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Construction of bacterial plasmids containing sequences complementary to chicken α-tropomyosin mRNA

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ABSTRACT

Recombinant plasmids have been constructed which contain sequences complementary to the mRNA coding for skeletal muscle α-tropomyosin. These recombinants were detected initially using a selective cDNA probe and subsequently using a messenger RNA selection assay. α-TM plasmids hybridize to a single mRNA species smaller than 18S ribosomal RNA and found only in skeletal muscle. Cross-hybridization with mRNA's coding for other tropomyosins could not be detected under normal conditions. However, under conditions of reduced stringency α-TM plasmids cross-hybridize with an RNA species in heart muscle which may code for cardiac tropomyosin.

INTRODUCTION

Tropomyosins are a family of related proteins which are widely distributed in nature (1-4). In muscle tropomyosin occurs bound to actin in the thin filament and serves to mediate the effect of Ca^{++} on the actin:myosin interaction. Skeletal muscle contains two types of tropomyosins, α-TM and β-TM, the proportions of which vary with fibre type (5,6). Amino-acid sequence analysis of a mixture of α-TM and β-TM from rabbit skeletal muscle revealed only 14 positions out of 142 in the C-terminal region where sequence differences could be observed (7), suggesting that α-TM and β-TM shared a high degree of sequence homology. However, the functional significance of these distinct tropomyosins in skeletal muscle remains obscure.

Tropomyosins are also found in non-muscle tissues (1-4) such as brain. Since these non-muscle tropomyosins share similar physical and biological characteristics with muscle tropomyosins it is presumed that they also serve a similar physiological function in non-muscle tissue. Peptide mapping studies of tropomyosins from muscle and non-muscle sources demonstrate that these proteins share common sequences although there are

also clear structural differences (4). It has been suggested that the tropomyosins comprise a family of related proteins which have evolved from a common ancestral gene (4).

In order to elucidate the nature of the structural differences between α-TM and β-TM in particular and within the tropomyosin multi-gene family in general it would be desirable to have suitable recombinant DNA probes. In this paper I describe the construction of recombinant bacterial plasmids containing sequences complementary to the mRNA coding for chicken skeletal muscle α-TM.

MATERIALS AND METHODS

Isolation and fractionation of RNA

RNA was isolated from embryonic chicken tissue using the guanidinium chloride extraction procedure followed by ethanol fractionation and phenol extraction as described previously (8,9). Poly A- containing RNA was isolated by chromatography on oligo dT-cellulose (10) and fractionated on a 5-20% sucrose gradient in 100 mM NaCl, 10 mM TRIS-HCl pH 7.5, 1 mM EDTA, 0.1% SDS. The RNA sample was denatured with CH_3HgOH prior to loading as described previously (9). Gradients were centrifuged at 40,000 rpm for 5 hours. 20 fractions were collected from each gradient and the fractions obtained from all six gradients were pooled. An aliquot of each fraction was ethanol-precipitated and mRNA activity assayed using cell-free translation (Fig. 1). Appropriate fractions of the gradient were pooled, adjusted to 35% ethanol and applied to a 1.0 cm x 5.0 cm CF-11 cellulose column (11) equilibrated in 100 mM NH_4 acetate containing 35% ethanol. After exhaustive washing with this buffer RNA was eluted from the column with water and lyophilized.

Cell free translation and protein analysis

Messenger RNA was translated in a micrococcal nuclease-treated reticulocyte lysate cell free system (12) as described previously. Proteins synthesized *in vitro* were analyzed on one-dimensional SDS polyacrylamide gels (13) or on two-dimensional gels using NEPHGE(non-equilibrium pH gradient electrophoresis) in the first demension (14) as described (8). Immunoprecipitation was performed using a rat antiserum against adult chicken leg muscle tropomyosin and protein A-Sepharose as immunoadsorbent as described (8). ^{35}S-labelled proteins were detected after fluorography (15) and exposure to prefogged film at -80°C (16).

