INVOLVEMENT OF RNA-BINDING PROTEINS IN THE FORMATION OF INFORMOSOMES *IN VIVO*

S. M. ELIZAROV, A. S. STEPANOV, P. E. FELGENHAUER+ and E. V. CHULITSKAYA+

A. N. Bakh Institute of Biochemistry, USSR Academy of Sciences, Moscow and *+N. K. Koltsov Institute of Developmental Biology, USSR Academy of Sciences, Moscow, USSR*

Received 16 June 1978

1. Introduction

RNA-binding proteins, first revealed in rat liver and HeLa cell extracts [1–4], are a special class of eukaryotic cell proteins (reviewed [5]). These proteins can interact specifically with exogenous RNA added to the extracts and forming stoichiometric informosome-like complexes with a buoyant density of 1.4 g/cm^3 in CsCl [1–4,6]. This property of the proteins of binding to RNA encourages the assumption that in vivo these proteins are responsible for the formation of informosomes [2,4]. Up to the present, however, this assumption was based only on indirect results obtained in studies of RNA-binding proteins in vitro.

Our previous communication reported the isolation of RNA-binding proteins from frog (*Rana temporaria*) oocyte extract by affinity chromatography on poly(U)–Sepharose and their labelling in vitro by reductive methylation using sodium [^{3}H]-borohydride [7]. Here the technique of microinjection of the labelled RNA-binding protein preparation into the frog oocytes has been used. It has been shown that the protein injected becomes incorporated into informosomes during oocyte cultivation.

2. Materials and methods

2.1. *Preparation of RNA-binding proteins for injection into frog oocytes*

Frog females were stimulated by intraperitoneal injections of *R. temporaria* hypophysis suspension (3 hypophyses/female). After 20–24 h the oocytes were removed and thoroughly washed in the Ringer solution for cold-blooded animals (6.5 g NaCl, 0.25 g KCl, 0.2 g $NaHCO_3$, 0.3 g $CaCl_2$ per liter H_2O) with antibiotics (500 penicillin units and 250 streptomycin units/ml). These oocytes were used for isolation of RNA-binding proteins and for injection of labelled protein preparations into them.

The procedures of homogenization of the oocytes, preparation of the ribosome-free extract and affinity chromatography of the extract on poly(U)–Sepharose columns have been detailed [7]. The RNA-binding proteins eluted from the poly(U)–Sepharose column were labelled by reductive methylation using sodium [^{3}H]borohydride (416 μCi/mM) [8]; the rechromatography of the labelled proteins on poly(U)–Sepharose was done as in [7]. Prior to injections the [^{3}H]protein preparations were dialyzed against the buffer containing 0.01 M triethanolamine–HCl, 0.01 M KCl, 0.005 M $MgCl_2$ and 0.006 M mercaptoethanol, pH 7.8 (standard buffer) with 0.10 M NaCl. The preparations for injection usually contained about 0.5 mg [^{3}H]protein/ml with spec. act. 45–75 $\times$ 10^3 cpm/μg. The RNA-binding activity in these preparations was assayed by the technique of retention of the formed ribonucleoprotein complexes on nitrocellulose filters as in [1,4]. 23 S *Escherichia coli*, 1 μg, was adsorbed on the filter in the presence of 1 μg protein preparation.

2.2. *Technique of protein microinjection and conditions of oocyte cultivation and homogenization*

The injection of [^{3}H]protein preparations into the oocytes was done with a pneumatic micromanipulator

by the method in [9,10]. Protein solution, 0.064 μl or 0.096 μl, was injected into 2.5–3 μl of an oocyte. The oocytes (150–350) were then cultivated in the Ringer solution with antibiotics for 16–20 h, at 18°C. After cultivation the oocytes were washed in 500-fold vol. standard buffer with 0.25 M sucrose and disrupted by centrifugation at 40 000 × *g* for 15 min. The lipid film was discarded and the supernatant fraction (extract) was collected for the subsequent analysis. All the steps of the procedure were performed at 4°C.

