rough microsomes by either of the above treatments was analyzed for poly(A)-containing RNA by affinity chromatography on columns of oligo(dT)-cellulose, the results of which are summarized in Table II. From these data it is apparent that poly(A)-containing RNA was associated with the microsomal membranes regardless of which treatment was used to "strip" the rough microsomes. The relative percentage of the poly(A)-containing RNA in the membrane fraction was approximately the same as that found in the released material, i.e., between 1 and 2% of the total RNA present in that particular fraction, indicating no significant enrichment for poly(A)-containing RNA associated with the microsomal membranes. It is also noteworthy from Table II (experiment 1) that the released

Table II: Recovery of Membrane-Associated or Released Poly(A)-Containing mRNA from Oligo(dT)-Cellulose Columns[a]

expt	treatment	RNA per fraction (A_{260} units) total rough microsomes	total applied to column: released	total applied to column: membrane	RNA bound to oligo(dT)-cellulose (A_{260} units): released	RNA bound to oligo(dT)-cellulose (A_{260} units): membrane	% total poly(A)-containing RNA: released	% total poly(A)-containing RNA: membrane
I	EDTA	572	488	91	5.1 (1.0)	1.3 (1.4)	80	20
	puromycin + 0.5 M KCl	675	604	52	6.2 (1.0)	1.04 (2)	86	14
II	EDTA		47.5	49.1	0.3 (0.6)	0.2 (0.4)		
	puromycin + 0.5 M KCl		59.9	54	1.1 (1.8)	0.65 (1.2)		

[a] Total RNA extracted from either microsomal membranes or released material was chromatographed on identical columns of oligo(dT)-cellulose, as described under Methods, and poly(A)-containing RNA was eluted from the columns as previously described (Blobel & Dobberstein, 1975a). Total membrane-bound polysomal mRNA in experiment I was defined as being equal to the sum of the poly(A)-containing RNA in the membrane and released fractions. Numbers in parentheses represent the percentage of the total RNA in that sample.

fraction had approximately 5–6 times as much poly(A)-containing RNA as the stripped membranes.

In order to characterize the microsomal membrane-associated poly(A)-containing RNA as functional mRNA, it was assayed for "messenger" activity in the wheat germ cell-free protein synthesizing system. Figure 1 shows that the poly-(A)-containing RNA which was associated with the microsomal membranes, as well as that isolated from released material, was very active in protein synthesis. The membrane-associated poly(A)-containing RNA, isolated following treatment of rough microsomes with puromycin and 0.5 M KCl, was as active in protein synthesis as the dog pancreas total mRNA. However, the mRNA obtained from released material or the membrane fraction following EDTA treatment of rough microsomes was consistently less active in protein synthesis than that obtained by using the puromycin and high salt procedure—the reason for this difference is unclear at present. The results presented in Figure 1 demonstrated that the membrane-associated poly(A)-containing RNA was indeed an active template for protein synthesis and did not represent, for example, degradation products of mRNA which had somehow become associated with microsomal membranes. To determine any functional significance for the membrane-associated mRNA, the translation products were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in an attempt to detect specific enrichment for particular pancreatic polypeptides. Thus, the translation products from membrane-associated mRNA were compared to those using mRNA isolated from released material or total pancreatic mRNA—the results of which are shown in Figure 2. The results demonstrate that the translation products from each mRNA preparation, regardless of origin, are qualitatively similar with respect to the *major* polypeptides synthesized in vitro and are identical with those previously described (DeVilliers-Thiery et al., 1975; Scheele et al., 1978). However, densitometric analysis of this autoradiograph (Figure 3) did reveal that the translation products obtained from EDTA-released material were significantly lower in amylase and procarboxypeptidase precursors than those from the other mRNAs (compare regions A and B in tracks 4 and 5). This could be due to partial degradation or inactivation of the mRNA since, as shown (Figure 1), this EDTA fraction was less active in supporting protein synthesis than mRNA from the other fractions. Nevertheless, there was no enrichment for specific pancreatic polypeptides in the translation products of the mRNA preparations isolated from either the released or the membrane fractions; compare tracks 1, 3, and 5, for example.