Synthesis and cloning of double-stranded cDNA

Double-stranded cDNA was synthesized using the procedure of Wickens et al. (17) except that the incubation temperature for synthesis of the second strand was 25°C. The product was treated with S1 nuclease (18) to cleave hairpins and remove single-stranded regions. Internal Eco R1 sites in the duplex DNA were then methylated in a 50 µl reaction containing 100 mM TRIS-HCl pH 7.8, 5 mM EDTA, 0.01 mM S-adenosyl methionine, 1 µg of double-stranded cDNA and 70 units of Eco R1 methylase (19). The mixture was incubated at 37°C for 30 minutes, phenol-extracted and applied to a Sepharose 4B column equilibrated in 10 mM NH_4 acetate. Fractions containing cDNA molecules longer than 500 base pairs were pooled and lyophilized. Ligation to synthetic Eco R1 linkers (20) was carried out in a 20 µl reaction containing 60 mM TRIS-HCl pH 7.9, 8 mM Mg acetate, 2 mM DTT, 1 mM ATP, 0.2 mM each of d ATP, dGTP, dCTP, and TTP, 2 µg of T4-ligase, 0.5 µg of DNA polymerase, 0.2 µg of methylated duplex cDNA, 0.02 µg of Eco R1 linkers phosphorylated as described previously (21). The reaction was left at room temperature overnight, diluted with one volume of 200 mM NaCl, 100 mM TRIS-HCl pH 7.5, 6 mM Mg acetate and heated at 70°C for 15 minutes. The mixture was cooled on ice and then digested with 350 units of Eco R1 at 37°C for 30 minutes. The reaction was phenol extracted and again chromatographed on Sepharose 4B.

Ligation into phosphatase-treated vector (22) transformation and selection of ampicillin resistant colonies was as described (9,23).

RNA electrophoresis and blotting

Poly A-containing RNA was adjusted to 50% formamide, 1 mM EDTA and heat-denatured at 65°C for 5 minutes. Samples were quick-cooled and applied to a composite gel (24) containing 1% agarose, 2% acrylamide, 0.1% bisacrylamide in 0.1% SDS, TAE buffer (40 mM TRIS-acetate pH 7.4, 20 mM Na acetate, 2 mM EDTA) (25). Electrophoresis was carried out at 10V/cm for 3 hours. RNA was transferred to DBM-paper as described (26).

Preparation of probes

High specific activity ^{32}P-labelled cDNA was synthesized as described (27) using as template RNA from pooled sucrose gradient fractions 11 + 12. The RNA template was destroyed by incubation in alkali, the cDNA solution neutralized, and diluted directly into hybridization buffer; 50% formamide 5 x SSC, 10 x Denhardt solution, 500 µg/ml of carrier DNA and 2.5% dextran sulfate (28). Hybridization was carried out overnight at 42°C, followed by extensive washing in the absence of probe in 0.1 x SSC, 0.1% SDS at

67°C. Hybridization under conditions of reduced stringency was performed in the same buffer at 35°C. Papers were washed in 1 x SSC, 0.1% SDS at room temperature.

The ^{32}P-labelled cDNA probe was made specific for leg muscle RNA sequences by preincubation with a 10-fold excess by weight of heart muscle poly A-containing RNA. The preincubation was carried out in a small volume (100 µl) of 50% formamide 5 x SSC,, 0.1% SDS at 42°C for 16 hours. The mixture was diluted directly into hybridization buffer and used as described above.

Plasmid DNA probes were prepared by nick translation using DNA polymerase (29).

α-TM insert sequences were purified by Eco R1 cleavage of recombinant plasmids followed by preparative electrophoresis and labelled by "filling-in" the cohesive termini using reverse transcriptase (9).

Purification of mRNA by hybridization to filter-bound plasmid DNA

Plasmid DNA was isolated from 20 ml cultures of chloramphenicol-treated cells using the alkaline SDS extraction procedure (30). The crude DNA preparation was denatured and dot-blotted onto nitrocellulose filters as described (31) and baked in vacuo at 80°C for several hours. Filters were pre-hybridized in 50% formamide, 500 mM NaCl, 40 mM Pipes pH 6.8, 1 mM EDTA, 0.1% SDS for 2 hours at 42°C. Hybridization was carried out overnight at 42°C in the same buffer containing 200 µg/ml of poly A-containing RNA from 20 day embryo leg muscle. The filters were washed, and eluted as described (32). mRNA activity was assayed using cell-free translation.

