PREPARATION AND CHARACTERIZATION OF MYOSIN COPY DNA

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Received December 2,1975

Summary: The AMV reverse transcriptase has been used to make a copy DNA utilizing purified myosin mRNA isolated from polysomes derived from 14 day embryonic chick leg muscle. The cDNA efficiently hybridizes to its template, but not to heterologous RNA's. When the probe is hybridized to mRNA isolated from presumptive myosin messenger ribonucleoprotein particles (mRNPs), the observed kinetics indicate that at least 80% of this mRNA contains identical or similar sequences found in the mRNA isolated from polysomes.

Introduction: Several eukaryotic messenger RNA's have been shown to act as efficient templates for the AMV reverse transcriptase (1,2). The resultant cDNAs have been successfully used as probes for detecting both the presence and amounts of the specific mRNAs in various tissues and cell types during development (3,4).

Myosin mRNA has previously been isolated in small quantities from the polysomes of 14 day embryonic chick leg muscle. This poly A containing mRNA sediments at 26S on sucrose gradients and 32S on polyacrylamide gels (5,6) Tryptic digests of the products synthesized in a cell-free system demonstrate that this mRNA directs the synthesis of the myosin heavy chain (5).

Recently, myosin mRNA has been isolated from a 70-90S mRNP found in the polysomes derived from 12 day embryonic chick leg muscle (8). At this stage in development, the large myosin synthesizing polysomes seen in 14 day leg muscle are completely lacking. The mRNA isolated from these mRNPs is found to contain poly A and shows a sharp peak at 26S on SDS-sucrose gradients. When the mRNA was tested for its ability to direct the synthesis of myosin in the reticulocyte lysate cell-free system, the only protein product to be made was myosin, based on its ability to go through a two step purification with carrier myosin and its subsequent co-migration with the carrier myosin on SDS acrylamide gels.

It was of interest to produce and characterize a cDNA probe to polysomal myosin mRNA in order to elucidate, by the technique of RNA-DNA hybridization, the exact relationship of the mRNP-mRNA with respect to the myosin mRNA derived from the 14 day polysomes. This report presents the methods used for synthesis and characterization of the myosin cDNA. In addition, the kinetics demonstrate that when the probe is hybridized to the mRNP-mRNA, the bulk of this mRNA is, indeed, identical, or very similar to the sequences found in the myosin mRNA isolated from the polysomes.

Materials and Methods:

Isolation of myosin polysomal mRNA and myosin mRNP-mRNA. The myosin synthesizing polysomes were isolated from 14 day embryonic chick leg muscle and the mRNPs from 12 day embryonic muscle as previously described (7,8). The polysome or mRNP pellet was resuspended in SDS buffer [0.05 M Tris (pH 7.4), 0.002 M EDTA, 0.5% SDS] and the sample layered onto a 27 ml 10-30% sucrose gradient generated in the same buffer. The gradient was centrifuged for 20 hours at 24,000 rpm in an IEC-SB110 rotor at 4°. The light shoulder of the 28S ribosomal peak was collected, and the RNA precipitated in 0.5 M Tris (pH 7.2), and 70% ethanol at -20°. The RNA precipitate was collected by centrifugation and taken up in a small volume of 0.5 M KCl, 0.01 M Tris (pH 7.6), and bound to a 0.5 gm oligo dT column (Collaborative Research). The mRNA was eluted with 0.01 M Tris at the same pH. This procedure was repeated, and the eluted mRNA was made 0.1 M in NaAc (pH 5.4) and 2.5 volumes of ethanol was added. The precipitated RNA was taken up in SDS buffer, layered onto an 11 ml 10-30% sucrose gradient generated in the same buffer. After centrifugation for 14 hours at 36,000 rpm at 4° in an IEC SB283 rotor, the absorbance was continually monitored by a Gilford spectrophotometer and the fraction indicated was collected and precipitated with ethanol. Ribosomal markers were run on a parallel gradient. Under these conditions myosin mRNA sediments at 26S.

