CORRELATION OF SPECIFIC CODING SEQUENCES WITH SPECIFIC PROTEINS ASSOCIATED IN UNTRANSLATED CYTOPLASMIC MESSENGER RIBONUCLEOPROTEIN COMPLEXES OF DUCK ERYTHROBLASTS

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Received 3 March 1977

1. Introduction

Cytoplasmic messenger RNA exists in animal cells in the form of two types of mRNA–protein (mRNP) complexes: in polyribosomes containing the actively translated mRNA, and as free cytoplasmic mRNP with untranslated mRNA.

In the case of globin mRNA, these complexes were isolated and purified as a '15 S' particle from polyribosomes and as '20 S' free mRNP; we found that these two different functional forms of globin mRNP contain two extensively different sets of accompanying proteins [1].

Recently, we could demonstrate that the 20 S globin mRNA is untranslatable in vitro prior to deproteinization whereas, in contrast, the 15 S globin mRNP from polyribosomes is as readily translatable in vitro as the purified 9 S globin mRNA isolated from 20 S or 15 S particles [2]. This represents a biochemical demonstration of the concept of 'Informosomes' as 'masked forms of mRNA' proposed by Spirin [17].

The 15 S polyribosomal mRNP contains the same two major proteins of approximately 50 000 and 75 000 daltons found associated with polyribosomal mRNA in many different cell systems [3–5]. In addition, we found 6 minor proteins [6]. It is unknown, so far, if these are specific to globin mRNA or if they are associated with all other types of mRNA as well.

Differential stability, as found for example in the case of duck 9 S globin mRNA and 12 S mRNA [7] and regulation of mRNA in animal cells, calls theoretically for a mechanism of specific mRNA recognition; the mRNA associated proteins could possibly serve such a function [8].

Therefore we isolated, in addition to the pure globin 20 S free RNP, some 25–35 S free mRNP particles containing 12–14 S mRNA coding for two proteins of 22 000 and 26 000 daltons. We show here that globin mRNA and these 12–14 S mRNAs are associated with different sets of major and minor proteins in the mRNP complexes. The published 20 S protein composition [1] is confirmed and we show that these mRNP complexes are free from soluble cytosol and ribosomal proteins.

Thus, an experimental basis is given to postulate recognition of specific mRNA by specific proteins at the informosome level thereby satisfying a theoretical requirement of post-transcriptional regulation at cytoplasmic level [9].

Abbreviations: 9 S mRNA, mRNA containing globin mRNA sequences; mRNP, messenger ribonucleic-acid–protein complex; 15 S* mRNP, polyribosomal mRNP containing 9 S mRNA; 20 S* mRNP, free cytoplasmic mRNP containing globin sequences; 30 S* mRNP, free cytoplasmic mRNP containing non-globin mRNA sequences; SDS, sodium dodecyl sulfate; EDTA, ethylene diamino tetra-acetate disodium salt; M_r, apparent molecular weight of proteins in SDS.

* Sedimentation values are nominal only

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2. Material and methods

2.1. *Solutions*

SDS sample-buffer: Tris–HCl (pH 6.8) 80 mM, SDS 2%, β-mercaptoethanol 2%, saccharose 10% and bromophenol blue as tracing dye; TEK-buffer: triethanolamine–HCl (pH 7.4) 10 mM, KCl 50 mM, β-mercaptoethanol 5 mM.

2.2. *Techniques*

Free cytoplasmic mRNP were prepared from a post-polyribosomal supernatant of duck immature erythrocytes as previously described [1] and fractionated by zonal centrifugation on isokinetic 10–26.5% sucrose gradients [10] in TEK-buffer (Beckman zonal rotor Ti 15, 19 h, 32 000 rev/min 2°C).

The mRNP collected from different regions of such a gradient were concentrated by pelleting at high speed (365 000 × *g*, overnight) and resuspended in TEK-buffer for further analysis and purification on a second isokinetic gradient (Beckman rotor SW 41).

