PRESENCE OF HAEMOGLOBIN MESSENGER RNA IN THE POSTRIBOSOMAL SUPERNATANT OF RABBIT RETICULOCYTES AND CONDITIONS NECESSARY FOR ITS TRANSLATION

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1. Introduction

As an extension of our earlier work on the incorporation of liver ribosomal subparticles into reticulocyte polysomes [1] we have developed a heterologous cell-free system containing rat liver ribosomal subparticles and rabbit reticulocyte postribosomal supernatant in order to test the initiation factor requirements for the translation of exogenous haemoglobin mRNA. Contrary to our expectations we have found that this cell-free system synthesizes globin without addition of exogenous mRNA. A number of earlier observations in the literature [2, 3] suggested the presence of mRNA in the postribosomal supernatant and we now present evidence that the amount of mRNA in the supernatant may be considerable. Moreover, the mRNA is not associated with small ribosomal subparticles and codes almost exclusively for the α-chains of globin. In addition we have found that the factors necessary for the translation *de novo* of haemoglobin mRNA are also present in the postribosomal supernatant.

2. Methods and materials

2.1. *Preparation of subcellular components*

2.1.1. Lysate and 0.5 M KCl ribosomal wash factors

The 1:2 lysate from rabbit reticulocytes and the high salt wash of ribosomes were prepared as previously described [1]. The salt wash will be referred to in the text as reticulocyte factors. This crude ribosomal wash is known to include factors necessary for the initiation of protein synthesis in a fractionated cell-free system [4].

2.1.2. Postribosomal supernatant

Aliquots (6 ml) of lysate were centrifuged for 3 hr at 150,000 *g* in the 8 × 25 ml angle rotor (MSE Ltd., Crawley, England) at 4°. The postribosomal supernatant (PRS) was obtained by carefully aspirating the top 4 ml.

2.1.3. Rat liver ribosomal subparticles

Ribosomal subparticles were prepared from the free rat-liver polysomes (sucrose washed) and dissociated with high salt in the presence of puromycin according to the method of Blobel and Sabatini [5]. The separated subparticles were precipitated with 0.7 vol of ethanol in the presence of high concentrations of magnesium ion [6]. These subparticles were resuspended in buffer (50 mM Tris-HCl, pH 7.6, 80 mM KCl, 2.5 mM $MgCl_2$) at a concentration of 50 and 20 A_{260} units/ml for the large and small subparticles, respectively. All preparations of lysate, PRS, factors and subparticles were stored in aliquots in liquid nitrogen and used once only.

2.1.4. Labelled subparticles

The subparticles were prepared as described above. The label was introduced by intraperitoneal injection of 62 μCi of [^{14}C]orotic acid (specific radioactivity 61 mCi/mmole) into a rat (100 g body wt) 18 hr previously.

2.2. *Cell-free incubations*

Each incubation contained (per ml): 0.40 ml lysate or PRS containing a mixture of 0.34 mg large and 0.17 mg small liver ribosomal subparticles (estimated by absorbancy at 260 nm where 10 A_{260} units = 1 mg),

0.16 ml of 0.5 M KCl factors containing 1–2 mg protein, 2.5 μmoles magnesium acetate, 80 μmoles KCl, 20 μmoles Tris-HCl, pH 7.6, 1.0 μmole dithiothreitol, 15 μmoles creatine phosphate, 1.0 μmole ATP, 0.25 μmole GTP, 30 μg creatine kinase, 0.1 μmole of each of the 20 protein amino acids minus leucine and 2 μCi of [^{14}C]leucine (5.84 nmoles).

50 μl aliquots were incubated at 30° for 1 hr and the incubation was stopped by the addition of 0.4 ml of 0.3 N NaOH; the mixtures were incubated for a further 15 min at 37° and precipitated with 10% trichloroacetic acid (TCA). The precipitates were filtered onto 3 cm Whatman GF/C glass fibre papers and washed with 5% TCA. The filters were glued on to planchettes, dried and counted in a Nuclear Chicago low background machine at approx. 20% efficiency.