Synthesis of the cDNA. The reactions were normally carried out in a volume of 50 µl containing: 2.5 µmoles Tris (pH 7.9), 0.5 µmoles $MgCl_2$, 7 µmoles KCl, 0.3 µmoles DTT, 0.02 µmoles of TTP, dCTP, dATP and 2.5 µg Actinomycin D. [^{3}H]-GTP was added in amounts indicated in the figure legends. The mRNA was pre-incubated with equimolar amounts of oligo-dT_{12-18} in 0.1 M NaCl at 37° for 15 minutes, and then added to the reaction in the amounts indicated in the figure legends. The reaction mixtures were started by the addition of 0.12 units of the AMV reverse transcriptase, purified according to Kacien et al (9), and incubated for 90 minutes at 40°.

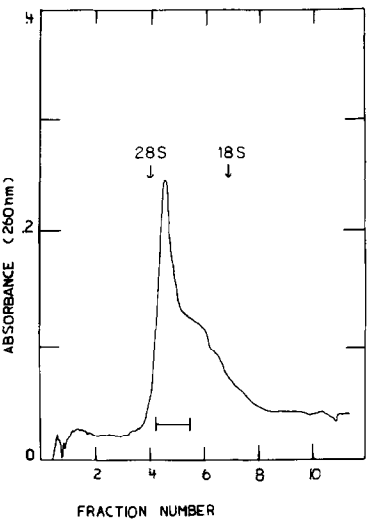

Figure 1. Isolation of Myosin Polysomal mRNA.

Determination of the Transcript's size

The reactions were carried out as indicated above and terminated by the addition of 1/10 volume of 3 M KOH. Alkaline hydrolysis was carried out at 37° for 18 hours, the samples neutralized and placed on a 1 x 58 cm G-100 Sephadex column. The sample was eluted with double distilled water, and the radioactive peak detected in the void volume was collected and precipitated in 0.1 M NaAc (pH 5.4) and 70% ethanol. The cDNA precipitate was collected by centrifugation, taken up in 0.5 ml of 0.9 M NaCl, 0.001 M EDTA, and 0.1 N NaOH and layered onto an 11 ml, 5-20% sucrose gradient generated in the same buffer. The gradients were centrifuged in an IEC SB283 rotor at 35,000 rpm for 14 hours at 4°. 0.3 ml fractions were collected, neutralized and the radioactivity determined in Aquasol (New England Nuclear).

Hybridization Reactions

The transcriptase reaction was carried out as indicated above. After alkaline hydrolysis, the cDNA was phenol extracted (10) before being passed through the Sephadex column. The precipitate collected from the void volume was brought up in double distilled water. Hybridizations were carried out with approximately 1000 cpms of cDNA in 5 µl containing: 50% deionized formamide, 0.4 M NaCl, 0.05 M HEPES (pH 6.8), 0.001 EDTA, 0.1% SDS and RNA as indicated in the figure legends. The samples were placed in glass capillaries, sealed, and placed in a 90° water bath for 5 minutes. Hybridizations were carried out at 52° to the indicated $R_o t$ values, and halted by rinsing the capillaries into 1.0 ml of 0.03 M NaAc (pH 4.6), 0.3 M NaCl, 0.0015 M $ZnSO_4$, 5% glycerine and 20 µg of sonicated, heat

Table 1. Parameters of the Transcriptase Reaction

Reaction	Picomoles dGMP incorporated	
	Myosin polysomal mRNA	Myosin mRNP mRNA
Complete	21.3	10.5
-oligo dT	6.9	4.8
-RNA	1.5	-
-TTP	2.5	2.5
+RNase*	1.4	1.2

The reactions were carried out as described in Materials and Methods. 0.187 µg of polysomal, or 0.5 µg of mRNP mRNA was added. 0.0065 µmoles of [^{3}H] dGTP (sp. act. 1.3 Ci/mM) was present. The reactions were carried out at 40° for 90 minutes, and terminated by the addition of 2 ml of 10% TCA. 100 µg of carrier yeast RNA was added, and the tubes allowed to sit on ice for 5 minutes. Acid precipitable radioactivity was collected on millipore filters, dried and the radioactivity determined in a toluene base fluor.

