Analysis of tropomyosin cDNAs isolated from skeletal and smooth muscle mRNA²

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ABSTRACT

Several clones containing alpha tropomyosin sequences were isolated from cDNA libraries prepared from quail skeletal or smooth muscle RNA. All of these clones contain identical sequences coding for amino acids 81-257 of alpha skeletal muscle tropomyosin where they overlap, strongly indicating they are derived from the same gene. However, there are differences among these clones in sequences coding for the final 27 amino acids, as well as 3' untranslated sequences. In addition, S1 nuclease and Northern analyses indicate that coding sequences at the 5' end of the tropomyosin mRNA are also differentially expressed in smooth and skeletal muscle. These data point to the likelihood that differential splicing at both the 5' and 3' ends of a single tropomyosin gene results in the appearance of multiple tissue-specific transcripts.

INTRODUCTION

Contractile proteins are the primary components of muscle cells. Most of these proteins are expressed as members of multigene families and the expression of specific isoforms of each contractile protein in different muscles plays a major role in determining muscle fiber structure and function. Furthermore, during myogenesis, different developmental and muscle-specific isoforms of these genes are expressed. Thus formation of specific muscle types such as smooth, skeletal, and cardiac muscle is associated with the differential accumulation of specific contractile protein isoforms.

Although many of the contractile protein genes have been isolated and characterized, very little is known about the tropomyosin gene family in vertebrates. Tropomyosins are a closely related family of proteins which are bound to actin and troponin and serve to mediate the effect of calcium on muscle contraction (1,2,3). Skeletal muscle contains two tropomyosin subunits, alpha and beta, which are present in different molar ratios in individual muscle types, and at different times during development (4,5). Mammalian cardiac muscle apparently contains only an alpha type subunit (5),

although the situation is not as clear in avians. Smooth muscle contains both alpha and beta type subunits, but very little is known about the function of these proteins since contraction in smooth muscle is thought to be independent of tropomyosin (6,7).

In Drosophila, there are three tropomyosin genes, two of which are expressed in muscle and one which is expressed only in non-muscle tissue (8,9,10). One of the muscle genes, mTM I, utilizes differential splicing to express two sets of tissue-specific mRNAs - one in embryos and cell cultures, and one in thoracic flight muscle of the adult. Much less is known about the vertebrate tropomyosin genes, although there is evidence that both smooth and skeletal muscle tropomyosin isoforms may be derived from the same gene (11). Thus it is possible that regulation of different tropomyosin isoforms in different muscle types may result from the transcription of multiple mRNAs from the same gene, as has been shown to occur in troponin T (12,13). We have isolated tropomyosin cDNA clones from both alpha skeletal and smooth avian muscle RNA. In this report we show that it is likely that several transcripts are produced from a single alpha tropomyosin gene by differential splicing at both the 5' and the 3' ends of the gene.

MATERIALS AND METHODS

RNA Isolation

Cultures of quail myoblasts and myofibers were prepared, and RNA isolated as described earlier (14). For isolation of RNA from quail muscles, tissue was sterilely dissected, minced, rinsed in saline, and stored at -80°C until used. The tissue was then thawed and fragmented in a Waring blender using 20 volumes of 20mM sodium acetate, pH 5.1, 250mM NaCl, 10mM EDTA, 1% SDS, 50% phenol, and 500ug/ml heparin. The homogenate was centrifuged at 12,000g for 20 minutes and the supernatant extracted twice with equal volumes of phenol and chloroform. After precipitation in ethanol, the RNA was dissolved in 10mM Tris, pH 8.0, 1mM EDTA, and precipitated in 2M LiCl. In some cases poly(A) RNA was isolated as described earlier (14).

Preparation and Screening of cDNA Libraries

The preparation and screening of a plasmid cDNA library constructed from myofiber poly(A) RNA has been described earlier (14). Phage cDNA libraries were prepared using poly(A) RNA from either late embryo breast muscle, heart muscle, or gizzard muscle tissue. The lambda gtl0 libraries were basically prepared and screened as described by Huynh et al. (15).

