THE TRANSLATIONAL INHIBITORY 10 S CYTOPLASMIC RIBONUCLEOPROTEIN OF CHICKEN EMBRYONIC MUSCLE IS DISTINCT FROM MESSENGER RIBONUCLEOPROTEINS

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1. Introduction

A class of cytoplasmic RNA species, about 70–90 nucleotides in size, and sedimenting as 4 S in sucrose gradients, which are potent inhibitors of in vitro mRNA translation, have been isolated from chick embryonic muscle [1–3]. The inhibitory RNA differs from the (U)-rich low molecular mass (M_r) RNA species of chick embryonic muscle, which inhibits specific mRNA translation (referred to as translational control RNA, tcRNA) [4–6] with respect to size, base composition, lack of oligo(U) tract, and its ability to inhibit the translation of a variety of mRNAs in a nondiscriminatory manner [1–3]. The association of tcRNA with specific mRNA (e.g., mRNA is complexed with specific tcRNA to form a translationally repressed mRNP) has been postulated as a negative control of translation [4–6]. A cytoplasmic 10–15 S RNP particle containing a 4.5 S RNA from chick embryonic muscle which inhibits mRNA translation in vitro was isolated in [7]. It was concluded that except for the presence of a single polypeptide, M_r 36 000 in the 10–15 S RNP, the protein patterns of the inhibitory RNP and mRNP are very similar [7]. It was suggested that the inability of some free mRNP to be translated in vitro may be related to the presence of 10–15 S RNP [7]. However, neither the inhibitory RNA was characterized, nor the RNP was purified in [7]. We describe here the characterization of a purified 10 S cytoplasmic RNP (iRNP) containing an inhibitory 4 S RNA (iRNA) species from chick embryonic muscle. The properties of iRNA indicate that it is unrelated to cytoplasmic mRNP. Furthermore, the inhibitory action of iRNP and the deproteinized iRNA on mRNA translation is not due to the double-stranded nature of iRNA.

2. Materials and methods

The postpolysomal particles (255 000 × g, 4 h) prepared from the leg and breast muscle of 14-day-old chick embryos as in [8,9] were used as the source of 10 S iRNP. About 30 A_{260} units of the postpolysomal particles suspended in TM buffer (10 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA) containing 0.5 M KCl were fractionated by centrifugation through a 28 ml 5–20% linear sucrose gradient in the same buffer at 24 000 rev./min for 17 h in a Spinco SW 25.1 rotor as in [10]. Fractions 4–7, indicated by the bar (fig.1 inset) and containing 10–12 S particles were pooled, and concentrated by ultrafiltration on Amicon UM 05 membrane filters at 4°C. The resulting material was resuspended in TM buffer containing 0.1 M KCl at 4–5 A_{260} units/ml and was further purified by centrifugation through a 12 ml linear 7–20% sucrose gradient in the same buffer at 41 000 rev./min for 6 h in a Spinco SW 41 rotor. Fractions 5–7 indicated by the bar (fig.1) were pooled as the source of 10 S iRNP and concentrated by ultrafiltration.

For the preparation of iRNA, gradient fractions containing iRNP (fig.1) were extracted with $CHCl_3$/phenol/isoamyl alcohol (50:50:1) [11]. After 2 cycles of precipitation with ethanol and 0.1 M potassium acetate at pH 5.0 at –20°C the RNA was further purified by chromatography on hydroxyapatite as in [12]. The RNA eluted at 0.04 M potassium phos-

phate (fig.2A) was precipitated with ethanol, desalted on Sephadex G-25 columns and lyophilized.

