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ISOLATION OF MYOSIN MESSENGER RIBONUCLEOPROTEIN PARTICLES WHICH CONTAIN A PROTEIN FRACTION AFFECTING MYOSIN SYNTHESIS

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SUMMARY: Myosin mRNP particles were purified using metrizamide buoyant density gradient centrifugation. This mRNP particle contains only the 26S myosin mRNA and, therefore, is a purified mRNP with regard to mRNA composition. A protein fraction, eluting from DEAE-cellulose between 0.15M KCℓ and 0.28M KCℓ, was isolated from the purified myosin mRNP particle. A significant stimulation of myosin heavy chain synthesis is observed when the isolated fraction is added to an unfractionated wheat germ cell-free system containing myosin mRNA. This protein fraction was found to have no effect on the translation of TMV RNA. In addition, this protein fraction does not bind TMV RNA, but is associated with myosin mRNA during translation.

INTRODUCTION

It is currently believed that mRNAs exist in the cytoplasm of a wide variety of eukaryotic cells as protein-bound complexes, free of ribosomes (1 - 6). These free or stored mRNAs appear to be maintained in an inactive form for a period of time in the cytoplasm. Thus far, except as reported here, it has not been possible to obtain purified mRNPs with regard to mRNA content. Proliferating myoblasts synthesize little, if any, myosin (2). However, following cell fusion and myotube formation the myosin mRNA appears in the polysomes and its half-life is considerably decreased. The significance of translational control RNA (tcRNA) in the inactivation of stored mRNAs, at least for myosin mRNPs, has been reported (7 - 9). However, the biological function of the proteins associated with the stored cytoplasmic mRNAs, still reamins ill-defined. As part of an effort to understand this, a protein fraction was isolated from purified myosin mRNP particles and tested for its ability to stimulate myosin synthesis in a wheat germ cell-free system.

MATERIALS AND METHODS

Myosin mRNPs were prepared from 12 day embryonic chick muscles (2). The collected ribosomes containing the myosin mRNP were pelleted by centrifugation for 5 hours at 200 000 x g. Reductive alkylation of myosin mRNPs from 12 day embryonic chick muscle (i.e. myoblasts) prior to metrizamide gradient centrifugation, was carried out as previously described (10), using [^{14}C] formaldehyde (New England

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Nuclear, 44 Ci/mol). The labelled sample was dialyzed overnight against 0.1M KCℓ, 0.003M $MgCℓ_2$, 0.01M β Mercaptoethanol, 0.01M Tris-HCℓ (pH 7.4) and subsequently the preparation was added to 5 mℓ metrizamide [2 - (3-acetamido-5-N-methylacet= amido-2,4,6-triodobenzamido)-2-deoxy-D-glucose] (Nyegaard & Co., Norway) gradients (40 %; W/V) in the above buffer, mixed thoroughly and centrifuged at 150 000 x g for 54 hours at 4 °C using a Beckman SW65L rotor. The gradient was analyzed for radioactivity and refractive index (Fig 1).

The protein factors were subsequently extracted from unlabelled mRNPs, (bands are visible), followed by centrifugation on sucrose density gradients (1). The fractions were collected, concentrated by vacuum dialysis, using Schleicher and Schull ultra-thimbles, and chromatographed on DEAE-cellulose (1). The protein fraction eluted at 0.28M KCℓ was concentrated and dialysed against 0.1M KCℓ, 0.003M Mg $Cℓ_2$, 0.006M β Mercaptoethanol, 5 % (v/v) glycerol, 0.02M Tris-HCℓ (pH 7.4) overnight.

The wheat germ (gift from D. Yaffe, Weizmann Institute, Israel) extract was prepared as described (1). Conditions for cell-free protein synthesis were as described (1), except that 0.25 nMoles each of 20 amino acids containing 5μCi[^{3}H] -labelled amino acid mixture (New England Nuclear) was added. Unless otherwise indicated, 5 μg of the mRNP protein and 10 μg mRNA were added to the reaction mixtures. The mRNP protein fraction and mRNA were pre-mixed prior to addition to the incubation mixture. Incubation was terminated after 1 hour at 25 °C, 50 μg carrier myosin was added and the myosin purified as previously described (11). Myosin synthesis was analyzed by polyacrylamide gel electrophoresis (8) and the incorporated radioactivity was determined by liquid scintillation counting after the acrylamide gels were sliced (11).