To determine if the association of mRNA with the microsomal membranes was by ionic linkage and to further investigate the nature of this interaction, stripped microsomal

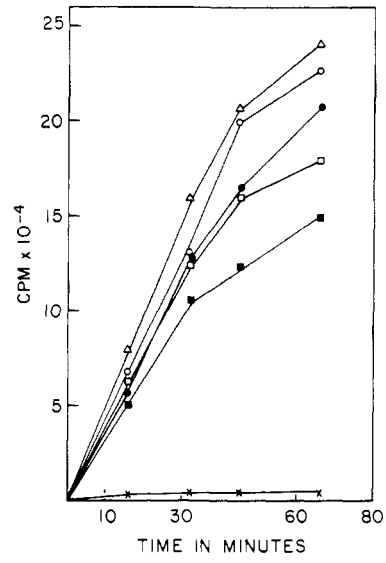

FIGURE 1: Time course of protein synthesis by using poly(A)-containing mRNA isolated from membrane-associated or released material. mRNA, at a final concentration of 1 A_{260} units/mL, was translated in the wheat germ cell-free protein synthesizing system by using conditions previously described (see Methods), containing 250 μCi/mL of [^{35}S]methionine. Aliquots (5 μL) of the incubation mixture were spotted onto 3 MM Whatman filter paper disks and assayed for hot acid precipitable material after 60 min of incubation (see Methods). Radioactivity was determined in a Beckman LS-8000 scintillation counter by using a toluene–liquifluor scintillant. (△) Dog pancreas total mRNA. Puromycin and 0.5 M KCl treatment: (○) membrane-associated mRNA; (●) released mRNA. EDTA treatment: (□) membrane-associated mRNA; (■) released mRNA. (×) Endogenous mRNA.

membranes were prepared by using puromycin plus high salt and then subjected to a second treatment with the same concentration of high salt and puromycin. The re-treated microsomal membranes were also isolated by flotation in sucrose gradients and subsequently analyzed for poly(A)-containing RNA as described above—the results of this experiment are shown in Table III. In agreement with the data of Table II, most of the membrane-bound polysomal mRNA was released by the first treatment with puromycin and 0.5 M KCl. However, a small amount of mRNA (3.6%) remained attached to the microsomal membranes. Re-treatment of the microsomal membranes with puromycin and high salt resulted in removal of 50% of the residual membrane-associated mRNA; consequently, less than 2% of the mRNA remained bound to the microsomal membranes (Table III).

Discussion

In agreement with results obtained from a variety of experimental systems (Grubman et al., 1977; Lodish & Fro-

Table III: Treatment of Stripped Microsomal Membranes with Puromycin and 0.5 M KCl[a]

fraction	total RNA (A_{260} units)	% total RNA per sample	oligo(dT)-cellulose chromatography: total applied (A_{260} units)	total bound (A_{260} units)	% bound	% total mRNA per sample
rough microsomes	2886	100				
released I	2532	87.7	1030	9.8	0.95	87.6
membranes I	71.2	2.5	71.2	1.0	1.40	3.6
released II	36.0	1.2	36.0	0.7	1.94	2.5
membranes II	63.6	2.2	63.6	0.5	0.79	1.8

[a] Rough microsomes (5000 A_{260} units) were treated with 40 mL of 1 mM puromycin dissolved in high-salt buffer (see Methods), and the stripped microsomal membranes were isolated by flotation in sucrose gradients. These stripped microsomal membranes (750 A_{260} units) were re-treated with 14 mL of 1 mM puromycin and 0.5 M KCl and reisolated by buoyant density centrifugation. RNA was then extracted from each sample and analyzed for poly(A)-containing RNA (see Methods). Total rough microsomal poly(A)-containing mRNA was estimated as being equivalent to 0.95% of the total cellular RNA, i.e., (2886/1030 × 9.8) = 27.5 A_{260} units; all values were calculated as a percentage of this figure.

