The inhibitor ribonucleoprotein of poly(A)-containing non-polysomal messenger ribonucleoprotein of *A. salina* cryptobiotic embryos

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The inhibitor present in poly(A)-containing non-polysomal mRNP preparations of *A. salina* cryptobiotic embryos has been purified by sucrose density gradient centrifugation, gel filtration on Sephacryl S200 and ion-exchange chromatography on DEAE-cellulose.

The inhibitor is a ribonucleoprotein sedimenting at 5–6 S with a M_r of $\sim 84000 \pm 6000$. The particle is composed of a protein with M_r of 64000 and an RNA with a length of 85 ± 2 nucleotides and is a very potent inhibitor of the translation of mRNA. The inhibition is a property of the RNA but the inhibitor ribonucleoprotein is more efficient in the inhibition of poly(A) containing mRNA than its extracted RNA.

iRNP *mRNP* *Protein synthesis*

1. INTRODUCTION

Free cytoplasmic mRNP exists as untranslatable as well as translatable protein-RNA particles (for review see [1]). Although proteins can be effective in the repression of stored mRNP [2] the presence of small inhibitory 4–4.5 S RNA has been demonstrated in the inactive mRNP [3–6]. Small RNA affecting the translation of mRNA has been observed in many eukaryotic cells and seems to be of a universal nature (for review see [7]). Different functional classes of small inhibitory RNA exist: Inhibitory RNA associated with mRNP [3–6] and other inhibitory RNA not considered to be part of free mRNP and isolated from a ribosomal salt wash [7] or from the cytosol [8].

The small inhibitor RNA is associated with proteins to form inhibitor ribonucleoproteins functional in the inhibition of protein synthesis [8–10]. The protein composition of these particles has led to conflicting results. In embryonic chicken muscle Bag et al. [8] reported that in the complex protein pattern of small inhibitor RNP only the M_r 36000 protein is different from the proteins of non-polysomal mRNP. In contrast, others [10] concluded that the particle is composed of ~ 30 proteins. The main proteins have M_r of 82000, 80000, 66000, 48000, 44000 and 14000. Duck globin inhibitor RNP is composed of 10 proteins. The main proteins have $M_r < 30000$ [9].

So far regulation of translation by inhibitor ribonucleoproteins associated with non-polysomal mRNP has not been described.

In *A. salina* cryptobiotic embryos the poly(A)-containing mRNP exists in a repressed form due to the presence of an inhibitor of protein synthesis [11]. In this communication we describe the isolation and characterization of the inhibitor and demonstrate its ribonucleoprotein nature.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; iRNA, inhibitor ribonucleic acid; iRNP, inhibitor ribonucleoprotein; mRNP, messenger ribonucleoprotein; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol

2. EXPERIMENTAL PROCEDURE

2.1. *Materials*

Artemia salina cryptobiotic embryos (Macau, Bresil) were obtained from the Artemia reference centre (University of Ghent, Belgium); oligo(dT)-cellulose from Collaborative Research (Waltham,

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MA, USA); [^{3}H]poly(U) (50 Ci/mmol), [^{35}S] methionine (720 Ci/mmol) and rabbit reticulocyte lysate from Amersham International (Buckinghamshire, England); ultrafiltration filters from Amicon (Oosterhout, The Netherlands); Sephacryl S200, poly(U)-Sepharose 4B and low molecular-weight marker proteins from Pharmacia Fine Chemicals (Uppsala, Sweden).

2.2. *Methods*

The postmitochondrial supernatant from *A. saline* cryptobiotic embryos was prepared in buffer A (10 mM Hepes pH 7.2, 100 mM KCl, 0.1 mM DTT, 0.1 mM PMSF) containing 150 mM sucrose as in [11]. Poly(A)-containing mRNP was isolated from the postmitochondrial supernatant by affinity chromatography on oligo(dT)-cellulose in buffer B (10 mM Hepes pH 7.2, 250 mM KCl, 0.1 mM DTT, 0.1 mM PMSF) at 4°C. After extensive washing of the column with buffer B bound mRNP was eluted with 10 mM Hepes pH 7.2, 0.1 mM DTT, 0.1 mM PMSF at 36°C. Template active poly(A) containing mRNP was prepared in buffer C (10 mM Hepes pH 7.2, 1 mM EDTA, 250 mM KCl, 0.1 mM DTT, 0.1 mM PMSF) as above. Poly(A)-containing mRNA of *A. saline* was prepared by affinity chromatography on poly(U)-Sepharose as described [12].