2.3. *Sedimentation analysis of the extract in the sucrose gradient*

The material for the analysis was layered on a 15–25% sucrose gradient, with a 0.75 ml 50% sucrose cushion, prepared in standard buffer with 4% formaldehyde. Centrifugation was done at 45 000 rev./min in a Spinco model L5-50 ultracentrifuge using SW-50.1 rotor; centrifugation time is indicated in the figure legends. Sedimentation coefficients of the fractions were estimated from their positions relative to the *E. coli* 70 S ribosomes.

2.4. *Analysis of the preparations in the CsCl density gradient*

CsCl gradient analysis was done for fractions of the extract isolated from the sucrose gradient or for the original extracts fixed with formaldehyde as in [4]. Preparation of CsCl solutions to form the gradients and determination of CsCl density in the fractions after centrifugation are described in [11]; the formation of stepwise CsCl gradients before centrifugation is also detailed in [12]. Centrifugation was done in a Spinco L5-50 centrifuge using a SW-50 rotor at 38 000 rev./min for 36–40 h, 4°C. The radioactivity of the fractions was measured in the trichloroacetic acid-precipitated material applied on nitrocellulose filters (0.40 μm pores) in the standard toluene–PPO–POPOP scintillation solution.

3. Results

The cell extracts obtained from oocytes 20 h after injection with RNA-binding [^{3}H]proteins were fractionated by centrifugation in the sucrose gradient. As seen in fig.1, the radioactive material sediments not only in the zone of free RNA-binding proteins (5–7 S), but also in the 50–70 S zone. The analysis in the CsCl gradient given in fig.2a shows that the [^{3}H]protein-containing material of the 50–70 S zone represents particles with a buoyant density of about 1.4 g/cm^3 characteristic of informosomes.

CsCl gradient centrifugation of the unfractionated extract reveals that the material containing [^{3}H]-protein is distributed in two bands with buoyant densities ~1.4 g/cm^3 and ~1.3 g/cm^3 (fig.2b). The proportion of the label in these bands depends on the RNA-binding activity of the injected [^{3}H]-proteins as well as on the absolute amount of [^{3}H]-proteins inected into the oocyte. On the average,

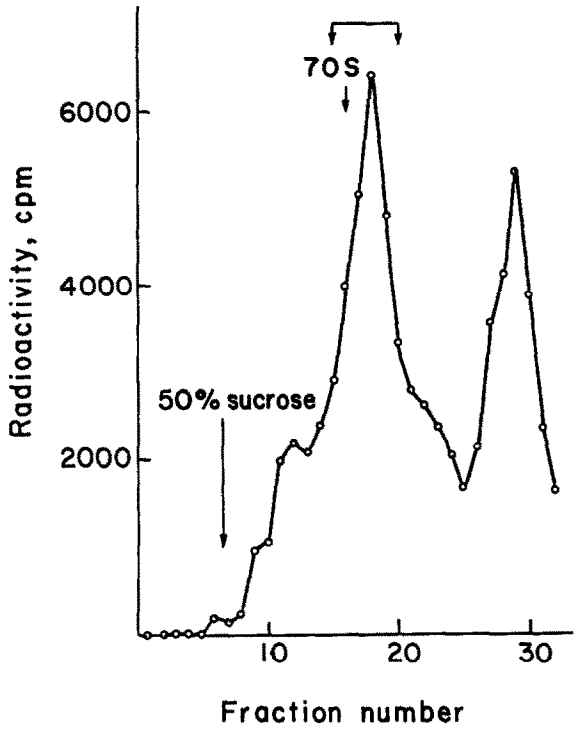


Fig.1. Sedimentation distribution in the sucrose gradient of [^{3}H]protein-containing material of the oocyte extract prepared 20 h after injection with RNA-binding [^{3}H]protein. Extract, 0.2 ml containing no more than 8 A_{260} units, was layered on the sucrose gradient prepared as indicated in section 2.3. Centrifugation was done for 90 min. Arrow shows position of *E. coli* ribosomes in a parallel tube.