Electrophoresis of RNA samples in 99% formamide was carried out at 400 V at room temperature using 8% gels [13]. The gels were stained with 2 μg ethidium bromide/ml and photographed in UV light. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins of 10 S RNP, after alkylation with iodoacetamide [10] was done using 5–15% linear gradient slab gels [8,9]. The buoyant density of formaldehyde-fixed RNP was determined by centrifugation in a preformed CsCl gradient [8]. The base composition of iRNA was determined by high-performance liquid chromatography (HPLC) of the neutralized alkaline hydrolyates obtained by incubating RNA samples with 0.3 N KOH at 37°C for 18 h. The nucleotides were separated on a μBondapak C_{18} column using 1.5% acetonitrile in 0.1 M triethyl ammonium bicarbonate (pH 7.5) as the solvent.

Hyperchromicity of RNA samples with increasing temperature was measured in a Cary Model 15 spectrophotometer. The RNase sensitivity of iRNA and iRNP was tested by incubating samples containing 100 μg RNA/ml with 10 μg insoluble pancreatic RNase/ml (Sigma Chemicals) in 50 mM Tris–HCl (pH 7.2) 0.1 M KCl, 10 mM $MgCl_2$ at 30°C for 3 h. The reaction was monitored by measuring hyperchromicity of the incubation mixture at 260 nm. After pelleting the insoluble RNase by centrifugation, the supernatant from incubations containing iRNA was extracted with phenol/$CHCl_3$/isoamyl alcohol [11] after addition of equivalent amounts of embryonic muscle tRNA as carrier. The RNA after 2 cycles of precipitation with ethanol was tested for biological activity. With reaction mixtures containing iRNP the supernatant was precipitated at −20°C with 2 vol. ethanol at 0.1 M NaCl.

The inhibition of mRNA translation by iRNP and iRNA was monitored in micrococcal nuclease-treated reticulocyte lysate [14] programmed with poly(A)$^+$ mRNA of chick embryonic muscle and rabbit globin mRNA. Details of the incubation mixture and assay procedure are given in the legends. The in vitro translation products were analyzed by fluorography [15].

Cytoplasmic free and polysomal mRNP particles of chick embryonic muscle were isolated and characterized as in [8,9]. Chromatography of RNP on columns of oligo(dT)-cellulose and oligo(dA)-cellulose for detecting (A)-rich and (U)-rich tracts capable of binding to the columns, was done as in [9].

3. Results and discussion

When subribosomal particles present in the pooled gradient fractions sedimenting at 10–12 S (fig.1, inset) were recentrifuged through a second sucrose gradient, a major peak sedimenting at 10 S with A_{260}/A_{280} ratio of ~1.2 and constituting ~65% of the total UV-absorbance was obtained (fig.1; –,●). In contrast, RNA extracted from the pooled 10 S peak fraction sedimented as 4 S in an identical sucrose gradient (○,––). Preliminary experiments indicated that both 4 S RNA (iRNA) and the 10 S particles (iRNP) inhibited strongly muscle poly(A)$^+$ mRNA and globin mRNA translation in micrococcal nuclease-treated reticulocyte lysate. Chromatography of iRNA on hydroxyapatite column gave a single major peak eluted at 0.04 M potassium phosphate (fig.2A: ●,–). Chick embryonic muscle tRNA was eluted from the column at 0.075 M potassium phosphate under identical conditions (not shown). This is illustrated by chromatography of a mixture of equal amounts of tRNA and iRNA which gave quantitative resolution into 2 peaks (○,––). These two peaks corresponded to those obtained with samples of tRNA and iRNA, run separately on the same column.