MATERIALS

Reverse transcriptase was the generous gift of J. Beard. DNA polymerase and T4-ligase were purified from the lysogens constructed by N. Murray (33,34). Eco R1 endonuclease and Eco R1 methylase were purified from E. coli RY15 (obtained from R. Roberts). S-adenosylmethionine (Sigma) was further purified by chromatography on Dowex to remove S-adenosyl homocysteine (35).

RESULTS AND DISCUSSION

Preparation of RNA template

Poly A-containing RNA from 20 day embryo leg muscle was fractionated on linear sucrose gradients under non-denaturing conditions. The sample

was pre-treated with CH_3HgOH to disperse stable aggregates which otherwise cause broadening of the peaks and loss of resolution. The mRNA activity of individual fractions was assayed using an mRNA dependent reticulocyte lysate cell-free system (Fig. 1). Fractions 11 + 12 were pooled, purified by adsorption to CF-11 cellulose (11) and used without further purification for the construction of recombinant bacterial plasmids.

The translatable activity of the partially purified RNA was compared to that of unfractionated 20 day embryo leg muscle RNA and of embryonic

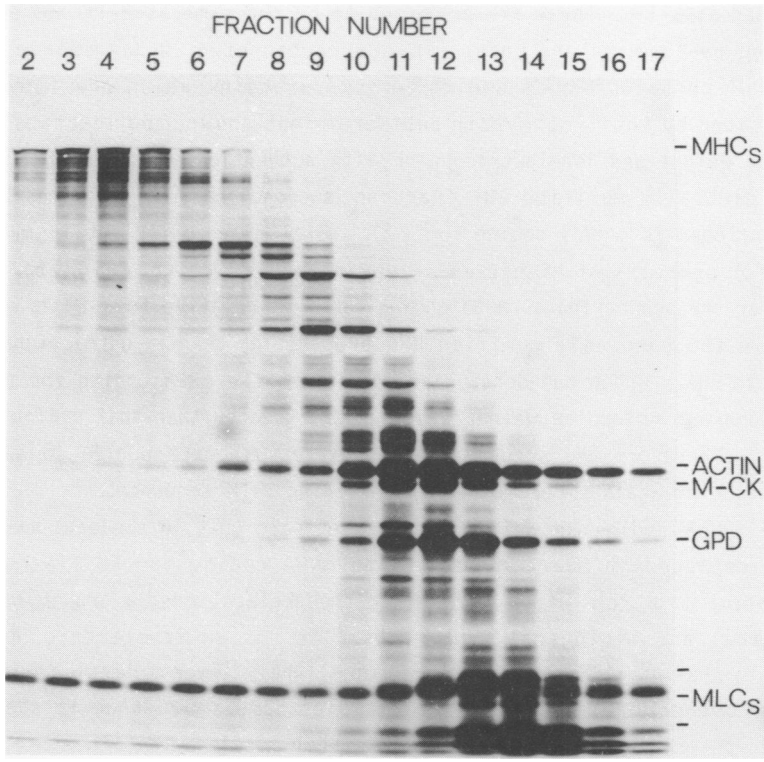

Figure 1. Sucrose gradient fractionation of poly A-containing RNA from 20 day embryo leg muscle. 300 µg of RNA was applied to each of six gradients in 13 ml. SW40 tubes and centrifuged at 40,000 rpm. for 5 hours. Messenger RNA activity was assayed by cell-free translation and proteins synthesized *in vitro* were examined on an SDS, 10% polyacrylamide gel. Markers shown are MHC_S (skeletal muscle myosin heavy chain), actin, M-CK (skeletal muscle creatine kinase), GPD (glyeraldehyde-3-phosphate dehydrogenase) and MLC_S (skeletal muscle myosin light chains). Fractions 11+12 were pooled and used without further purification for the construction and identification of recombinant plasmids.

