PURIFICATION OF MYOSIN mRNP TRANSLATIONAL CONTROL RNA AND ITS INHIBITION OF MYOSIN AND GLOBIN MESSENGER TRANSLATION

Doris S. KENNEDY, Eric SIEGEL and Stuart M. HEYWOOD
Genetics and Cell Biology Section, University of Connecticut, Storrs, CT 06268, USA

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1. Introduction

Since oligo(U)-containing tcRNA was first found in embryonic muscle [1] it has also been observed in *Artemia salina* [2] and connective tissue [3]. In all cases this tcRNA has been shown to inhibit protein synthesis. Only in the studies from muscle cells has this inhibition been shown to have a high degree of discrimination between messengers. In this case, highly purified oligo(U)-containing tcRNA was shown to interact stoichiometrically with myosin mRNA and inhibit its translation while having little effect on the translation of other messengers [4]. The oligo(U)-containing tcRNA, isolated from *Artemia salina* [2] may, inhibit protein synthesis in a non-selective manner by acting directly on the ribosomes [2]. Oligo(U)-containing tcRNA, isolated from connective tissue, inhibits both globin and collagen mRNA translation [3]; however, the inhibition of collagen synthesis is significantly greater than that of globin. Our studies have suggested that the sequence of addition of the reactants to the protein synthesizing system is important in determining the mode of action of this tcRNA [4,5]. We have further examined this in an attempt to correlate these apparently conflicting reports concerning the inhibitory effect of oligo(U)-containing tcRNA. In this report we verify that myosin oligo(U)-containing tcRNA is associated with myosin mRNPs and not with ribosomes co-sedimenting with the mRNPs. In addition, we find that it is necessary both to use stoichiometric amounts of mRNA and tcRNA and to add tcRNA and mRNA in the proper sequence to the reaction mixture in order to observe a selective inhibition of messenger translation. These results support our original suggestion that oligo(U)-containing tcRNA interacts selectively with messenger, thereby inhibiting its translation.

2. Experimental

The isolation of the 70–120 S sucrose density gradient fraction of 13 day embryonic chick muscle has been described [6]. This fraction contains myosin mRNPs as well as free ribosomes [6,7]. Myosin mRNPs were separated from the co-sedimenting ribosomes in the following manner. The 70–120 S sucrose density gradient fractions were centrifuged for 2 h at 240 000 × *g*. The resulting pellets are resuspended in 0.2 M NaCl, 0.05 M Tris–HCl (pH 7.8) and heated to 37°C for 5 min. After rapid cooling, the sample was applied to an oligo(dT)–cellulose column equilibrated with the same buffer. The column was washed with the 0.2 M NaCl buffer until all the unbound ribosomes were removed as determined by monitoring the elution at 260 nm. Subsequently myosin mRNPs were eluted with 0.01 M Tris–HCl (pH 7.8) containing 50% formamide. Both the ribosomal and mRNP samples were pelleted by centrifugation at 240 000 × *g* for 90 min. RNA was extracted from the resulting pellets by the procedure in [8]. After precipitation for 24 h in 2 vol. ethanol at –20°C the RNA samples were dissolved in water and subsequently reprecipitated in ethanol to remove all traces of phenol. Finally, 2.5 mg rRNA (material not binding to the oligo(dT)–cellulose column) and 30–35 μg poly(A)-containing RNA (from oligo(dT)–cellulose bound mRNPs) were each dissolved in 0.005 M

