Cytoplasmic Low Molecular Weight Ribonucleic Acid Species of Chick Embryonic Muscles, a Potent Inhibitor of Messenger Ribonucleic Acid Translation in Vitro†

Malcolm G. Pluskal and Satyapriya Sarkar*

ABSTRACT: A low molecular weight ribonucleic acid (RNA) species [inhibitory ribonucleic acid (iRNA)] has been isolated from the 0.5 M KCl wash of the 100000*g* pelleted material of 14-day-old chick embryonic muscles by a combination of DEAE-cellulose and hydroxylapatite chromatography. The iRNA does not bind to oligo(dT)- or oligo(dA)–cellulose; it shows three major bands in the 70–90 nucleotide range by polyacrylamide gel electrophoresis, and it has a base composition as follows: 40.9% A, 17.6% C, 20% G, and 21.5% U. The iRNA at a range of 40–60 ng strongly inhibits the translation of chick embryonic muscle poly(A+) messenger ribonucleic acid (mRNA) in a micrococcal nuclease treated reticulocyte lysate. Only at higher concentrations, 5 μg and above, does iRNA partially inhibit polysome-directed amino acid incorporation. The kinetics of inhibition of mRNA translation and examination of the translation products by fluorography suggest that iRNA acts primarily at the initiation step, and this mode of inhibition does not involve mRNA discrimination. The properties of iRNA indicate that it is distinct from the inhibitory RNA species of chick embryonic muscles described in the literature [Heywood, S. M., & Kennedy, D. S. (1976) *Biochemistry 15*, 3314–3319] which display specificity in the inhibition of mRNA translation in vitro. The iRNA also appears to be different from other low molecular weight RNA species which have been described in the literature as being involved in modulation of in vitro protein synthesis. Hyperchromicity measurements of iRNA with increasing temperature and loss of biological activity of iRNA after treatment with pancreatic ribonuclease (RNase) indicate that iRNA is not a double-stranded molecule, suggesting that its potent inhibitory effects are not due to the presence of double-stranded RNA. The presence of iRNA as a translational inhibitor in the cytoplasm suggests that it may play a subtle role in posttranscriptional regulation of protein synthesis in embryonic muscles.

According to several reports, a class of cytoplasmic ribonucleic acids (RNAs) in the molecular weight range of 6000–12 000 isolated from various eukaryotic cells such as rabbit reticulocytes (Fuhr & Natta, 1972; Bogdanovsky et al., 1973; Berns et al., 1975; Fuhr & Overton, 1975; Raymondjean et al., 1976), *Artemia salina* embryos (Lee-Huang et al., 1977; Slegers et al., 1977; DeHerdt et al., 1979), newborn rat calvaria (Zeichner & Breitkreutz, 1978), and chick embryonic muscles (Heywood et al., 1974; Bester et al., 1975; Kennedy et al., 1978) are involved in the modulation of protein synthesis in cell-free systems. The RNA isolated from reticulocytes stimulates translation of messenger ribonucleic acids (mRNAs) in a nonspecific manner (Berns et al., 1975). Other RNA species isolated from rat calvaria (Zeichner & Breitkreutz, 1978) and *Artemia salina* embryos (Slegers et al., 1977; DeHerdt et al., 1979) show an inhibitory effect on in vitro translation of both homologous and heterologous mRNAs. A class of oligo(U)-rich inhibitory RNA species isolated from chick embryonic muscles (referred to as translational control RNA or tcRNA) has been reported to inhibit the translation of specific mRNAs in a discriminatory manner (Heywood & Kennedy, 1976; Kennedy et al., 1978). According to these reports, myosin heavy chain mRNA remains associated with a specific tcRNA in a translationally repressed nonpolysomal messenger ribonucleoprotein (mRNP)[1] particle, presumably due to specific interaction of the tcRNA with the mRNA. The tcRNA of embryonic muscles acts at the initiation step (Kennedy et al., 1974), while similar oligo(U)-rich RNAs of *Artemia salina* embryos preferentially block the elongation step (Lee-Huang et al., 1977). It has been proposed that the two RNA species present in *A. salina*, one with stimulatory and the other with inhibitory properties on in vitro protein synthesis, play a dual regulatory role during various stages of embryonic development (Lee-Huang et al., 1977).

Recent studies from a number of laboratories have suggested that in addition to the transcriptional control (John et al., 1977; Strohman et al., 1977; Paterson & Bishop, 1977) translational control may also be involved as a subtle regulatory mechanism during muscle growth and differentiation (Bag & Sarkar, 1975, 1976; Jain & Sarkar, 1979; Heywood & Kennedy, 1976). In order to probe for possible translational controls which may operate during myogenesis, we have searched for RNA species in chick embryonic muscles which act as modulators of translation. We report here the isolation and partial characterization of a class of cytoplasmic RNA species, from the ribosomal 0.5 M KCl wash of chick embryonic muscle, which acts as potent inhibitors of mRNA translation in vitro. The properties of this iRNA species indicate that it is quite distinct from previously reported mRNA-discriminatory tcRNA isolated from chick embryonic muscles as well as other

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[1] Abbreviations used: iRNA, inhibitory RNA species; A_{260} unit, the quantity of material contained in 1 mL of a solution which has an absorbance of 1 at 260 nm when measured in a cell of 1-cm path length; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; mRNP, messenger ribonucleoprotein; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; oligo(dT), oligo(thymidine); oligo(dA), oligo(deoxyadenylic acid).

