INTERACTION OF HAEMOGLOBIN MESSENGER RNP WITH THE SMALL SUBPARTICLE OF RETICULOCYTE RIBOSOMES

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1. Introduction

When reticulocyte polysomes that have been isolated from rabbits injected 18 hr previously with ^{32}P phosphate are dissociated with EDTA, a highly labelled component is released [1–3]. This material sediments at approximately 14 S and has been shown to contain 9 S RNA, which is believed to be the messenger for haemoglobin [4–8], together with proteins [3].

There exists considerable evidence of rapidly-labelled ribonucleoprotein complexes in other cells, including liver [9–11], thyroid [12], and L cells [13]. Whether these complexes have any physiological significance remains to be seen, but they are thought to be the form in which mRNA is transferred from the nucleus to the polysomes. These complexes bear a remarkable resemblance to informosomes, which are polysome-bound and can be translated [14].

The binding of mRNA to the small ribosomal subparticle is a prerequisite for the initiation of protein synthesis both in *E. coli* [15,16] and in mammalian cells [17]. Recently [18] it has been shown that 9 S RNA can be isolated from the small subparticle, derived by dissociation of reticulocyte polysomes with pyrophosphate.

We wish to report that a messenger ribonucleoprotein complex can be released from the small ribosomal subparticle by treatment with EDTA and that under certain conditions it can bind to the 40 S ribosomal subparticle. These subparticles have been obtained by a mild procedure involving the release of nascent protein with puromycin followed by dissociation. The method is a modification of that used by Lawford [19] with rat liver subparticles.

2. Methods

Preparation of reticulocytes and isolation of polysomes was as described previously [20]. ^{32}P-labelled messenger ribonucleoprotein was isolated according to the method of Huez et al. [1].

Preparation of derived subparticles: Polysomes were incubated 40 min at 37°, as described under the relevant diagrams, cooled to 4°, and immediately layered onto a G-25 Sephadex column, 30 cm × 2 cm, pre-equilibrated with TKM buffer (mM) (50 tris-HCl, pH 7.6; 150 KCl; 1 $MgCl_2$) [19]. The load on this column did not exceed 16 mg polysomes in 5 ml and the flow rate was approximately 20 ml/hr. The ribosomes were collected approximately 1 hr later and immediately layered onto 5–20% sucrose gradients in TKM buffer. They were centrifuged at 21,000 rpm for 13 hr in the MSE 3 × 65 rotor (60,000 *g*) at 4°.

Gradient fractionation and radioactivity measurements: Fractions were collected as previously described [20]. Radioactivity was determined after addition of carrier bovine serum albumin (0.5 mg) and precipitation with cold 5% TCA. Samples were filtered on Oxoid membranes and counted in the low-background Nuclear Chicago Gas Flow Counter at 50% efficiency.

3. Results

3.1. *Preparation of ribosomal subparticles*

Ribosomal subparticles were prepared as shown in fig. 1. Peak tubes corresponding to the 40 S subpart-

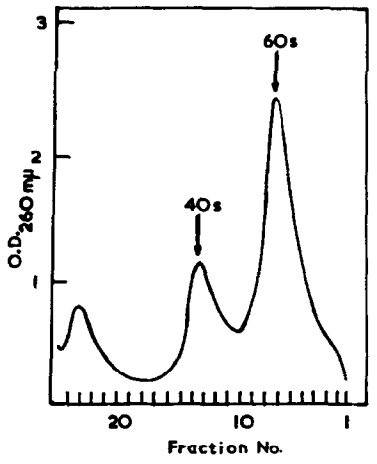

Fig. 1. Preparation of ribosomal subparticles. Incubation mixture contained per ml (μmoles) 50 tris-HCl, pH 7.6 at 20°; 50 KCl; 8 $MgCl_2$; 0.7 puromycin; 5 glutathione; 0.125 of each of 20 L-amino acids and 3 mg (30 $OD_{260m\mu}$ units) polysomes. Samples were incubated, pre-equilibrated with TKM buffer, and layered onto gradients as given in the Methods section.

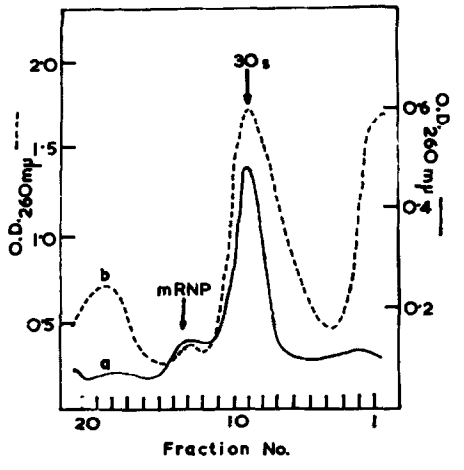

Fig. 2. Release of mRNP from derived 40 S subparticles. (a) Small subparticles (prepared as described in 3.1) were resuspended in magnesium-free buffer (10 mM sodium phosphate, pH 7.0) [3]. EDTA (7 μmoles/$OD_{260m\mu}$ unit) was added immediately before layering the mixture onto sucrose gradients (15–30% in magnesium-free buffer) and centrifuging at 30,000 rpm for 16 hr at 4° in the MSE 50 ultracentrifuge. (b) Polysomes (4 mg) were treated in exactly the same manner as in (a).

icle were combined and the subparticles were pelletted. It should be noted that the ratio of 60 S:40 S is not exactly 2:1, probably due to 40 S dimerization in the 60 S region (unpublished results and see [22,23]).

