STORED MYOSIN MESSENGER IN EMBRYONIC CHICK MUSCLE

S. M. HEYWOOD, D. S. KENNEDY and A. J. BESTER*

Genetics and Cell Biology Section, U-125 The University of Connecticut, Storrs, Connecticut 06268 USA

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1. Introduction

Prior to the formation of multinucleated myotubes, differentiating skeletal muscle goes through a phase of rapid cell proliferation [1–3]. These proliferating myoblasts synthesize little, if any myosin. During embryogenesis, as the number of proliferating cells in chick leg muscle decreases, both the number of multinucleated cells and the amount of myosin per DNA increases [4]. At fourteen days myosin polysomes are easily detected by analysis on sucrose density gradients [5]. Myosin messenger has been isolated and characterized from these polysomes [6–8]. At earlier stages of embryogenesis, with myoblasts in the proliferative stage predominating, the amount of myosin mRNA found in polysomes is considerably less [5,9].

In cell culture it is well established that the onset of myosin synthesis noticeably occurs after myoblast fusion and myotube formation [1,10,11]. However, a number of studies have suggested that myosin messenger transcription may actually precede cell fusion. If myoblast cell cultures are treated with actinomycin D shortly before the onset of fusion the synthesis of myosin is not inhibited [10]. Buckingham et al. [9] have observed the accumulation of 26S myosin mRNA in dividing myoblast cell cultures which sediments as a 70–90S mRNP upon sucrose density gradient centrifugation. After cell fusion this RNA species appears in the polysomes and its half-life is considerably increased. Chacko and Joseph [12] have recently reported that precardiac mesodermal cells from stage 7 were still capable of expressing the differentiated phenotype of cardiac cells in culture when treated with 5-bromodeoxyuridine. From these studies, it would appear that in the determined myoblast the transition to be differentiated state of the myotube or carciad cell is, at least in part, a result of the activation of pre-transcribed and stored messenger RNA.

The studies reported here suggest that the twelve day chick embryonic leg muscle (at a time preceding extensive fusion) contains a considerable amount of myosin messenger stored in a 70–90S ribonuclear-protein particle (myosin mRNP).

2. Experimental

Myosin mRNPs were prepared from 40–45 g of 12 day embryonic chick muscle. The tissue was dissected and homogenized with a loose fitting Dounce type homogenizer in 0.25 M KCl, 0.005 M $MgCl_2$, 0.02 M Tris–HCl (pH 7.4). The 10 000 *g* supernatant was subsequently layered on six 27 ml, 15–30% sucrose density gradients (same buffer) and centrifuged at 25 000 rev/min for 1.5 hr in an I.E.C. SB 110 rotor. A typical ribosomal–polysomal profile is shown in fig.1. The area shown by the horizontal arrow (fig.1) under the 80S ribosomal peak was collected from the six gradients. The collected ribosomes containing the myosin mRNP were pelleted by centrifugation for four and one half hours at 200 000 *g*. The pellets were then resuspended in 2 ml of 0.05 M K^+ phosphate buffer (pH 6.8) containing 5 mM EDTA and dialyzed against 15 vol of the same buffer for 4 hr at 2°C to remove myosin translational control RNA (tcRNA) (to be published elsewhere).

*Permanent Address: Tygerberg Hospital, Cape Town, South Africa.