2.3. *Product analysis by carboxymethylcellulose chromatography*

After incubation of the cell-free preparation for 1 hr at 30°, aliquots (volumes according to the figure legends) were added dropwise to 10 vol of 10% HCl in acetone at approx. −20°. The precipitates were washed twice with acetone, dried *in vacuo* and resuspended in dilute buffer (see below). The globin solution was absorbed on to a column of carboxymethylcellulose (25 cm × 0.9 cm dia.). The α- and β-chains were separated by elution with a linear gradient of pyridine (0.02 → 0.2 M)–formic acid (0.2 → 2 M) according to the method of Dintzis [7]. Fractions (6 ml) were collected and the absorbancy at 280 nm was measured. Protein was precipitated by adding TCA to a final concentration of 10% and the precipitates were filtered on to 2.5 cm Whatman glass fibre filters. The precipitates were subsequently washe with 5% TCA followed by ether–alcohol (3:1 by vol)

Table 1
Cell-free incorporation of [^{14}C]leucine into TCA-precipitable polypeptide.

Cell-free system	Incorporation of [^{14}C]leucine (pmoles/50 μl incubation)	
	Without factors	With factors
Reticulocyte supernatant alone	0.48	0.53
Reticulocyte supernatant + liver subparticles	3.34	5.68
Unfractionated reticulocyte lysate	47.43	58.45

The incubations were performed as described in the Methods section.

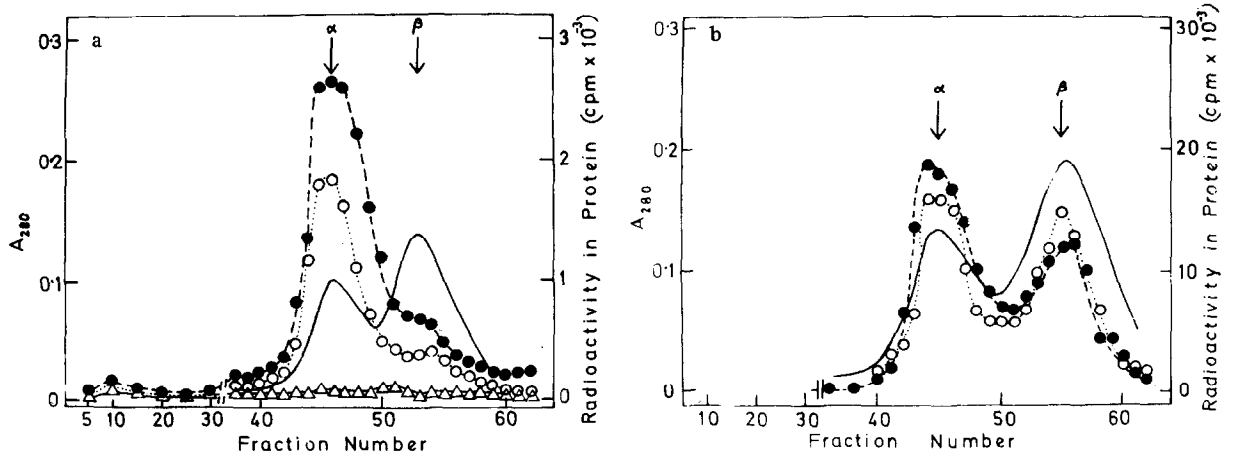

Fig. 1. Product analysis of cell-free systems by chromatography on carboxymethylcellulose. Incubation mixtures (0.5 ml) were extracted and processed as described in the Methods section. a) Postribosomal supernatant alone (▲—△—△); postribosomal supernatant + liver subparticles (○····○····○); postribosomal supernatant + liver subparticles + reticulocyte factors (●----●----●); A_{280} (——). b) Unfractionated lysate alone (no additions) (○····○····○); unfractionated lysate + reticulocyte factors (●----●----●); A_{280} (——).

Table 2
Incorporation of [^{14}C]leucine into protein and globin chains.