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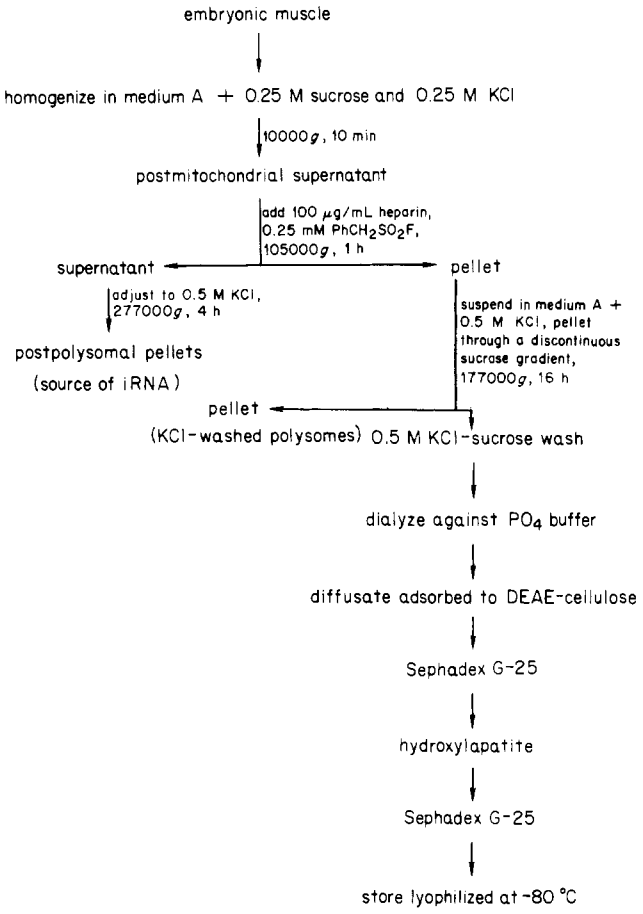
Scheme I



inhibitory RNA species described in the literature.

Experimental Procedures

Isolation of Subcellular Fractions. All preparative operations were carried out at 0–4 °C unless otherwise specified. Standard precuations to prevent nuclease activity, viz., use of acid-washed glassware and sterilized solutions, were taken throughout all operations. The scheme used for the isolation of subcellular components is shown in Scheme I. Details of some of the preparative procedures used in this scheme have been previously reported (Bag & Sarkar, 1975, 1976; Jain & Sarkar, 1979). About 100–120 g of leg and breast muscles of 12–14-day-old chick embryos was homogenized in an equal volume of buffer A [10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT)] containing 0.25 M sucrose and 0.25 M KCl, and the homogenate was centrifuged at 12000*g* for 15 min (Sarkar & Cooke, 1970). The resulting postmitochondrial supernatant was supplemented with heparin (100 μg/mL) and phenylmethanesulfonyl fluoride (0.5 mM) used as nuclease and protease inhibitors, respectively, and was further fractionated by centrifugation at 105000*g* for 60 min. The supernatant thus obtained was adjusted to 0.5 M KCl and further centrifuged at 255000*g* for 4 h to yield a pellet of postpolysomal particles (Jain & Sarkar, 1979). Both the 105000*g* pellet (crude polysomes and ribosomes) and the pellet of postpolysomal particles could be stored at −80 °C for 1–2 months without any significant loss in the yield of iRNA.

The 105000*g* pellet was resuspended in buffer A containing 0.5 M KCl at a concentration of about 10 A_{260} units/mL. The material was then centrifuged through a discontinuous sucrose gradient containing three 5-mL layers of 1.35, 1.6, and 2.0 M sucrose in 0.5 M KCl-containing buffer A at 177000*g* for 16 h by using a Spinco Ti 60 rotor, as previously described (Jain & Sarkar, 1979). The supernatant and the sucrose layers were carefully collected, pooled, and dialyzed against 20 volumes of buffer B (50 mM potassium phosphate, pH 6.8, and 5 mM EDTA) for 4–16 h at 4 °C. The pellet of postpolysomal particles was also processed and dialyzed in a similar manner. The resulting dialysates obtained from the subcellular fractions were used for the isolation of iRNA by DEAE-cellulose chromatography.

Isolation of iRNA. The dialysate of the subcellular fractions was slowly stirred in batches of 2 L for 4 h at 4 °C with 7 g of DEAE-cellulose (DE-52, Whatman, Ltd.) previously equilibrated with buffer B. The DEAE-cellulose was allowed to settle, and the slurry was packed in a column (25 × 1 cm) and washed extensively with the above buffer. The bound material was eluted by using a linear 0–0.75 M KCl gradient in buffer B. Fractions containing RNA (Figure 1) were pooled, lyophilized, and then desalted by using a Sephadex G-25 (120 × 1 cm) column equilibrated with autoclaved distilled water. The RNA fractions were then lyophilized and subjected to a second cycle of DEAE-cellulose chromatography and desalting. The RNA samples thus obtained were further fractionated by hydroxylapatite chromatography.

Hydroxylapatite powder (Bio-Rad Ltd.) was hydrated with 6 volumes of buffer C (1 mM potassium phosphate, pH 6.8), the fines were removed by decantation, and the slurry was packed into a 1 × 25 cm column. RNA samples dissolved in buffer C at a concentration of 5–10 A_{260}/mL were applied to the column. The column was washed with buffer C, and the bound material was eluted with a linear gradient of 0–0.2 M potassium phosphate, pH 6.8, in buffer C (Figure 2). Any tightly bound material, which usually ranged at about 15–20% of the samples applied to the column, was removed by step elution with 1 M potassium phosphate, pH 6.8. The RNA samples were concentrated by lyophilization and desalted by Sephadex G-25 chromatography, as described above. In some cases, the RNA samples were dialyzed by using Spectraphore no. 6 dialysis membranes (1000 molecular weight cutoff, Spectrum Medical Industries) to remove salts.

