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Messenger RNA for Globin in the Postribosomal Supernatant of Rabbit Reticulocytes

(mouse ascites tumor extract/α chain mRNA)

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ABSTRACT **10S RNA has been isolated from the postribosomal supernatant of rabbit reticulocyte lysate. This RNA is associated with protein in a ribonucleoprotein particle that sediments at approximately 20S. The 10S RNA extracted from this particle codes predominantly for α globin chains when it is translated by a mouse ascites cell-free extract. The product of the cell-free synthesis has been characterized by column chromatography and electrophoresis of the tryptic peptides. About 20% of the total α chain mRNA is present in the postribosomal supernatant, whereas β chain mRNA is almost exclusively associated with polyribosomes.**

Several authors have suggested that messenger RNA (mRNA) not associated with polyribosomes may be present in the postribosomal supernatant of eukaryotic cells (see ref. 1 for references). This putative mRNA is associated with proteins to form ribonucleoprotein particles, which have been designated "informosomes" by Spirin (1). However, no defined species of mRNA has been isolated from informosomes and shown to promote the synthesis of the corresponding protein. There is evidence that informational molecules, presumably mRNA, are present in the postribosomal supernatant of reticulocytes. Shapira *et al.* (2), by incubating reticulocyte ribosomes of one species with a supernatant fraction from another species, were able to synthesize hemoglobin of both species.

Here we report the isolation from rabbit reticulocyte lysate of a RNP that contains globin mRNA. This RNA promotes predominantly the synthesis of rabbit α chain by a mouse ascites cell-free extract. β chain mRNA is thus found almost exclusively in polyribosomes, whereas α chain mRNA is distributed between supernatant and polyribosomes. This finding makes it possible to purify α chain mRNA from β chain mRNA by extraction of mRNA from postribosomal supernatant.

METHODS

Cell-Free Incubation. The mouse ascites cell-free system used is that described by Housman *et al.* (3), except that the Mg^{2+} concentration was 3 mM. This concentration gave maximal stimulation by added globin mRNA.

Analysis of the Product of the Cell-Free System. Aliquots of the incubation mixtures were precipitated with 5% trichloroacetic acid and counted. Samples analyzed by column chromatography on carboxymethyl (CM)-cellulose (4) were dialyzed against 0.02 M pyridine–0.2 M formic acid (pH 2.7) after the addition of 5 mg of rabbit globin uniformly labeled with [^{14}C]tyrosine. Rabbit globin was eluted as described (5). An aliquot of each fraction was evaporated to dryness, redissolved in 0.5 ml of 0.1 N NaOH, neutralized by the addition of 0.05 ml of acetic acid, and counted after the addition of 5 ml of a 1:2 (v/v) solution of Triton X-100 and 1.2% butyl-PBD (C.I.B.A.) in toluene.

Fractions containing globin chains were pooled and lyophilized. The protein was redissolved in 20% formic acid and dialyzed against distilled water. The pH was then adjusted to 8.0–8.5 with 5% trimethylamine (phenolphthalein was used as an indicator) and the protein was digested (6) with trypsin. Aliquots of the digest were fractionated directly by high-voltage paper ionophoresis at pH 6.5 (3). The papers were cut into 1-cm strips, which were eluted with 1 ml of 0.1 N NaOH by shaking for 2 hr at 37°C. The samples were then counted as described above.

Preparation of Globin mRNA. mRNA was isolated by two successive 24-hr centrifugations at 26,500 rpm (Spinco SW27 rotor) of RNA obtained from the polyribosome pellet (7). Linear 15–30% sucrose gradients in 0.1 M NaCl–0.5% sodium dodecyl sulphate–1 mM EDTA–10 mM Tris·HCl (pH 7.4) were used. The peak of RNA that sediments at about 10 S is globin mRNA (8).

RESULTS

Fractionation of reticulocyte lysate and isolation of RNA

Reticulocyte lysate was fractionated by sucrose gradient centrifugation (see Fig. 1). A broad peak sedimenting at about 20S was consistently observed between the 40S ribosomal subunit and hemoglobin (fraction *c*, Fig. 1). Fractions corresponding to this peak (called here 20S RNP), to the 40S subunit peak, and to intermediate fractions were pooled, and the RNA was precipitated by the addition of NaCl (to 0.1 M) and of three volumes of ethanol.