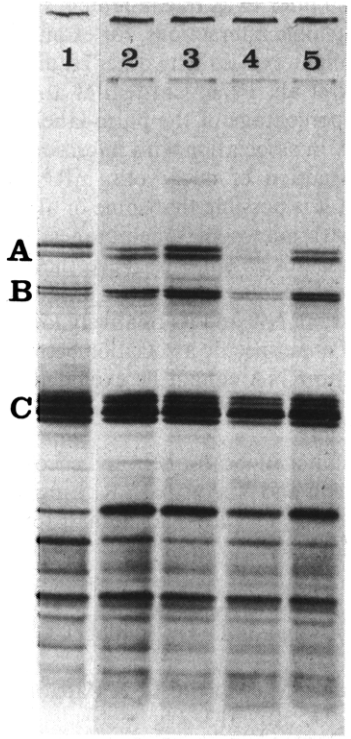

FIGURE 2: Analysis of the transition products synthesized in the wheat germ system using membrane-associated or released mRNA. Following 60 min of incubation 10–25-μL aliquots of each incubation mixture equivalent to approximately 250000 cpm of radioactivity per sample were prepared for gel electrophoresis (Blobel & Dobberstein, 1975a). The samples were then analyzed by gel electrophoresis by using gradients of 10–15% acrylamide in the presence of 0.1% sodium dodecyl sulfate as previously described (Blobel & Dobberstein, 1975a). After electrophoresis the gels were stained, fixed, and dried—shown are the translation products analyzed by autoradiography—by using the following mRNAs. Track 1: control total dog pancreas mRNA. Track 2: mRNA from released material. Track 3: microsomal membrane-associated mRNA following treatments with puromycin and 0.5 M KCl. Track 4: mRNA from released material. Track 5: mRNA associated with microsomal membranes following treatment with EDTA. Letters refer to putative precursors: A, amylase, lipase; B, procarboxypeptidases; C, serine protease zymogens.

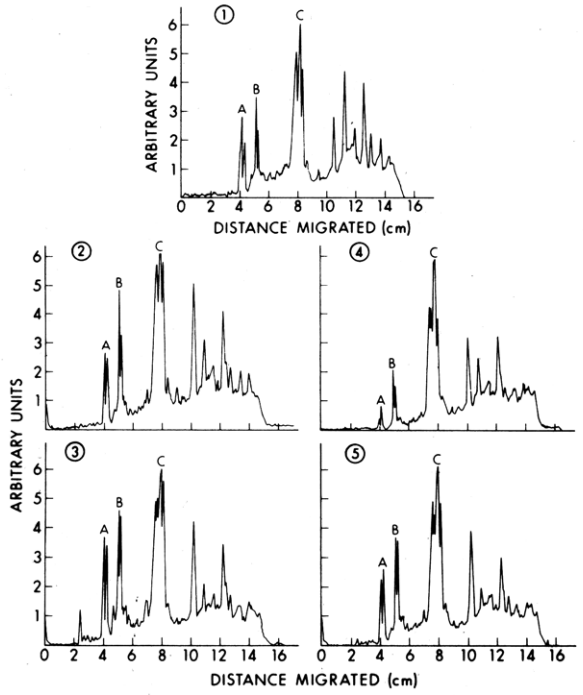

FIGURE 3: Densitometric analysis of the translation products synthesized in the wheat germ system by membrane-associated and released mRNA. The autoradiograph shown in Figure 2 was subjected to densitometric scanning by using a Joyce-Loebl (Gateshead, England) double-beam densitometer, Model IIIC. The numerals correspond to the appropriate track of the autoradiograph in Figure 2 of the cell-free translation products, and the letters A, B, and C refer to regions in the gel where the putative precursors for amylase, procarboxypeptidase, and serine protease zymogens migrated, respectively.

shauer, 1977; Kruppa & Sabatini, 1977; Smith et al., 1978), in the dog pancreas most of the poly(A)-containing mRNA from membrane-bound polysomes was not directly associated with the membrane of the endoplasmic reticulum (Tables I and II) but was readily released following dissociation of the polyribosomes into subunits and mRNP. However, a variable but small percentage of the total membrane-bound polysomal mRNA remained associated with microsomal membranes following treatment with either EDTA or puromycin and high salt. From Tables II and III it can be seen that between 2 and 20% of the total cellular mRNA remained attached to microsomal membranes following treatment of rough microsomes with puromycin and 0.5 M KCl or EDTA, respectively. It is noteworthy that Adelman et al. (1973) found that approximately 15% of membrane-bound polysomes could only be released from microsomal membranes by detergents or by using KCl concentrations significantly higher than those employed for these experiments. In the results reported here less than 2% of the mRNA isolated from rough microsomes was present attached to microsomal membranes following re-treatment of stripped microsomal membranes with puromycin and high salt (Table III).