Oligo(dA)- and Oligo(dT)-Cellulose Chromatography of iRNA. The iRNA samples (10–15 A_{260} units) were dissolved in 10 mM Tris-HCl, pH 7.5, containing 1 M NaCl and were applied to a column (5 × 0.6 cm) containing 0.5–0.6 g of oligo(dA)–cellulose (Collaborative Research, Inc.) equilibrated with the same buffer. Under these conditions, optimized by trial runs, about 50 A_{260} units of poly(U) were bound per gram of oligo(dA)–cellulose. After the column was thoroughly washed with the binding buffer, step elutions were carried out with 10 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl followed by 10 mM Tris-HCl, pH 7.5. Chromatography of iRNA samples on oligo(dT)–cellulose (type T_3; Collaborative Research, Inc.) was carried out in a similar manner except 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl was used as the binding buffer.

Nucleotide Composition of iRNA. The base compositions of iRNA samples were determined by ion-exchange chromatography (Katz & Comb, 1963) of the neutralized alkaline hydrolysates (0.3 N KOH, 18 h, 37 °C). In several cases, the base compositions were determined by high-pressure liquid chromatography (high-pressure LC) of the neutralized hydrolysates. High-pressure LC was carried out by using a Waters Associates apparatus. A μBondapak C_{18} resin column with 1–5% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, solvent system at room temperature was used to separate the nucleotides. The effluent was monitored at 260 nm, and the nucleotides present in the peak fractions were identified and estimated from their molar extinction

coefficients at pH 7.0.

Polyacrylamide Gel Electrophoresis of RNA. RNA samples were dissolved in 50% deionized formamide, heated to 65 °C for 30 s and quickly chilled (Lehrach et al., 1977). Electrophoresis of the samples was carried out at 600–800 V for 6–8 h at room temperature by using 8 or 12% acrylamide gels polymerized in 7 M urea and 90 mM Tris–borate, pH 8.3 (Lockard et al., 1978). The running buffer was 90 mM Tris–borate, pH 8.3, and 4 mM EDTA. A purified sample of yeast tyrosine transfer RNA with a size of 78 nucleotides was run as a marker. The gels were stained with 1 μg/mL ethidium bromide for 30 min followed by thorough washing in water. The stained gels, rendered fluorescent by placing on a UV transilluminator (model C-61, UV Products, Ltd.), were photographed by using Polaroid high-speed (type 107) film.

UV Absorption Spectra and Hyperchromicity Measurements of RNA Samples. The UV absorption spectra of RNA samples dissolved in 10 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, and 1 mM EDTA were recorded in a Beckman Model 25 spectrophotometer at 25 °C. For hyperchromicity measurements, RNA samples were dissolved in 0.1 M NaCl and 20 mM sodium phosphate, pH 7.0, at a concentration of 0.5 A_{260} units/mL. After the absorbance at 260 nm was measured by using a stoppered cuvette fitted with a temperature probe in a Cary Model 15 spectrophotometer, the temperature of the samples was increased stepwise by 5–10 °C, and the absorbance was recorded.

Isolation of Poly(A)-Containing RNA. RNA was extracted from KCl-washed free muscle polysomes (Scheme I) with phenol–chloroform–isoamyl alcohol (50:50:1) according to the procedure of Aviv & Leder (1972), as previously described (Bag & Sarkar, 1976). The final aqueous phase after three successive extractions was adjusted to 0.2 M potassium acetate, pH 5.0, and RNA was precipitated with 2.5 volumes of ethanol at −20 °C. The pelleted RNA was resuspended in autoclaved distilled water and the precipitation step repeated twice to remove traces of $NaDodSO_4$ and heparin. Poly(A)-containing RNA was isolated by oligo(dT)–cellulose chromatography of samples of total polysomal RNA as previously described (Bag & Sarkar, 1976; Jain & Sarkar, 1979). Column fractions containing poly(A) RNA were adjusted to 0.1 M LiCl, and RNA was precipitated with 2 volumes of ethanol at −20 °C.

Translation of mRNA. Micrococcal nuclease-treated reticulocyte lysate, prepared and assayed by the method of Pelham & Jackson (1976), was used for the translation of mRNAs. The optimal assay conditions such as K^+ and Mg^{2+} requirements and concentrations of mRNAs were determined by preliminary trial runs. The incubation mixture in a total volume of 25 μL contained the following: 12 μL of lysate, 15 μM hemin, 4 mM creatine phosphate, 4 μg of creatine phosphokinase, 0.4 mM magnesium acetate, 0.1 mM of 19 L-amino acids, 3 μCi of L-[3,4,5-^{3}H(N)]leucine (specific activity 150 Ci/mmol, New England Nuclear), 0.2–0.5 μg of mRNA or 0.5 A_{260} unit of KCl-washed muscle polysomes, and indicated amounts of iRNA. Incubation was started by the addition of lysate to the mixture of reaction components which were kept at 0 °C. In incubations containing iRNA, the latter was added just prior to the addition of the lysate. Samples (5 μL) of the incubation mixtures were processed for the estimation of Cl_3CCOOH-insoluble protein-bound (95 °C) radioactivities by liquid scintillation counting (Ernst et al., 1978).

For the analysis of translation products, the incubation mixture was adjusted to 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM dithiothreitol, and 2% $NaDodSO_4$ and then heated to 95 °C for 3 min. The samples were cooled, adjusted to 5% β-mercaptoethanol, and analyzed by using 12–18% gradient $NaDodSO_4$ slab gels. The gels were stained in 0.1% Coomassie blue, 5% Cl_3CCOOH, 5% sulfosalicylic acid, and 40% methanol. After the gels were destained, they were fluorographed by using prefogged X-ray film (Bonner & Laskey, 1974).