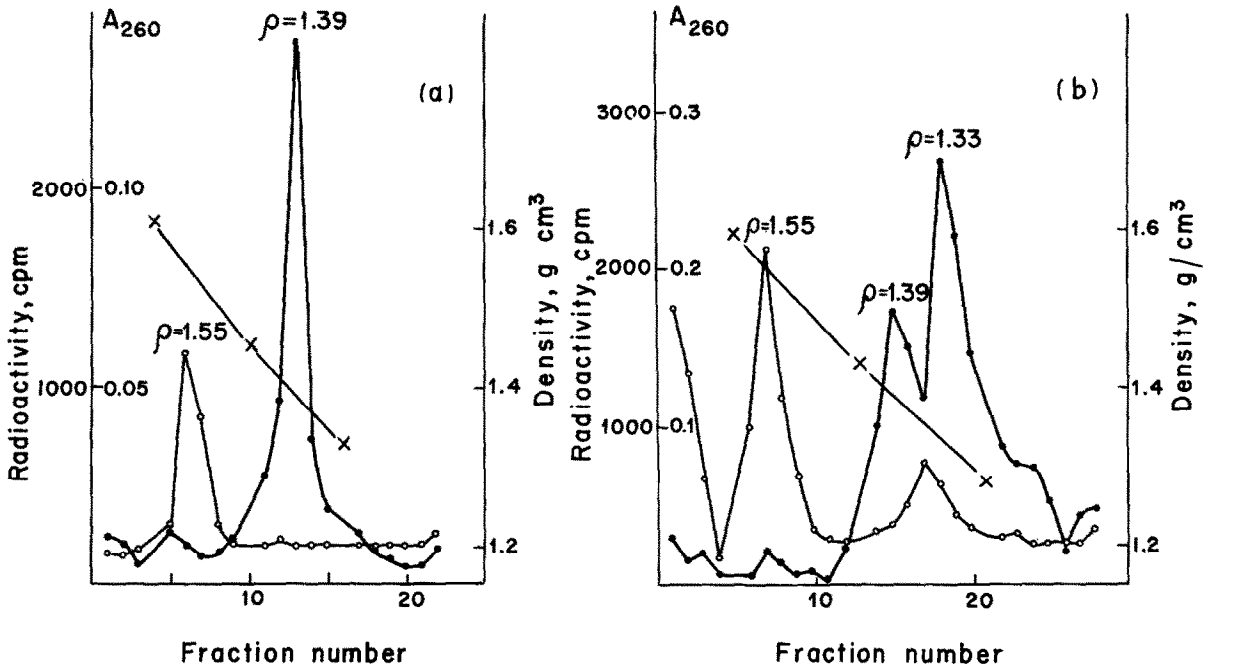


Fig. 2. Density distribution in the CsCl gradient of: (a) fraction of the extract (50–70 S zone) denoted by brackets in fig. 1, and (b) the same unfractionated extract as in fig. 1. Formaldehyde fixation of the samples to be analyzed and centrifugation was done as indicated in section 2.4. ^{3}H-radioactivity (—•—); A_{260} (—o—); CsCl density (—x—).

when the dilution of the pool of free RNA-binding proteins of the recipient oocyte by the injected proteins is not significant (5–20%), no less than 20–30% ^{3}H-labelled material introduced into the CsCl gradient is detected in the informosome band ($\rho \simeq 1.4$ g/cm^3).

Figure 3 shows the results of sucrose and CsCl gradient centrifugation analysis of the extract pre-treated with RNase. It is seen that this treatment results in the practically complete disappearance of the material containing [^{3}H]protein from the 50–70 S zone (fig.3a); correspondingly, the component with buoyant density 1.4 g/cm^3 becomes absent in the CsCl gradient profile (fig.3b).

Thus, the data provide evidence that after 18–20 h from the time of their injection into oocytes the RNA-binding [^{3}H]proteins are found to be incorporated into ribonucleoprotein particles with the sedimentation and density characteristics of informosomes. The particles are sensitive to RNase which is also typical of informosomes.

Some special controls are required in order to exclude:

(i) A metabolic transfer of the radioactive label to the proteins of pre-existing informosomes of the recipient oocytes;

(ii) A non-specific exchange of the [^{3}H]protein with the protein of pre-existing informosomes during or after homogenization of the recipient oocytes.

To check the first possibility, cytosol [^{3}H]proteins purified from RNA-binding proteins by affinity chromatography on poly(U)–Sepharose, were injected into the oocyte and then the latter was incubated in the same manner as in the above experiments; in this case the labelled component with buoyant density ~1.4 g/cm^3 was not observed in the oocyte extracts (fig.4). Thus, the appearance of ^{3}H-labelled informosomes in oocytes injected with RNA-binding [^{3}H]-proteins is the result of the specific interaction of just these proteins with the RNA component of the recipient oocyte.