heart muscle using two dimensional gel electrophoresis (Fig. 2). NEPHGE (non-equilibrium pH gradient electrophoresis) was used in the first dimension to facilitate the comparison of the one-dimensional and two-dimensional patterns (14). Adult chicken skeletal muscle contains two types of tropomyosins which have nearly identical isoelectric points but differ slightly in molecular weight (not shown). The component of higher molecular weight is defined as β-TM since it is this component which is reduced in abundance in breast muscle, a fast twitch muscle, compared with leg muscle (not shown). Similarly the component of lower molecular weight is defined as α-TM. These are presumed to be the same as α-TM and β-TM described by Cummins and Perry (5). Unfractionated 20 day embryo leg muscle RNA contains mRNA's coding for two proteins which are immunoprecipitated by antitropomyosin antiserum (not shown) and identical in molecular weight and isoelectric point with authentic α-TM and β-TM (Fig. 2a).The partially purified RNA fraction (sucrose gradient fractions 11 + 12) is enriched in mRNA's coding for α-TM and β-TM and deficient in mRNA's coding for proteins of higher and lower molecular weight (Fig. 2b). In particular the proportion of mRNA coding for β-TM relative to α-TM is much higher in the partially purified RNA preparation than in unfractionated leg muscle RNA. Under non-denaturing conditions the mRNA coding for β-TM has an apparent molecular weight which is 25% greater than that coding for α-TM (8). Therefore the sucrose gradient fractions were pooled so as to avoid selecting against recombinants containing β-TM sequences.

The mRNA's coding for α-TM and β-TM are found only in skeletal muscle and are replaced in heart muscle by an mRNA coding for TM_c (cardiac tropomyosin) (Fig. 2c). TM_c is similar to the skeletal muscle tropomyosins in physical and biological properties. It has an isoelectric point similar to that of α-TM and β-TM but is of slightly lower molecular weight (8,36). Furthermore, immunological studies have shown that it shares antigenic determinants in common with skeletal muscle α-TM (37).

Preparation of probes

cDNA probes to be used in subsequent colony hybridization assays were first characterized by hybridization to RNA blots of poly A-containing RNA from leg muscle and from heart muscle. RNA was electrophoresed on composite agarose:acrylamide gels and transferred to DBM-paper as described (26). ^{32}P-labelled cDNA probe was synthesized using the partially purified RNA preparation as template (sucrose gradient fractions 11+12). Hybridization of this probe to an RNA blot of poly A-containing

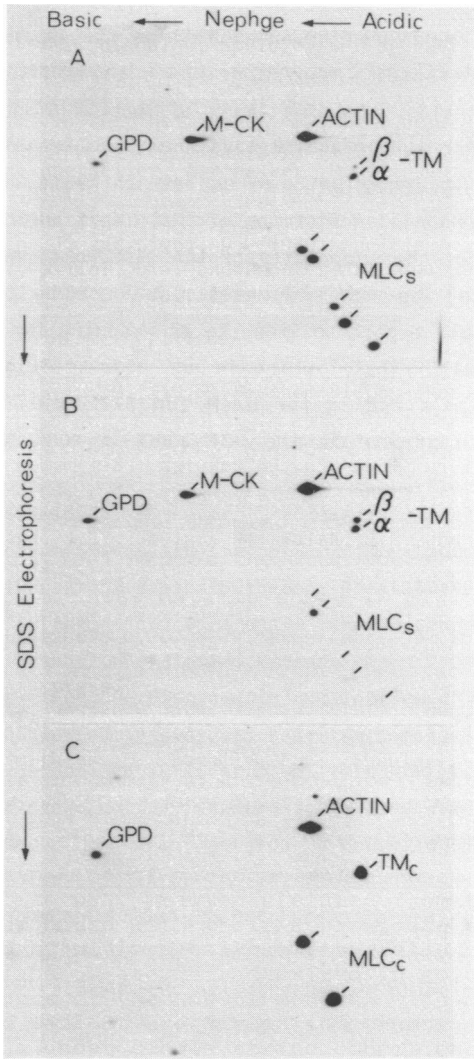

Figure 2. Two dimensional gel analysis of cell-free translation products. Proteins were synthesized in vitro using RNA from A) unfractionated 20 day embryo leg muscle B) sucrose gradient fractions 11 +12 C) embryonic heart muscle, and displayed on two-dimensional gels using NEPHGE (non-equilibrium pH gradient gel electrophoresis) in the first dimension. Protein spots were identified by immunoprecipitation in the case of GPD, M-CK, α-TM, β-TM and TMc and co-migration with purified markers in the case of actin and MLC.