Aminoacylation of tRNA. This was carried out by using a pH 5 enzyme system (Moldave, 1963), prepared from chick embryonic muscles, in a volume of 25 μL containing the following: 50 mM Tris-HCl, pH 7.6, 50 mM KCl, 2 mM dithiothreitol, 4 mM ATP, 3 mM magnesium acetate, a mixture of 19 amino acids (minus leucine) at 20 nM of each amino acid, 10 nM L-[3,4,5-^{3}H(N)]leucine (specific activity 100 Ci/mmol), and 0.2 mg of pH 5 enzyme. Incubation was carried out at 37 °C for 20 min, and the reaction was terminated by adding a 20-μL sample to 2 mL of Cl_3CCOOH containing 1 mg/mL leucine. The acid-insoluble (4 °C) material was collected by filtration on Whatman GF-C filters, and the radioactivity was determined by liquid scintillation counting. The remaining 5 μL was then processed for the estimation of Cl_3CCOOH-insoluble protein-bound counts by heating at 90 °C for 10 min. The protein-bound counts (about 150–200 cpm) were subtracted from the total acid-insoluble (4 °C) counts to obtain the radioactivities incorporated in tRNA.

Materials. Unlabeled amino acids, dithiothreitol, heparin, phenylmethanesulfonyl fluoride, creatine kinase, creatine phosphate, hemin, and nucleoside triphosphate were purchased from the Sigma Chemical Co. Oligo(dT)–cellulose (type T_3) was obtained from Collaborative Research, Inc. Acrylamide, *N,N'*-methylenebis(acrylamide)(electrophoresis purity grade), and hydroxylapatite were purchased from Bio-Rad Laboratories. Ultrapure RNase-free urea and sucrose were obtained from Schwarz/Mann. Highly purified yeast tyrosine transfer RNA (78 nucleotides) was a gift from the laboratory of Dr. U. L. RajBhandary of the Massachusetts Institute of Technology.

Results

Isolation of iRNA. The 0.5 M KCl wash of the 100000*g* pelleted material and the postpolysomal particles (Scheme I) were used separately as the source for the isolation of iRNA. The dialysate obtained from these subcellular fractions (see Experimental Procedures) was adsorbed to DEAE-cellulose and the bound material eluted with a linear KCl gradient. A typical elution profile obtained with the dialysate from the KCl wash of 100000*g* pellet is shown in Figure 1. Two peaks, one eluted at about 0.15 M KCl and the other at about 0.45–0.5 M KCl (panel A), accounted for about 90% of the bound material. Only the material present in the second peak gave a typical UV spectrum for RNA and a positive orcinol test. Essentially identical elution profiles were obtained when dialysate from the postpolysomal particles was used (results not shown). The pooled fractions (indicated by the bar) of three to four column runs were diluted with 10–12 volumes of buffer B to lower the ionic strength and then subjected to a second cycle of DEAE-cellulose chromatography. About 85% of the material was recovered in a sharp peak eluted at about 0.5 M KCl (panel B; indicated by the bar). The fractions were pooled, lyophilized, and desalted on a Sephadex G-25 column. The materials present in the excluded fractions were concentrated by lyophilization and then further purified by hydroxylapatite chromatography, which was found to be capable of

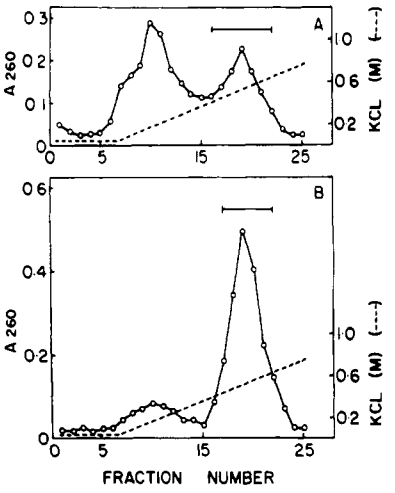


FIGURE 1: Isolation of iRNA by DEAE-cellulose chromatography of the dialysate from the 0.5 M KCl wash of the 100000*g* pelleted material of chick embryonic muscles. For details, see Experimental Procedures and Scheme I. (A) Typical elution profile of the bound material from the dialysate of the 0.5 M KCl wash of the 100000*g* pelleted material by using a 100-mL linear 0–0.75 M KCl gradient. Fractions (volume 2.3 mL) indicated by the bar were pooled, lyophilized, and subjected to a second cycle of DEAE-cellulose chromatography. (B) Rechromatography of pooled fractions (panel A) on DEAE-cellulose. Fractions indicated by the bar were pooled and used for further purification.

resolving iRNA from other low molecular weight cellular RNAs such as tRNA. Since subcellular fractions (Scheme I) similar to those used in this study are reported in the literature to contain tRNA (Hampel et al., 1972), it was necessary to further purify iRNA. About 85% of the bound RNA was eluted in a sharp peak at about 0.05–0.06 M potassium phosphate (Figure 2). The remaining bound material was released from the column by stepwise elution with 1 M phosphate. When a sample of chick embryonic muscle tRNA was run on the same column, it was eluted between 0.1 and 0.14 M potassium phosphate. Furthermore, if equal amounts of iRNA and tRNA were applied to the same column, they were clearly resolved into two distinct peaks, one corresponding to that of iRNA (Figure 2) and the other being eluted at 0.1–0.14 M phosphate (results not shown). These results suggest that about 15% of the material which was retained by the column and subsequently eluted with 1 M phosphate was due to other RNA species, presumably tRNA. Because of the large volume of the fractions in which this material was released, further characterization of this RNA proved to be difficult. The amounts of iRNA recovered from the 0.5 M KCl wash of 100000*g* pellets and the postpolysomal particles were about 160 and 100 μg, respectively, starting with about 120 g of muscle. The studies reported in the following sections were carried out with iRNA isolated from the salt wash of the 100000*g* pellet, unless indicated otherwise.