Myosin mRNA was prepared from myosin synthesizing polysomes (12) and mRNPs essentially as previously described (13), followed by centrifugation of the 26S myosin mRNA on 10 - 30 % sucrose density gradients (2). Gel electrophoresis of RNA samples on sodium dodecylsulphate-polyacrylamide gels was performed as pre= viously described (8).

RESULTS

If the proteins associated with the stored cytoplasmic mRNAs are to have a translational role in the cell, they must be shown to affect the translation or activation of mRNAs isolated from the same mRNP particle, free of ribosomes. For this reason we have isolated myosin mRNPs from 12 day embryonic chick muscle; thus, ribonucleoprotein particles from muscle tissue before extensive myoblast fusion. Subsequent fractionation of the isolated ribonucleoprotein particles was achieved by using metrizamide buoyant density centrifugation (Fig 1). Metrizamide, a tri-iodinated benzamido derivative of glucose has no ionizable groups, therefore eli= minating fixation of protein-nucleic acid complexes prior to isopycnic banding. This property is important, since the protein factors can be extracted with buffers containing 0.5M KCℓ. However, due to the high molar absorption of metrizamide in the ultraviolet region below 300 nm due to the iodinated aromatic nucleus (14), the ribonucleoprotein particles were labelled with [^{14}C] formaldehyde prior to metri= zamide density gradient centrifugation (Fig 1). The mRNP particles, isolated from tissue prior to cell fusion, banded at two distinct refractive indexes of 1.42 and 1.38. Labelled native monosomes (80S), included in a separate gradient as a marker, banded at a refractive index of 1.42. In an attempt to define the RNA species

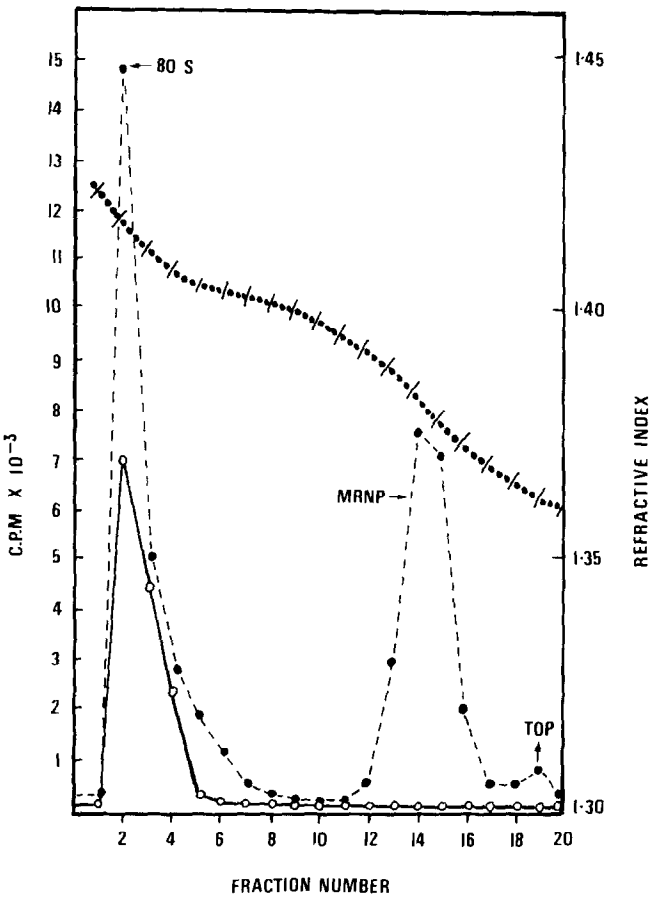

Figure 1 Myosin mRNPs and 80S ribosomal particles were labelled with [^{14}C]-Formaldehyde and analyzed on metrizamide gradients as described in the Methods section. Fractions (19 drops per fraction) were collected from the bottom of the tubes. Radio= activity was determined by mixing 10 mℓ of Packard Insta Gel with 200 µℓ aliquots of each fraction, followed by counting in a Packard Tricarb Scintillation counter. The remainder of each fraction was used to determine the refractive index by means of an Abbe Refracto= meter. Myosin mRNPs from day 12 (●——●) and 80S ribosomal particles (o——o). Refractive index (/ .../).

present in the mRNPs banded at a refractive index of 1.38, RNA was extracted as described and electrophoresed on 2.6 % acrylamide gels (Fig 2). Only 26S RNA was detectable.