Cell-free system	Product				
	Acid-acetone extracted protein (cpm)	Globin chains (cpm)	Globin α-chain (cpm)	Globin β-chain (cpm)	Ratio α:β
PRS + liver subparticles (fig. 1a)					
(a) No factors added	16,480	11,530	9,195	2,335	3.9
(b) Reticulocyte factors added	21,200	20,710	17,580	3,380	5.0
Unfractionated lysate (fig. 1b)					
(a) No factors added	182,000	145,710	78,000	67,710	1.15
(b) Reticulocyte factors added	248,000	182,090	112.190	69,900	1.60
PRS + liver subparticles (fig. 3)					
(a) No factors added	9,680	4,210	3,310	900	3.7
(b) No factors added + globin mRNA	18,680	11.860	6,311	5,550	1.1
(c) + Globin mRNA (incorporation due to supernatant mRNA has been subtracted)	9,000	7,650	3,001	4,650	0.645

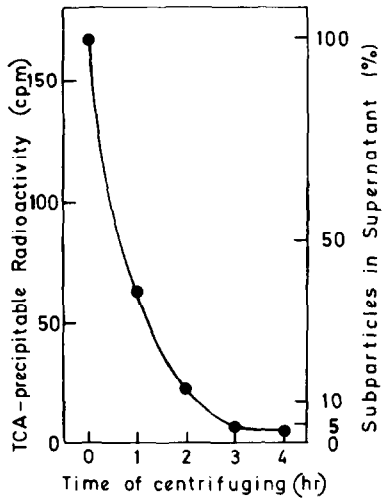

Fig. 2. Sedimentation of ^{14}C-labelled rat liver small ribosomal subparticles from reticulocyte lysate. Labelled rat liver polysomes were dissociated into subparticles (for method of labelling with ^{14}C and preparation of subparticles see details in Methods section). 2.0 A_{260} units of small subparticles (5,500 cpm) were added to aliquots of a lysate (6 vol) and centrifuged for various times at 150,000 *g*. The top 4 ml of the supernatant were carefully aspirated and dilute acetic acid was added to adjust the pH to 5.2 in order to precipitate the ribonucleoprotein, which was sedimented by centrifugation at 2000 rpm for 10 min. The precipitates were resuspended in 2.0 ml 100 mM Tris-HCl buffer (pH 7.6). 0.1 ml aliquots were precipitated with 10% trichloroacetic acid and counted in a low background (2 cpm) Nuclear Chicago counter. Radioactivity (cpm) in 0.1 ml aliquots (●—●—●).

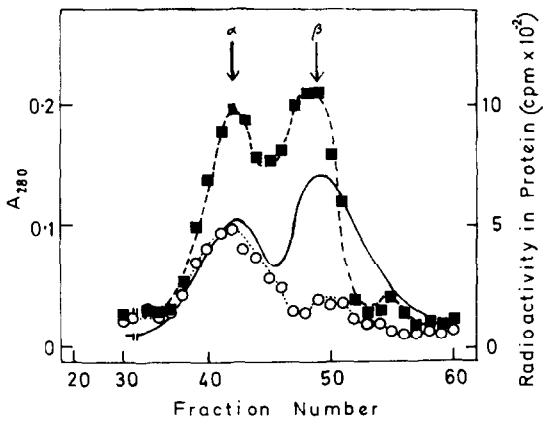

Fig. 3. Analysis of the protein synthesized by liver subparticles by chromatography on carboxymethylcellulose. Cell-free incubations (0.375 ml) were carried out and processed as described in the Methods section. Postribosomal supernatant + liver subparticles (○···○···○); postribosomal supernatant + liver subparticles + rabbit globin mRNA (5.45 μg) (■----■----■); A_{280} (——).

and counted with approx. 80% efficiency in a Packard Tricarb liquid scintillation spectrometer.

2.4. *Materials*

[U-^{14}C]leucine and [6-^{14}C]orotic acid were purchased from the Radiochemical Centre, Amersham, Bucks., England). Carboxymethylcellulose (CMC-32) came from Whatman Biochemicals (Maidstone, Kent, England).