RNA from leg muscle and from heart muscle is shown in Fig. 3a. One of the striking features of the hybridization pattern is the apparent complexity. This suggests the presence of many more RNA species than are apparent using *in vitro* translation (Fig. 2B). Most of these RNA species appear to be common to both heart muscle and skeletal muscle. Pre-hybridization of the ^{32}P-labelled cDNA probe with an excess of heart muscle poly A-containing RNA eliminates hybridization to the heart muscle track (Fig. 3b) and greatly reduces the complexity of the pattern of hybridization to leg muscle RNA. Among the mRNA sequences visualized by the leg muscle-specific probe we would expect to find the mRNA coding for M-CK (skeletal muscle-specific creatine kinase) which is not expressed in heart muscle. Detection of the mRNA's coding for α-TM and β-TM will depend on the ability of Tm_c mRNA to compete out the α-TM and β-TM cDNA sequences in the

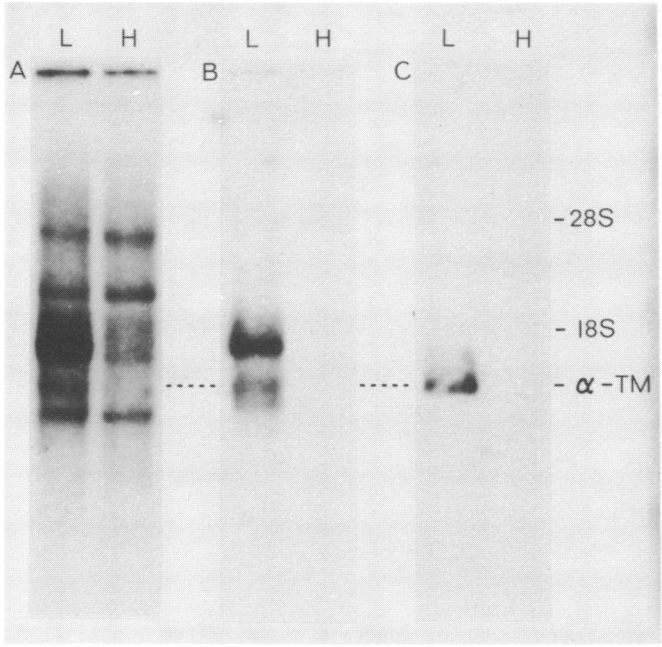

Figure 3. Characterization of cDNA and α-TM plasmid probes using RNA blots. Poly A-containing RNA from 20 day embryo leg muscle and from embryonic heart muscle was fractionated by electrophoresis on a composite agarose: acrylamide gel and transferred to DBM-paper. Hybridization probes used to visualize RNA bands were A) ^{32}P-labelled cDNA prepared using RNA from sucrose gradient fractions 11 + 12 B) the same ^{32}P-labelled cDNA pre-hybridized with excess heart muscle poly A containing RNA C) pTM1 DNA labelled by nick-translation. The leg muscle RNA tract is labelled L; the heart muscle RNA track is labelled H.

labelled probe. One of the bands detected in the leg muscle pattern using the prehybridized cDNA probe co-migrates with a leg muscle-specific RNA species which hybridizes to a plasmid containing an α-TM insert. (see below). (Fig 3c). No hybridization to heart muscle RNA was observed using the plasmid probe. The α-TM plasmid was selected by colony hybridization using the prehybridized cDNA probe and contains an insert of 600 base pairs (Fig. 6). Therefore the mRNA coding for TM_c competes very poorly with the α-TM cDNA sequences in the cDNA probe and conversely α-TM plasmids selected using this probe fail to hybridize to the mRNA coding for TM_c.