The precipitate was dissolved in 0.4 ml of buffer (see *Methods*) and fractionated on sucrose gradients (Fig. 2) by the same procedure used to isolate globin mRNA. A peak sedimenting at about 10S was observed in the gradient prepared from the 20S RNP (Fig. 2*C*); this is the sedimentation value expected for globin mRNA (8). A trace amount of 10S RNA was observed in the gradient when the fractions sedimenting between the 20S RNP and the 40S ribosomal subunit were analyzed (Fig. 2*B*). The 10S RNA could not be detected in the gradient analysis of the 40S RNP, although a small amount of 10S RNA could possibly have escaped detection because

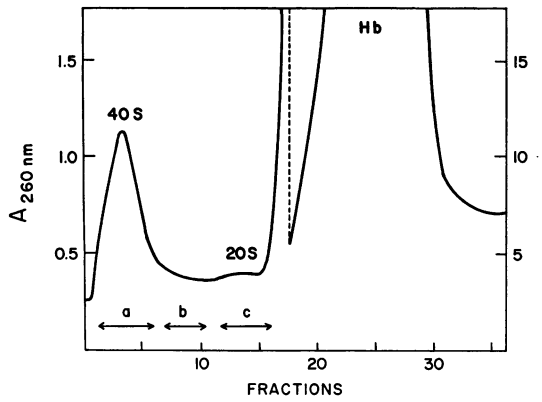

FIG. 1. Fractionation of reticulocyte lysate by sucrose density gradient centrifugation. For each gradient, 2 ml of lysate were diluted with 7 ml of 10 mM Tris·HCl (pH 7.5), 20 mM KCl, 1.5 mM $MgCl_2$ (TKM buffer) and applied over a 28 ml, 20–40% sucrose gradient in TKM buffer. The gradients were spun 24 hr at 27,000 rpm and 3° in a Spinco SW27 rotor. Preliminary experiments had shown that in this time the 40S ribosomal subunit sediments close to the bottom of the tube. The A_{260} was analyzed in a Gilford continuously recording spectrophotometer, and the indicated fractions were pooled.

TABLE 1. *Stimulation of protein synthesis by RNA fractions*

RNA fraction	cpm/μg RNA
Ia	1,070
IIa	6,680
Ib	20,800
Ic	24,300
IIc	3,750

The A_{260} of RNA fractions isolated from the gradients shown in Fig. 2 was measured. The RNA was then precipitated with ethanol, washed with ethanol, dried, and redissolved in 50 μl of distilled water. 5 μl of RNA solution was added to an ascites cell-free extract to a final volume of 120 μl. Cold amino acids and 15 μCi of [^{3}H]tyrosine (43 Ci/mmol) were added to each incubation mixture as described (3). The stimulation of protein synthesis by RNA fractions has been calculated by subtraction of the incorporation obtained without added RNA (endogenous incorporation) from the values obtained, and normalizing for the amount of RNA added. The values reported are total net incorporation for each incubation mixture. Between 0.34 and 2.7 μg of RNA was added to each incubation mixture. Incubations were 60 min at 30°.

the large peak of 18S RNA has two shoulders sedimenting at about 12S and 14S (Fig. 2*A*). The 10S RNA, and a corresponding fraction from all the gradients, was precipitated with ethanol; the RNA sedimenting around 7S (*IIb*, Fig. 2*B*) and that sedimenting around 14S (*Ia*, Fig. 2*A*) were also precipitated.

Translation of RNA and characterization of the product

Translation of globin mRNA in heterologous cell-free extracts has been described by several authors (3, 8, 11). We have used the mouse ascites cell-free extract described by Housman *et al.* (3) to establish whether the RNA isolated from the 20 S RNP is globin mRNA. The ascites cell-free extract was stimulated significantly by the addition of this RNA, whereas RNA isolated from other regions of the gradients shown in Fig. 2 had little stimulatory activity (Table 1). In particular, the RNA isolated from the 10S region of the gradient prepared from the RNA of the 40S peak (fraction *IIa* of Fig. 2*A*) had very little activity.

The product of translation of the 10S RNA isolated from 20S RNP has been analyzed by column chromatography on CM-cellulose (Fig. 3). This method separates the α and β chains of rabbit globin (4). Rabbit globin labeled with [^{14}C]-tyrosine was used as a carrier. Of 26,600 cpm of [^{3}H]tyrosine-labeled protein, 19,000 cpm were recovered under the α chain peak, whereas only 2040 cpm were recovered under the β chain peak. The specific activity (^{3}H/^{14}C) of the α chain peak was nine times higher than that of the β chain peak.