It could be assumed that the incorporation of

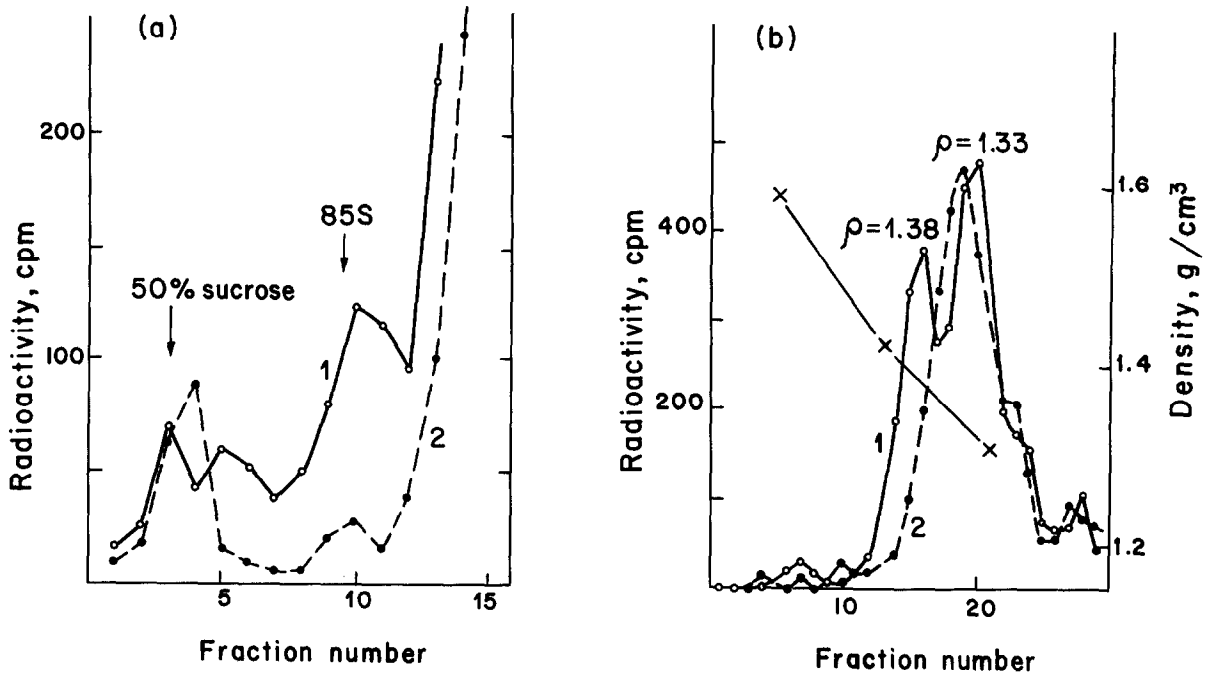
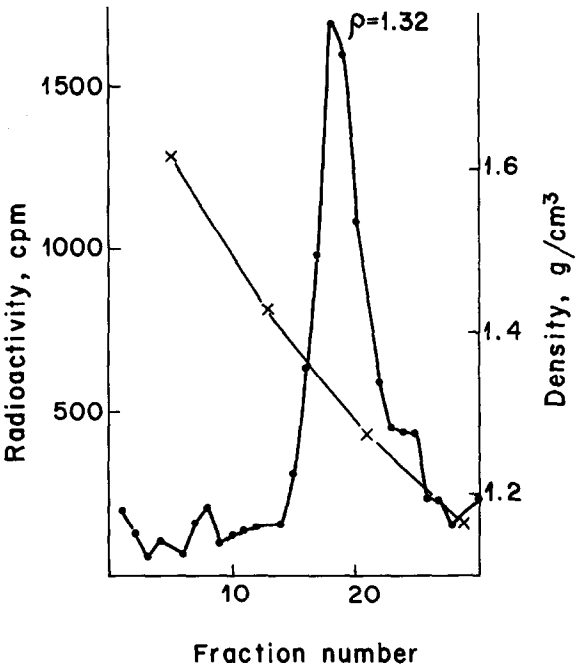


Fig.3. Distribution of material containing [^{3}H]protein of the oocyte extract prepared 18 h after injection with RNA-binding [^{3}H]protein. (a) sucrose gradient; (b) CsCl density gradient. Curve 1, original extract (—o—); curve 2, the same extract pre-incubated for 60 min at 20°C in the presence of 25 μg/ml RNase (- -•- -). Centrifugation was done for 60 min.