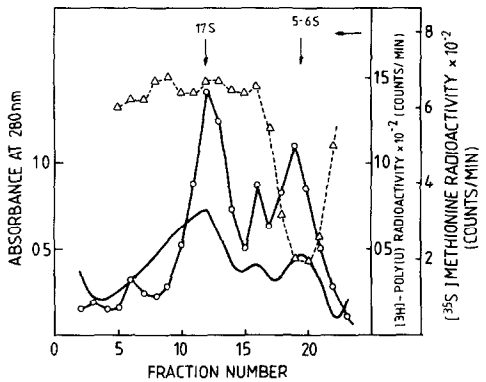

Fig. 1. The inhibitor of mRNP translation is a 5–6 S particle. 15 A_{280} poly(A)-containing mRNP is centrifuged on a 10–30% (w/v) sucrose density gradient in a Beckman SW27 rotor at 96 300 g for 17 h and at 4°C and fractionated in 1.6 ml fractions. The direction of centrifugation is indicated. Absorbance at 280 nm (——). Poly(A)-sequences were detected by hybridization with [^{3}H]poly(U) (15) (○—○). 6 μl of each fraction was mixed with 1 μg mRNA before addition to rabbit reticulocyte lysate. Incorporation of [^{35}S]methionine into protein (Δ---Δ).

In vitro protein synthesis was carried out in a rabbit reticulocyte lysate. [^{35}S] Methionine (10 μCi), 1 μg tRNA (0.05 mg/ml) and amino acids (without methionine) to a final concentration of 150 mM were dried in a vacuum centrifuge (Savant Instruments, Hicksville, USA) and dissolved in 12 μl lysate. 6 μl of the fractions to be tested for the effect on protein synthesis were premixed with 1 μl mRNA (1 μg/μl) at 4°C and 1 μl salts to a final concentration of 10 mM Hepes pH 7.4, 75 mM KCl, 2 mM $MgCl_2$. After 15 min lysate was added. Assays of 20 μl contained 12 μl lysate and a final concentration of 117 mM KCl and 2 mM $MgCl_2$. Incubation was at 25°C for 60 min. An aliquot of 10 μl was taken to determine the incorporation of [^{35}S]methionine into protein. Hybridization with [^{35}H]poly(U) and isopycnic centrifugation in sucrose was as in [13]. Ion-exchange chromatography on DEAE-cellulose (DE52) was done in buffer D (20 mM Tris–HCl pH 7.5, 50 mM KCl, 0.1 mM DTT).

3. RESULTS AND DISCUSSION

We have demonstrated previously that mRNP prepared from *A. salina* cryptobiotic embryos in the presence of $MgCl_2$ is not translated in a rabbit reticulocyte lysate whereas mRNP prepared in the presence of EDTA is used as a template although less efficiently than its extracted RNA [11]. In comparison with the results obtained by Heywood [14] we have proposed that the repression is due to the association of an inhibitor RNA to mRNP. The latter inhibitor is partially dissociated from the complex in the absence of $MgCl_2$ [12].

Poly(A)-containing mRNP was prepared by affinity chromatography on oligo(dT)-cellose in buffer B and purified by sucrose density gradient centrifugation in buffer A (fig. 1). The majority of mRNP sedimented at 17 S. Integrity of mRNP was measured by hybridization with [^{3}H]poly(U). Approximately 65% of the poly(A)-sequences were located in the 17 S mRNP. The sucrose gradient was assayed for the presence of inhibitors of translation by addition of excess exogeneous mRNA to the gradient fractions before translation in a rabbit reticulocyte lysate. A potent inhibitor was localized in the 5–6 S region of the gradient.

Table 1

Binding of the 5–6 S inhibitor to nitrocellulose filters.

The inhibitor containing fractions (No. 19–20) of the sucrose gradient of fig. 1 were filtered through nitrocellulose (Millipore HA, 0.45 μm). Increasing amounts of the filtrate and of unfiltered fractions were mixed with 1 μg mRNA before addition to a rabbit reticulocyte lysate. In the absence of inhibitor 39 500 cpm was incorporated into protein. The results were corrected for endogenous activity of the lysate.

$A_{260} \times 10^{-3}$/assay	cpm	
	before filtration	after filtration
14	31 400	37 000
20	15 800	33 000
26	7 000	29 000

The ribonucleoprotein nature of the 5–6 S inhibitor was demonstrated from nitrocellulose filter binding and from the inhibition of translation of poly(A)-containing mRNA by RNA extracted from the inhibitory fractions of the sucrose density gradient.

The inhibitor containing fractions of fig. 1 were filtered through nitrocellulose and the filtrate was assayed for the presence of an inhibitor. The initial inhibition of 82% obtained with 0.026 A_{260} of the 5–6 S gradient fractions was reduced to 25% after filtration (table 1). The RNA present in the inhibitor containing fractions was extracted by phenolization.