The identity of the α chain synthesized by this cell-free extract was confirmed by high-voltage ionophoresis of the

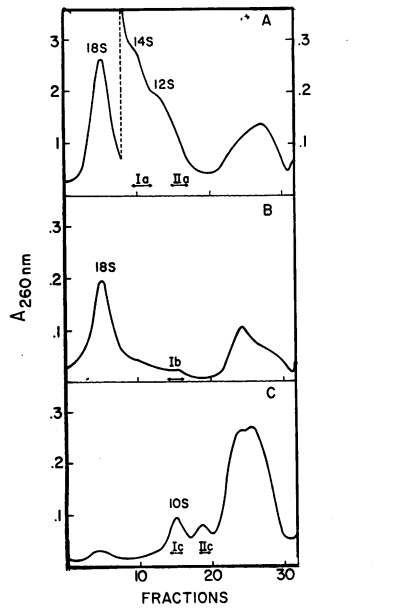

FIG. 2. Fractionation of the RNA obtained from the gradients shown in Fig. 1. Corresponding fractions from three gradients were combined, precipitated with ethanol, and redissolved in buffer (see *Methods*). The RNA was fractionated by centrifugation for 24 hr at 26,500 rpm (see *Methods*). The indicated fractions were combined and precipitated with ethanol.

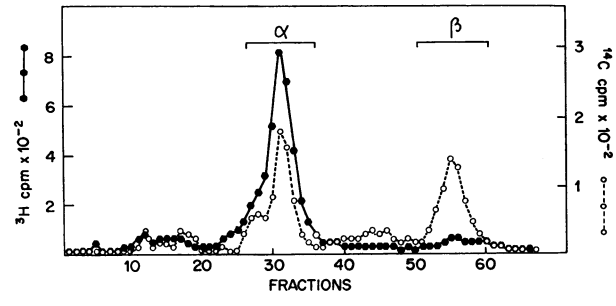

FIG. 3. Chromatography of the cell-free synthesis product of RNA isolated from 20S RNP. 5 μg of RNA obtained from peak *Ic* of Fig. 2 was added to 25-μl of ascites cell-free extract containing 10 μCi of [^{3}H]tyrosine. After 1 hr of incubation at 30°, the sample was processed (*Methods*) for CMC-chromatography. 5-ml Fractions were collected, and 2-ml aliquots were counted.

tryptic peptides. Three tyrosine-containing peptides are present in each rabbit globin chain; they can be separated by ionophoresis at pH 6.5 (3). The peptides of the α chain isolated by column chromatography showed a perfect correspondence with the tryptic peptides of the carrier α chain (Fig. 4*A*). The migration of β chain peptides is shown for reference (Fig. 4*B*). β chain synthesized by the cell-free extract could not be analyzed in this way, since only a small amount of ^{3}H-labeled protein was recovered under this peak.

Estimate of mRNA in 20S RNP

To establish whether the 10S RNA isolated from 20S RNP is as effective in directing globin synthesis as is globin mRNA isolated from polyribosomes, it has been necessary to characterize the response of the ascites cell-free extract to globin mRNA. The stimulation of protein synthesis by globin mRNA is linearly related to the input of RNA, at least to 0.5 μg of RNA per 120-μl reaction mixture (Fig. 5). In the experiment described in Table 1, less than 0.5 μg of RNA isolated from the gradient fractions shown in Fig. 2 was added, except in the case of fraction *Ia*. Since this fraction was heavily contaminated with 18S ribosomal RNA, 2.7 μg of RNA was added to the incubation mixture. Control experiments showed that the addition of up to 2.5 μg of 18S RNA to a reaction mixture containing 0.5 μg of globin mRNA had no inhibitory effect on protein synthesis.

The concentration of RNA in the fractions tested in the cell-free extract was estimated by measurement of the A_{260nm} before ethanol precipitation. At the same time, globin mRNA was isolated from the polyribosome pellet of the gradients used to prepare 20S RNP (Fig. 1), and its concentration was determined in the same way. These data allowed us to calculate the amount of 10S RNA present in 20S RNP relative to that in 10S RNA isolated from polyribosomes, and to compare their stimulatory activity.