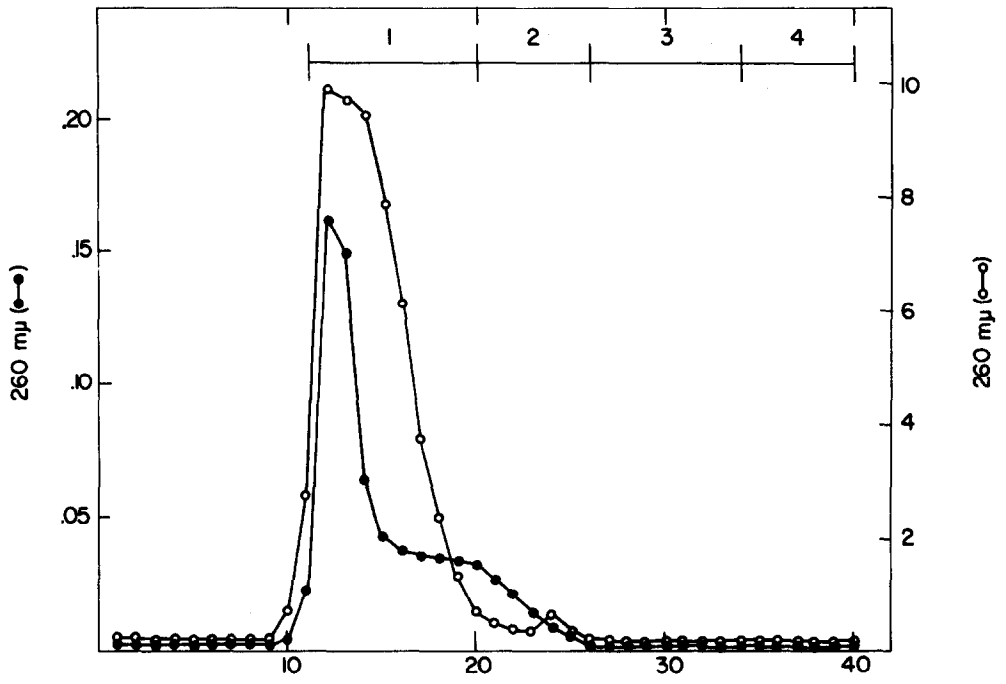

Fig.1. CL–Sepharose 4B chromatography of ribosomal RNA and myosin mRNP–RNA. The ribosomal and mRNP–RNAs were obtained from the 70–120 S region of sucrose density gradients prepared with chick muscle extracts. Ribosomes and myosin mRNPs were separated from each other as in section 2. 2.5 mg ribosomal RNA (o—o) and 35 μg myosin mRNP–RNA (●—●) were applied to the column. The column was developed as in section 2. Fractions were pooled into 4 composite fractions as indicated for subsequent identification of poly(A)- and oligo(U)-containing RNAs.

EDTA, heated to 60°C for 2 min and rapidly chilled. Each sample was applied to a 1 × 50 cm CL–Sepharose 4B (Pharmacia) column, pre-equilibrated with 0.005 M EDTA. This procedure is a modification of that in [3]. The column was developed at 2°C while collecting 1 ml fractions and monitoring the elution at 260 nm. Typical elution profiles for both myosin mRNP–RNA and rRNA are shown in fig.1. Affinity chromatography of the fractions eluted from the CL-Sepharose 4B column using oligo(dT)–cellulose for messenger and oligo(dA)–cellulose of oligo(U)-containing tcRNA were performed as in [4,6]. Briefly, the pooled fractions, shown in fig.1, were applied to an oligo(dT)–cellulose column. The material not binding to the oligo(dT)–cellulose subsequently chromatographed on oligo(dA)–cellulose columns to obtain oligo(U)-containing tcRNA while the bound poly(A)-containing RNA was eluted with low salt buffer. The mRNA and tcRNA fractions were finally precipitated in 2 vol. ethanol containing 0.24 M ammonium acetate. The determination of oligo(U)-containing tcRNA by ^{3}H-labeled poly(A) hybridization was performed as in [5].

The conditions for the preparation of myosin and globin mRNAs, the preparation of the wheat germ lysate, and the translation of these messengers in this system has been described [6,9]. In all experiments reported here no additional initiation factors from muscle or reticulocytes were present in the reaction mixtures. The amounts of messenger and tcRNA present in the incubation mixtures is indicated in the table and figure legends.