3.2. *Release of mRNP from derived subparticles*

Using identical conditions to Huez et al. [1], it can be seen that a complex sedimenting at about 14 S is released by treatment of polysomes with EDTA (fig. 2 (b)). This complex has been shown to contain 9 S RNA, the mRNA for haemoglobin [2–4]. Treatment of the derived 40 S ribosomal subparticles in exactly the same manner yields a complex cosedimenting with the 14 S complex (fig. 2 (b)). If isolated ^{32}P-mRNP marker, prepared as described in the Methods Section, is added after addition of EDTA to the small subparticle, it cosediments with the released complex (fig. 3).

3.3 *Binding of isolated ^{32}P-mRNP to derived subparticles*

Polysomes were incubated with puromycin as described in Methods. ^{32}P-mRNP was added to the in-

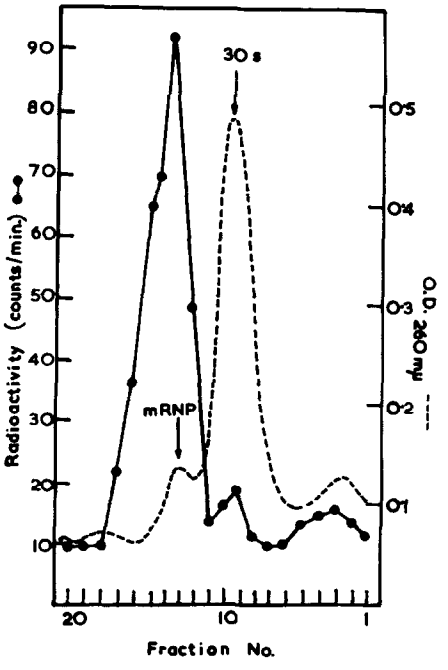

Fig. 3. Cosedimentation of mRNP with RNP released from derived subparticles. Subparticles were treated as in fig. 2. ^{32}P-labelled mRNP was added as marker after treatment with EDTA and the mixture was centrifuged in a 15-30% sucrose gradient.

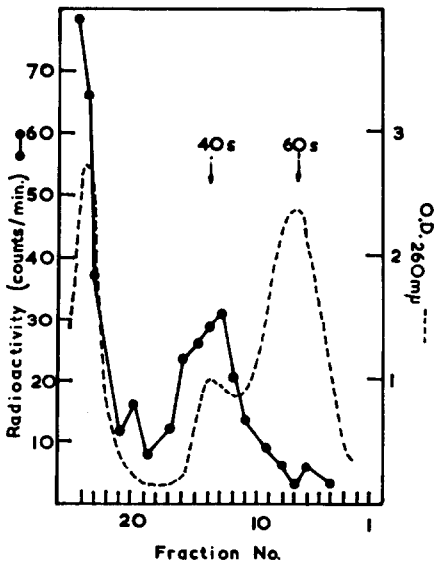

Fig. 4. Binding of ^{32}P-labelled mRNP to the small subparticle. ^{32}P-labelled mRNP (20 μg, 700 cpm) was added to the incubation as described in 3.3. The mixture was layered onto a 5–20% sucrose gradient in buffer L and centrifuged as described in 3.1.

cubation immediately before layering onto the G-25 Sephadex column. The subunit preparative gradient was fractionated and radioactivity determined as described in Methods. Binding of ^{32}P-mRNP appears to be specifically to the small subparticle and there is virtually no binding to the large subparticle (fig. 4).

4. Discussion

Incubation of reticulocyte polysomes with puromycin in order to remove nascent protein has been found to facilitate their complete conversion into subparticles at moderately high salt concentrations (150 mM KCl, 1 mM $MgCl_2$), as previously shown by Lawford [19] with liver ribosomes. No release of messenger ribonucleoprotein was observed during this dissociation (fig. 1), but the messenger can be released subsequently from the isolated 40 S ribosomal subparticle by treatment with EDTA (figs. 2 and 3). We have found it necessary to use freshly prepared subparticles for these experiments because of their instability on storage. Recently, Holder and Lingrel [18] have reported that treatment of reticulocyte polysomes with 2.2 mM pyrophosphate also yields subparticles, together with monoribosomes, without release of 9 S messenger RNA. It is thus evident that the messenger ribonucleoprotein complex is released only by drastic removal of magnesium with EDTA, possibly because this treatment results in the unfolding of ribosomal subparticles [24].

Under the relatively mild conditions used in our experiments, mRNA either remains bound or, if initially released, rebinds to the 40 S subparticle. The latter interpretation of our results is favoured by the experiment showing that free ^{32}P-labelled messenger ribonucleoprotein can be bound to the 40 S subparticle (fig. 4), as well as by the observation that the messenger appears to be associated with only one subparticle. Thus, it is found to be present in the 40 S fraction, whereas a complex consisting of messenger and several small ribosomal subparticles would be expected to have a sedimentation coefficient greater than 60 S.

These experiments are thus consistent with an initial dissociation of polysomes followed by the attachment of one small ribosomal subparticle to a site on the messenger for which it has a particularly high affinity. It seems likely that this is the site where protein synthesis is initiated, although it remains to be demonstrated that this messenger-subparticle complex can participate in the cell-free synthesis of haemoglobin. It will also be of interest to investigate the proteins present in the messenger ribonucleoprotein released from the small ribosomal subparticle in order to determine whether they are identical with those found in the complex obtained directly from polysomes by similar treatment with EDTA.

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