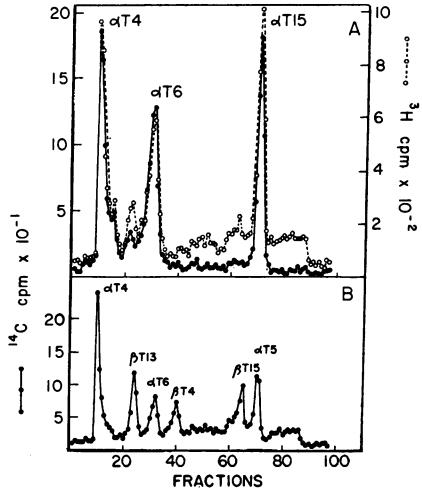

FIG. 4. Ionophoresis of the tryptic peptides of the α chain synthesized with mRNA obtained from postribosomal supernatant. *A*, the α-chain peak of Fig. 3 was concentrated and digested with trypsin; the tryptic peptides were separated and counted as described under *Methods*. *B*, ionophoresis of the tryptic peptides of rabbit globin uniformly labeled with [^{14}C]-tyrosine, shown for comparison. For identification of the peptides, see Housman *et al.* (3).

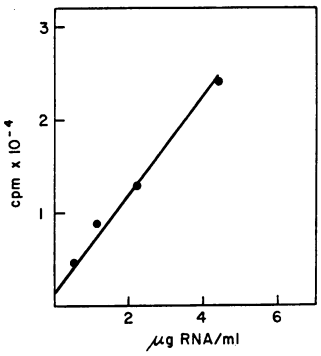

FIG. 5. Stimulation of protein synthesis by 10S RNA isolated from polyribosomes. Reaction mixtures identical to those described in the legend of Table 1 were used. 10S RNA isolated from polyribosomes was dissolved at a concentration of 110 μg/ml; aliquots of this solution were added to the incubation mixtures. Cpm are calculated as described in Table 1.

In two separate experiments 73 and 66 μg of 10S RNA were isolated from the polyribosome pellet, obtained from 6.0 ml of lysate, whereas 9.7 nd 9.8 μg of 10S RNA were isolated from 20S RNP (12% of the total mRNA). However, the 10S RNA isolated from 20S RNP (peaks *Ib* + *Ic* of Fig. 2) stimulated the ascites cell-free extract only 40% as efficiently as 10S mRNA (Table 1 and Fig. 5).

DISCUSSION

We have shown that globin mRNA can be isolated from the postribosomal supernatant of reticulocyte lysate. We suggest that this RNA is found in a ribonucleoprotein particle sedimenting at about 20S. The resolution in our gradients is not sufficient to allow us to estimate the sedimentation value of this RNP with any degree of accuracy. A RNP with similar sedimentation characteristics has also been obtained by dissociation of reticulocyte polyribosomes with Mg^{2+} chelating agents by Lebleu *et al.* (19). These authors have reported that two proteins, of molecular weights 68,000 and 130,000, are complexed with the RNA (9).

We do not have any evidence that the globin mRNA in the 20S RNP is complexed with specific proteins. Nonspecific complexes of mRNA with cytoplasmic proteins are easily formed (10) and, thus, it cannot be excluded that a RNP results from this type of artifactual interaction. Attempts to sediment the RNP and to resuspend and analyze it further have failed because of the tendency of the RNP to aggregate and the difficulty of bringing it back into solution.

We have established that the 20S RNP contains predominantly α chain mRNA. As a control that our cell-free extract does not preferentially translate α chain mRNA, we have also analyzed the product of translation of mRNA isolated from polyribosomes. We have observed that the ratio of α to β chains synthesized is 0.48, thus confirming the observation of Housman *et al.* (3) and of Temple and Housman*, who have reported ratios of 0.67 and 0.4, respectively, for polyribosomal rabbit globin mRNA translated in ascites cell-free extracts. Similar findings have been reported by Mathews *et al.* (11), who have obtained an α to β chain ratio of 0.57 for mouse globin mRNA in the ascites cell-free extract and by

* Temple, G. & Housman, D. (1972) *Proc. Nat. Acad. Sci. USA* 69, in press.

Nienhuis *et al.* (12), who have obtained a ratio of 0.67 for rabbit globin mRNA in a reticulocyte cell-free extract made dependent on mRNA. Also, Lane *et al.* (13) have observed that more β chain than α chain is synthesized upon injection of rabbit globin mRNA into frog oocytes.

These observations indicate that β chain mRNA is translated more efficiently than α chain mRNA. Several explanations seem possible for this finding. However, the observation of Hunt and Hunter (14) that in rabbit reticulocytes α chains are synthesized on polyribosomes smaller than those synthesizing β chains, together with the recent demonstration by Lodish (15) that the rate of elongation is identical for these chains, suggests that the rate of initiation of the β chain is faster than that of the α chain (15).