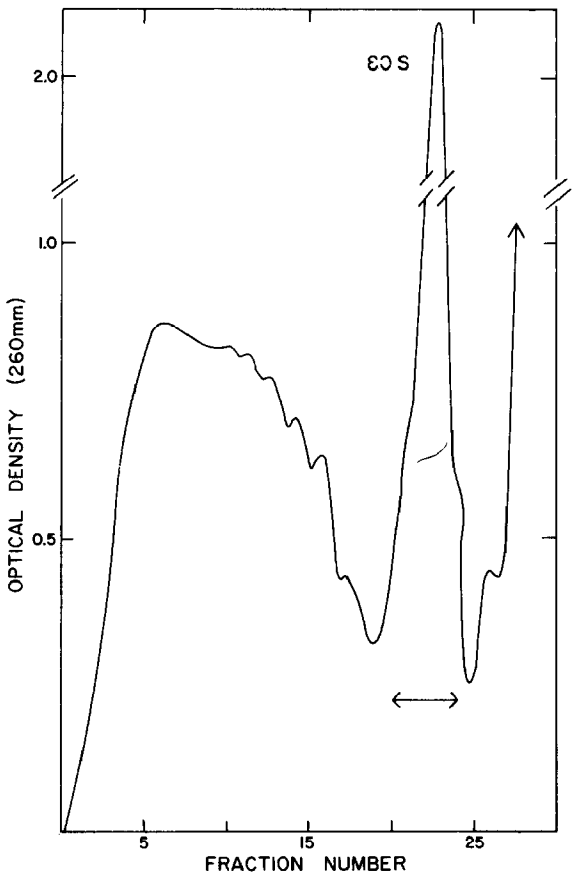

Fig.1. Sucrose density gradient analysis of 12 day embryonic chick leg muscle. The gradient was analyzed on a Gilford spectrophotometer by continuous monitoring. Fractions were collected as indicated by the horizontal arrow from six similar gradients and pooled. Centrifugation was performed as described in Experimental section.

After dialysis the ribosome–mRNP fraction was phenol extracted by the method of Aviv and Leder [13] and then precipitated in two vol of ethanol. Poly(A) containing mRNA was isolated from the total RNA by its ability to bind to oligo-(dT)-cellulose [13].

The preparation of rabbit reticulocyte lysate and the conditions for cell-free protein synthesis were as previously described [14]. The reaction mixtures (70 μl) contained 5 μg myosin mRNA (isolated from 70–90S mRNP) and 15 μg muscle initation factor 3 (IF3). After incubating for 45 min at 30°C, 250 μg carrier myosin was added to each reaction mixture. Myosin was subsequently purified by low ionic strength precipitation followed by DEAE-cellulose chromatography as previously described [7]. The amount of myosin synthesis was determined by measuring the radioactivity co-electrophoresing with the purified myosin on 7% SDS-acrylamide gels [4]. The electrophoretograms were as previously described using this procedure [7].

3. Results

3.1. *Sucrose gradient analysis of 70–90S mRNP mRNA*

After oligo-(dT)-cellulose chromatography of the phenol extracted RNA, the RNA fractions eluting from the column at 0.5 M KCl and at low salt (0.01 M Tris–HCl pH 7.5) were precipitated in two vol of ethanol at −20°C. These RNA fractions were analyzed by sucrose density gradient centrifugation as shown in fig.2. As can be seen in fig.2A, the RNA washed from the column at the high salt concentration is essentially all ribosomal RNA which sediments at 28S and 18S. The poly(A) containing RNA, eluting from the column at low salt, has a major peak of optical density desimenting at 26S (fig.2B). This is the characteristic sedimentation value of myosin mRNA [6–8]. Normally, using 40–45 g of tissue one can obtain 15–20 μg of 26S mRNA from these mRNPs. This is considerably more than is obtainable from myosin synthesizing polysomes which requires the use of 18–24 gradients for a similar amount. It is not known at the present time if myosin is the only mRNA sedimenting at 26S that is obtained from these 70–90S mRNPs. Preliminary results from the analysis of SDS-acrylamide gels containing the total protein synthesized in the reticulocyte lysate after the addition of the 26S mRNA suggests there may be a slight contamination by other mRNAs (unpublished). However, a small degree of premature termination of myosin synthesis would yield similar results and therefore cannot be ruled out.