Construction of recombinant plasmids

Double-stranded cDNA was synthesized as described in Methods using the partially purified RNA preparation (sucrose gradient fractions 11 + 12) as template for first strand synthesis. To allow insertion of duplex cDNA into the plasmid vector cohesive termini were generated by blunt-end ligation of synthetic Eco R1 linkers followed by cleavage with Eco R1 endonuclease. Methylation of Eco R1 sites occurring within the duplex DNA prevents internal Eco R1 endonuclease cleavage and avoids selection against recombinants containing one or more internal Eco R1 sites.

This method of cloning offers several advantages. The inserts can be purified free of vector DNA by Eco R1 cleavage and preparative gel electrophoresis. The insert can be labelled and used as a probe to detect overlapping clones in the same library. Since the inserts already contain Eco R1 cohesive termini they can be easily recloned into the M-13 vector, M-13 mp2, for rapid DNA sequence analysis if desired (38). In addition the use of Eco R1 methylase should, in theory, allow the cloning of "full-length" recombinants even if Eco R1 sites are present within the sequence. In practice this appeared not to be necessary in the case of α-TM as no internal R1 sites were detected in any of the clones.

Identification of α-TM recombinants

Bacterial clones harbouring recombinant plasmids were identified by colony hybridization (39) using the cDNA probes described previously. In an initial screen of 300 recombinants eight clones were selected as positive using the ^{32}P-labelled cDNA probe prehybridized with heart muscle RNA. Crude plasmid DNA was prepared from each of these denatured and immobilized on millipore filters as described (31). After hybridization with unfractionated leg muscle poly A-containing RNA, specifically bound mRNA was eluted and assayed using cell-free translation (Fig. 4). Of the

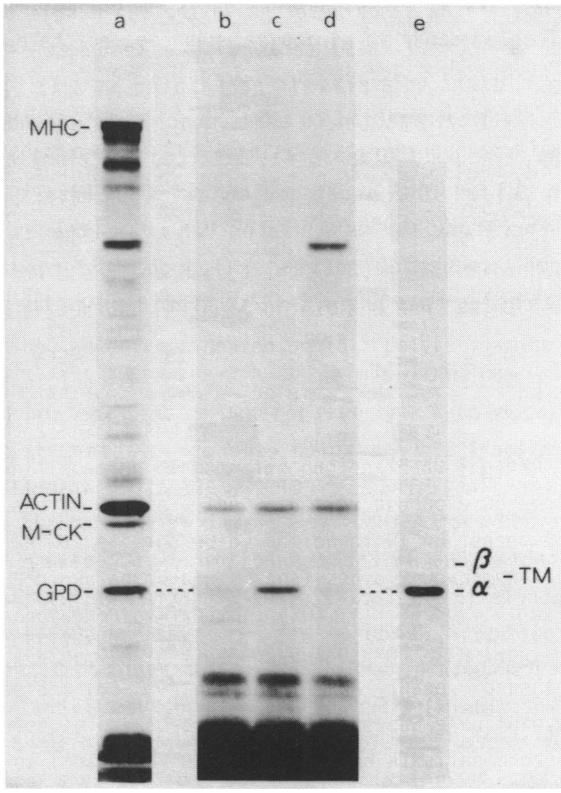

Figure 4. Identification of a recombinant plasmid containing α-TM sequences. The translation products of RNA selected by hybridization to immobilized plasmid DNA from clones b) p32 c) pTM1 d) pG were compared to the translation products of unfractionated 20 day embryo leg muscle RNA (a). The mRNA selected by hybridization to pTM1 DNA codes for a protein which co-migrates with α-TM. This protein is also immuno precipitated by antiserum to adult chicken leg muscle tropomyosin (e). Proteins were analyzed on an SDS, 10% polyacrylamide gel. Track (e) is from a separate gel.

eight recombinants tested only one, pTM1, yielded a detectable protein product after in vitro translation and this product co-migrated with α-TM (Fig. 4c). The in vitro translation products of mRNA hybridized to two different plasmids selected using another probe is shown for comparison (Fig. 4,b,d). The low frequency of occurrence of plasmids yielding positive signals by in vitro translation is consistent with the previous observation that the RNA population revealed by hybridization is much more complex than the RNA population seen using cell-free translation. Thus it

would appear that only a small number of the RNA species present are efficiently translated into protein products by the reticulocyte lysate cell-free system.