The data reported here contrast somewhat with those of other workers (Milcarek & Penman, 1974; Lande et al., 1975; Mechler & Vassali, 1975; Cardelli et al., 1976; Adesnick et al., 1976) who found significantly higher amounts of mRNA

associated with the membrane of the endoplasmic reticulum. In those experiments, mRNA was specifically radioactively labeled in the presence of inhibitors of protein and/or rRNA synthesis. Consequently, the radioactively pulse-labeled mRNA would most likely have represented only a small percentage of the polysomal mRNA and therefore may not have been representative of the overall distribution of the total mRNA population. In the experiments described here, membrane-bound polysomal mRNA was isolated without prior administration of metabolic inhibitors. Furthermore, in contrast to previous reports, the steady-state distribution of the total rather than pulse-labeled poly(A)-containing membrane-associated mRNA was investigated relative to the total membrane-bound polysomal mRNA. In other studies (Milcarek & Penman, 1974; Adesnick et al., 1976; Lande et al., 1975; Cardelli et al., 1976) the membrane-associated mRNA was not characterized for specific products by, for example, hybridization to defined cDNA probes or by translation in a cell-free protein synthesizing system. It is thus unclear whether the pulse-labeled mRNA associated with microsomal membranes represented active mRNA or merely degradation products containing poly(A) regions. This report presents the results of an alternative approach.

Total mRNA was isolated from microsomal membranes following stripping of the rough microsomes by two different procedures and translated in a cell-free protein synthesizing system. Both membrane-associated and released mRNA was almost as active as total dog pancreas mRNA in supporting protein synthesis (Figure 1). Analysis of the translation products of the released and membrane-associated mRNAs and comparison to those obtained by using total pancreatic mRNA showed them to be similar to each other (Figure 2), at least with respect to the major pancreatic secretory products, and qualitatively identical with those recently described (Scheele et al., 1978). Although densitometric tracing of the autoradiograph (Figure 3) showed that the EDTA-released mRNA translation products had reduced amounts of precursors to amylase and procarboxypeptidase (regions A and B, track 4), in general the products of both the membrane-associated and released mRNAs were quantitatively very similar. The diminished amylase and procarboxypeptidase in track 4, Figures 2 and 3, are probably due to partial mRNA degradation since this fraction was the least active in protein synthesis. It is noteworthy that the released and membrane-associated material, prepared by puromycin and high-salt treatment, had virtually identical amounts of these two products. Furthermore, no significant enrichment for any of the major pancreatic secretory products was found in either membrane fraction. If the mRNA–microsomal membrane interaction was somehow involved in providing topological conditions to discriminate between mRNAs translated on free or membrane-bound ribosomes, for example, it might be expected that the membrane fraction would be enriched for particular species of mRNA and might thus code for specific proteins. The data presented here show that this is not the case. Since not all mRNAs are translated with equal efficiency in different cell-free protein synthesizing systems and since mRNA translation can vary significantly depending on the ionic conditions, it could be argued that the mRNA isolated from the microsomal membranes might contain unique mRNA species which were poorly translated in the wheat germ system under these present conditions. However, this is probably not the case since the cell-free system employed here was optimized for a variety of different mRNAs coding for translation products from 15 000 to 100 000 daltons, a range which encompasses most of the products of dog pancreas mRNA. Thus, it is unlikely that there might be a membrane-associated mRNA which would only be translated efficiently under significantly different ionic conditions from those used in these experiments. In addition, translating the various mRNAs in the mRNA-dependent rabbit reticulocyte lysate yielded identical results with those obtained with the wheat germ system (data not shown). Consequently, it is felt that these data probably do reflect the quantitative distribution of mRNA in these various fractions, although hybridization analysis will be necessary to definitively prove this.

Although this present study cannot exclude the possibility of cellular poly(A)-containing mRNA interacting directly with microsomal membranes, it does rule out the involvement of an ionic or salt linkage in this process. It is possible, however, that a small percentage of the total mRNA could be transiently membrane bound prior to its role in protein synthesis, perhaps via weak hydrophobic interactions, for example. Should this be the case, it might explain why other studies (Lande et al., 1975; Adesnick et al., 1976; Cardelli et al., 1976) found a relatively high percentage of the pulse-labeled de novo synthesized mRNA in association with microsomal membranes. A significant fraction of eucaryotic mRNA is not polyadenylated, and it is possible that some or all of this fraction could interact with microsomal membranes; experiments are in progress to test this possibility. Nevertheless, on the basis of the absence of unique translation products from membrane-associated mRNA and its relatively low concentration, the possibility of a nonspecific association between microsomal membranes and mRNA cannot be excluded.