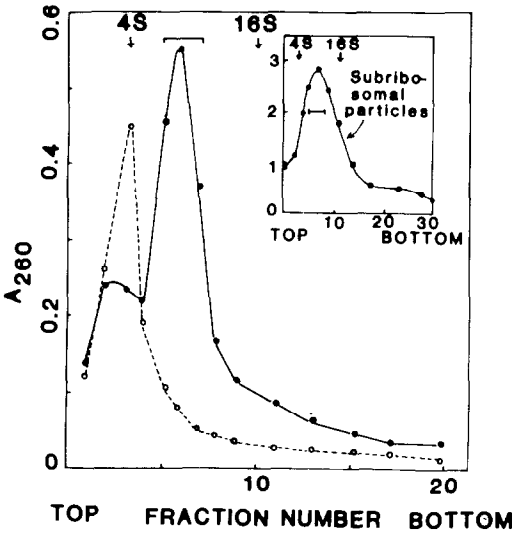
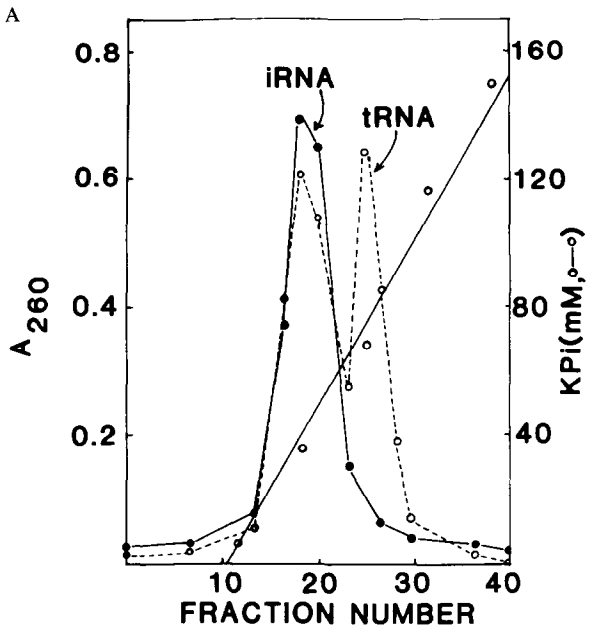


Fig.1. Purification of 10 S iRNP particles. For details see section 2 and text. Inset: Fractionation of subribosomal particles by sucrose gradient centrifugation. Fractions 4–7, indicated by bar and containing crude 9–12 S particles were pooled for further purification of iRNP: (●) centrifugation of 9–12 S particles; (○) RNA isolated from 10 S particles (peak fractions 5–7, indicated by bar) run in an identical gradient.

The UV spectrum of hydroxyapatite-purified iRNA (fig.2B) is very similar to that of a sample of chick muscle tRNA with a characteristic maximum at 255 nm and a minimum at 230 nm (fig.2B). Interestingly, iRNP particles gave a strikingly different spectrum with a peak at 230 nm and a broad shoulder in the 255–280 nm range. When formaldehyde-fixed 10 S particles were centrifuged to equilibrium in CsCl gradients, ~90–95% of the UV-absorbance was recovered in a single peak at 1.402 g/cm^3 (not shown). This buoyant density is characteristic of RNP particles and corresponds to a protein content of ~74.5% [16]. PAGE of the hydroxyapatite-purified iRNA in the presence of 99% formamide gave a heterogeneous pattern with 3 major bands (fig.3A, gel 2) in the 70–80 nucleotide size range, as judged by comparison with mobility of a sample of yeast tyrosine tRNA (size 78 nucleotides; indicated as 4 S). Two additional bands, in the 90–100 nucleotide size range and constituting ~15–20% of the total RNA were also present. The RNA nature of iRNA is also indicated by its A_{260}/A_{280} ratio of 2.0; and the loss of biological activity and quantitative conversion to ethanol-soluble material after incubation with 0.3 N KOH at 37°C for 18 h. The base composition of iRNA was: AMP, 30.9%; GMP, 21.9%; CMP, 23.8%; and UMP, 23.4%. These values are somewhat different from those (AMP, 45.8%; CMP, 11.2%; GMP, 21.6% and UMP, 21.5%) obtained for the inhibitory RNA species isolated from 0.5 M KCl-wash of embryonic muscle ribosome [1–3] suggesting that the 2 RNA species may not be identical. When 10 S particles and iRNA samples were tested for binding to oligo(dT)-cellulose and oligo(dA)-cellulose columns, the material in both cases, was recovered quantitatively in the unbound fractions under conditions in which samples of poly(A) and poly(U) were quantitatively bound to the appropriate columns (not shown). Thus, both iRNP and iRNA lack (U)-rich and (A)-rich tracts of sufficient lengths capable of binding to cellulose-bound ligands.