To investigate whether the proteins associated with the purified myosin mRNPs contain any factor activity, these protein factors were extracted as described, followed by sucrose density gradient centrifugation and DEAE-cellulose chromato= graphy (Fig 3). The ability of the protein fraction, eluted between 0.15M KCℓ and 0.28M KCℓ, to stimulate myosin synthesis in a wheat germ cell-free system was tested

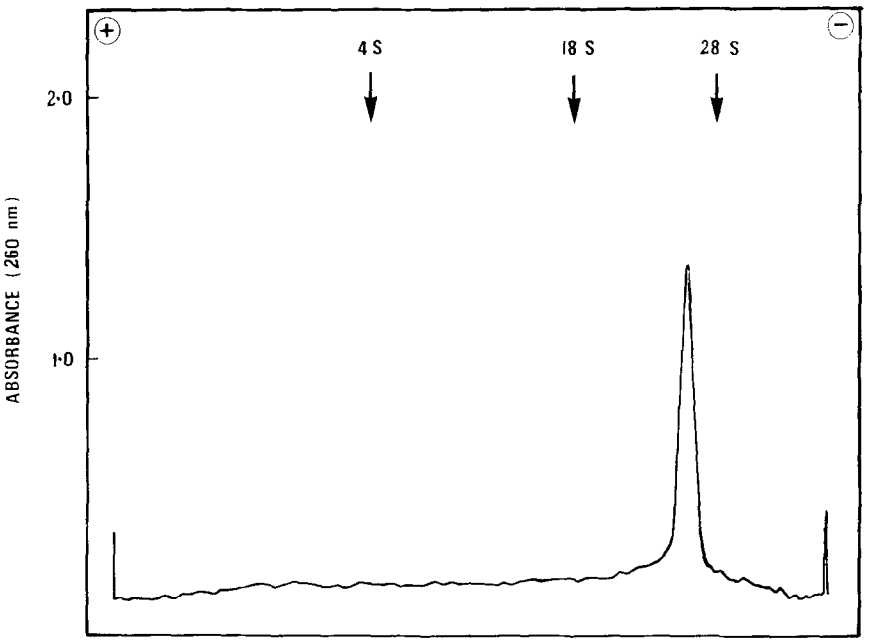

Figure 2 Electrophoretic analysis of 26S mRNA, isolated from myosin mRNPs (see Fig 1), on 2.6 % sodium dodecylsulphatepolyacrylamide gels. [^{3}H]-labelled 4S, 18S and 28S RNA were included as internal markers. Unstained gels were scanned at 260 nm in Quartz tubes, using a Unicam Spectrophotometer. The gels were subsequently sliced and radioactivity determined as described in the Methods section.

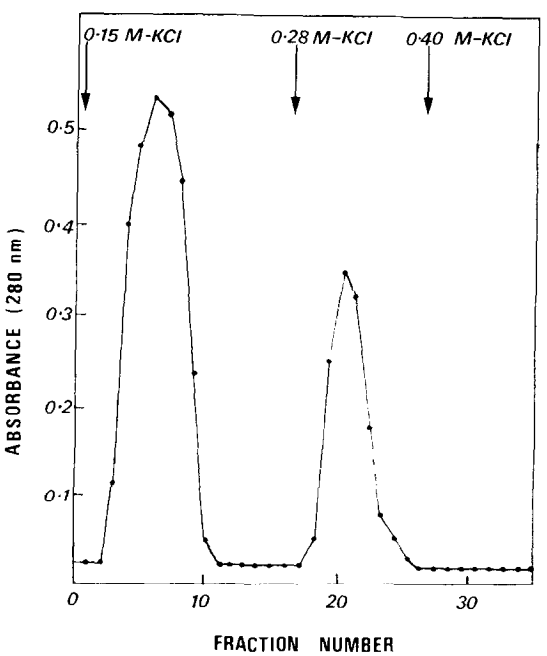

Figure 3 DEAE-cellulose chromatography of proteins obtained from high salt wash of myosin mRNPs.