Hybrid Selection

Plasmid DNA was isolated from cDNA clones and used to select RNA

essentially as described by Ricciardi *et al.* (17). DNA was denatured and bound to nitrocellulose paper, and allowed to hybridize overnight with 100ug of total myofiber RNA in 50ul of 50% formamide, 5X SET, and 0.1% SDS. Unhybridized RNA was removed by washing in the same solution and bound RNA eluted by the addition of boiling water. RNA was then precipitated with ethanol and translated in a wheat germ cell free translation system as described earlier (18). Antibody directed against adult quail breast muscle alpha tropomyosin was allowed to react with the extract in the presence of unlabelled carrier tropomyosin, and precipitated product analyzed on SDS gels (19).

S1 Nuclease Mapping

The S1 mapping studies were done as described by Favaloro *et al.* (20). End-labelled restriction fragments were hybridized with 30 ug of total RNA in 80% formamide, 0.4M NaCl, 0.04M Pipes, 1 mM EDTA. Hybridization was at 52°C for 16 hours, followed by digestion with 150 units of S1 nuclease. Products were analyzed on 8% polyacrylamide-urea gels (21).

Northern Analysis

Northern blot analysis was done basically as described earlier (14,22). Ten micrograms of total RNA was separated on a 1.5% gel containing formaldehyde. RNA was transferred to a nylon membrane and individual lanes probed with nick-translated restriction fragments (10⁶CPM per lane). Specific DNA fragments were isolated for nick-translation in the following manner. Restriction fragments were excised from low melting gels and macerated. Following addition of an equal volume of phenol, the mixture was frozen and thawed. The phases were separated by centrifugation and the aqueous phase extracted twice with phenol/chloroform. DNA was precipitated with ethanol and used for nick-translation.

DNA Sequencing

Restriction fragments were excised from soft agar gels and cloned into mpl8 and mpl9 cloning vectors for sequence analysis, which was performed according to Sanger *et al.* (23), using ³⁵S-dATP. In some cases a series of overlapping deletions in mpl9 were prepared and sequenced as described by Dale *et al.* (24).

RESULTS

Isolation of Skeletal Muscle Tropomyosin cDNA Clones

A plasmid cDNA library constructed from myofiber poly(A) RNA was previously screened with labelled myoblast and myofiber RNA. Seventy two

Figure 1. Identification of cDNA Clones Containing Tropomyosin Sequences by Hybrid Selection.

Myofiber RNA was allowed to hybridize with filter-bound DNA from three cDNA clones: TM171, TM394, and TM682. Unhybridized RNA was washed off and bound RNA eluted from the filter and translated in a wheat germ cell-free translation system. An antibody directed against skeletal muscle tropomyosin, as well as carrier tropomyosin, was added to the extract and radiolabelled immunoprecipitable material analyzed on 12.5% SDS polyacrylamide gels. In a control experiment shown in the left lane, total myofiber RNA was added to the wheat germ system, followed by the addition of carrier tropomyosin and anti-tropomyosin antiserum.

different clones were identified which hybridized strongly to myofiber RNA but not at all to myoblast RNA (14). Twelve of these were selected at random and analyzed by hybridization/selection as described in Materials and Methods. Of these twelve, one selected a mRNA which, when assayed in a wheat germ cell-free translation system, produced a protein which was precipitable with antibody directed against alpha tropomyosin and co-migrated with alpha tropomyosin on SDS polyacrylamide gels. This clone (TM682) was labelled by nick-translation and used to screen the entire cDNA library of 1000 clones. Two other clones hybridized strongly with TM682. These two DNAs (TM394 and TM171) were also assayed by hybridization selection, and both

Figure 2. Schematic Diagrams of the cDNA Clones TM171, TM394, and TM682 derived from DNA sequence analysis.

The shaded regions specify amino acid coding sequences and the clear regions specify 3' untranslated sequences. TAA represents the position of the termination codon and ATAAA represents the position of consensus poly A addition signals.

selected a mRNA which produced a protein precipitable with anti-tropomyosin antiserum. The hybridization selection data is shown in Figure 1.