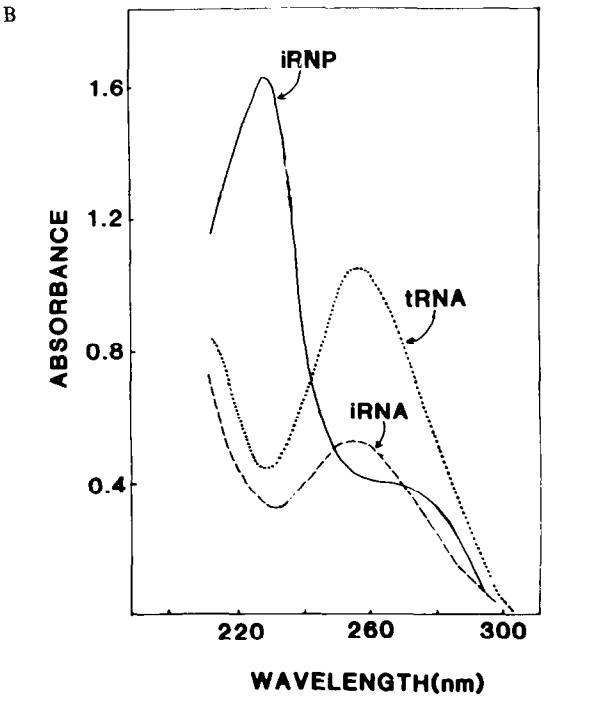


Fig.2A. Elution profiles of iRNA and a mixture of iRNA and tRNA on hydroxyapatite columns. For details see section 2 and text. RNA samples (6–8 A_{260} units) dissolved in the binding buffer, 1 mM potassium phosphate (pH 6.8) were applied to a 1.5 cm × 15 cm column packed with hydroxyapatite (Bio Rad) equilibrated with the binding buffer. After washing the column with the binding buffer, the bound material was eluted with 100 ml linear gradient of 0–0.2 M potassium phosphate (pH 6.8). Fractions (2 ml each) were monitored for UV-absorbance. Two separate runs using iRNA (•) and a mixture of iRNA and tRNA (○) are shown. The position of the tRNA peak was also independently confirmed by running a sample of chick muscle tRNA. Fig.2B. UV-absorption spectra of samples of iRNP, iRNA and tRNA measured at 25°C in 10 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate and 1 mM EDTA.