The process governing polyribosome size is the frequency of initiation events and the rate of translation. β chains are synthesized predominantly on polyribosomes made up of five ribosomes, whereas α chains are synthesized predominantly on polyribosomes made up of three to four ribosomes; to achieve the balanced synthesis of α and β chains that is observed in reticulocytes (16), there should be 1.4 times more α chain mRNA than β chain mRNA in polyribosomes (15). Even though the globin mRNA obtained from polyribosomes presumably contains more α than β chain mRNA, the product of the different cell-free extracts mentioned above gives a β to α chain ratio close to 2:1. This finding suggests that under these conditions, the β chain mRNA is almost three times more active in directing protein synthesis than is α chain mRNA. It seems possible that this is due to preferential initiation of β chain mRNA.

We have used 10S RNA obtained from polyribosomes to construct a calibration curve used to estimate the activity of 10S RNA obtained from the postribosomal supernatant. However, since this RNA codes predominantly for α chains, we have to introduce a correction factor that takes into account both the relative amount of α and β chain mRNA in polyribosomal 10S RNA and their relative activity in the cell-free extract. We have thus calculated that pure α chain mRNA should be roughly half as active as an equal amount of polyribosomal 10S RNA, a value close to that found experimentally. We can thus conclude that 10S RNA isolated from either polyribosomes or postribosomal supernatant may be equally good in directing protein synthesis in a cell-free extract. However, we cannot say whether in intact cells the 10S RNA in the supernatant participates in protein synthesis and is as active as polyribosomal 10S RNA.

It is interesting to point out that if ribosomes translate mRNA with random probability, one might expect to find some mRNA not associated with ribosomes. Using a Poisson's distribution, and taking three and five as the average number of ribosomes in polyribosomes synthesizing α and β chains, respectively, we have calculated that 5% of the α and 0.5% of the β chain mRNA might not be associated with ribosomes by chance. However, since the α chain mRNA isolated from the supernatant represents about 20% of the total α chain mRNA (12% of the total mRNA, corrected for the different amount of α and β chain mRNA present in polyribosomes), such a random event cannot account entirely for the presence of α chain mRNA in the postribosomal supernatant. It remains to be established whether this RNA has any specific function; for instance, that of storage. The finding that an RNA moiety coding for a defined protein is present in the postribosomal supernatant supports the idea that mRNA can be stored in the cytoplasm of eukaryotic cells.

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1. Spirin, A. S. (1969) *Eur. J. Biochem.* **10**, 20–35.
2. Shapira, G., Padieu, P., Maleknia, N., Kruh, J. & Dreyfus, J. C. (1966) *J. Mol. Biol.* **20**, 427–446.
3. Housman, D., Pemberton, R. & Taber, R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2716–2719.
4. Dintzis, H. M. (1961) *Proc. Nat. Acad. Sci. USA* **47**, 247–261.
5. Housman, D., Jacobs-Lorena, M., RajBhandary, V. L. & Lodish, H. F. (1970) *Nature* **227**, 913–918.
6. Baglioni, C., LaVia, M. & Ventruto, V. (1965) *Biochim. Biophys. Acta* **111**, 479–484.
7. Evans, M. J. & Lingrel, J. B. (1969) *Biochemistry* **8**, 3000–3005.
8. Lockard, R. E. & Lingrel, J. B. (1969) *Biochem. Biophys. Res. Commun.* **37**, 204–212.
9. Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A. & Chantrenne, H. (1971) *Eur. J. Biochem.* **19**, 264–269.
10. Baltimore, D. & Huang, A. S. (1970) *J. Mol. Biol.* **47**, 263–273.
11. Mathews, M. B., Osborn, M. & Lingrel, J. B. (1971) *Nature New Biol.* **233**, 206–209.
12. Nienhuis, A. W., Laycock, D. G. & Anderson, W. F. (1971) *Nature New Biol.* **231**, 205–208.
13. Lane, C. D., Marbaix, G. & Gurdon, J. B. (1971) *J. Mol. Biol.* **61**, 73–91.
14. Hunt, R. T., Hunter, A. R. & Munro, A. J. (1968) *Nature* **220**, 481–483.
15. Lodish, H. F., *J. Biol. Chem.*, in press.
16. Colombo, B. & Baglioni, C. (1966) *J. Mol. Biol.* **16**, 51–66.