Sequencing of cDNA Clones

Each of the three cDNAs was sequenced in its entirety. Schematic diagrams of the three clones are shown in Figure 2. TM171 contains sequences coding for the first 165 amino acids of alpha skeletal muscle tropomyosin, when compared with the published amino acid sequence of rabbit alpha skeletal muscle tropomyosin (25). TM394 contains DNA sequences coding for the last 26 amino acids of alpha skeletal muscle tropomyosin as well as 288 bases of 3' untranslated sequence. The clone contains a stretch of 26 As at the 3' end, indicating that it represents the 3' end of a mRNA. TM682 has a somewhat surprising sequence arrangement. It contains sequences coding for amino acids 119 through 257 of alpha skeletal muscle tropomyosin, as well as the first 205 bases of 3' untranslated sequence. However, it is missing DNA coding for the last 27 amino acids, as well as DNA coding for the last 83 bases of the 3' untranslated region. It does contain a poly(A) stretch, indicating that this clone also represents the 3' end of a mRNA. Where they overlap, these three clones have identical sequences, including wobble positions and 3' untranslated regions, implying that they are derived from the same gene.

Another implication of the sequencing data is that two different 3' non-translated ends are associated with alpha skeletal muscle tropomyosin mRNA. In the case of TM682, there are 205 bases present beyond the TAA termination

codon, with a consensus poly(A) addition site located 18 bases from the end of the clone. This exactly correlates with sequence data from a chicken alpha skeletal muscle cDNA clone (26). In addition to this sequence, TM394 contains an additional 83 bases and a second poly(A) addition site located 20

Figure 3. Tissue-specific Expression of TM394 and TM682 type mRNAs.

Thirty micrograms of total cellular RNA was isolated from various tissues and allowed to hybridized to an end-labelled HindIII fragment from TM682 which contains 275 bases of coding sequence and 65 bases of 3' untranslated sequence. After digestion with S1 nuclease the products were separated on a 8% denaturing polyacrylamide gel. The presence of a 65 base band indicates an RNA population which differs from TM682 in the region immediately prior to the termination codon. The presence of a 340 base band indicates an RNA population which is colinear with TM682. DNA molecular weights were determined using a 123 base ladder. In one lane (Probe) the end-labelled DNA alone was added. In another lane (no RNA) a mock hybridization was done in which tRNA was used instead of muscle RNA.

bases from the end of the clone. Both clones terminate in a stretch of poly As.

More surprisingly, there also appear to be two different classes of alpha skeletal mRNA, with differences in the carboxy terminal region of the protein. One class of mRNA, as exemplified by TM394, codes for a protein with a similar amino acid sequence to that of rabbit tropomyosin. The other class, represented by TM682, is similar to alpha skeletal muscle tropomyosin until amino acid 257. However, the last 27 amino acids are not present. To eliminate the possibility of a sequencing artifact TM682 was cloned into mp18 and sequenced three separate times.

Tissue-specific Distribution of pTM682

To determine in which muscle type a TM682-like mRNA might be found, this

Figure 4. Developmental Expression of TM394 and TM682 type mRNAs.

Thirty micrograms of total cellular RNA was isolated from breast muscle at various times during development and allowed to hybridize to the same 360 base HindIII probe described in Figure 3. Conditions of S1 nuclease analysis and interpretation of the results were also as described in Figure 3.

clone was used for S1 analysis. A 340 base pair Hind III fragment was labelled with polynucleotide kinase and hybridized to RNA from different muscle types. If mRNA similar to TM682 is present, a fully protected RNA of 340 bases would be expected. On the other hand, if the "normal" alpha tropomyosin mRNA represented by pTM394 were present, there would only be a 65 base protected fragment due to the looping out and S1 cleavage of the RNA coding for the final 27 amino acids. RNA was isolated from late embryonic skeletal muscle (breast and leg), smooth muscle (gizzard and aorta), cardiac muscle (heart), and liver and used in the S1 analysis. As shown in Figure 3, the 3' untranslated end of TM682 does not hybridize to RNA from smooth or cardiac muscle, nor to non-muscle tissues such as liver.