Myosin and globin synthesis were determined by analysis of the reaction mixtures on sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described [6]. The addition of each messenger individually to the cell-free system resulted only in the appearance of a radioactive peak upon electro-

phoresis which migrated with its respective protein marker (200 000 mol. wt for myosin and 18 000 mol. wt for globin). Radioactivity was determined by liquid scintillation counting after slicing the gels and dissolving each 1 mm slice in hydrogen peroxide.

3. Results

The various fractions from the CL–Sepharose 4B chromatography of both rRNA and myosin mRNP–RNA were pooled is indicated in fig.1. The presence of oligo(U)-containing RNA was determined by ribonuclease protection of ^{3}H-labeled poly(A) following hybridization to the different fractions eluting from the column [5]. In addition the percentage of poly(A)-containing RNA and oligo(U)-containing RNA were determined from the elution of the oligo(dT)– and oligo(dA)–cellulose columns (table 1). Fraction 1 of the myosin mRNP–RNA includes most of the poly(A)-containing RNA of the sample while fraction 3 includes the majority of the oligo(U)-containing RNA. The chromatographic fractions of the rRNA have neither poly(A)- nor oligo(U)-containing RNAs. When the total poly(A)-containing RNA from (fractions 1, 2, table 1) and the total oligo(U)-containing RNA (fractions 2, 3, table 1) were analyzed on sucrose density gradients all of the poly(A)-containing RNA sediments at 26 S (fig.2A) while the oligo(U)-containing tcRNA sediments at less than 4 S (fig.2B). These sedimentation values are characteristic of myosin heavy chain mRNA and tcRNA, respectively. The oligo(U)-containing tcRNA

Table 1
CL–Sepharose chromatography of myosin mRNP–RNA

Fraction[a]	% Poly(A) RNA	% Oligo(U) RNA
1	85	0
2	15	12
3	0	88
4	0	0

[a] Fractions are as shown in fig.1

Poly(A) RNA from myosin mRNP, 32μg, was chromatographed on the column. Oligo(U)-containing tcRNA, 1.2 μg, was recovered. No poly(A) or tcRNA was found in ribosome fractions chromatographed on the column

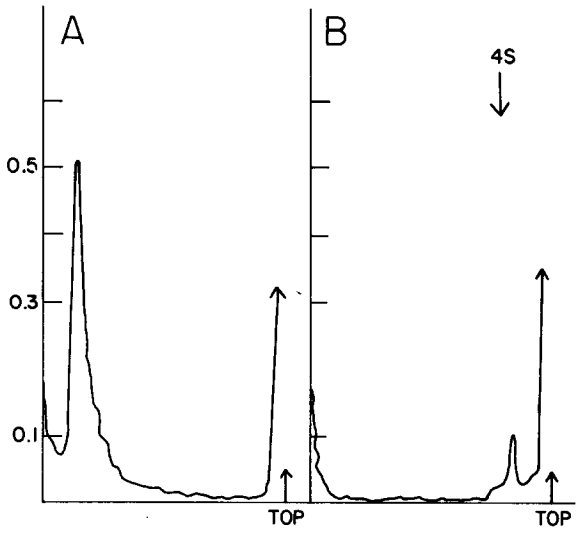

Fig.2. Sucrose density gradient analysis of poly(A)- and oligo(U)-containing RNA obtained from CL–Sepharose 4B chromatography of myosin mRNP–mRNA. (A) Poly(A)-containing RNA from fractions 1, 2 (fig.1). The single peak of RNA sediments at 26 S. (B) Oligo(U)-containing RNA from fractions 3, 4 (fig.1). The single peak of low molecular weight RNA sediments at less than 4 S. The sucrose density gradients were 10–30% sucrose containing 0.05 M Tris–HCl (pH 7.6), 0.005 M EDTA and 0.5% sodium dodecyl sulfate. Centrifugation was for 22 h at 40 000 rev./min in an IEC, SB-283 rotor.