After precipitation of purified mRNP in 0.1 M NaCl by addition of two vol. ethanol and redissolution in SDS sample-buffer, gel electrophoresis of the proteins was performed. The best resolution in the separation of the mRNP proteins and the comparison with ribosomal proteins was obtained by superposition of a 5% stacking-gel onto a 10–15% exponential-gradient polyacrylamide slab-gel [11]. The buffer system was derived from Laemmli [12], except that the electrophoresis buffer contained Tris 0.0375 M, Glycine 0.3 M and SDS 0.1%.

Phosphorylase B (90 000 M_r), BSA (68 000 M_r), hexokinase (50 000 M_r), DNAase (31 000 M_r), α-chymotrypsinogen (25 000 M_r) and cytochrome *c* (12 500 M_r) were used as mol. wt markers.

The RNAs from the mRNP were isolated, after dissociation of the particles by SDS-treatment, either on SDS–DOC sucrose-gradients [3] or by the hot phenol-extraction procedure [13]. In vitro biological mRNA activity was essayed in a wheat-germ extract cell-free protein synthetizing system [14,2].

After gel electrophoresis of the incubation mixture, the gels were stained (0.1% Coomassie), destained, and dried for fluorographic detection of radioactivity [15].

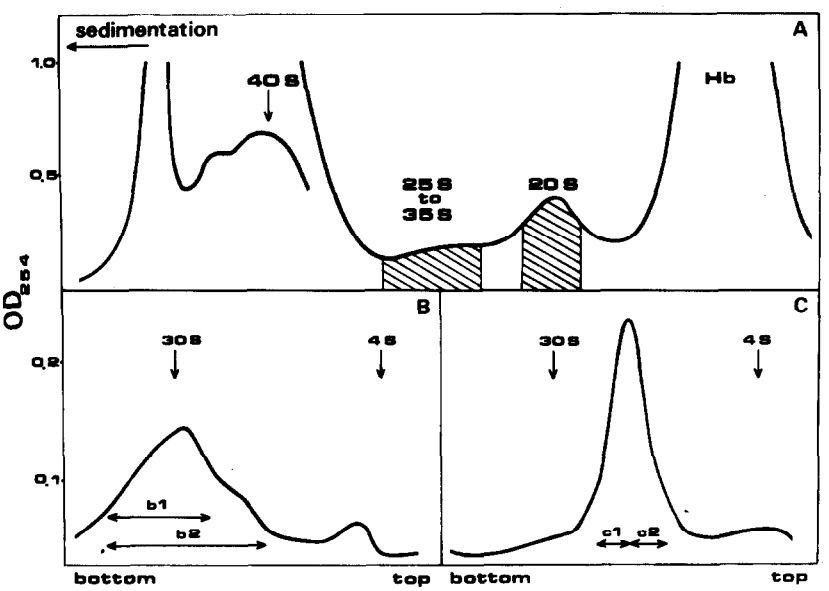

Fig.1. Preparation and analysis of free mRNP particles on sucrose-gradients. Post-polyribosomal particle pellets were resuspended in TEK-buffer and 200 mg were fractionated on a 10–26.5% (w/w) isokinetic sucrose-gradient in TEK-buffer (Beckman zonal rotor Ti 15, 32 000 rev/min, 2°C, 19 h). The hatched zones of this gradient-profile (fig.1A), corresponding to the 20 S and 25–35 S sedimentation zones, were pooled and concentrated before recentrifugation on a second sucrose-gradient (Beckman rotor SW 41, 41 000 rev/min, 2°C, 14 h). (A) Preparative zonal-gradient. (B) and (C) Analytical gradients. (B) 25–35 S pool (C) 20 S pool.

3. Results

3.1. *Preparation and purification of cytoplasmic mRNP*

Cytoplasmic mRNP were prepared by fractionation of the post-polyribosomal supernatant through isokinetic zonal sedimentation-gradients. The typical A_{254}-profile obtained after such a centrifugation (fig.1A) shows two distinct peaks of particles sedimenting between haemoglobin and the 40 S ribosomal subunits: a peak of 20 S globin mRNP [1,2] and a peak of particles sedimenting in the 25–35 S zone of the gradient.