TABLE I The effect of protein factors from myosin mRNPs on myosin synthesis; myosin mRNA was isolated from myosin mRNPs (mRNP-mRNA) and from myosin synthesizing polysomes (MSP-mRNA) and cell-free protein synthesis performed as described in methods section

Experiment	myosin mRNP protein factors	CPM per myosin heavy chain
Control	-	120
mRNP-mRNA	-	1 580
mRNP-mRNA	+	4 100
mRNP-mRNA*	+*	4 950
MSP-mRNA	-	1 530
MSP-mRNA	+	3 830
MSP-mRNA*	+*	4 200

*10 µg of protein factors was added to the incubation mixture

(Table 1). A significant stimulation of myosin heavy chain synthesis is observed when the isolated protein fraction is added to incubation mixtures containing the indicated myosin mRNA populations. It was previously reported that the mRNA, isolated from myosin synthesizing polysomes (MSP) and from myosin mRNPs are identical as judged by hybridization experiments (1, 15). On increasing the amount of added mRNP-protein factors from 5 µg to 10 µg, a further stimulation is observed. On the other hand, when adding this isolated protein fraction to a wheat germ cell-free system containing TMV-RNA (Tobacco Mosaic Virus RNA) no stimulatory effect is noticeable (Table II).

To investigate whether this protein factor is associated with the 40S-mRNA complex, as well as with the myosin mRNA during translation, the protein fraction was labelled with [^{14}C] formaldehyde and added to a wheat germ cell-free system, containing myosin mRNA. TMV RNA was included in a separate system as a control. After 15 minutes at 25 °C, the incubations were terminated by the addition of cycloheximide (50 µg/mℓ) and analyzed on 10 to 30 % linear sucrose density gradients (Fig 4). It is apparent from figure 4 that radioactivities were associated with the heavy poly-

TABLE II The effect of protein factors from myosin mRNPs on the translation of TMV-RNA and myosin mRNA derived from mRNP particles in a wheat germ cell-free system

Experiment	Myosin mRNP protein factors	*C.P.M.	CPM per myosin heavy chain
Control	-	2 750	180
TMV-RNA	-	22 950	210
TMV-RNA	+	21 680	230
mRNP-mRNA	-	32 430	2 010
mRNP-mRNA	+	38 250	4 700

*Hot Trichloroacetic acid precipital counts per minute

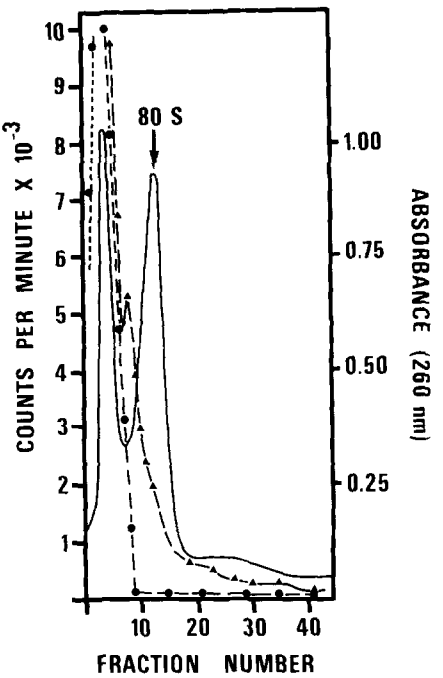

Figure 4 Sucrose density gradient analysis of wheat germ cell-free incubations. The reaction mixtures contain myosin mRNA (▲——▲) and TMV RNA (●——●) respectively. Reaction conditions were as described, except that the labelled amino acids were replaced by a complete mixture of unlabelled amino acids (0.25 nMoles of each amino acid) and that a [^{14}C]-Formal= dehyde labelled protein fraction were used. After 15 min of incuba= tion at 25 °C, the reactions were terminated by the addition of cycloheximide (50 µg/mℓ) and layered onto 10 - 30 % sucrose gradients. Centrifugation was carried out for 1 hour at 40 000 r.p.m. at 4 °C in an I.E.C. 283 rotor. The gradients were analyzed by continuous monitoring and the radioactivity determined as described in figure 1.