[^{3}H]protein into the informosomes is either an artefact of homogenization or the result of a non-specific exchange ([^{3}H]protein + RNA·protein⇌[^{3}H]-protein·RNA + protein) occurring equally during incubation of living recipient oocytes and in their homogenates. The results of the control experiments on homogenization of non-injected oocytes in the presence of RNA-binding [^{3}H]proteins (fig.5a) and on the incubation of RNA-binding [^{3}H]proteins in oocyte homogenates (fig.5b) refute both the first and the second assumptions. Thus, this proves that the incorporation of RNA-binding proteins into informosomes takes place in the living cell.

Fig.4. Density distribution in the CsCl gradient of [^{3}H]-proteins of the extract obtained from oocytes 20 h after injection of labelled total cytosol protein. Cytosol protein was labelled by reductive methylation as indicated in section 2.1 after preliminary removal of the RNA-binding protein fractions by affinity chromatography. ^{3}H-radioactivity (—o—); CsCl density (—x—).

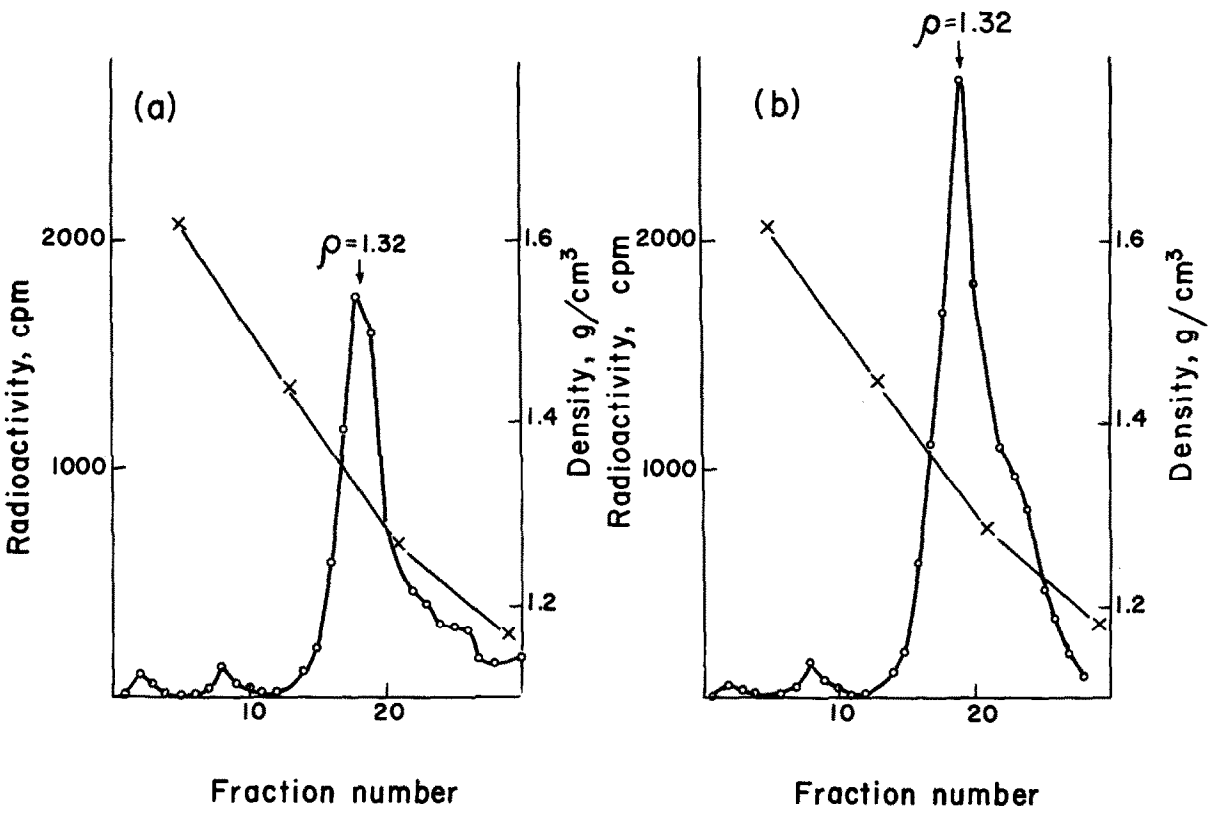


Fig.5. Density distribution in the CsCl gradient of RNA-binding [^{3}H]proteins added to extracts prepared from non-injected oocytes. The oocytes were disrupted in the presence of RNA-binding [^{3}H]protein added in an amount equal to that injected into the oocytes in the other experiments; (a) the extract was treated immediately with formaldehyde and analyzed in CsCl; (b) the mixture was incubated for 16 h at 4°C, fixed with formaldehyde and analyzed in CsCl. ^{3}H-radioactivity (—o—); CsCl density (—x—).

4. Conclusion

The results reported in this communication are the first direct experimental proof of the hypothesis that the function of RNA-binding proteins in vivo is to form informosomes. It is shown that RNA-binding proteins isolated from ribosome-free extracts of frog oocytes by affinity chromatography and radioactively labelled in vitro are incorporated into informosomes in the process of 18–24 h incubation after their injection into oocytes. Informosomes, containing labelled RNA-binding proteins isolated from recipient oocytes, change neither their sedimentation nor density characteristics both in the presence of excess free RNA and on sucrose gradient centrifugation without formaldehyde (data not given). In other words, the RNA-binding proteins injected are capable of forming normal stable informosomes in vivo.