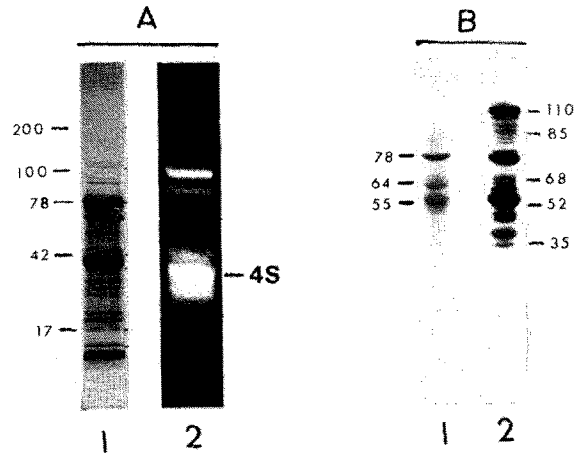


Fig.3. PAGE of RNA and protein components of iRNP particles. For details see section 2 and legends to fig.1 and 2A. (A) Gel 1, SDS–PAGE of proteins of iRNP. Proteins (80 μg) were run in a gradient 5–15% slab gel and stained with Coomassie blue. The mobilities of the following marker proteins: myosin heavy chain, Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum, transferrin, actin, and troponin C, of known molecular mass ($\times 10^{-3}$) are represented. Gel 2, electrophoresis of 5 μg iRNA in 99% formamide. The mobility of yeast tyrosine tRNA (78 nucleotides) run as a marker is shown at the right. (B) SDS–PAGE of proteins of polysomal mRNP (gel 1; 48 μg protein) and free mRNP (gel 2; 80 μg protein) purified by oligo (dT)-cellulose chromatography [8,9]. Gels of 8% were used. The molecular masses ($\times 10^{-3}$) of the major bands of mRNP-proteins, as calculated from a standard curve obtained with marker proteins run in a parallel gel, are shown.

SDS–PAGE of protein moieties of iRNP and highly purified free and polysomal mRNP particles of chick embryonic muscle indicates the following points: A complex set of proteins, M_r 12 000–100 000 is present in iRNP (fig.3A, gel 1). Both free and polysomal mRNP particles show strikingly different protein patterns (fig.3B, gels 1,2). The low molecular mass proteins (M_r 12 000–35 000) observed in iRNP, are absent in mRNP particles. There are also distinct differences in the high molecular mass proteins (M_r 50 000–100 000) present in iRNP and mRNP particles. Among the 2 types of mRNP particles the polysomal mRNP shows a relatively simpler pattern of 3 major proteins (M_r 78 000, 64 000, 55 000) which appear to be present also in free mRNP in agreement with [8,9]. These results indicate that iRNP is clearly distinct from mRNP particles. This is in contrast to [7] where a preparation of crude 10–15 S inhibitory RNP of chick embryonic muscle was very similar, almost identical in protein patterns, to free mRNP particles of chick embryonic muscles. In [7] fluorograms of in vitro labeled proteins obtained by reductive alkylation of crude RNP particles by radioactive formaldehyde were used in contrast to Coomassie blue-stained gels of purified preparations used here. Since reductive alkylation involves reaction with exposed amino groups in proteins [17], the conclusion in [7] does not necessarily reflect the true protein patterns of different RNP particles.

Both iRNP and iRNA strongly inhibited the translation of poly(A)$^+$ mRNA of chick embryonic muscle and globin mRNA, as shown by fluorography of the translation products (fig.4A,B). The degree of inhibition, calculated from [^{3}H]leucine incorporation into CCl_3COOH-insoluble protein-bound counts was found to be concentration-dependent over 10–80 ng iRNA or equivalent amount of RNP, a final level of ~95% inhibition being observed for both types of mRNA with 60–80 ng RNA. Chick embryonic muscle