However, both breast and leg muscle contain these sequences. Bands of 340 bases and 65 bases were detected in each tissue, indicating that both types of mRNA are present. The intensity of the bands were quantified as described earlier (6). Although it is difficult to obtain accurate values due to the material trapped at the top of the wells, both mRNAs appear to be present in roughly equal amounts in late embryonic skeletal muscle.

Developmental Analysis of Tropomyosin mRNAs

To determine when these mRNAs are made during development, RNA from 8 day embryo, 12 day embryo, 18 day embryo, newly hatched, and 10 day old quail breast, leg, heart, and gizzard muscle was used for S1 analysis. As expected, the 3' end of the TM682 did not hybridize to any tissue except skeletal muscle (data not shown). As shown in Figure 4, both types of mRNA were detected in low amounts in 8 day quail embryo breast, a time when muscle fusion is just beginning *in ovo*. The amount of each mRNA increases as development proceeds, with both mRNAs being present in roughly equal amounts at all times assayed.

Thus, not only does TM682-like mRNA exist, but it appears to be present in roughly the same amount as "normal" tropomyosin mRNA. Both types of mRNA are found only in skeletal muscle tissue, and are present in the same ratio at all developmental times examined.

Isolation of Additional Skeletal Muscle Tropomyosin cDNA Clones from a Phage Library

Since neither TM394 nor TM682 extends to the 5' end of the coding region of tropomyosin it is not possible to determine if these two clones share a common 5' coding sequence, or even how closely the 5' end of the mRNAs represented by these clones corresponds to the known amino acid sequence of alpha skeletal tropomyosin. Nor is it possible to know what kind of 3' seq-

Met Asp Ala Ile Lys Lys Lys Met Gln Met Leu Lys Leu Asp Lys Glu Asn Ala Leu Asp 20
 TGTGCCGCGGGACGCGCCCTAACCCACCCGCCCATGG ATG SAT GCC ATC AAG AAA AAG ATG CAG ATG CTG AAA CTG GAG GAG AAT GCC TTG GAC

Asp Ser
 Arg Ala Glu Gln Ala Glu Ala Asp Lys Lys Ala Ala Glu Glu Arg Ser Lys Gln Leu Glu Asp Glu Leu Val Ala Leu Gln Lys Lys Leu 50
 AGA GCC GAG CAA GCC GAA GCG GAC AAG AAG GCA GCG GAG GAG AGG AGC AAG CAG CTG GAG GAC GAG CTG GTG GCT CTG CAA AAG AAG CTG

Ala Glu
 Lys Gly Thr Glu Asp Glu Leu Asp Lys Tyr Ser Glu Ser Leu Lys Asp Ala Gln Glu Lys Leu Glu Leu Ala Asp Lys Lys Ala Thr Asp 80
 AAG GGC ACT GAG SAT GAG CTG GAC AAA TAT TCC GAG TCC CTT AAA SAT GCA CAG GAA AAG TTG GAA CTG GCT GAC AAA AAG GCC ACA GAT

Ala Asp
 Ala Glu Ser Glu Val Ala Ser Leu Asn Arg Arg Ile Gln Leu Val Glu Glu Glu Leu Asp Arg Ala Gln Glu Arg Leu Ala Thr Ala Leu 110
 GCT GAG AGT GAG GTA GCT TCC CTG AAC GAA GCG ATC CAA CTG GTT GAG GAA GAG TTG SAT CCG GCT CAG GAG CCG TTG GCT ACT GCC CTG

Ser
 Gln Lys Leu Glu Glu Ala Glu Lys Ala Ala Asp Glu Ser Glu Arg Gly Met Lys Val Ile Glu Asn Arg Ala Gln Lys Asp Glu Glu Lys 140
 CAG AAG CTG GAA GAG GCT GAG AAG GCT GCA SAT GAG AGT GAA AGA GGA ATG AAG GTC ATT GAA AAT AGA GCC CAG AAG GAT GAA GAG AAG