These two types of mRNP particles were purified by a second round of sucrose gradients (fig.1B and 1C). Ribosomal subunits prepared by dissociation of polyribosomes with EDTA were used as sedimentation markers on parallel gradients. The 20 S particles sediment on this purification gradient as a single peak free of haemoglobin contamination. The 25–35 S particles sediment in a broader zone centered around the 30 S position with a shoulder in the 20 S region of sedimentation. No contamination by ribosomal subunits could be detected on these gradients. By definition, we will call the particles of the 25–35 S zone '30 S free mRNP'.

3.2. *Translation of mRNA isolated from 20 S globin mRNP and the 30 S free mRNP particles*

In the wheat-germ protein synthesizing system, the incorporation of labelled methionine into polypeptides is stimulated by the addition of mRNA from free 20 S globin mRNP or from 30 S free mRNP particles. The products of translation coded for by RNA from the two species of mRNP were separated according to their mol. wt on polyacrylamide SDS-gels and visualized by fluorography (fig.2). As previously shown [2], mRNA extracted from 20 S mRNP directs exclusively the synthesis of globin chains. On the contrary, mRNA isolated from heavier particles (30 S mRNP) directs the synthesis of longer peptides (fig.2, Slots 4 and 6). Using mRNA prepared exclusively from the faster sedimenting 30 S mRNP particles (Fraction b1 of fig.1B), contamination by globin mRNP could be avoided (fig.2, Slot 4). Sequences coding for two predominant peptides of unknown nature, mol. wt 22 000 and 26 000, are present in this mRNA.

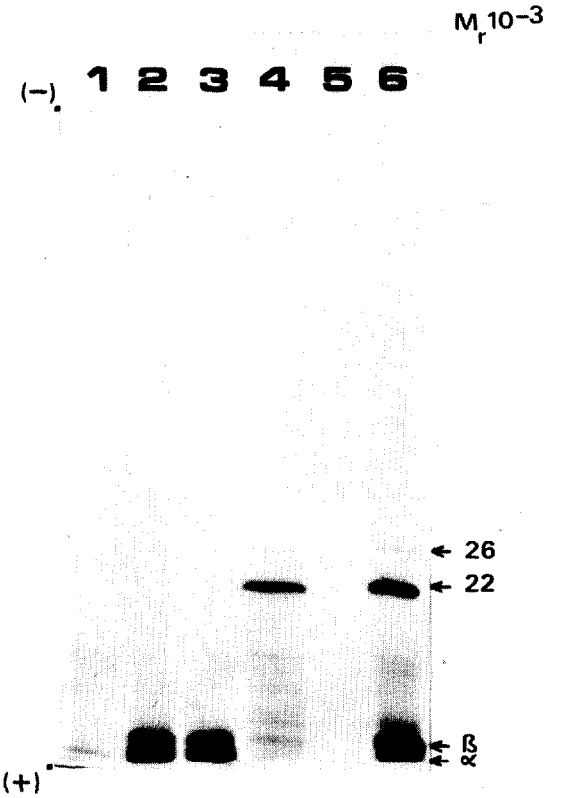

Fig.2. Analysis of the products of in vitro translation of the purified mRNP messengers. The wheat-germ extract cell-free translation mixtures, containing mRNA isolated by deproteinisation of the different purified mRNP were incubated for 2 h at 30°C and immediately chilled at 0°C [14]. Aliquots of about 8000 TCA-precipitable cpm were boiled in SDS sample-buffer and loaded onto 10 cm long SDS–acrylamide (13% w/v) slab-gels. Non-radioactive globin chains and standard Sigma proteins were used as M_r markers. The gels were briefly stained, destained and processed for fluorography according to the technique of Laskey and Mills [15]. (1) No added mRNA. (2) Polyribosomal 9 S mRNA. (3) mRNA from purified 20 S mRNP. (4) mRNA from purified 30 S mRNP (fraction b1 fig.1B). (5) Mol. wt markers. (6) mRNA from total 30 S mRNP (fraction b2 fig.1B).

