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ARTICLE *in* FEBS LETTERS · NOVEMBER 1971

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(71)80413-1 · Source: PubMed

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PROTEINS ASSOCIATED WITH GLOBIN MESSENGER RNA IN AVIAN ERYTHROBLASTS: ISOLATION AND COMPARISON WITH THE PROTEINS BOUND TO NUCLEAR MESSENGER-LIKE RNA

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Received 18 August 1971

1. Introduction

There is evidence that the polyribosomal messenger RNA (mRNA) in animal cells is specifically associated with proteins [1–7]. In the case of cytoplasmic [5, 8] or nuclear [9] messenger-like RNA (mlRNA) similar RNA–protein (RNP) complexes have also been described. Contrary to the claim that such associations may be non-specific [10, 11], adequate control experiments suggest that they pre-exist in the cell and are not artefacts produced during lysis [5, 8, 12].

The exact physiological significance of these structures is not known. They may play a variety of roles such as: protection against RNase attack [5, 8], RNA transport [1, 8, 9], cleavage of giant mlRNA and selection of the RNA molecules to be transferred to the cytoplasm [5, 9], or initiation of protein synthesis [7]. In particular, two reports [13, 14] claim that the protein bound to the giant nuclear mlRNA is the same as that associated with mRNA in polyribosomes, indicating a possible function in mRNA transport.

We have studied these mRNP particles in a highly differentiated system: duck immature red blood cells. In contrast to the hemoglobin producing cells in mammals, duck red cells are nucleated, making possible the investigation of the ml- and mRNA complexes in the nucleus and in the cytoplasm of a cell where a specific mRNA can be identified.

We found that the polyribosomal globin-mRNA prepared by EDTA dissociation is associated with 2 predominant proteins of 73,000 and 49,000 MW, and that these proteins differ from those associated with nuclear mlRNA.

2. Materials and methods

2.1. *Substances*

Sucrose: RNase-free from Mann Research Laboratories (USA). Sodium deoxycholate: from Fluka (Switzerland), purified according to [5]. The other chemicals were reagent grade.

2.2. *Solutions*

Lysis buffer (LB): 0.01 M triethanolamine (TEA) 0.02 M KCl, 0.002 M $MgCl_2$, 0.005 M 2-mercaptoethanol, pH 7.4. Suspension buffer (SB): 0.25 M glycerol, 0.01 M TEA, 0.02 M KCl, 0.002 M $MgCl_2$, 0.005 M 2-mercaptoethanol, pH 7.4. TEK: 0.01 M TEA, 0.01 M KCl, pH 7.4. SDS-DOC buffer: 0.01 M TEA, 0.1 M NaCl, 0.01 M EDTA, 0.2% SDS, 0.2% DOC, pH 7.4.

2.3. *Isolation of polyribosomal mRNP*

Duck immature red blood cells were isolated as described [15]. Cells were lysed in 5 volumes of LB and after 3–5 min isotonicity was restored by addition of 2 M sucrose in LB. The post-mitochondrial supernatant was centrifuged at 360,000 *g* for 40 min (acceleration included) through 15% (w/v) sucrose in LB. The polyribosome pellet was resuspended in SB

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North-Holland Publishing Company – Amsterdam

at a concentration of 5–10 mg/ml. 100–200 mg of polyribosomes were dissociated [16] by addition of 0.5 M EDTA (pH 7.3) to a final concentration of 7 μmoles/mg of ribosomes and charged on a Beckman Ti 15 zonal rotor. After centrifugation the gradient was collected, the mRNP containing fractions pooled, concentrated by DIAFLO membrane ultrafiltration (Aminco PM-30), and recentrifuged through a SW 27 gradient. The purified mRNP was used for further analysis.

2.4. *Isolation of nuclear mlRNP*

Crude nuclei were cleaned by detergent [15] or by centrifugation through 2 M sucrose. Nuclear mlRNP was extracted by the technique of Samarina et al. [9], but at 37°, the extract was centrifuged on a sucrose gradient and the mono-particles [9] in the 35 S sedimentation zone were pooled.