Asp
 Met Glu Ile Gln Glu Ile Gln Leu Lys Glu Ala Lys His Ile Ala Glu Glu Ala Asp Arg Lys Tyr Glu Glu Val Ala Arg Lys Leu Val 170
 ATG GAA ATC CAA GAG ATC CAG CTT AAA GAA GCT AAG CAC ATT GCT GAA GAG GCT GAC CSC AAG TAT GAA GAG GTG GCT CGT AAG CTT GTG

Ser Gly
 Ile Ile Glu Gly Asp Leu Glu Arg Ala Glu Glu Arg Ala Glu Leu Ser Glu Ser Lys Cys Ala Glu Leu Glu Glu Glu Lys Thr Val 200
 ATC ATT GAG GGT GAC CTG GAG CCG GCT GAG GAA CBT GCT GAA CTA TCA GAA AGC AAA TGT GCT GAG CTT GAA GAG GAG TTG AAA ACT GTG

Glu Ser
 Thr Asn Asn Leu Lys Ser Leu Glu Ala Gln Ala Glu Lys Tyr Ser Gln Lys Glu Asp Lys Tyr Glu Asp Glu Ile Lys Val Leu Thr Asp 230
 ACC AAC AAC CTG AAG TCG CTG GAG GCT CAG GCT GAG AAG TAC TCG CAG AAA GAA GAC AAA TAT GAA GAG GAG ATT AAA GTT CTA ACT GAC

Glu Ser
 Lys Leu Lys Glu Ala Glu Thr Arg Ala Glu Phe Ala Glu Arg Ser Val Thr Lys Leu Glu Lys Ser Ile Asp Asp Leu Glu Asp Glu Leu 260
 AAA CTG AAG GAG GCT GAG ACC CGT GCT GAA TTT GCT GAG AAG TCA GTA ACC AAG CTG GAG AAG AGC ATT GAT GAT CTA GAA GAT GAG CTT

END
 Tyr Ala Gln Lys Leu Lys Tyr Lys Ala Ile Ser Glu Glu Leu Asp His Ala Leu Asn Asp Met Thr Ser Ile
 TAT CAT CAG AAA CTG AAG TAC AAA GCC ATC AGC GAG GAG CTG GAC CAT GCT CTC AAG GAT ATG ACT TCC ATA TAA-TTTTCTTTGCTTCACTTCT

CAAGACATCTTCATCGGGTTGGATAAAACATCTTTCCAGCTTTTGGATGTTTCATGGCTCCCTGCTGCTCTTCTAGCTTACTTGGCTATCTCTGGCAGCTGTTTCAGAACAT
 T T - - A A T

GCTCTTTGTG-CTCTATTGTACAGAACTCTACATCTCTAAATGTAATAAATCCACCTGCTATTCCTTTTGGCTATCCCTTGCCCTTTAATTATTCAAATAGCTATTTTASTTA
 A C- G

TTAATAAAAAATATAATGTTGTATG6GTT (POLY A)
 ----- (POLY A)

Figure 5. Complete sequence of SK74.

Differences between SK74 and a chicken cDNA clone (17) are indicated, with the chicken nucleotides shown underneath the quail sequence. Dashes indicate the absence of a nucleotide. Potential poly A addition sites are underlined. Differences in amino acid sequence between quail and rabbit alpha skeletal muscle tropomyosin are also indicated, with the rabbit amino acids shown above the quail.

ences are found on TM171, a clone which has "normal" 5' sequences.

Therefore, a phage cDNA library was prepared from late embryo breast muscle mRNA and screened using TM171, TM394, and TM682 as probes. Several clones were isolated which spanned the entire coding region and also contained both

Figure 6. Hybridization of Smooth and Skeletal Muscle RNA with SK74.

Ten micrograms of total cellular RNA was isolated from breast and gizzard muscle of early (#1), mid (#2) and late (#3) embryo, as well as 2 day (#4), 10 day (#5), and 21 day (#6) post-hatched quail. RNA was separated on a denaturing gel, transferred to nylon paper, and hybridized to nick-translated SK74. The size of the hybridizing RNA was determined using lambda and 1KB ladder standards purchased from BRL, and the position of 18s and 28s ribosomal RNA denoted by arrows.