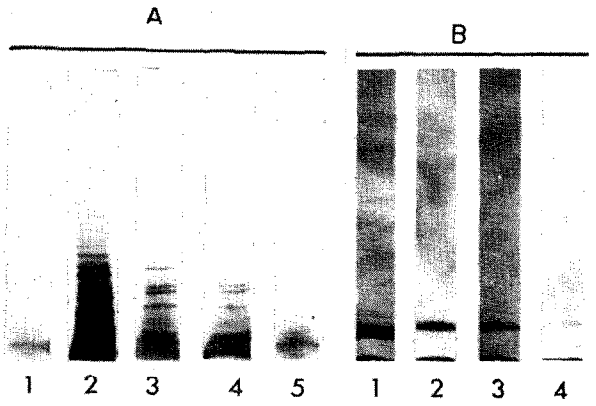


Fig.4. Inhibition of translation of muscle poly(A)$^+$ mRNA and globin mRNA in micrococcal nuclease-treated reticulocyte lysate by iRNP and iRNA. The reaction mixture contained in 25 μl total vol.: 12 μl lysate, 15 μM hemin, 12 mM creatine phosphate, 7.5 μg creatine kinase, 0.4 mM magnesium acetate, 100 mM KCl, 0.6 mM each of 19 L-amino acids except leucine, 3 μCi L-[3,4,5-^{3}H(*N*)]leucine (spec. act. 150 Ci/mmol, New England Nuclear), 0.4 μg mRNA and indicated amounts of iRNA or iRNP. After incubation at 30°C for 60 min, 20 μl samples were used for fluorography [15]. (A) Lane 1, nuclease-treated lysate; lane 2, muscle poly(A)$^+$ mRNA; lane 3, incubation containing muscle poly(A)$^+$ mRNA and 25 ng iRNA; lanes 4,5, incubations containing muscle poly(A)$^+$ mRNA and iRNP equivalent to 25 and 80 ng iRNA, respectively. (B) Lane 1, globin mRNA; lane 2, globin mRNA + 25 ng iRNA; lanes 3,4, incubations containing globin mRNA and iRNP equivalent to 25 ng and 75 ng iRNA, respectively.

tRNA at 200 ng/assay did not cause any inhibition. The iRNA was also inactive when tested both as a substrate or inhibitor of aminoacylation using a pH 5 enzyme system from chick embryonic muscle (not shown). The iRNP and iRNA were equally effective as inhibitors of translation of both types of mRNA. The time course of amino acid incorporation indicated that both iRNP and iRNA inhibited in a concentration-dependent manner without any lag. The fluorograms show that the intensities of the major bands are inhibited in a non-selective manner indicating that translation of both homologous and heterologous mRNAs is blocked in a nondiscriminatory manner.

Hyperchromicity measurement of iRNA with increasing temperature indicated that iRNA melts over 40–80°C in a non-cooperative manner giving rise to ~25% hyperchromicity (fig.5A; ○). Chick muscle tRNA showed a larger value of hyperchromicity over 70–80°C (fig.5; ●), which agrees with the view that tRNA contains considerable secondary structure [18]. The melting curve of iRNA was found to be indistinguishable from that obtained with a sample of poly(A) (not shown) which is known to melt like single-stranded RNA [18]. These results indicate that the observed gradual melting of iRNA reflects primarily unstacking of bases and lack of significant levels of double-stranded structure.

Incubation of samples of tRNA and iRNA with insoluble pancreatic RNase over 2 h gave rise to ~25% and 40% hyperchromicity, respectively (section 2) (fig.5B). The RNase-treated iRNA, after reisolation by phenol-extraction (section 2) was totally inactive as inhibitor of mRNA translation (table 1). Control experiments in which iRNA was incubated in the absence of RNase and similarly processed, did not show any loss of biological activity. The neutralized alkaline hydrolyzate of iRNA obtained by incubating with 0.3 N KOH at 37°C for 18 h was also biologically inactive (table 1). Interestingly, iRNP was resistant to RNase treatment, as judged by both lack of hyperchromicity during RNase treatment (fig.5B) and retention of biological activity of the reisolated iRNP (section 2) (table 1). Heating iRNA at 80°C for 3 min followed by quick cooling (a procedure which melts double-stranded structure) did not cause any change in the inhibitory property of iRNA (table 1). These results, considered together with the hyperchromicity profile of iRNA with increasing temperature (fig.5A) indicate that the potent inhibitory action of iRNA on mRNA translation is not due to double-stranded nature of iRNA. Furthermore, the association of the protein moieties to iRNA does not influence the biological activity of iRNA per se (fig.4). Both iRNA and iRNP do not have any nuclease activity associated with them, as judged by their inability to cleave labeled poly(A), poly(A,C), 23 and 16 S rRNAs and globin mRNA (not shown).