2.5. *Preparation of the protein samples for electrophoresis*

To the ml- or mRNP samples an equal volume of 8 M urea–6 M LiCl solution was added and stirred overnight at 2°. After centrifugation at 10,000 *g*, 30 min, precipitation of the supernatant by cold 20% TCA, washing with 70% ethanol and drying, the material was redissolved in the appropriate buffer for SDS [17] or pH 4.5 urea gels [9, 18], pH readjusted and dialysed overnight against 1000 volumes of the same buffer. To completely reduce any S–S bridges, samples were heated at 100° for 1 min in 0.14 M 2-metcaptoethanol [19, 20] before charging on the gels.

2.6. *General techniques*

Isokinetic sucrose gradients [21] for conventional and zonal rotors were calculated [22] using the procedure described by Noll [21]. RNA was analysed either by SDS-sucrose gradients or by electrophoresis in polyacrylamide exponential gels [23]. Protein con-

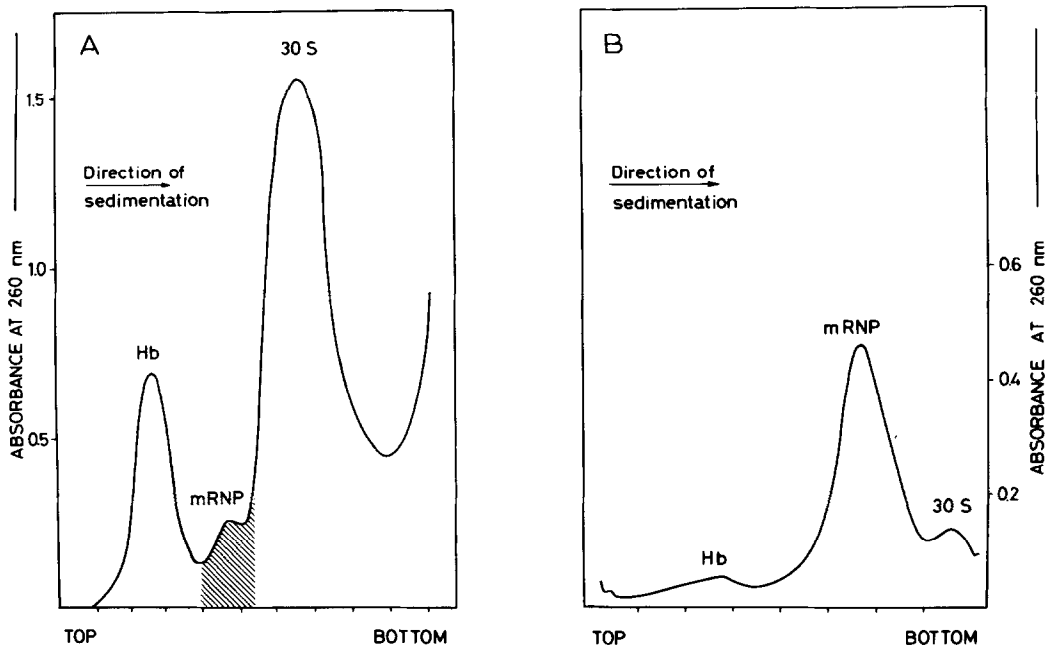

Fig. 1. Preparation of polyribosomal globin mRNP. (a) 150 mg of polyribosomes in 20 ml were dissociated by EDTA (cf. Methods) and charged on an exponential glycerol gradient (mixing chamber: 650 ml of 25% (w/w) glycerol in TEK; reservoir: 45% (w/w) glycerol in TEK) in a Beckman Ti 15 zonal rotor. 230 ml of TEK buffer were injected as overlayer. Centrifugation: 24 hr, 35,000 rpm, 2°. The mRNP region contained 3 mg of material. (b) After concentration by ultrafiltration, 2.2 mg of mRNP were recovered in 10 ml and recentrifuged on four 15–25.5% (w/w) isokinetic sucrose gradients in a SW 27 rotor (27,000 rpm, 37 hr, 2°).

centration was determined according to Lowry et al. [24]. Protein gel electrophoresis was performed either in SDS gels for the determination of MW [17], or in urea gels at pH 4.5 [9, 18].