3.3. *Protein composition of free cytoplasmic mRNP containing globin or other mRNA*

3.3.1. Protein composition of the purified free cytoplasmic globin mRNP

To control the homogeneity of the purified 20 S globin mRNP, we compared the protein composition of two different fractions c1 and c2 of the gradient

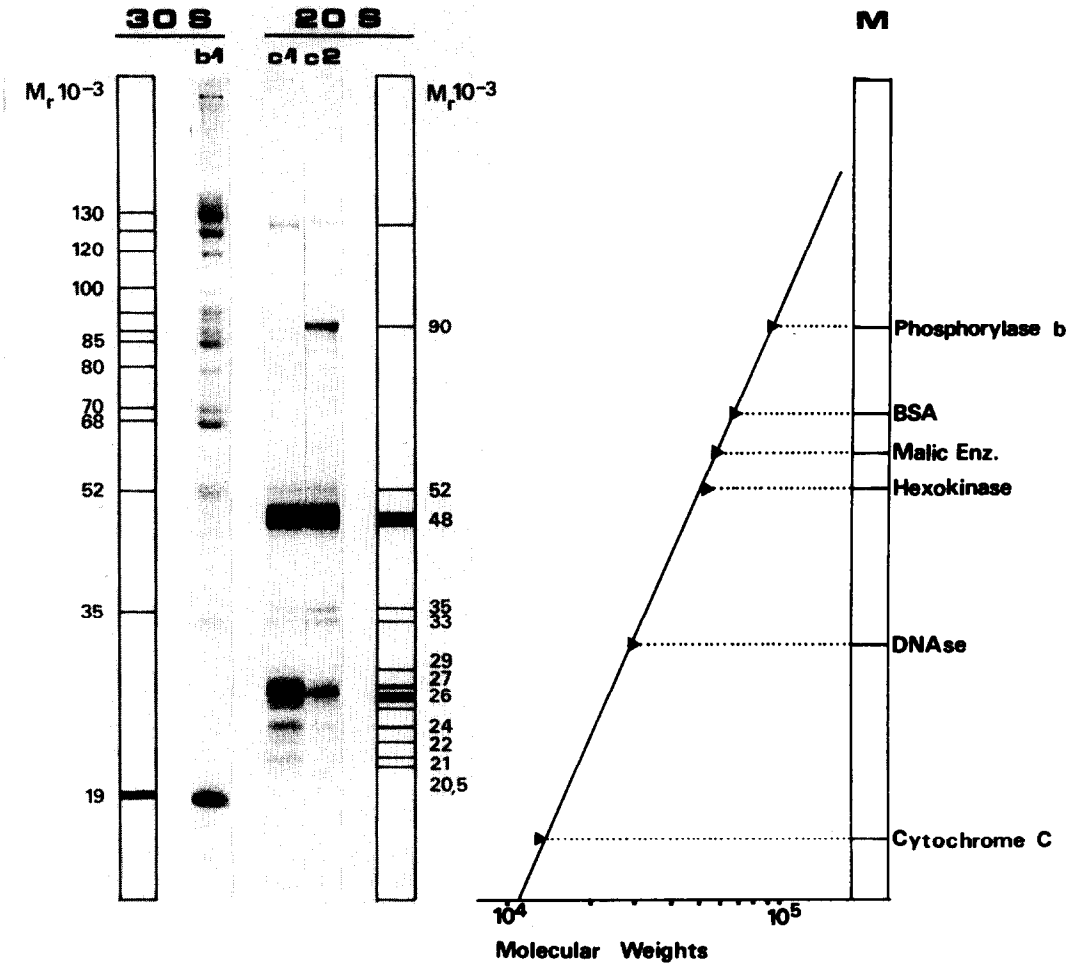

Fig.3. Comparative polyacrylamide gel electrophoresis of free cytoplasmic mRNP–proteins and the determination of their molecular weights. mRNP particles (10–15 μg) contained in the peak fractions shown in fig.1 were precipitated with ethanol in 0.1 M NaCl. The precipitate was dissolved in 20 μl SDS sample buffer, boiled and charged on a 20 cm long 8–15% exponential polyacrylamide gel-slab containing SDS. Gels were stained with Coomassie Brilliant Blue in TCA 12% and destained in 7.5% acetic acid with continuous stirring. Electrophoresis was at 25 mA for 6 h at 18°C. (30 S) Purified 30 S mRNP (fraction b1 of fig.1B). (20 S) Purified 20 S mRNP (fig.1C). (c1) Fraction c1. (c2) Fraction c2. (M) Marker positions (original gel not shown). Phosphorylase *b* (M_r 90 000). Bovine serum albumin (M_r 68 000), hexokinase (M_r 50 000), DNAase (M_r 31 000), cytochrome *c* (M_r 12 500) (Sigma), malic dehydrogenase (M_r 56 000) (gift by A. G. Goodridge).

shown in fig.1C. The electrophoretic pattern of proteins contained in these purified 20 S mRNP are shown in fig.3. Some proteins between 70 000 and 120 000 daltons, present in crude particles, disappear during purification (not shown). An additional 