Poly(A)-containing mRNA was mixed with increasing amounts of the extracted RNA before translation in rabbit reticulocyte lysate. As shown in fig. 2 the inhibition is a property of the RNA present in the 5–6 S fractions. The ribonucleoprotein nature of the inhibitor was further demonstrated by isopycnic centrifugation in sucrose [13]. The translational repressor was localized by the inhibitor assay at an equilibrium density of 1.28 g/cm^3 (data not shown). This density is slightly higher than the density of 1.25–1.27 g/cm^3 measured for the 17 S poly(A)-containing mRNP of *A. salina* and below the density of 1.35 g/cm^3 measured for

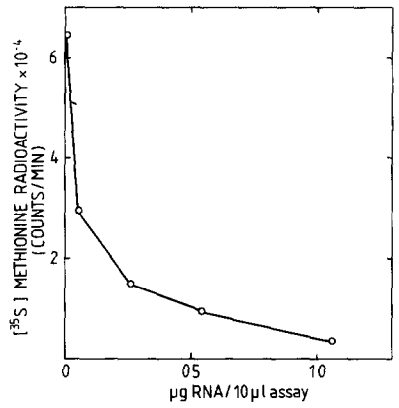

Fig. 2. Inhibition by RNA present in the 5–6 S particle. 500 μl of the 5–6 S inhibitor containing fractions (No. 19–20) of fig. 1 were phenolized. Increasing amounts of the extracted RNA were mixed with 1 μg mRNA before addition to rabbit reticulocyte lysate. The incorporation of [^{35}S]methionine into protein was corrected for the endogenous activity of the lysate (7000 cpm).

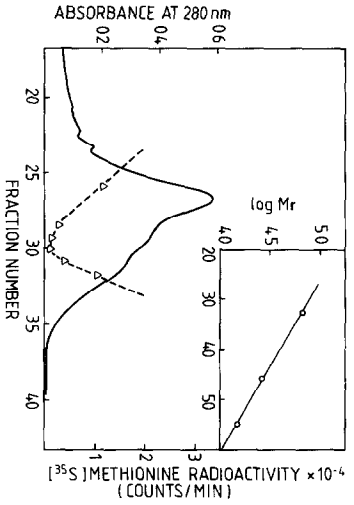

Fig. 3. Purification of iRNP by gel filtration. The 5–6 S inhibitor containing fractions (No. 18–21) of two identical gradients as shown in fig. 1 were chromatographed on Sephacryl S-200 (2.5 × 60 cm) in buffer A containing 5% glycerol. Fractions of 1.1 ml were collected. The column was calibrated with bovine serum albumin (M_r 68 000), chymotrypsin (M_r 25 000) and cytochrome *c* (M_r 12 500) (insert). Absorbance at 280 nm (——). 6 μl of each fraction was mixed with 1 μg mRNA before translation in a rabbit reticulocyte lysate. Incorporation of [^{35}S]methionine into protein was corrected for the endogenous activity of the lysate (4000 cpm) (Δ---Δ). In the absence of inhibition 46 500 cpm was incorporated into protein.

80 S ribosomes [13]. From the density position in sucrose a RNA/protein ratio of 1:2.5 was calculated for the inhibitor.

A modification of the method of Mukherjee and Sarkar [15] was used to purify the inhibitor of mRNA translation. The 5–6 S fractions of fig. 1 were pooled, concentrated by ultrafiltration and applied to a column of Sephacryl S-200 previously equilibrated with buffer A containing 5% (v/v) glycerol (fig. 3). Fractions were assayed for the presence of the inhibitor. The inhibitor was eluted in fractions 26–32 with a maximum around fraction 29. From the latter fraction a M_r of 84000 ± 6000 is calculated.

The inhibitor was further purified by ion-exchange chromatography on DEAE-cellulose. The inhibitor containing fractions of the Sephacryl S-200 column were dialysed against buffer D containing 10% (v/v) glycerol and applied to DEAE-cellulose. After extensive washing with buffer D the bound material was eluted with a linear salt gradient. Two peaks of absorbance were eluted at 250 and 500 mM KCl, respectively (fig. 4). Fractions were dialysed overnight against buffer B and assayed for the presence of the inhibitor. The latter was detected in fractions eluted at ~500 mM KCl.

The iRNP purified by gel filtration and ion-exchange chromatography has a A_{260}/A_{280} ratio of 1.5 further supporting the ribonucleoprotein nature of the inhibitor. The particle is very potent in the repression of protein synthesis. Translation of 1 μg RNA was completely inhibited by 0.250×10^{-3} A_{260} iRNP. The inhibition was much more efficient as compared to the RNA of iRNP. 1 μg