was further analyzed by acrylamide gel electrophoresis under denaturing conditions. As can be seen in fig.3, the tcRNA migrates as a single peak. Although the exact molecular weight cannot be determined from the analysis, it can be approximated at about 10 000 from its relative mobility to 5 S rRNA and 4 S tRNA. This is in agreement with our determination of the molecular weight of oligo(U)-containing myosin tcRNA [4]. Because oligo(U)-containing tcRNA is not found in the chromatographic fractions of rRNA it is likely that this tcRNA is only found associated with mRNPs as suggested [4].

Earlier experiments have indicated that some degree of messenger discrimination existed in the inhibitory activity of oligo(U)-containing tcRNA [1,4,5]. This was demonstrated by the fact that tcRNA isolated from myosin mRNP-rich fraction of sucrose density gradients inhibited myosin synthesis without affecting globin synthesis when both mes-

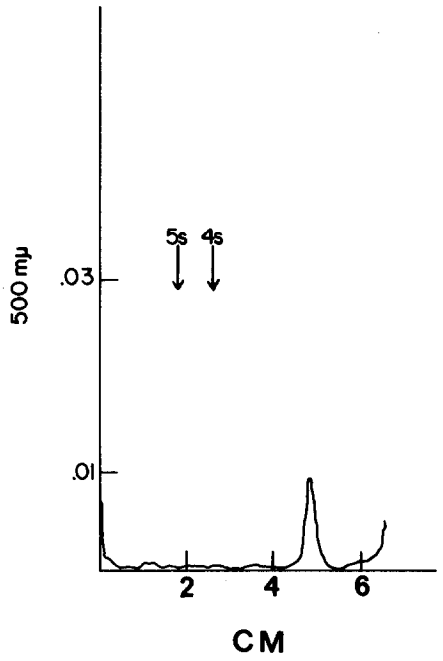

Fig.3. Acrylamide gel electrophoresis of oligo(U)-containing tcRNA isolated from myosin mRNPs. Electrophoresis under denaturing conditions and gel scanning have been described [4]. The markers, 5 S rRNA and 4 S tRNA, were run on parallel gels.

sengers were present in the cell-free system. In this case, stoichiometric amounts of myosin mRNA and oligo(U)-containing tcRNA were premixed prior to addition to the reaction mixture [4]. Several reports have suggested that oligo(U)-containing tcRNA does not have discriminatory properties in its inhibition of messenger translation [2,3]. Therefore, we have reinvestigated our findings concerning this discriminatory property of myosin mRNP–tcRNA. The sequence of addition of the reactants to the reaction mixture was considered as a possible explanation of the reported discrepancies. For this reason a number of experiments were performed in order to determine if either the sequence of addition of mRNA and tcRNA to the reaction mixture or the premixing of these reactants has an effect on the translation of myosin and globin mRNAs. In all cases premixing of the mRNAs and oligo(U)-containing tcRNA was performed in 4 μl incubation buffer [6] for 5 min at 2°C. In the experiments reported in table 2, a 2-fold excess of myosin mRNP–tcRNA was used over stoichiometric values with regard to myosin mRNA. The premixing of myosin mRNP–tcRNA with either myosin or globin mRNA results in a significant inhibition of translation of both messengers (lines 3, 4, table 2) as compared to control values (lines 1, 2,

Table 2
Sequence of addition of tcRNA to cell-free amino acid incorporating system

	Experiment[a]	Myosin (cpm)	Globin (cpm)	% Inhibition	
				Myosin	Globin
1	M→	1928	–	–	–
2	G→	–	14 776	–	–
3	t + M→	152	–	92	–
4	t + G→	–	5794	–	60
5	t + M→ G	175	16 998	91	0
6	t + G→ M	646	10 376	66	30
7	t + M + G→	560	13 682	70	8
8	t ⇌ M + G	296	4566	85	70