3. Results and discussion

3.1. *mRNP purification [25, 26]*

The results of a typical zonal rotor centrifugation and second gradient purification are shown in fig. 1. The purified mRNP has an S value of about 20, and is free of ribosomal or hemoglobin contamination. The characteristics of this mRNP are similar to those of the mRNP isolated from rabbit reticulocytes [3].

3.2. *Analysis of the RNA from the mRNP*

When analysed on SDS-sucrose gradients the RNA moves as a homogeneous band of 9–10 S (fig. 2A), while on exponential acrylamide gels one can detect 3 major bands in the MW range of $1.8–2.2 \times 10^5$ daltons (fig. 2B). This RNA has biological activity in a cell-free protein synthesizing system [27]. Recently, it has been shown that mouse globin mRNA can be fractionated into two species which direct the synthesis of alpha and beta chains, respectively, in a similar cell-free system [28]. Thus it is possible that our 3 peaks correspond to the individual messengers for the 3 duck globins.

3.3. *Analysis of the proteins of the mRNP*

In SDS gels, 2 major and a few minor bands were detected (fig. 3). Molecular weight determinations gave values of 73,000 and 49,000 daltons for the main bands. It is interesting to note that in rabbit reticulocytes Lebleu et al. [7] have also found 2 proteins in globin mRNP of MW 130,000 and 68,000 daltons.