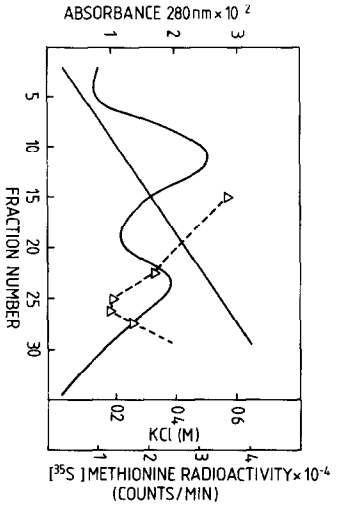

Fig. 4. Purification of iRNP by ion-exchange chromatography. The inhibitor containing fractions (No. 28–31) of fig. 3 were chromatographed on a DEAE-cellulose column (1.2 × 3 cm) in buffer D. Bound material was eluted with a linear gradient of 50 mM (15 ml) to 600 mM (15 ml) KCl. 1-ml fractions were collected. Absorbance at 280 nm (——). After extensive dialysis against buffer D each fraction was assayed for the presence of the inhibitor. 6 μl was mixed with 1 μg mRNA before translation in rabbit reticulocyte lysate. Incorporation of [^{35}S]methionine into protein was corrected for endogenous activity of the lysate (8800 cpm) (Δ---Δ). 48000 cpm was incorporated in the absence of inhibition.

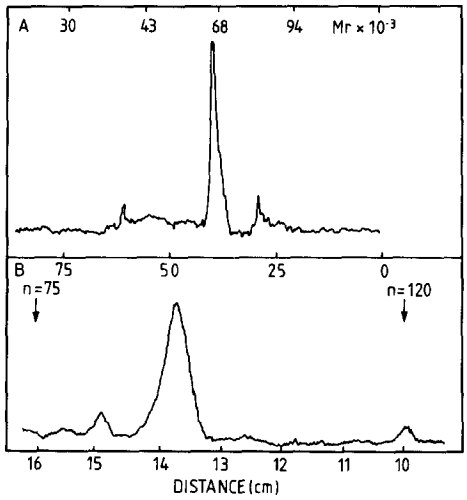

Fig. 5. Analysis of purified iRNP by polyacrylamide gel electrophoresis. (A) Protein composition of iRNP: Electrophoresis of 0.3 μg iRNP on SDS-polyacrylamide gel was as in [11]. Laser densitogram of silver-stained gel. (B) RNA composition of iRNP: RNA was extracted from approximately 25 μg iRNP by phenolization. 7 μg RNA was 3′ labelled with [5′-^{32}P]pCp, precipitated with ethanol and dissolved in 3 μl of 20 mM Tris-HCl pH 7.5, 8 M ureum, 1 mM EDTA, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue. After heating at 60°C for 30 s RNA was electrophorized on a 12% (w/v) polyacrylamide gel (0.38 × 400 mm). Gels were pre-run overnight at 700 V. Electrophoresis was at 1700 V. The autoradiogram was scanned with a laser densitometer. The molecular weight was calculated using 5 S rRNA (120 nucleotides), tRNA (75 nucleotides) and xylene cyanol FF (position of 50 nucleotides) as standards.

mRNA is inhibited to the same extent by 3.3×10^{-3} A_{260} iRNA (data not shown).

The composition of iRNP was determined by polyacrylamide gel electrophoresis. The main protein of the purified particle has a M_r of 63 500–64 000 (fig. 5A). The iRNA was extracted from iRNP, 3′-end labelled with [5′-^{32}P]pCp [16] and analyzed by polyacrylamide gel electrophoresis as described by Peattie [17] (fig. 5B). A length of 85 ± 2 nucleotides was measured for the main RNA component of iRNP. Several minor RNAs were also detected in the preparation.

Different control experiments indicate that iRNA is distinct from poly(A) also present in the 5–6 S fractions of the sucrose gradient (fig. 1) and from tRNA similar in size to iRNA: (i) migration of iRNA on polyacrylamide gels is different from that of tRNA and poly(A) both with a nucleotide length of 75 nucleotides, (ii) tRNA does not affect translation if added in the same amount as iRNA, (ii) a poly(A)-binding protein is not detected in purified iRNP, (iv) preliminary sequence data indicate the absence of poly(A)sequences, (v) iRNA is uridylic acid rich and can be bound to oligo(dA)-cellulose.

The RNA component of iRNP has a similar length to other inhibitor RNAs reported in literature. However, the latter inhibitors are complexed with much more protein and sediment at 10 S. Except for tcRNA these inhibitors of translation have not been purified from mRNP [7,8]. The latter iRNAs also have different properties from tcRNA: they inhibit translation in a nonselective manner, seem to lack oligo(U) sequences and differ in their inhibition mechanism of the initiation of protein synthesis [18]. Apparently iRNA of purified 5–6 S iRNP has similar properties to tcRNA. The characterized iRNP seems to be involved in the regulation of translation of stored mRNP. Further work is in progress to demonstrate the association of iRNP with the repressed poly(A) containing mRNP and to determine the mechanism of repression.

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