[a] *Abbreviations:* M, 5 μg myosin mRNA; G, 0.5 μg globin mRNA; t, 0.05 μg tcRNA

(→) indicates when addition to wheat germ lysate occured. For example, in experiment number 5, (t + M→G) represents tcRNA premixed with myosin mRNA and then added to the wheat germ lysate already containing globin mRNA

The reaction mixtures were incubated for 1 h at 25°C

table 2). However, when myosin mRNP–tcRNA is premixed with myosin mRNA and subsequently added to the reaction mixture containing globin mRNA, only myosin mRNA translation is inhibited (line 5, table 2). If the reverse experiment is performed by premixing tcRNA with globin mRNA (line 6, table 2), the translation of both messengers is inhibited; however, myosin synthesis is inhibited to a greater extent than globin synthesis. After premixing both messengers with tcRNA and subsequently adding the mixture to the cell-free system only a slight inhibition of globin mRNA translation occurs while myosin synthesis is inhibited 70% (line 7, table 2). If myosin mRNP–tcRNA and the messengers are individually added to the reaction mixture without regard to the sequence of addition, the translation of both messengers is inhibited (line 8, table 2). However, even in this experiment we routinely observe a greater inhibition of myosin synthesis as compared to globin synthesis. We assume that these latter results (line 8, table 2) were performed in a similar manner to those in [2,3].

The *Artemia salina* oligo(U)-containing tcRNA inhibited poly(U)-directed phenylalanine incorporation [2]. We have been unable to demonstrate such an inhibition in the wheat germ cell-free system at 10 mM Mg^{2+} using oligo(U)-containing tcRNA isolated from myosin mRNPs (unpublished results). The reason for this discrepancy is not clear. Therefore, with the exception of the experiment utilizing synthetic messenger, our results are in agreement with those of both [2] and [3]. However, our results indicate that in order to observe a discriminatory function of oligo(U)-containing tcRNA attention must be paid to the sequence of addition of both the messenger and the tcRNA to the reaction mixture.

In addition to the premixing of the RNA species and the order to addition of the reactants, attention should also be given to the stoichiometry of the reactants. As shown in fig.4, when both myosin and globin mRNA are present in the reaction mixture with myosin mRNP–tcRNA in a 1:1 mole ratio with myosin mRNA there is a complete inhibition of myosin mRNA translation with no inhibition of globin synthesis. As the quantity of tcRNA is increased to a 5-fold excess over the stoichiometric amount required for complete inhibition of myosin synthesis a significant inhibition of globin synthesis also occurs.

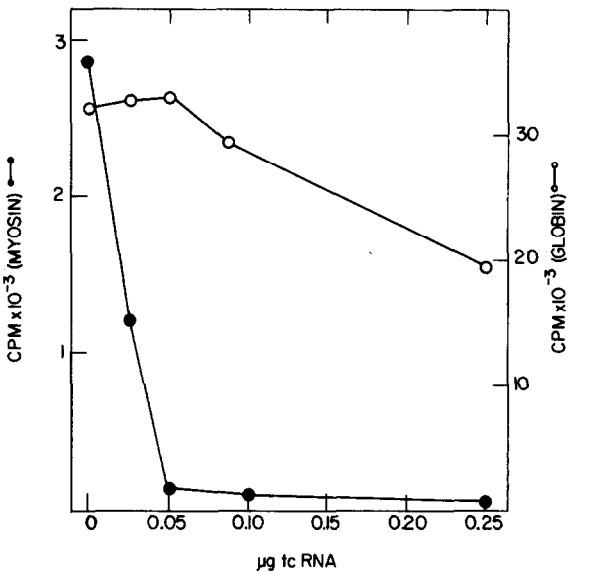

Fig.4. Stoichiometric relationship between myosin mRNA and myosin mRNP–tcRNA as measured by the inhibition of myosin and globin synthesis. Prior to addition to each reaction mixture 10 μg myosin mRNA and 2 μg globin mRNA were premixed with the indicated amount of myosin mRNP–tcRNA as in section 2. Cell-free synthesis and methods of analysis for myosin and globin synthesis have been described [6,11]. (•—•) Radioactivity incorporated into myosin heavy chain. (○—○) Radioactivity incorporated into globin.