*Heat treated pancreatic RNase was added at a concentration of 50 µg per ml during the 15 minutes 37° incubation period.

denatured calf-thymus DNA. Four units of S1 nuclease were added (11), and the sample incubated at 45° for 45 minutes. 2.0 ml of 10% TCA was added.and the sample was passed through a millipore filter. Acid-precipitable radioactivity was determined in a Triton, toluene based fluor. The amount of cDNA hybridized in each sample was expressed as a percentage of the acid precipitable radioactivity determined from a sample that had been incubated without the nuclease.

Results: The absorbance profile of the polysomal myosin mRNA is shown in Figure 1 and represents material pooled from approximately 280 dozen 14 day embryonic chick legs. In contrast to the mRNA isolated from the mRNP's, the polysomal myosin mRNA does not show a sharp 26S peak. Although only the material indicated in Figure 1 is collected, it is possible that this material is slightly contaminated with sequences other than those found in the myosin mRNA.

The basic parameters of the transcriptase reaction are shown in Table 1. Of the two templates used, it is apparent that the polysomal myosin mRNA is the more efficient template. Although the reason for this difference is not clear,

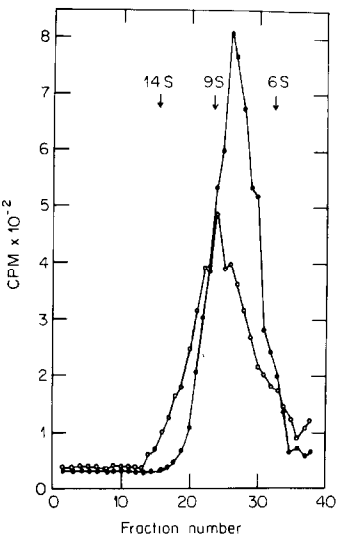

Figure 2. Size of the cDNA Transcripts. 0.3 µg of polysomal, or 0.5 µg of mRNP mRNA was added to each reaction mixture. 0.01 µmoles of [^{3}H] dGTP (sp. act. 4 Ci/mM) was present. The S values were determined by the method of Dingman (15). (O—O) cDNA prepared from the polysomal-mRNA template. (●—●) cDNA prepared from the mRNP-mRNA template.

it is possible that the mRNP-mRNA may be contaminated with tcRNA (12). This could be expected to inhibit the reverse transcriptase. The reaction with both templates is not completely dependent upon addition of the oligo-dT primer, although a marked stimulation is seen upon its addition.

Figure 2 indicates that under the conditions of relatively high substrates concentrations, the polysomal myosin mRNA generates large fragments that can be resolved into three discrete size classes, the S values of which are given in Table 2. The largest fragments made in appreciable amounts sediment at approximately 14 S, indicating a length of about 3,500 nucleotides. These transcripts probably contain between 50-60% of the nucleotide sequence of the myosin mRNA.

The hybridization of the cDNA to its template, polysomal myosin mRNA is shown in Figure 3. The reaction is characterized by a R_0t 1/2 of 5.3. The specificity of the probe to myosin mRNA is shown by its inability to hybridize to ribosomal RNA, even at very high R_0t's.

Table 2. Size of cDNA Transcripts

Template	S_{w20}	Length (in nucleotides)
myosin mRNA (polysomal)	9.46	1,432
	8.47	1,086
	7.80	884
myosin mRNA (mRNP)	7.36	764
	6.50	560

S_{w20} values were obtained from the alkaline gradients (see Fig. 2). The number of nucleotides was determined by the method of Studier (16).

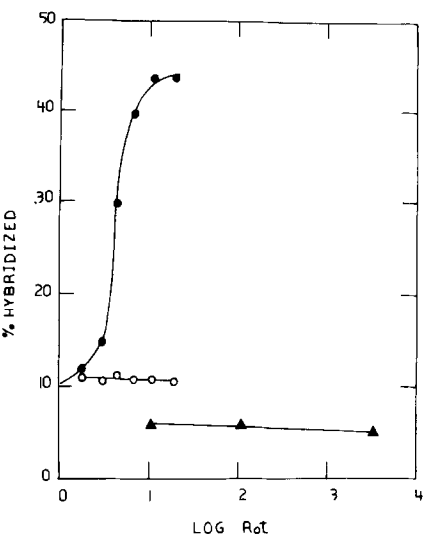

Figure 3. Hybridization Reactions. The hybridization reactions were carried out as described in Materials and Methods. S-1 resistant background levels were determined from reactions incubated without added RNA. R_0t = initial concentration of RNA (in moles liters^{-1}) x seconds. (●—●) template myosin polysomal, mRNA. (▲—▲) E. coli ribosomal RNA. (○—○) no RNA.