3. Results

3.1. *The presence of messenger RNA activity in the PRS*

The incorporation of [^{14}C]leucine into TCA-precipitable material by the rabbit reticulocyte PRS was very low but increased seven fold when rat liver subparticles were added, even in the absence of factors (table 1). The observed subparticle-dependent activity corresponds to approx. 6% of the activity of the original lysate. The addition of factors had little effect on the incorporation by reticulocyte PRS without ribosomal subparticles, but stimulated the subparticle-dependent incorporation by 70%. The original lysate was also stimulated by the same factor preparation, but only by 20%.

Analysis of the incubation products by chromatography on CMC-columns (figs. 1a and 1b) showed that the majority of the radioactivity was incorporated into complete globin chains. It should be noted that the low level of incorporation obtained in the absence of subparticles (table 1) was apparently not due to synthesis of globin chains. In the PRS-subparticles system the ratio of α:β chain incorporations was 3.9 in the absence and 5.0 in the presence of added reticulocyte factors (fig. 1a). In order to compare the synthesis of globin chains by the PRS and the lysate from which it was derived, an equivalent amount of lysate (containing the same amount of ribosomes as the PRS system) was incubated under identical conditions (fig. 1b). In this case the α:β globin ratio was 1.15 and this value was increased to 1.6 by the addition of reticulocyte factors. We conclude therefore that the reticulocyte PRS contains mRNA which codes predominantly for the α-chain of globin and this messenger can be translated by heterologous subparticles. The α-chains synthesized by the reconstituted systems correspond to about 12% of the total α-chains produced by a complete lysate under the same conditions. On the assumption that the efficiency of mRNA translation is comparable in both systems we estimate that there is 0.25–0.5 μg mRNA per ml of PRS.

3.2. *Examination of the PRS for presence of subparticles*

The question arises whether the mRNA in the PRS could be present in association with any residual small ribosomal subparticles. The following experiment designed to give an estimate of the amount of small subparticles remaining in the PRS under our conditions of centrifugation was therefore carried out. Small ribosomal subparticles were labelled with ^{14}C and added to the lysate prior to centrifugation. The amount of radioactivity remaining in the supernatant was determined after various times (fig. 2). It can be seen that less than 4% of the added label remains in the supernatant after centrifugation for 3 hr. This value is almost certainly an overestimate since the amount of label remaining in the supernatant after centrifugation for 4 hr was similar and can thus probably be attributed to the presence of some acid-insoluble radioactivity other than small subparticles. If this 4 hr figure is considered to represent residual background radioactivity arising, for example, from solubilized ribosomal proteins, then the actual amount of subparticles remaining in the supernatant after 3 hr may be as low as 1%, corresponding to less than 0.08 μg of 40 S subparticle/ml of PRS. This amount of subparticles would bind only approx. 0.02 μg of globin mRNA (assuming the ratio of the relevant molecular weights to be 10^6:2.5×10^5), but even the higher figure (4%) indicates that at most only 0.08 μg of mRNA could be associated with subparticles in the 3 hr PRS preparation. Using the previous estimate for the amount of mRNA in PRS (see sect. 2.1.1.) it is concluded that there is a considerable excess of mRNA over any subparticles present, the lowest estimate being 3-fold and the highest 25. These results strongly suggest that the mRNA in the PRS is not bound to subparticles and that if it is present as an mRNP particle it has a sedimentation value of considerably less than 40 S.

3.3. *The translation of exogenous mRNA*

The synthesis of a large excess of globin α-chains

by our cell-free system can be explained most easily by the presence of excess α-chain mRNA in the PRS. The possibility exists, however, that the cell-free system translates α-chain mRNA far more efficiently than any β-chain mRNA which may be present. To investigate this possibility, we added exogenous rabbit globin mRNA to the cell-free system (fig. 3). Both α- and β-globin chains were synthesized in the ratio 0.64:1 under these conditions indicating clearly that the cell-free system can translate both mRNA species. Thus, we conclude that the observed excess α-chain synthesis is due to the presence of mainly α-chain mRNA in the PRS. These results also confirm that the PRS contains all the required factors for polypeptide chain initiation, elongation and release.

