A POOL OF NATURALLY OCCURRING RAT LIVER RIBOSOMAL SUBUNITS CAPABLE OF TRANSLATING EXOGENOUS MESSENGER RNA

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1. Introduction

A post microsomal fraction of rat liver (PMS) has been prepared which exhibits very little endogenous capacity for protein synthesis, but is highly active in translating messenger RNA isolated from rabbit reticulocytes. Analysis of this fraction by sucrose density centrifugation indicates that it contains few if any polysomes; it consists of both large and small ribosomal subunits as well as a soluble component which is required for protein initiation. The behavior of PMS toward exogenous messenger RNA is compared with that of rat liver-free polysomes, membrane-attached polysomes, and polysomes freed from the endoplasmic reticulum by detergent treatment. PMS, is contrast to the other ribosomal fractions tested, does not require preincubation in order to translate the globin messenger RNA and is considerably more active in responding to added exogenous messenger. PMS probably represents a pool of naturally occurring ribosomal subinits plus all of the factors necessary to initiate protein synthesis.

2. Materials and methods

2.1. *Preparation of ribosomal fractions*

2.1.1. The post microsomal supernate

The post microsomal supernate (PMS) was prepared from the livers of fasted rats by homogenizing the livers in 2.5 vol of: 0.25 M sucrose; 25 mM Tris–HCl, pH 7.6; 75 mM KCl; 5 mM magnesium acetate and 6 mM 2-mercaptoethanol. The homogenate was centrifuged in the Sorvall SS-34 rotor for 15 min at 30 000 *g* and in the Beckman #40 rotor at 105 000 *g* for 30 min, discarding the pellets.

PMS was further fractionated by centrifugation for 2 hr at 368 000 *g* in the Beckman 65 rotor. The pellet was suspended in 0.25 M sucrose, 25 mM Tris–HCl, pH 7.6, 75 mM KCl, 5 mM magnesium acetate for RNA quantitation and for analysis on sucrose gradients. The supernate was used as a suspension medium for a rough endoplasmic reticulum fraction or for polysomes, to provide the soluble factors necessary for protein synthesis. This supernate had no protein synthetic activity of its own, either in the presence or absence of exogenous messenger. PMS and its isolated fractions could be rapidly frozen in a methanol–dry ice bath and stored at −70°C for at least three days, but a loss of up to 50% of its response to exogenous messenger occasionally occurred under these conditions.

For analysis, PMS and the 368 000 *g* pellet derived from PMS, were layered on linear 10%–40% gradients of sucrose in 0.5 M KCl, 25 mM Tris–HCl, pH 7.4 and 5 mM magnesium acetate. Gradients were centrifuged in the Beckman SW 40 rotor for 3.5 hr at 284 000 *g*. Authentic rat liver ribosomal subunits, prepared by the method of Blobel and Sabatini [1], as well as native rat liver polysomes, were used as markers.

2.1.2. Membrane bound- and free polysomes

Membrane bound- and free polysomes were prepared by a modification of the procedure of Ragland et al. [2]. Livers from fasted rats were homogenized in 2 vol of 0.44 M sucrose in TKM buffer (50 mM Tris–HCl, pH 7.4; 25 mM KCl; 5 mM magnesium acetate). A post-mitochondrial supernate, prepared by centrifuging the homogenate at 10 000 rpm in the

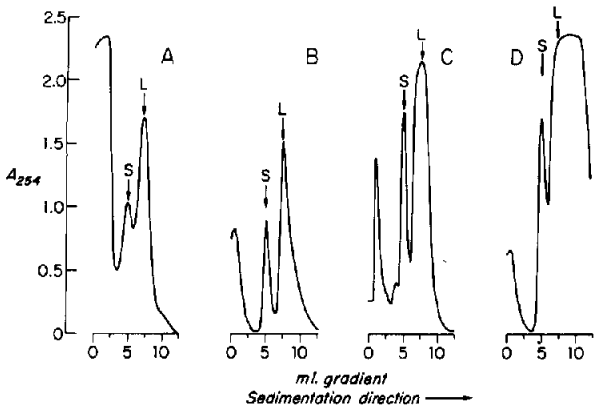

Fig. 1. Sucrose density gradient profiles: A) 0.5 ml PMS; B) PMS pellet, 8.2 A_{260} units; C) ribosomal subunits derived from free polysomes, 23 A_{260} units; D) polysomes, 23 A_{260} units. Gradients were linear, 10%–40% sucrose in 500 mM KCl; 25 mM Tris–HCl, pH 7.4; 5 mM magnesium acetate, and were centrifuged for 3.5 hr in the Beckman SW-40 rotor at 40 000 rpm. Gradients were analysed with an Isco Density Gradient Fractionator. Positions of the small and large ribosomal subunits are denoted by the letters S and L, respectively. Other details are given in Materials and methods.