90 000 dalton protein found in the fraction c2 (cf. fig.1C) represents the only qualitative difference found consistently in the protein composition of the two fractions of purified 20 S particles. The importance of this protein will be discussed elsewhere (Vincent et al. in prep.).

Thus, the 20 S globin mRNP contains clearly identifiable proteins (or protein subunits) ranging from 20 000–52 000 daltons. This protein composition, slightly different from previous results [1], confirms that the proteins associated with globin

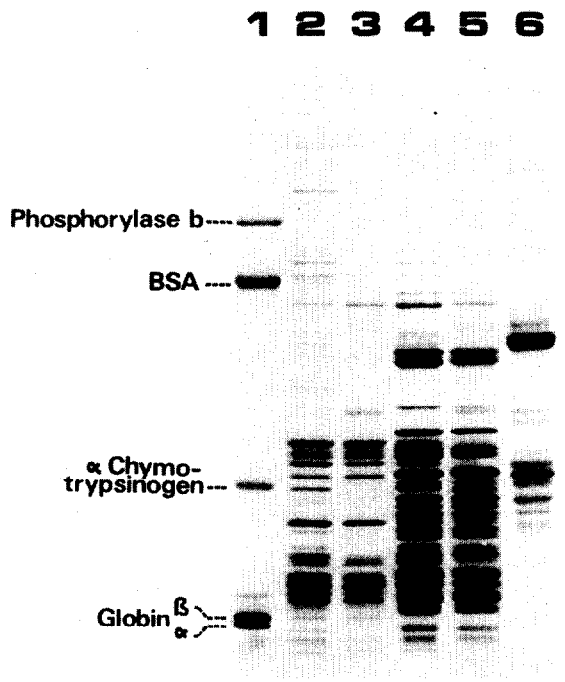

Fig.4. Comparison of purified 20 S globin mRNP proteins with purified ribosomal subunit proteins. RNA protein particles in TEK-buffer were pelleted by high speed centrifugation, redissolved in SDS sample-buffer and charged on 10 cm long 10–15% exponential polyacrylamide slab-gels. Electrophoresis was at 25 mA for 4 h at 18°C. (1) Mol. wt markers. (2) and (4), 50 S and 30 S ribosomal subunits derived by EDTA dissociation of polyribosomes. (3) and (5), native 40 S and 60 S ribosomal subunits. (6), Purified 20 S mRNP (fractions c1 + c2 of fig.1C).

mRNA in the free cytoplasmic mRNP are different from those found in polyribosomal globin mRNA [6]: none of these proteins comigrates within globin markers (fig.4). This demonstrates the absence of contamination of the 20 S mRNP by cytosol soluble proteins. Furthermore, these results exclude an association in untranslated globin mRNP, of globin mRNA with its translation product as has been suggested for immunoglobulin [16].