4. Discussion

4.1. *The translation of globin messenger by rat-liver ribosomal subparticles*

In an earlier paper [1] we reported that 40 S subparticles derived from rat liver ribosomes are incorporated into polysomes in an unfractionated lysate of rabbit reticulocytes by a process which has many of the characteristics of protein synthesis. It was not rigorously proved, however, that the liver subparticles which become attached to the polysomes are active in hemoglobin synthesis. The present experiments provide direct evidence that recombined 40 S and 60 S subparticles obtained from rat liver ribosomes are able to translate globin messenger RNA in a cell-free system containing the ribosome-free supernatant fraction of a rabbit reticulocyte lysate. The ribosomal subparticles were prepared by treatment of rat liver ribosomes with high salt and puromycin according to the method of Blobel and Sabatini which would be expected to remove initiation factors and endogenous messenger RNA [5]. The postribosomal supernatant was found to have only a very low activity in cell-free protein synthesis indicating the almost complete removal of ribosomes and subparticles by the conditions of centrifugation used by us. The virtual absence of subparticles in the postribosomal supernatant after centrifugation at 150,000 *g* for 3 hr is confirmed by the demonstration that labelled 40 S subparticles are almost completely sedimented from the lysate (fig. 2). The product of the subparticle and messenger-dependent protein synthesis has been characterized as α- and β-globin chains by chromatography on carboxymethylcellulose and we conclude, therefore, that heterologous ribosomal subparticles can translate globin messenger.

4.2. *The presence of initiation factors in the post-ribosomal supernatant*

The results presented in figs. 1 and 3 show that the supernatant is capable of translating both endogenous and exogenous globin mRNA, producing complete α- and β-chains of rabbit globin. It is clear, therefore, that the supernatant contains all the factors necessary for the *de novo* synthesis of globin. Previously, initiation factors have been shown to be present in the high salt wash of reticulocyte ribosomes [4], but there is no a priori reason to assume that they may not be present also in the supernatant. Indeed supernatant initiation factors have been isolated from wheat embryos [8] and Artemys (shrimp) embryos [9]. Our results are consistent with the presence of initiation factors in the ribosome-free supernatant, as well as in the high salt wash of ribosomes, and thus support the view that these factors may be released from ribosomes during or soon after the formation of the initiation complex and commencement of polypeptide synthesis [10].

4.3. *The presence of globin α-chain messenger in the postribosomal supernatant*

A comparison between the protein synthesizing ability of the postribosomal supernatant (supplemented with an excess of rat liver subparticles) and the lysate from which it was derived can be seen in table 2. Messenger RNA for globin α-chains appears to be present in the PRS in 4- to 5-fold excess over β-chain messenger as shown by the analysis of the product synthesized in a cell-free system capable of translating both mRNA species (fig. 3). Similar results were obtained at a higher (5 mM) magnesium concentration (unpublished results). It is interesting to note (fig. 1) that the addition of reticulocyte factors to the cell-free system appears to increase both total protein synthesis and the proportion of globin α-chains. The presence of globin mRNA in the factor preparations has been confirmed [11] and it has been shown that it is mainly α-chain mRNA [12]. Since the preparation of the reticulocyte lysate involves only a simple

hypotonic lysis of the cells it is unlikely that the presence of mRNA in the PRS is an artefact of the preparation. It is more likely, for example, that the supernatant mRNA is present as a result of an imbalance between chain elongation and initiation. Another possibility is that the supernatant mRNA appears as a result of reticulocyte maturation. The presence of excess α-chain mRNA is intriguing and could be due to a number of events. Thus, the β-chain mRNA may be more unstable, or may not be released free but remain bound to ribosomes at the cessation of protein synthesis. Whatever the mechanism, our findings indicate that extraction of mRNA from the postribosomal supernatant of reticulocytes may be a useful method for obtaining preparations greatly enriched with biologically active globin α-chain mRNA.

Similar conclusions concerning the presence of mRNA in reticulocyte supernatant based, however, on a different experimental approach have been reported in a recent publication [13] which appeared whilst this manuscript was in preparation.

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