Sorvall SS-34 rotor for 10 min and discarding the pellet, was adjusted to 1.35 M sucrose TKM. This was overlayered by 0.44 M sucrose TKM. The gradients were centrifuged in a Beckman 60 Ti rotor at 360 000 *g* for 16 hr. Rough endoplasmic reticulum was collected from the interface between 2.0 M sucrose and 1.38 M sucrose, while free polysomes were pelleted below the 2.0 M sucrose.

Membrane-bound polysomes were removed from rough endoplasmic reticulum by diluting the gradient fraction with TKM to 0.5 M sucrose and adjusting the suspension to 0.5% w/v in sodium deoxycholate. Polysomes were pelleted in the Beckman 50 Ti rotor at 225 000 *g* for 1 hr. The pellets were resuspended in 0.25 M sucrose TKM and repelleted before use.

2.1.3. Preparation of messenger RNA

Rabbit reticulocyte mRNA and 28-S RNA were prepared by the methods of Lockard and Lingrel [3] and Schutz et al. [4]. 28-S RNA has no messenger activity and was used to test for non-specific response of the system to added RNA.

2.1.4. Assay for protein synthesis

Protein synthesis pre-incubations (0.2 ml) contained magnesium acetate, 1 μmole; KCl, 10 μmoles; Tris–HCl, pH 7.6, 6.25 μmoles; 2-mercaptoethanol, 1.5 μmoles; ATP, 0.25 μmoles; GTP, 0.025 μmoles; 19 unlabelled amino acids excluding leucine, 4 nmoles each; creatine phosphokinase, 25 μg and phosphocreatine, 3 μmoles; plus 0.1 ml of ribosomal fraction. Following preincubation, the messenger RNA (2.5 μg), 28-S RNA (21#μg) or polyuridylic acid (20 μg) were added, followed by either 50 μl (50 μCi) of [^{3}H]-leucine (38 Ci/mmole), 50 μl (2.5 μCi) of [^{14}C]-leucine (311 μCi/μmole); or 50 μl (2.5 μCi) of [^{14}C]-phenylalanine (492 μCi/μmole). Mixtures were incubated for 30 min, and duplicate 0.1 ml aliquots were applied to discs of Whatman 3 MM filter paper which were processed for determination of radioactivity by the method of Mans and Novelli [5].

Table 1
Effect of preincubation on the response of PMS to added RNA.

Preincubation time (min)	[^{3}H]leucine incorporation (pmole/mg RNA)		
	PMS alone	+Globin mRNA	+28-S RNA
0	8.16	28.96	12.44
10	2.68	27.44	5.36
20	1.40	16.28	3.64

3. Results and discussion

Fig. 1 shows the sucrose density gradient profiles of PMS; of the pellet derived from PMS by centrifugation for 2 hr at 368 000 *g*; of authentic rat liver ribosomal subunits [1] and of rat liver polysomes. Gradient details are given in Materials and methods. PMS (fig. 1A) contains both ribosomal subunits, but there is no indication of significant amounts of a species larger than the large subunit. Fig. 1B shows that both subunits are removed from PMS by centrifugation of this fraction for 2 hr at 368 000 *g*. Native polysomes (fig. 1D) undergo some degradation in this high salt gradient, and some of the larger polysomes sediment to the bottom of the tube. PMS yields no pellet under these conditions.

The response of PMS to exogenous messenger RNA

Table 2
Response of rat liver ribosomal preparations to exogenous messenger RNA.

Ribosomal preparation	[^{3}H]leucine incorporation, pmoles/mg ribosomal RNA				Stimulation of incorporation by mRNA
	Unpreincubated	Preincubated 5 min			
		Endogenous	+28-S RNA	+Globin mRNA	
Free polysomes	50.9	6.15	8.45	17.4	x 2.83
ER membrane-bound polysomes	89.7	19.8	19.1	27.8	x 1.40
Polysomes detached from ER membrane	179.0	17.5	21.7	15.4	0
PMS	8.56	4.52	12.96	122.0	x 27.0

is shown in table 1. PMS has very little endogenous activity, but is highly active in the synthesis of rabbit globin in the presence of the messenger. SDS-gel electrophoresis showed that the messenger directed the synthesis of both the alpha- and the betaglobin chains. PMS does not require preincubation to show stimulation by the globin messenger, but preincubation lowers the endogenous activity still further, magnifying the effect of added messenger. For the following experiments, a 5 min preincubation was routinely used.