4. Discussion

We have isolated the oligo(U)-containing tcRNA from a myosin mRNA rich fraction of sucrose density gradient by a dialysis procedure [4]. It is possible that this procedure limited the size of the recoverable tcRNA. The use of CL–Sepharose 4B allows for the first time the elution of nucleic acids by molecular weight distribution from Sepharose [10]. Therefore, we have used this procedure to isolate oligo(U)-containing tcRNA from myosin mRNPs. The results presented in this report demonstrate that this tcRNA can be separated from mRNA by chromatography on such a column. Furthermore, the oligo(U)-containing tcRNA found in the 70–120 S fraction of a muscle homogenate binds to oligo(dT)–cellulose prior to deproteinization of the sample. This suggests that the oligo(U)-containing tcRNA is associated with myosin mRNPs (containing 26 S myosin mRNA) and, there-

fore, is not associated with ribosomes. The myosin mRNP–tcRNA can be isolated by oligo(dA)–cellulose chromatography of the mRNP–RNA which does not bind oligo(dT)–cellulose. It is of interest that oligo(dT)–cellulose chromatography of mRNPs allows the tcRNA to remain associated with the poly(A)-containing RNA while after deproteinization and subsequent heating and rapid chilling the tcRNA is no longer retained by the oligo(dT)–cellulose. This suggests that the protein components of mRNPs are involved with the tcRNA–mRNA interaction.

The molecular size of myosin mRNP–tcRNA as judged by both sucrose density gradient analysis and denaturing acrylamide gel electrophoresis agrees with [4]. This suggests that both the dialysis and chromatographic procedures for its isolation yield similar results with regard to the molecular weight of myosin mRNP–tcRNA.

The effect of the sequence of addition of oligo(U)-containing tcRNA and mRNA as well as the stoichiometric relationship of these reactants with regard to the messenger discriminatory function of tcRNA has been investigated. The results presented here are in agreement with those who have claimed the inhibitory effect of tcRNA on messenger translation is non-discriminatory [2,3]. This is accomplished by adding the oligo(U)-containing tcRNA to the reaction mixture without premixing with an mRNA isolated from the same mRNP. However, if myosin mRNA and myosin tcRNA are allowed to 'find' each other in the absence of other components of the protein synthesizing system a substantial degree of messenger discrimination is observed. In addition, if more than stoichiometric amounts of oligo(U)-containing tcRNA, with regard to its mRNA, are present in the reaction mixture interference with the translation of other messengers is observed (in this case globin synthesis). These results suggest that myosin mRNA and myosin mRNP–tcRNA interact such that the hybrid formed both makes the myosin mRNA untranslatable and removes tcRNA so that it cannot inhibit protein synthesis in a non-specific manner as suggested [2]. If mRNPs containing different messages also contain different tcRNAs it may also be necessary to purify the mRNPs prior to isolation of the oligo(U)-containing tcRNA in order to demonstrate the messenger discriminatory function of these molecules. Fortunately, the myosin mRNPs we have isolated contain at least 80% myosin mRNA [11] and, therefore, are not likely to be highly contaminated by other tcRNA species.

In summary, the oligo(U)-containing tcRNA is found associated with mRNPs and a direct interaction between mRNA and tcRNA can result in a discriminatory inhibition of translation of the messenger. These results support our hypothesis concerning the role of oligo(U)-containing tcRNA in the translational control of protein synthesis [1].

Acknowledgements

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