The mechanism of informosomes formation is not considered in this report. However, some results (such as homogeneity of labelled informosomes during sedimentation, the absence of labelled RNA-binding proteins in polyribosome-bound informosomes and the absence of exchange of [^{3}H]proteins with proteins of pre-existing informosomes during incubation of the extracts) suggest that RNA-binding proteins in the oocyte form complexes only with newly synthesized RNA. At present this assumption is undergoing direct experimental tests.

Acknowledgements

We would like to express our gratitude to Professor A. S. Spirin for constant attention to our work and discussion of results and to Professor T. A. Detlaf and Dr A. S. Voronina for helpful consultations.

References

[1] Ovchinnikov, L. P., Voronina, A. S., Stepanov, A. S., Belitsina, N. V. and Spirin, A. S. (1968) Molekul. Biol. 2, 752–763.
[2] Spirin, A. S. (1969) Eur. J. Biochem. 10, 20–35.
[3] Baltimore, D. and Huang, A. S. (1970) J. Mol. Biol. 47, 263–273.
[4] Stepanov, A. S., Voronina, A. S., Ovchinnikov, L. P. and Spirin, A. S. (1971) FEBS Lett. 18, 13–18.
[5] Preobrazhensky, A. A. and Spirin, A. S. (1978) Prog. Nucl. Acid Res. Mol. Biol. 21, 1–37.
[6] Preobrazhensky, A. A. and Ovchinnikov, L. P. (1974) FEBS Lett. 41, 233–237.
[7] Elizarov, S. M. and Stepanov, A. S. (1978) Biokhimiya 42, No. 8.
[8] Reboud, A.-M., Buisson, M., Arpin, M. and Reboud, J.-P. (1977) Biochim. Biophys. Acta 474, 562–578.
[9] Detlaf, T. A., Felgenhauer, P. E. and Chulitskaya, E. V. (1977) Ontogenesis (USSR) 8, 478–486.
[10] Felgenhauer, P. E. and Chulitskaya, E. V. (1977) Ontogenesis (USSR) 8, 197–199.
[11] Belitsina, N. V., Ovchinnikov, L. P., Spirin, A. S., Gendon, Yu. Z. and Chernos, V. N. (1968) Molekul. Biol. 2, 727–735.
[12] Preobrazhensky, A. A. and Elizarov, S. M. (1975) Bioorg. Khimiya 1, 1633–1638.