Table 2 compares the endogenous protein synthetic activity of several rat liver ribosomal fractions and their response to added globin messenger. All fractions except PMS are very active in endogenous protein synthesis. As expected, preincubation lowers the native activity, and the addition of globin messenger stimulates protein synthesis to varying degrees. Free polysomes exhibit a 3-fold stimulation of incorporation upon messenger addition; rough endoplasmic reticulum (ER) responds less well and plysomes detached from the ER membrane do not respond at all. In comparison, Sampson et al. report a 2 to 3-fold stimulation using a 30 000 *g* supernatant fraction of mouse liver [6] and up to 6-fold stimulation using polysomes isolated by detergent treatment of this supernatant fraction [7]. PMS, on the other hand, displays greater than 25-fold stimulation over its preincubated endogenous activity and almost 15-fold stimulation over the unpreincubated value. The degree of stimulation was variable with a range of about 10-fold to 40-fold, and could drop to 5-fold following overnight storage at 0°C or, freezing and thawing. The failure of detached membrane-bound polysomes to the messenger may reflect the loss of one or more initiation factors during preparation. In this connection, Schreier and Staehelin [8] have reported that the efficiency of translation of globin messenger in liver systems is markedly improved by the addition of reticulocyte initiation factors.

PMS can be fractionated into its ribosomal and soluble components by centrifugation for 2 hr at 368 000 *g*. Table 3 shows that neither fraction is capable by itself of responding either to globin mRNA or to polyuridylic acid. Resuspension of the pellet in the supernate, however, restores full activity. These experiments were performed using frozen preparations,

Table 3
Fractionation of PMS.

Component	[^{14}C]leucine or phenylalanine incorporation (pmoles)		
	Endogenous	+Globin mRNA	+Poly-U*
PMS	0.68	5.45	8.57
PMS 368 000 *g* pellet	1.18	1.14	1.4
PMS 368 000 *g* supernate	0.66	0.68	0.74
Pellet + supernate recombined	0.77	5.81	8.43

* For Poly-U directed polyphenylalanine synthesis, magnesium acetate concentration was 16 mM.

which accounts for the relatively lower level of response to added mRNA. In order to ensure the presence of tRNA and the soluble factors necessary for protein synthesis where the supernate has been omitted, the pH 5 enzyme fraction of rat liver [9] was added to the incubation mixtures. It thus appears that components involved in peptide initiation remain soluble upon centrifugation. The ribosomal subunits obtained in PMS are presumably equivalent to the 'native' subunits which have been described as participants in the ribosome cycle during protein synthesis [10–13].

Although we are at present unable to discuss in detail the interaction of the various factors involved in the control of peptide initiation in mammalian cells, the apparent presence in PMS of all the conditions necessary for initiation in vitro should make it useful in such studies. The simplicity of preparation of PMS should also make it attractive as an assay medium for evaluating the messenger activity of RNA preparations.

Acknowledgements

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References

[1] Blobel, G. and Sabatini, D. (1971) Proc. Natl. Acad. Sci. U.S. 68, 390–394.
[2] Ragland, W.L., Shires, T.K. and Pitot, H.C. (1971) Biochem. J. 121, 271–278.
[3] Lockard, R.E. and Lingrel, J.B. (1969) Biochem. Biophys. Res. Commun. 37, 204–212.
[4] Schutz, G., Beato, M. and Feigelson, P. (1972) Biochem. Biophys. Res. Commun. 49, 680–689.
[5] Mans, R.J. and Novelli, G.D. (1961) Arch. Biochem. Biophys. 94, 48–53.
[6] Sampson, J., Mathews, M.B., Osborn, M. and Borghetti, A.F. (1972) Biochemistry 11, 3636–3640.
[7] Sampson, J. and Borghetti, A.F. (1972) Nature New Biol. 238, 200–202.
[8] Schreier, M.H. and Staehelin, T. (1973) J. Mol. Biol. 73, 329–349.
[9] Von der Decken, A. and Campbell, P.N. (1962) Biochem. J. 121, 683–694.
[10] Henshaw, E.C., Guiney, D.G. and Hirsch, C.A. (1973) J. Biol. Chem. 248, 4367–4376.
[11] Hirsch, C.A., Cox, M.A. Van Venrooij, W.J.W. and Henshaw, E.C. (1973) J. Biol. Chem. 248, 4377–4385.
[12] Ayuso-Parilla, M., Henshaw, E.C. and Hirsch, C.A. (1973) J. Biol. Chem. 248, 4386–4393.
[13] Ayuso-Parilla, M., Hirsch, C.A. and Henshaw, E.C. (1973) J. Biol. Chem. 248, 4394–4399.