somes, as well as with the 80S monosomes and 40S ribosomal subunits when myosin mRNA was used as a template for protein synthesis. However, no detectable radioactivity was demonstrable in a similar gradient when TMV RNA was included in the wheat germ cell-free system.

DISCUSSION

Although it is currently believed that mRNP particles exist in the cytoplasm of a variety of eukaryotic cells, free of ribosomes, the specificity and function of the associated proteins are still undefined.

Heywood *et al.* (16) have suggested the transfer of proteins from messenger ribo= nucleoprotein particles to eIF-3 during the first initiation of an activated stored mRNP. A prerequisite for such a study is the isolation of an mRNP particle containing only one messenger RNA, because the structural complexity of the initiation sites of natural mRNAs may result in different affinities for these proteins. Further, the isolation of such an mRNP particle should be done under non-ionic or low-salt condi=

tions, without prior fixation of the mRNA-protein complex. This is important, since all the initiation factors can be extracted with buffers containing 0.5M KCℓ.

We have demonstrated here the separation of myosin mRNP particles, free from ribosomal subunits, using metrizamide (non-ionic) buoyant density centrifugation. Only myosin mRNA is detectable in this particle, when total RNA is extracted and electrophoresed on sodium dodecylsulphate-polyacrylamide gels.

When adding the protein fraction, eluting from a DEAE-cellulose column between 0.15M KCℓ and 0.28M KCℓ, to an unfractionated wheat germ cell-free system containing myosin mRNA, a significant stimulation in myosin heavy chain synthesis is observed. The stimulatory effect of this protein fraction on myosin synthesis is demonstrable irrespective of the population of myosin mRNA used, i.e. isolated from myosin synthesizing polysomes or myosin mRNPs. However, when adding this fraction to a wheat germ cell-free system in the presence of TMV RNA no stimulatory effect is noticeable. It therefore seems evident that this fraction contains factors that not only stimulate the translation of mRNA, but in addition are discriminatory. Further evidence for the discriminatory role of this protein fraction is also presented in figure 4. These results may also indicate that the protein fraction isolated from myosin mRNPs, not only binds the mRNA during the initiation of protein synthesis, but remains associated with the myosin mRNA during translation.

It was earlier suggested that eIF-3, a large complex factor made up of multiple subunits, could function in a non-discriminatory manner and subsequently become discriminatory upon the addition of modular proteins; thus becoming a mixture of discriminatory and non-discriminatory eIF-3 molecules (16). Trachsel *et al.* (17) suggested that the eIF-3 complex may have some interchangeable or modifiable subunits which could be responsible for specific messenger selection and binding. Several examples of this type of differential mRNA recognition have been reported (18 - 20). The complexity and heterogeneity of eIF-3 is demonstrated by sodium dodecylsulphate-polyacrylamide gel electrophoresis as well as by sedimentation velocity studies (1, 21). From these studies it does not appear that eukaryotic eIF-3 have identical compositions. However, a "core" eIF-3 is likely to be identical in all eukaryotic cell types (16). It is tempting to speculate that this protein fraction, isolated from stored myosin mRNA-protein particles and showing a stimulatory activity for myosin synthesis, is transferred during the first initiation of the stored myosin messenger to the "core" eIF-3, thereby modulating the specificity of muscle eIF-3 for specific myosin messenger selection. In the light of the specific binding of proteins from the initiation factor complex and of proteins associated with the 40S ribosomal subunits to the methylated 5' end (cap-containing sequences) of various mRNAs we speculate that this discriminatory factor may be associated with the cap-sequence of myosin mRNA and may recycle with myosin mRNAs during translation.

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