Properties of iRNA. The hydroxylapatite-purified material failed to give a positive reaction with ninhydrin and biuret reagents. Alkaline hydrolysis of iRNA (see Experimental Procedures) led to quantitative conversion to nucleotides as judged by the inability of the hydrolysate to be eluted in the void volume during Sephadex G-25 chromatography and to be precipitated with ethanol. Quantitative analysis with the orcinol reagent showed that 1 A_{260} unit of the material contained 40–41 μg of RNA (Table I), indicating that the material was a relatively pure preparation of RNA. The UV spectrum of iRNA was indistinguishable from that obtained with a sample of muscle tRNA (Figure 3). Base analysis of the neutralized alkaline hydrolysates indicated that the material contained about 40% adenosine (Table I). High-pressure LC analysis of a neutralized alkaline hydrolysate of iRNA gave a simple pattern of peaks identical with a mixture of standard nucleotides. In contrast, a hydrolysate of embryonic muscle tRNA gave a more complex pattern, presumably due to the characteristic methylated bases. When samples of iRNA were chromatographed on oligo(dA)- and oligo(dT)-cellulose columns, the iRNA fractions were quantitatively recovered in the unbound fractions (Figure 4), under conditions where samples of poly(U) or poly(A) showed about 95–98% binding to the affinity columns. These results indicate that iRNA does not contain poly(A) or poly(U) tracts of sufficient lengths—about 20–30 nucleotides—which are capable of binding to these columns.

Electrophoretic analysis of the DEAE-cellulose-purified iRNA fractions by urea–polyacrylamide gel electrophoresis

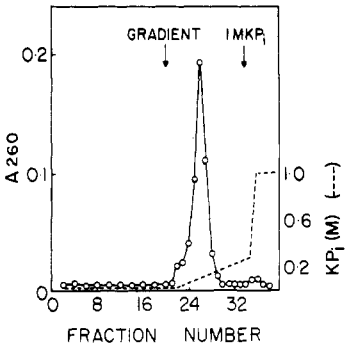


FIGURE 2: Hydroxylapatite chromatography of iRNA fractions (Figure 1). For details, see Experimental Procedures and Scheme I. The bound material was eluted by a 50-mL linear (0–0.2 M KP_i) gradient. The peak fractions (volume 2.3 mL) as indicated by the bar were pooled and desalted by Sephadex G-25 chromatography. The arrows indicate the positions where the gradient and step elutions with 1 M potassium phosphate were applied.

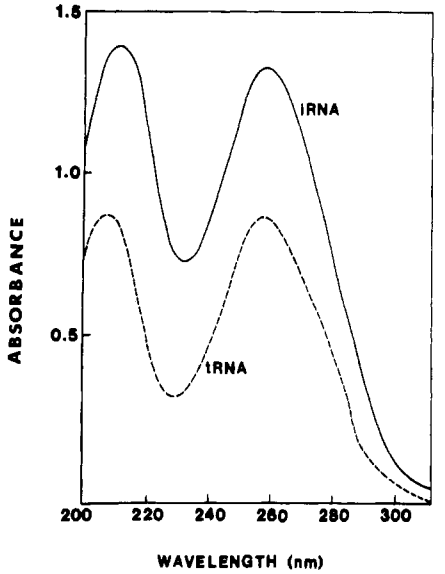


FIGURE 3: UV absorption spectra of iRNA (Figure 2) and a sample of yeast $tRNA^{Tyr}$. For details, see Experimental Procedures.

Table I: Summary of Properties of iRNA

A_{260}/A_{280}	2–2.2
orcinol reaction	quantitative reaction showing that 1 $A_{260} \equiv 40$ μg of RNA
av base composition	AMP, 40.9%
	CMP, 17.6%
	GMP, 20%
	UMP, 21.5%

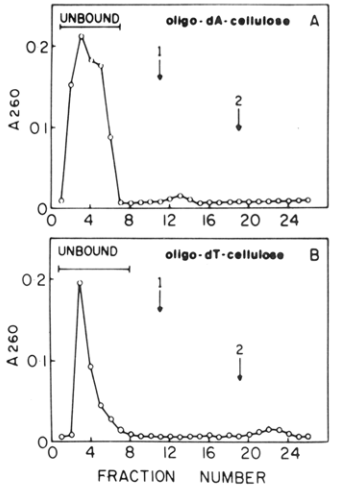


FIGURE 4: Elution profiles of iRNA (Figure 2) on oligo(dA)–cellulose (A) and oligo(dT)–cellulose (B). For details, see Experimental Procedures. Arrows 1 and 2 indicate the positions where step elutions were carried out with 10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.5, respectively.

on a 10-cm 12% gel (Figure 5, panel A) shows the presence of one major and two minor bands. On the basis of the mobility of a marker yeast tyrosine tRNA of 78 nucleotide size, the size of these bands was estimated approximately in the 70–90 nucleotide range. Electrophoresis of hydroxylapatite-purified RNA samples gave a gel pattern which was essentially similar to that shown in panel A, except in some preparations the slower moving bands (panel A) were found to be present in variable amounts (results not shown). When electrophoresis was carried out with 20-cm 8% gels, the major band (panel A) was further resolved into three to four components (panel B) migrating slightly faster than yeast tRNA marker. The slower migrating minor bands were also resolved into a number of components (panel B), indicating further the heterogeneity of iRNA. When electrophoresis of iRNA was carried out in the presence of 99% formamide, essentially the same gel pattern shown in panel B was obtained (results not shown).

Biological Activity of iRNA. The effect of iRNA on amino acid incorporation in micrococcal nuclease-treated reticulocyte lysate programmed with poly(A+) mRNA and 0.5 M KCl-washed free polysomes of chick embryonic muscles is shown in Figure 6. The iRNA inhibited strongly the translation of poly(A+) mRNA, about 85–90% inhibition being obtained at 40–60 ng of iRNA (panel A). In contrast, polysome-directed amino acid incorporation, which reflects mainly the elongation phase of nascent polypeptide chain synthesis, was not affected at these levels of iRNA. Only at much higher concentrations of iRNA was polysome-directed amino acid incorporation progressively reduced, about 55% inhibition being obtained with 6 μg of iRNA (panel B). Incubation in which tRNA of chick embryonic muscles was used at a concentration of 200 ng per assay did not show any inhibition of translation of poly(A+) mRNA. It was important to rule out the possibility that iRNA interferes with the aminoacylation process, and this accounts for the inhibitory activity of iRNA. With a pH 5 enzyme preparation of chick embryonic muscles for aminoacylation of tRNA, about 20-fold stimulation of [^{3}H]leucine incorporation above the zero time control was obtained (Table II). In the presence of 100 ng of tRNA, which was the same concentration as the level of iRNA required for complete inhibition of mRNA translation (Figure 6), the incorporation of leucine in tRNA was further increased by 12%. This is presumably due to the presence of sufficient amounts of endogenous tRNA in the pH 5 enzyme (Moldave,