RNA-DNA hybridizations, when carried out in large RNA excess, exhibit pseudo-first order kinetics, and are conveniently graphed using the double reciprocal transformation (13). From data treated in this manner, the theoretical saturation point at $R_0t = \infty$, and the R_0t 1/2 of the reaction can be accurately obtained. Figure 4 shows the hybridization data treated in this manner. The cDNA prepared

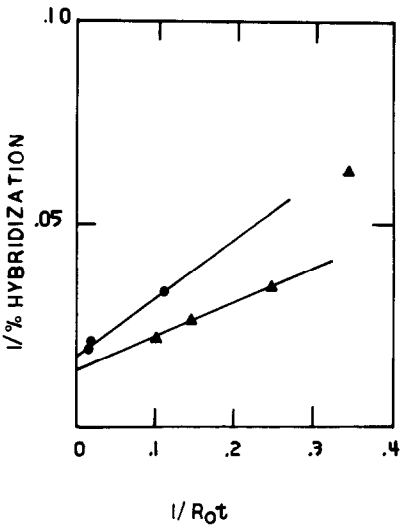

Figure 4. Reciprocal Plots of the Kinetics of Hybridization of cDNA Prepared from the Polysomal and mRNP Myosin mRNA's. The Y intercept occurs at infinite R_0t and indicates the theoretical saturation level (13). (▲—▲) polysomal myosin mRNA (●—●) mRNP myosin mRNA.

from the polysomal myosin mRNA template is back hybridized both to the template and the 26S mRNP-mRNA. The saturation point for the template is 80% and the R_0t 1/2 of 5.3 is obtained. The mRNP-mRNA shows a saturation value of 71% and this reaction is characterized by a R_0t 1/2 of 6.6. An estimation of the percentage of polysomal myosin mRNA by weight in the mRNA obtained from the mRNP was obtained using the relationship of Imaizumi et al (14);

$$\frac{R_0t \text{ 1/2 mRNA}}{R_0t \text{ 1/2 sample}} = \frac{\% \text{ sequences in sample}}{\% \text{ sequences in mRNA}}$$

Approximately 80% of the sequences in the mRNP-mRNA are similar or identical to those sequences in the myosin obtained from the polysomes.

Discussion: The myosin mRNA derived from the polysomes of 14 day embryonic chick leg muscle has been shown to code for the myosin heavy chain using the criteria of co-purification, co-migration with carrier myosin, and a peptide map of the tryptic digest of the products synthesized in a heterologous cell-free system (5). The mRNA obtained from the presumptive myosin mRNP fraction of 12 day embryonic

chick leg muscle has previously been shown to have similar characteristics to the myosin mRNA obtained from the polysomes; it contains poly A, upon centrifugation sediments at 26S and directs the synthesis of the heavy chain of myosin as determined by co-purification and SDS-acrylamide gel electrophoresis.

This report demonstrates that the bulk of the sequence(s) found in the mRNP-mRNA is indeed, myosin mRNA. Furthermore, the existence of an mRNP containing myosin is confirmed. It would appear that there is a functional compartmentalization in the developing chick; the myosin mRNA sequences found in the active polysomes of the 14 day chick can also be isolated from the translationally inactive mRNP's found in the 12 day embryo.

The use of a cDNA probe should prove useful in continuing developmental studies in the chick muscle system. The first appearance of the myosin mRNA can now be monitored in vivo and in culture conditions and the amount of myosin message can now be correlated with the amount of myosin in the cell. Such studies have broad implications relating to the current concepts of post-transcriptional and translational controls. These studies are now in progress.

Acknowledgements: The AMV was a generous gift received under the auspices of the Virus Cancer Program. This research was supported by a research grant from the NIH (PHS Grant No. HD 03316) and the eggs were paid for by a grant from the National Cancer Institute (CA 14733).

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