3.2. *Cell-free synthesis of myosin from the 26S mRNA isolated from 70–90S mRNPs*

In order to demonstrate that the 26S RNA obtained from the mRNPs is indeed myosin mRNA it was tested for its ability to direct the synthesis of

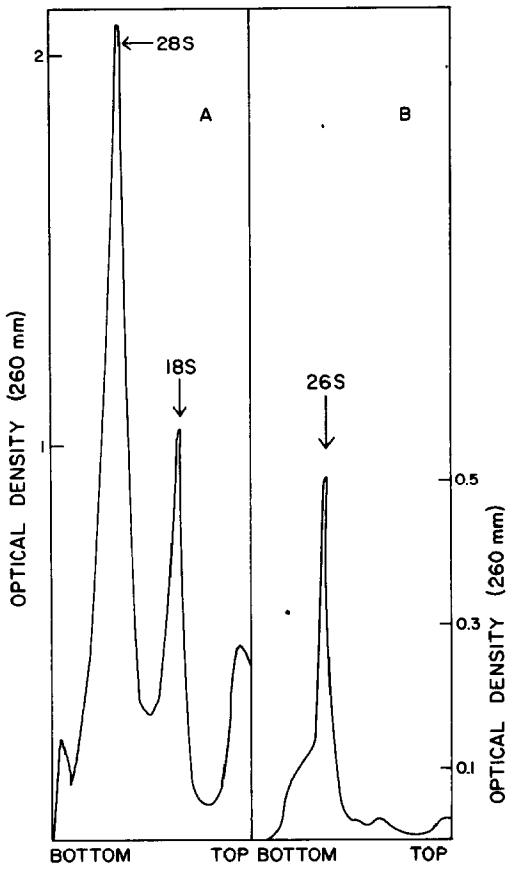

Fig.2. Sucrose density gradient analysis of the RNA obtained from the 70–90S RNP fraction (see fig.1). The 10–30% sucrose gradients in 0.05 M Tris–HCl (pH 7.4), 0.005 M EDTA, 0.5% sodium dodecylsulfate were centrifuged for 10 hours at 40 000 rev/min at 5°C in an I.E.C. 283 rotor. Each 10.5 ml gradient was layered with the RNA sample in the same buffer as in the sucrose gradient. A) One third of the RNA not binding to the oligo-(dT)-cellulose; B) the poly(A) containing RNA which binds to the oligo-(dT)-cellulose. The gradients were analyzed as indicated in fig.1.

myosin in a heterologous cell-free system. As can be seen in table 1, the addition of the 26S mRNA to the reticulocyte lysate cell-free system results in a synthesis of a protein judged to be myosin by its ability to go through the two steps of purification with carrier myosin and its electrophoretic behavior on SDS-acrylamide gels. Final proof that the 26S mRNA isolated from the myosin mRNP is identical

Table 1
The cell-free synthesis of myosin directed by 26S mRNA obtained from 70–90S mRNPs

Experiment	Myosin*	cpm Total
Control	50	105 760
Plus 26S mRNA	3140	93 185

*Determined from radioactivity co-electrophoresing with myosin after purification of the synthesized myosin by ionic precipitation and DEAE-cellulose chromatography. See [4] for typical electrophoretogram.

to 26S mRNA found on myosin synthesizing polysomes [6–8] will require competitive hybridization to copy DNA. These studies are in progress.

4. Discussion

As mentioned previously, several reports [9,10,12] have suggested that the transcription of myosin mRNA may precede its translation by a considerable time lag in developing muscle. In particular, myosin mRNA may be transcribed in the predetermined myoblast and subsequently be stored in the cytoplasm until myotube formation. The studies reported here lend support to this hypothesis. Results to be reported elsewhere suggest that a small RNA molecule (translational control RNA) obtained from the myosin mRNP will specifically inhibit the utilization of myosin mRNA, and, therefore, may be involved with the inactivation and storage of myosin mRNA during myoblast proliferation. Further studies demonstrating the activation of 70–90S myosin mRNPs and the subsequent incorporation of the 26S mRNA into polysomes during the terminal differentiation of muscle will be required to establish this point.

Acknowledgements

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