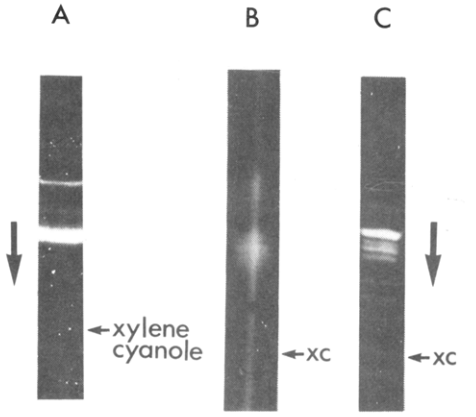


FIGURE 5: Polyacrylamide gel electrophoresis of iRNA. For details, see Experimental Procedures. (A) Electrophoresis of 10 μg of iRNA isolated by two cycles of DEAE-cellulose chromatography (Figure 1) by using a 10-cm 12% polyacrylamide–7 M urea slab gel. (B) Electrophoresis of 7.5 μg of iRNA after hydroxylapatite chromatography (Figure 2) by using a 20-cm 8% polyacrylamide–7 M urea slab gel. (C) Electrophoresis of 0.15 μg of yeast tRNA^{Tyr} run in a parallel slot of the same slab gel as in (B). Electrophoresis was from top to bottom as indicated by the vertical arrow. In all cases, the dye xylene cyanole, whose migration approximates 40–50 nucleotides in this gel system, was included in a parallel slot and is indicated with an arrow.

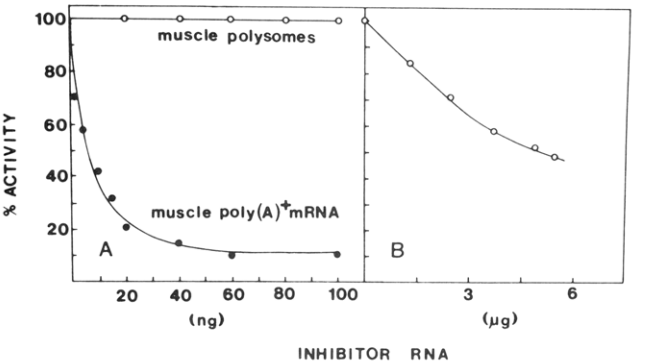


FIGURE 6: Effect of different concentrations of iRNA (Figure 2) on mRNA and polysome-directed translation in nuclease-treated reticulocyte lysate. Translation was carried out with saturating concentrations of muscle poly(A+) mRNA (0.16 μg) and salt-washed muscle polysomes (20 μg), giving the following incorporation of [^{3}H]leucine per 25-μL assay: control, 4500 cpm; + poly(A+) mRNA, 42 500 cpm; polysomes, 276 890 cpm. (A) Effect of iRNA over a range 0–100 ng/assay; (B) effect of a higher concentration range, 0–6 μg of iRNA, on muscle polysome-directed amino acid incorporation.

1963). Neither iRNA used alone, at a level of 100 ng, nor a mixture of tRNA and iRNA, 100 ng of each, had any effect on the total leucine counts incorporated (Table II). These results indicate that iRNA has no activity as either a substrate or an inhibitor of the aminoacylation step. When a sample of iRNA was digested with 10 μg/mL pancreatic RNase at 30 °C for 60 min and subsequently reextracted with phenol–chloroform–isoamyl alcohol after addition of carrier tRNA, the reisolated RNA sample was found to be totally inactive as an inhibitor of mRNA translation (results not shown). These results indicate that the inhibition by iRNA was not due to a contaminant nor was it due to an indirect effect on aminoacylation by iRNA.

The kinetics of amino acid incorporation in poly(A+) mRNA directed incubations in the absence and presence of iRNA samples are shown in Figure 7. It is clear that concentration-dependent progressive inhibition of translation by iRNA shown in Figure 6 was also observed during the linear part of the time course. The slower rate of amino acid in-

Table II: Effect of iRNA on the Aminoacylation of tRNA by a pH 5 Enzyme[a]

addition	[^{3}H]leucine counts incorporated into tRNA
complete system	39 900
complete system + 100 ng of muscle tRNA	45 000
complete system + 100 ng of iRNA	38 600
complete system + 100 ng of tRNA + 100 ng of iRNA	44 450

[a] Assay conditions were as described under Experimental Procedures. For details, see text. The zero time incorporation gave 1500 cpm.

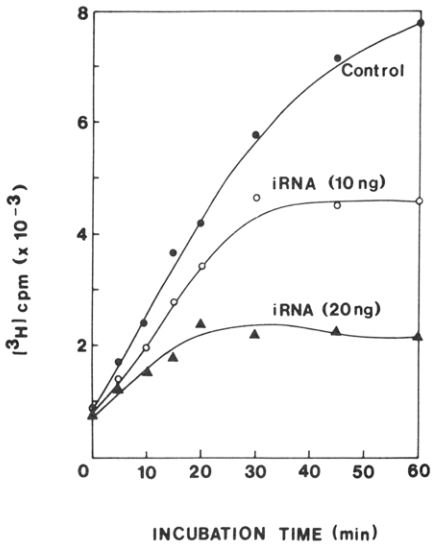


FIGURE 7: Inhibition of translation of muscle poly(A+) RNA by iRNA (Figure 2) in a nuclease-treated reticulocyte lysate system. For details, see Experimental Procedures. All incubations contained a saturating amount (0.16 μg) of poly(A+) mRNA. Procedure: (●) control; (○) 10 ng and (▲) 20 ng of iRNA/25-μL assay.

corporation between 30 and 60 min observed in incubations containing mRNA is presumably due to reinitiation of the mRNAs following run off. Interestingly, this phase was absent in incubations containing all levels of iRNA tested.