Acknowledgments

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Evidence for the Double-Sieve Editing Mechanism in Protein Synthesis. Steric Exclusion of Isoleucine by Valyl-tRNA Synthetases†

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ABSTRACT: Evidence is presented for a simple stereochemical model, the "double sieve", by which the exquisite fidelity of the genetic coding process is preserved at the level of charging tRNA with the correct amino acid. The high accuracy in the recognition of amino acids is achieved by the synthetic and hydrolytic (editing) sites on an aminoacyl-tRNA synthetase functioning as a pair of sieves, crudely sorting the amino acids according to size as well as chemical nature. The synthetic site rejects, at a tolerable level, amino acids larger than the specific substrate; the hydrolytic site destroys the reaction products of the amino acids which are smaller than (or isosteric with) the specific substrate. Testing hypothetical editing mechanisms by mapping out the range of misactivations catalyzed by the aminoacyl-tRNA synthetases is hampered by trace impurities of the specific amino acid in preparations of the nonspecific amino acids. An enzymic method is given for scavenging these impurities. It is found that at least 97% of the apparent isoleucine-dependent pyrophosphate exchange activity of three representative valyl-tRNA synthetases is attributable to residual traces of valine. The selectivity of the enzyme from *Escherichia coli* against isoleucine is greater than 6×10^4. Combined with the known ratio of concentrations of isoleucine and valine in vivo, an error rate of less than 3×10^{-6} is calculated for the mistaken activation of isoleucine for valine. Isoleucine is thus sieved out at the activation step and the rate of formation of Ile-tRNA^{Val} is so low as not to require subsequent editing.

The selection of amino acids during protein biosynthesis is very precise. The overall error rate for the misincorporation of valine for isoleucine, which is possibly the most difficult example, is only 1/3000 (Loftfield, 1963; Loftfield & Vanderjagt, 1972). In order to maintain this accuracy at the level of aminoacylation of tRNA, certain aminoacyl-tRNA synthetases have evolved "editing" or "proofreading" mechanisms whereby the products of misactivated amino acids are removed somehow by hydrolysis (Norris & Berg, 1964; Baldwin & Berg, 1966). It has now been shown that the editing of misactivated threonine and α-aminobutyrate by valyl-tRNA synthetases occurs after the tRNA^{Val} has been misacylated (Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a): the amino acid is activated by the enzyme (eq 1) and is transferred to the tRNA (eq 2) in the normal way but the misacylated tRNA is rapidly deacylated by a separate hydrolytic active site on the enzyme before the tRNA is released into solution (eq 3).

$$\text{E·Thr·ATP} \rightarrow \text{E·Thr–AMP} + \text{PP}_i \quad (1)$$

$$\text{E·Thr–AMP} + \text{tRNA}^{Val} \rightarrow \text{E·Thr–tRNA}^{Val} + \text{AMP} \quad (2)$$

$$\text{E·Thr–tRNA}^{Val} \rightarrow \text{E} + \text{Thr} + \text{tRNA}^{Val} \quad (3)$$

Our working hypothesis for predicting when misactivations will occur and where editing mechanisms are necessary is based on a combination of ideas of steric exclusion and goodness of fit (Fersht, 1977a). It is reasoned that amino acids larger than the specific substrate of an aminoacyl-tRNA synthetase will be excluded, at a tolerable level, from binding productively to the active site by steric hindrance. On the other hand, smaller amino acids must be able to bind productively and react but the reaction rates will be lower in proportion to the decrease in binding energy. It is suggested that editing is required only for rejection of the smaller amino acids—the "double-sieve" hypothesis (Fersht, 1977a; Fersht & Dingwall, 1978).

Whereas there is a simple theory to account for the binding of smaller substrates to a larger active site (Fersht 1974, 1977a), there is little quantitative knowledge of the steric hindrance to the binding of larger substrates. It is clear that phenylalanine, for example, will be completely excluded from the active site of the valyl-tRNA synthetase, but the degree of exclusion of isoleucine is unknown. It has been reported that the valyl-tRNA synthetase from *Escherichia coli* catalyzes the pyrophosphate exchange reaction in the presence of isoleucine with values of V_{max} as high as 10–22% of that with valine (Loftfield & Eigner, 1966; Yaniv & Gros, 1969). The enzyme from yeast has recently been reported to catalyze the isoleucine-dependent reaction with V_{max} = 57% of that for valine and K_M = 7.1 mM (compared with 0.17 mM for valine) (Igloi et al., 1978). If the latter is so, then an efficient editing mechanism is required to correct the mistaken activation of

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