The protein product identified using cell-free translation was confirmed to be α-TM by immunoprecipitation with antitropomyosin antiserum (Fig. 4e) and by co-migration with α-TM on two dimensional gels (Fig. 5a,b). No evidence for cross-hybridization with β-TM was observed using in vitro translation. Hybridization of nick-translated pTM1 DNA to the RNA blot of leg muscle RNA and of heart muscle RNA yielded the result shown in Fig. 3C.

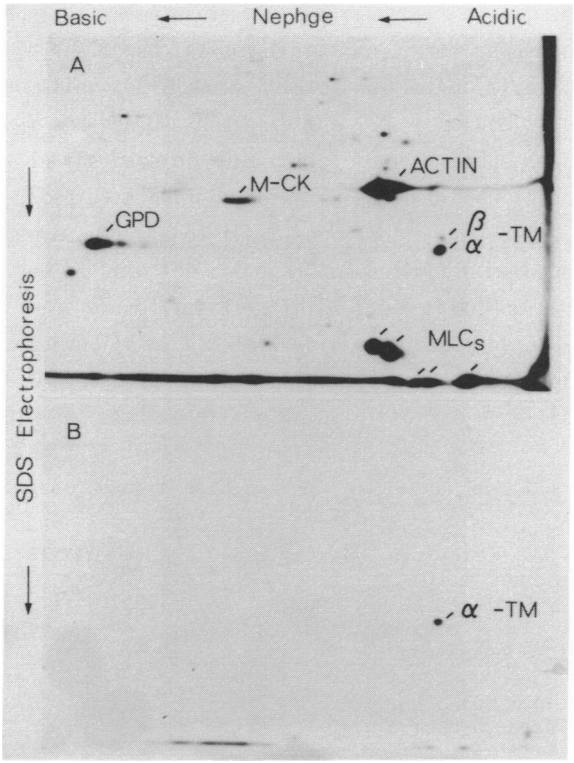

Figure 5. Identification of a recombinant plasmid containing α-TM sequences. Two dimensional gel analysis of cell-free translation products of A) unfractionated 20 day embryo leg muscle RNA B) RNA selected by hybridization to pTM1 DNA immobilized on a nitrocellulose filter. The RNA selected by hybridization to pTM1 DNA codes for one protein spot which co-migrates exactly with marker α-TM.

Plasmids containing sequences overlapping with pTM1 were identified by cross-hybridization. pTM1 DNA was cleaved with Eco R1 and the α-TM insert was purified by preparative gel electrophoresis. The cohesive ends were labelled by "filling-in" with ^{32}P-dATP and reverse transcriptase. A further 1400 recombinants were screened using this probe and 15 strongly hybridizing colonies were identified. The Eco R1 cleavage pattern of plasmid DNA from several of these clones is shown in Fig. 6. Inserts of 400-700 base pairs appeared to be the most frequent although some inserts of greater than 1000 base pairs were also detected. One particular plasmid pTM10 contained an insert of 1150 base pairs. Since this plasmid is expected to contain a very large fraction if not all of the coding