We have also tested the possibility that iRNA may inhibit selectively the translation of mRNAs coding for specific myofibrillar proteins in a manner analogous to the reported mode of action of tcRNA of chick embryonic muscles (Kennedy et al., 1978; Heywood & Kennedy, 1976). The translation products of poly(A+) mRNA directed incubations in the absence and presence of various concentrations of iRNA were examined by fluorography. Purified samples of the major chicken myofibrillar proteins, e.g., myosin heavy chain (200 $\times 10^3$ daltons), actin (42 $\times 10^3$ daltons), tropomyosin–troponin T [(35–37) $\times 10^3$ daltons], troponin C (18 $\times 10^3$ daltons), and myosin light chains [(27, 24, and 18) $\times 10^3$ daltons], were used as markers; the arrows indicate the positions of some of these bands. The fluorogram (Figure 8) indicates that all major bands, some of which correspond to the above-mentioned marker proteins, are equally inhibited in a nonselective manner, as judged by the lack of any apparent difference in the relative intensities of the bands. Furthermore, this mode of inhibition was observed with all levels of iRNA tested (lanes 3–6).

Hyperchromicity of iRNA at Increasing Temperature. Since double-stranded RNA is known to block protein synthesis by interfering with the initiation process [for a review, see Ochoa & DeHaro (1979)], it was of interest to test the possibility that the inhibitory action of iRNA may be due to its double-stranded nature. Hyperchromicity measurements with increasing temperature (Figure 9) indicate that the melting of iRNA (open circles) takes place over a wide temperature range, suggesting a noncooperative nature of the melting. The melting curve of iRNA was very similar to that of poly(A) (closed circles). Interestingly, yeast tyrosine tRNA gave a larger value of hyperchromicity (closed squares), which is in agreement with the view that tRNA contains considerable secondary structure [for a review, see Bloomfield et al. (1974)]. The hyperchromicity shown by iRNA, which was indistinguishable from poly(A) and different from tRNA, strongly suggests that iRNA is not a double-stranded molecule. This is also consistent with the observation that treatment with pancreatic RNase, which does not cleave double-stranded

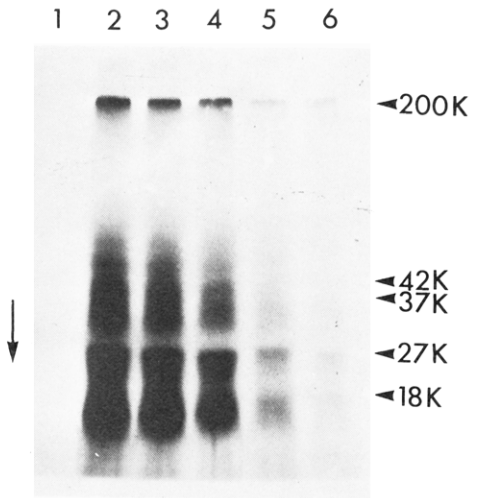


FIGURE 8: Fluorography of translated products obtained with muscle poly(A+) mRNA in the presence and absence of iRNA. For details, see Experimental Procedures and text. Nonradioactive myofibrillar proteins were run as markers on the same gel and located by staining with Coomassie blue. Arrows indicate the position of these marker proteins. (Lane 1) Nuclease-treated lysate; (lane 2) muscle poly(A+) RNA (0.16 μg); (lanes 3–6) incubations containing 0.16 μg of muscle poly(A+) RNA and 4, 8, 20, and 40 ng of iRNA, respectively.

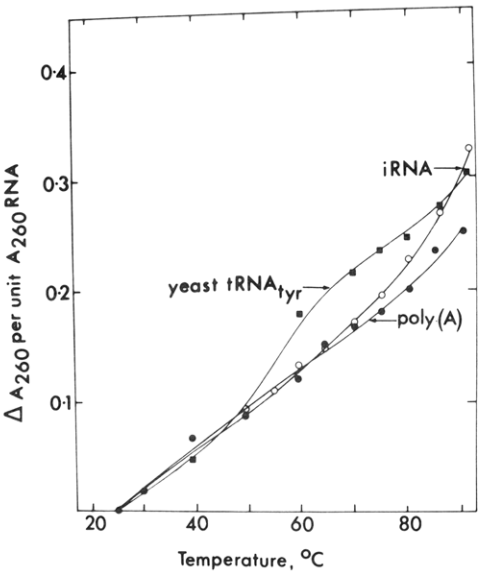


FIGURE 9: Hyperchromicity measurements with iRNA (Figure 2) as a function of increasing temperature. For details, see Experimental Procedures. (■) Sample of yeast tRNA^{Tyr}; (○) iRNA; (●) sample of poly(A). The ordinate is expressed in a normalized manner: Δ absorbance (i.e., absorbance of the samples at temperature t minus initial absorbance at 25 °C) divided by the A_{260} units of RNA of the samples at 25 °C.

RNA, results in a complete loss of biological activity of iRNA. The observed hyperchromicity of iRNA presumably reflects primarily the unstacking of bases [for a review, see Bloomfield et al. (1974)].