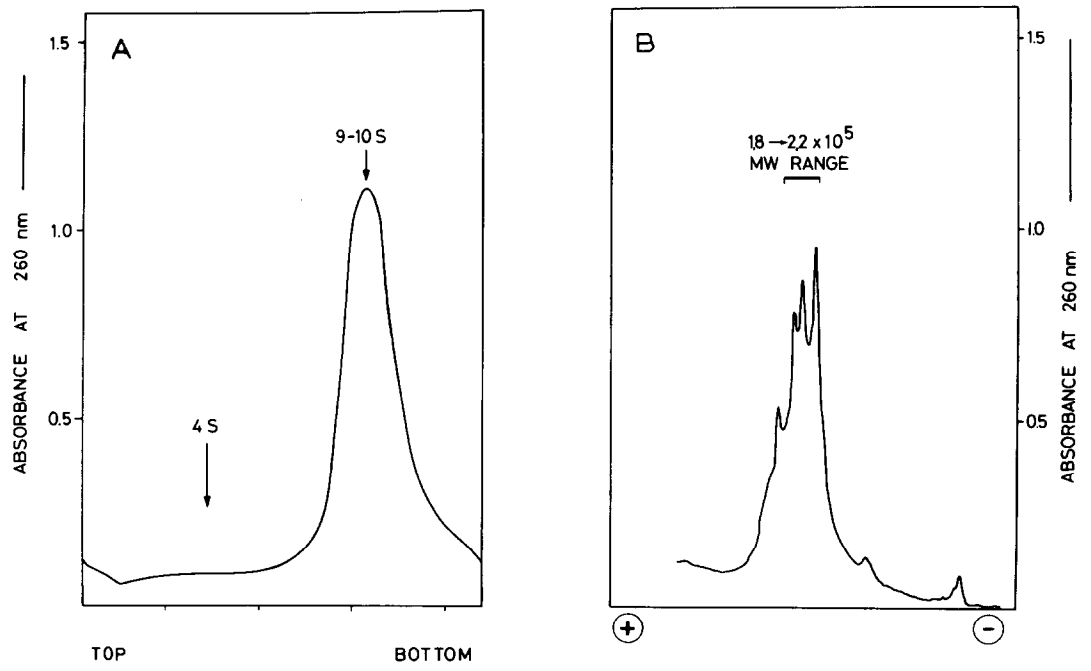

Fig. 2. Analysis of the RNA contained in polyribosomal mRNP. (a) On SDS-sucrose gradients: 0.8 mg of mRNP were suspended in 0.01 M NaCl, 0.01 M TEA, 0.01 M EDTA pH 7.4, 1% SDS, 1% DOC, warmed at 37° for 5 min and centrifuged on the SW 25.2 rotor on a 5–20.9% (w/w) isokinetic sucrose gradient in SDS-DOC buffer (45 hr, 25,000 rpm, 2°). (b) On experimental polyacrylamide gels: 0.5 A_{260} units of mRNP were suspended in 1 M urea, 1% SDS, 0.02 M Na acetate, 0.04 TEA, 0.002 M EDTA pH 7.4 and electrophoresis was carried out on a 2.5–15% exponential gel at 15° for 12 hr.

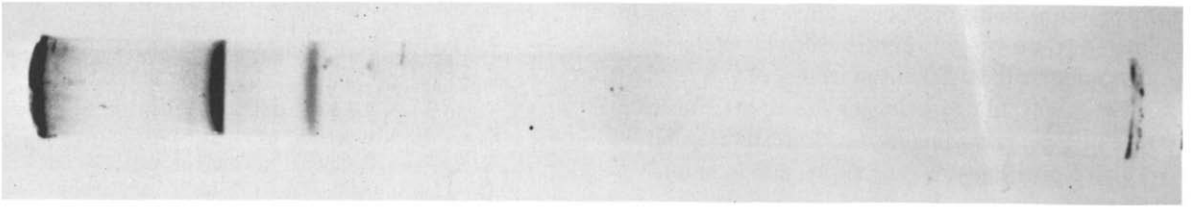

Fig. 3. Characterization of the proteins from polyribosomal mRNP by electrophoresis in the presence of SDS. 25 μg of protein was charged on a 10% polyacrylamide gel, 7 cm long. Electrophoresis: 8 mA/tube, 25°, 5 hr, 30 min. Marker dye moved 5.3 cm. Staining: Coomassie Brillant Blue.

3.4. *Comparison of polyribosomal mRNA-associated proteins with those associated with the nuclear mlRNA*

As is evident from fig. 4, the major protein bands associated with the polyribosomal mRNA and nuclear mlRNA are different, the nuclear protein moving faster than the mRNP protein bands. All ribosomal proteins have a more basic charge. In consequence, the zone of the gel where the mRNP proteins move is entirely free of contaminants. Thus the different nature of mRNP proteins compared to those of nuclear mlRNPs and ribosomes becomes unequivocal. Indirect evidence for this difference was obtained recently by Lukanidin et al. [29], comparing rabbit reticulocyte mRNA proteins to heterologous nuclear mlRNP proteins.

4. Comments

In contrast to recent claims using rat liver [13, 14], in avian immature red blood cells the proteins associated with the nuclear mlRNA are different from those associated with the polyribosomal mRNA. If the nuclear proteins had a function in transporting mRNA to the cytoplasm, ml- and mRNP would contain the same proteins. Thus the association of proteins with mlRNA in the nucleus can only be a transient one, if