The contrasting effects of RNase on iRNA and iRNP (fig.5B, table 1) suggest that a possible function

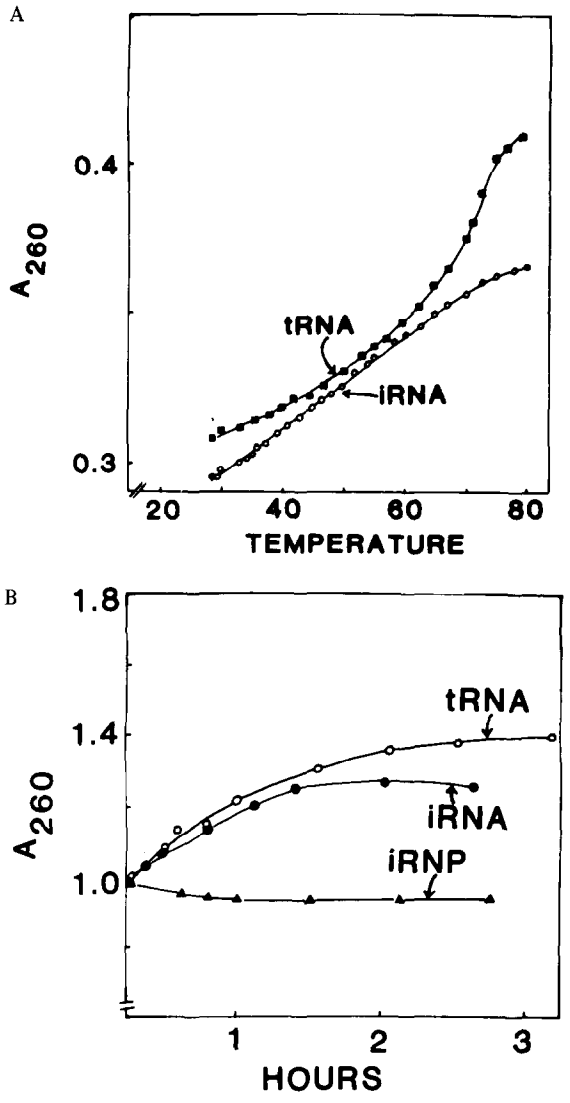


Fig.5A. Hyperchromicity of iRNA and chick muscle tRNA. For details see section 2. RNA samples were dissolved in 0.1 M NaCl, 20 mM sodium phosphate (pH 7.0). Fig.5B. Effect of insoluble pancreatic RNase on iRNA, tRNA and iRNP. For details see section 2.

Table 1
Effect of various treatments on the activity of iRNA and iRNP on translation of poly(A)$^+$ mRNA of chick embryonic muscle in micrococcal nuclease-treated lysate

Additions	cpm
Nuclease-treated lysate	95
+ poly(A)$^+$ mRNA	2712
+ poly(A)$^+$ mRNA + 40 ng iRNA	267
+ poly(A)$^+$ mRNA + 60 ng RNase-digested iRNA	2560
+ poly(A)$^+$ mRNA + 60 ng alkali-hydrolysed iRNA	2814
+ poly(A)$^+$ mRNA + 40 ng heated and quickly chilled iRNA	190
+ poly(A)$^+$ mRNA + 60 ng RNase-treated iRNP	220

Assays were done as in section 2 and fig.4

of the proteins of iRNP is to provide a RNase-resistant nucleoprotein structure to iRNA which is RNase-sensitive. Considered together with the fact that free and polysomal mRNP particles are extremely sensitive to RNase [8,19], our results indicate that the 10 S particles represent a novel class of translational inhibitory RNP, distinct from mRNP particles in protein patterns, size and nature of the RNA moieties. The iRNA also appears to be distinct from small nuclear RNAs (e.g., the well-characterized U_1SnRNA) [20,21] in size and base composition. The biological role of iRNP is not clear at present. One possible function of these particles, which are also distinct from tcRNA [4–6], is that they may be involved directly or indirectly in mRNA compartmentation between polysomes and free mRNP pools, which is known to exist in embryonic muscle [8,19,22,23].

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