3.3.2. Comparison of proteins associated with different types of mRNA in free cytoplasmic mRNP

The analysis in polyacrylamide SDS gels of the proteins associated with non-globin mRNA in the free cytoplasmic 30 S mRNP has given a very specific pattern (fig.3). A group of proteins ranging from 50 000–120 000 daltons with a major component of 19 000 daltons are detected on these gels. Comparing 20 S globin mRNP and 30 S non-globin mRNP it is evident that their respective protein compositions are totally different. Preliminary results (data not shown) demonstrate that these differences persist after high (0.5 M KCl) salt treatment of the respective particles.

4. Discussion

The main observation reported here concerns the evidence that in untranslated mRNP complexes free in the cytoplasm of an eukaryotic cell, mRNAs of different information content are associated with different populations of proteins. This situation is in marked contrast to that in the polyribosomes in which, in all cases so far analyzed, the different mRNAs are associated with the same two major proteins. Thus, a selective recognition of specific mRNA by accompanying proteins can be postulated exclusively at the level of the free mRNP, which in view of the repressed state of their mRNA [2], seem to constitute a cytoplasmic system of selective negative control of gene expression. Such a system, postulated in Spirin's Informosome Concept [17], and in our Cascade Regulation Hypothesis [8] could operate, hence, by the competition for a particular mRNA between non-specific polyribosomal mRNP proteins ('initiation factors', possibly) and specific informosome proteins acting directly or indirectly as mediators of repression.

The difference in protein composition of the two sets of free mRNP–proteins associated with mRNA of different information content (fig.2) seems well established (fig.3). Controls demonstrate that soluble proteins are absent from the 20 S mRNP and that ribosomal proteins are not detectable either, in 20 S or in 30 S mRNP (fig.4, and unpublished data). Furthermore, recent experiments in which the free complexes were subjected to treatment with high salt concentrations showed that at 0.5 M KCl characteristic release of certain proteins occurs. Nevertheless, a distinctive difference in the protein compositions of the two core particles still persists. A systematic

study of these salt-washed mRNP by two-dimensional gel electrophoresis is in progress.

An interesting observation concerns the absence in free particles of the specific poly(A)-binding protein of about 75 000 daltons which has been found on all polyribosomal mRNP so far analysed [18,19,20]. Since both free mRNPs contain polyadenylated mRNA (our laboratory, unpublished) it seems that the poly(A) sequence is somehow shielded by elements of secondary structure or by one or more of the specific mRNP proteins. In the latter case, since none of the proteins we have observed seems to be common to both types of specific mRNA examined, a combined recognition of poly(A) as well as of a mRNA specific sequence must be postulated. Such a situation is implied in the recent model proposed by Brawerman [21]. However, the model of Heywood, involving a tcRNA [22], could also fit such a structure.

A last striking feature of this preliminary analysis concerns the unambiguous demonstration that globins are not bound to their mRNA in the repressed mRNP. This excludes, for globin mRNA as well, the model of cytoplasmic repression of mRNA originally proposed by Williamson [16,23], operating by direct association of the messenger with its product. More complex mechanisms of control involving possibly specific proteins, cytoplasmic repressors, or tcRNA [22] must operate in the eukaryotic cell.

Acknowledgements

We thank Dr K. Maundrell and Dr M. T. Imaizumi for helpful discussions during this investigation, and for critical reading of this manuscript. One of us (O.C.) holds a fellowship of the Swiss Institute of Technology. This work was supported by the DGRST (Grant no. 7470574 and no. 7671190), the Fondation pour la recherche Medicale Francaise and the French CNRS (ATP No. 2118).

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