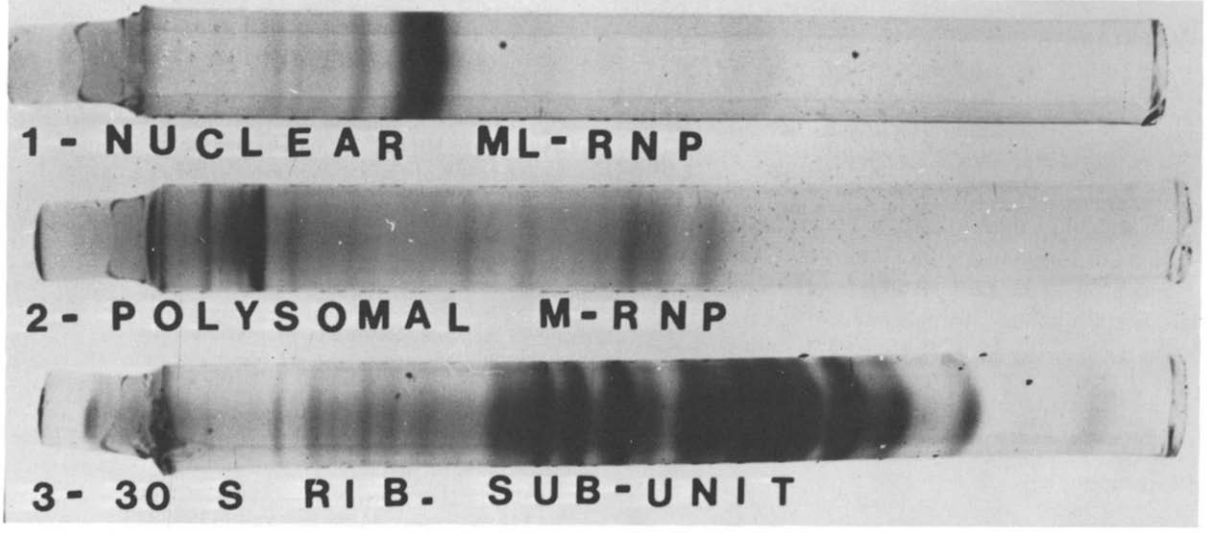

Fig. 4. Comparison of the proteins from polyribosomal mRNP, nuclear mlRNP and 30 S ribosomal subunits in urea gels at pH 4.5. 50 μg of nuclear mlRNP protein, 25 μg of polyribosomal mRNP protein and 100 μg of 30 S ribosomal subunit protein were charged on gels 1, 2 and 3, respectively. Gels were 7 cm long, 6 cm of small pore gel (10% acrylamide) and 1 cm of large pore spacer-gel (2.5% acrylamide). Electrophoresis: 3 mA/tube for 1 hr, then at 5 mA/tube for 3 hr, at 4°. Staining: Amido Black.

really mlRNA is a precursor to mRNA [6]. Then the exchange of proteins must occur during the transport of mRNA to the polyribosomes. Such an exchange could represent the mechanism of one of the regulational steps governing processing, transport and selection of mRNA, according to the "Cascade Regulation" hypothesis proposed for gene expression in animal cells [30].

Further it should be noted from our results as well as those of Lebleu et al. [7], that similar mRNAs associate with different proteins in different species. In contrast, the proteins associated with nuclear mlRNA seem to be identical in all cells [31]. Thus, these mRNP proteins must be very specific to a given mRNA. This fact, as well as their acidity (nuclear mlRNP proteins have an isoelectric point of about 7 [32], thus the slower moving mRNP proteins must be acidic), strengthens the claims concerning the non-artificial nature of mRNAs in the cell. It also makes theoretically possible their involvement in regulation. In view of the recent results of Lingrel et al. [33], demonstrating that mRNP complexes may function in an heterologous cell-free protein synthesizing system, it would be interesting to develop more defined *in vitro* systems, in which a possible specific biological function of these proteins could be tested.

Acknowledgements

We are grateful to Pierre-André Briand and Rita Müller for their excellent technical assistance and Remy Moret and Claude Germanier for the help in the preparation of the manuscript. We thank Drs. R. Eisenman, S. Modak and A. Stewart for critical reading of this manuscript. One of us (Carlos Morel) is indebted to the European Molecular Biology Organisation for a long-term fellowship. This work was supported by the Swiss National Foundation, grants nos. 3.164.69 and 3.322.70.

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