Discussion

The results presented here show that low molecular weight RNAs, about 70–90 nucleotides in size, isolated from the 0.5 M KCl wash of the 100000*g* pelleted material of chick embryonic muscles, are potent inhibitors of translation of poly(A+) mRNAs. The RNA nature of the hydroxylapatite-purified inhibitor is shown by (a) its alkali lability, (b) UV spectra, (c) base composition, (d) quantitative reaction with orcinol, and (e) loss of biological activity after digestion with RNase (Table I and Figure 3). Most of the studies reported here were carried out by using iRNA isolated from the crude ribosomal 0.5 M KCl wash. Our preliminary studies indicate that a similar inhibitory RNA is also present in the postpolysomal supernatant fraction, and these two iRNA fractions are very similar in size, electrophoretic mobility, and biological activity. Further work on the interrelationship of the cytoplasmic iRNA fractions is now in progress.

Although iRNA contains about 40% A, its inability to bind to oligo(dT)- and oligo(dA)–cellulose (Figure 4) indicates that it does not contain poly(A) or poly(U) tracts of lengths sufficient for binding to the affinity columns. Thus, iRNA is clearly distinct from the low molecular weight tcRNA of chick embryonic muscles previously reported in the literature (Heywood & Kennedy, 1976; Kennedy et al., 1978) with respect to size, base composition, lack of a detectable oligo(U) tract, and its ability to inhibit translation of muscle-specific mRNAs in a nonselective manner (Figure 8). We have recently observed that iRNA also inhibits efficiently the translation of both homologous and heterologous mRNAs (e.g., globin mRNA and myosin heavy chain mRNA), poly(A+) mRNA, and poly(A–) mRNA, thus further supporting the view that the inhibition by iRNA does not take place in a mRNA-discriminating manner (Pluskal et al., 1979; Sarkar & Pluskal, 1979), a feature which clearly distinguishes it from tcRNA. It should be pointed out that tcRNA inhibits the translation of specific homologous mRNA (e.g.,, myosin heavy chain mRNA is inhibited by myosin tcRNA), and this inhibition requires the presence of the poly(A) tract of the mRNA (Heywood et al., 1975).

With regard to the puzzling question of why iRNA, which is about the same size as tRNA, is dialyzable (see also Experimental Procedures), we have made the interesting observation that under our preparative conditions, namely, the hyperosmotic composition of the 0.5 M KCl–sucrose wash, about 50% of a sample of yeast tRNA was recovered in the dialysate. Also, about 80% of hydroxylapatite-purified iRNA dissolved in the 0.5 M KCl–sucrose buffer became dialyzable. However, the dialyzability of both 4S yeast RNA and iRNA is sharply reduced to about 10%, when the samples were dissolved in 0.1 M KCl or NaCl in the absence of sucrose. It appears that the dialyzability of iRNA fractions was strongly dependent on the osmolarity of the solution, which may also influence the hydrodynamic properties of iRNA.

Although iRNA is similar in size to tRNA (Figure 5), four lines of evidence indicate that it is distinct from tRNA. These are the following: differences in the elution properties from hydroxylapatite columns; the inability of iRNA to be aminoacylated; the inability of tRNA to inhibit the translation of poly(A+) mRNA under conditions in which iRNA shows quantitative inhibition; differences in the high-pressure LC analysis of neutralized alkaline hydrolysates which show that the tRNA gives a more complex pattern, presumably due to base modification.

Some clues regarding the mode of inhibition by iRNA are obtained from the following considerations: (a) inhibition of amino acid incorporation in polysome-directed and mRNA-programmed incubations requires strikingly different concentrations of iRNA (Figure 6); (b) the second slower phase of amino acid incorporation in mRNA-directed incubations is completely inhibited in the presence of iRNA (Figure 7). These suggest strongly that the inhibition by iRNA, particularly at low concentrations, takes place primarily at the initiation step. Furthermore, the ability of iRNA to inhibit the translation of mRNAs coding for various muscle proteins indicates that the inhibition by iRNA does not discriminate between mRNAs.

The site of action of iRNA, particularly on mRNA translation at low level, remains to be determined. It may involve an early event in initiation, possibly interference with mRNA binding to ribosomes. We are currently engaged in studies concerning the mechanism of action of iRNA on the initiation process.

The inhibitory action of iRNA on mRNA translation (Figures 6–8), considered together with other properties such as size, base composition, etc., clearly distinguishes it from other low molecular weight RNA species reported in the literature, such as the purine-rich stimulatory RNA of rabbit reticulocytes (Bogdanovsky et al., 1973), the activator and inhibitory RNAs of *Artemia salina* embryos (Lee-Huang et al., 1977; Slegers et al., 1977; DeHerdt et al., 1979), and the 7S poly(A+) RNA of chick embryonic heart which induces cardiac differentiation in vitro (Deshpande et al., 1977; Arnold et al., 1978). The relationship of iRNA to low molecular weight nuclear RNAs which have been reported in a variety of eukaryotic cells (Weinberg & Penman, 1968; Zieve & Penman, 1976; Reddy et al., 1974; Rao et al., 1977) remains to be clarified. One of the well-characterized nuclear RNAs, U_1, which has been sequenced, has a 5′ cap structure and an initiator AUG codon and markedly inhibits the translation of poly(A+) mRNA (Reddy et al., 1974; Rao et al., 1977). The iRNA appears to be different from U_1 nuclear RNA in its size and base composition. Currently, we are engaged in further resolving the iRNA bands with the ultimate goal of identifying the inhibitory components and hopefully sequencing the purified biologically active components.

The melting of iRNA, which appears to be noncooperative and takes place over a wide temperature range (Figure 9), considered together with the characteristic elution of iRNA from hydroxylapatite at relatively low salt concentration (Figure 2) and the loss of biological activity of iRNA following pancreatic RNase treatment, strongly suggests that the inhibitory action of iRNA is not due to double-stranded molecules. Thus, iRNA appears to be a novel class of translational inhibitors. Its biological function remains to be understood. It may be involved directly or indirectly in the distribution of mRNAs between polysomes and free mRNP pools in the cytoplasm and chick embryonic muscles, which has been reported by us and others (Bag & Sarkar, 1975, 1976; Jain & Sarkar, 1979; Dym et al., 1979).

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