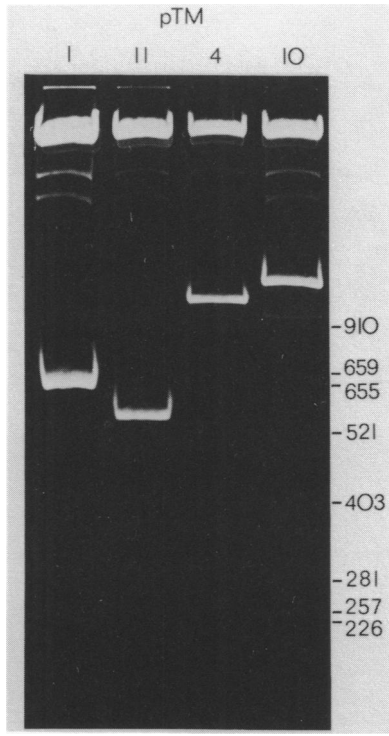

Figure 6. Restriction enzyme analysis of recombinant plasmids containing α-TM sequences. Plasmid DNA was prepared from clones pTM1 and 3 other clones pTM4, 10 and 11 which were selected by cross-hybridization with pTM1. DNA was digested with Eco R1, electrophoresed on a 5% polyacrylamide gel in TAE buffer and visualized by staining with ethidium bromide. Restriction fragments generated by cleavage of pBR322 with Alu I were used as molecular weight markers (41).

sequence of α-TM mRNA it was tested for cross-hybridization with the mRNA coding for TM_c. pTM10 DNA was nick-translated and hybridized under relatively non-stringent conditions to an RNA blot of poly A-containing RNA from leg muscle and from heart muscle (Fig. 7). As seen previously with pTM1 only a single major hybridizing species can be detected in leg muscle RNA. However, after very long exposures a single faint band can be detected in the heart muscle pattern. The mobility of the heart RNA species is very similar to that of the mRNA coding for α-TM suggesting that it could be the mRNA coding for TM_c.

Under the conditions of electrophoresis the mRNA coding for β-TM

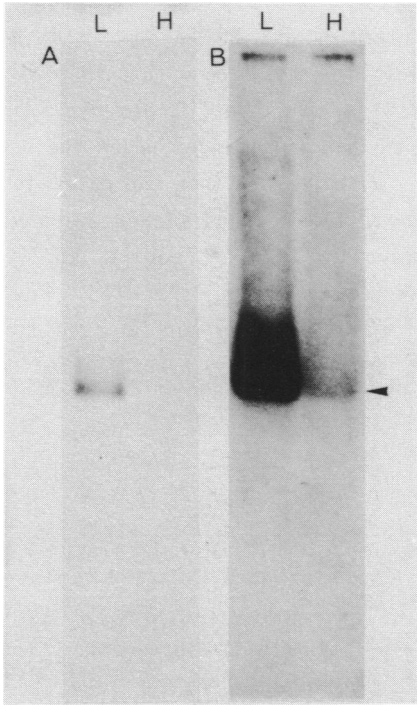

Figure 7. Hybridization of pTM10 to an RNA blot of poly A-containing RNA from 20 day embryo leg muscle and embryonic heart muscle. pTM10, which contains an insert of 1150 base pairs was nick-translated and hybridized to the RNA blot in a 50% formamide, 5 x SSC hybridization buffer at 35° for 48 hours. The blot was washed at room temperature in 1 x SSC wash buffer and exposed to X-ray film for A) 1 day B) 7 days. The leg muscle RNA track is labelled L; the heart muscle RNA track is labelled H. The arrow indicates the position of the cross-hybridizing RNA species in the heart muscle track.

migrates more slowly than the mRNA coding for α-TM (8). However, it is difficult to discern cross-hybridization of pTM10 to β-TM mRNA because of trailing of the very strong α-TM signal. If cross-hybridization between α-TM and β-TM sequences does occur it would appear to be very weak.

CONCLUSIONS

These results demonstrate that although the tropomyosins of skeletal muscle and heart muscle appear to be very similar in physical and biological properties the mRNA's coding for these proteins have considerably different primary sequences. Some sequence homology would be expected since these proteins share common peptides and antigenic determinants (4,37). Indeed some cross-hybridization could be observed between the α-TM cDNA plasmids and an RNA species in heart muscle which may be TM_c mRNA. No cross-hybridization with β-TM mRNA could be detected using *in vitro* translation. However, cross-hybridization is a very stringent test of sequence homology since, under the usual conditions of hybridization, sequences with greater than one base mismatch in six will fail to form stable hybrids (40). Thus sequence analysis of the mRNA's coding for these tropomyosins will be required to determine the exact nature of the differences between these molecules. Preliminary results suggest that the α-TM cDNA plasmids can be used to detect plasmids containing β-TM and TM_c sequences using hybridization conditions of very low stringency.

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