Sk = Skeletal Muscle RNA; Sm = Smooth Muscle RNA.

5' and 3' untranslated sequences. All but one of these contained identical sequences where overlap occurred and were precisely colinear with the rabbit amino acid sequence. Some of the clones terminated with a poly A sequence after 208 bases and others after 288 bases, indicating that the presence of the longer 3' untranslated sequence is not related to the presence or absence of the DNA coding for the last 27 amino acids. The sequence of the largest of these clones, SK74, is shown in Figure 5. Its coding sequence agrees almost exactly with a previously published chicken alpha skeletal muscle tropomyosin (26), differing in only 5 nucleotides. In addition, this clone contains 39 bases of 5' untranslated sequence.

Isolation of Tropomyosin Clones from a Smooth Muscle cDNA Library

The full length cDNA clone SK74 was hybridized with RNA isolated from

Quail Skel. Asp Glu Leu Tyr Ala Gln Lys Leu Lys Tyr Lys Ala Ile Ser Glu Glu Leu Asp His Ala Leu Asn Asp Met Thr Ser Ile End

Rat Smooth Glu Lys Gly Ala His Ala Lys Glu Glu Asn Leu Ser Met His Gln Met Leu Asp Gln Thr Leu Leu Glu Leu Asn Asn Met End

Quail Smooth Glu Lys Val Ala His Ala Lys Glu Glu Asn Leu Asn Met His Gln Met Leu Asp Gln Thr Leu Leu Glu Leu Asn Asn Met End

Quail Skel. GAT GAG CTT TAT GCT CAG AAA CTG AAG TAC AAA GCC ATC AGC GAG GAG CTG GAC CAT GCT CTC AAC GAT ATG ACT TCC ATA TAA

Rat Smooth GAG AAA GGT GCA CAT GCC AAA GAA GAA AAC CTT AGT ATG CAC CAG ATG CTG GAC CAG ACT TTA CTG GAG CTA AAC AAC ATG TGA

Quail Smooth GAG AAA GTC GCG CAT GCC AAA GAA GAA AAC CTT AGT ATG CAI CAG ATG CTG GAI CAA ACT TTA CTG GAG ITA AAC AAC ATG TGA

aaacctcttcagcagcaacacattgtttattttttacacctgcttgccgtgaagcccccataaacatgtcttttttttagtttcaccacagtcacacaacacacagctaa
 attatcaggcagggtcagggaagtaccacaaaagggaagctgtatgcgggttaagctatttcattatgtgttttaaaigtgccatttccccacaaaagtaccacatttg
 tagagattcagagattggaatgcttagtcttgggaataatctctgctctgtcaggtagcgtccaccctcaagtgccaacacagttttgaacagcatgatcacctc
 aaaaagatccagcacaagaaggtgctatttggatccaaaagctctgtaggggcttttattgtatcccgatgagctaggagctgttgagcagagtaatttattttatcagc
 attttatgctctgtgaggatttgacttttggctttgatttcaatagaacgaaggtgtgtgtgtcacaacagtaaaacaaaggttatgaagtttttaatttgaact
 tgcactcgtttacataagtgtaggtcaacatttccgcttgagttatttcttattgtatttttcatgtttcaaatgaagattc~~aa~~tttcaggagaaaacacaaatttga
 gaagagattgtttaaaatgtattttatttgtgtggagcttggggcggggtggaacttacaatcgatattcgctcactttatttttccaccttcttttgagcagtttaa
 gtatacctgtatgaacattgtataatgatata~~ga~~aaatgcacatttttaggaatttt

Figure 7. Comparison of Sequences Coding for the 3' end of Quail Skeletal, Quail Smooth, and Rat Smooth Muscle Tropomyosin.

Nucleotide sequences and derived amino acid sequences from quail clones SK74 (skeletal muscle), SM18 (smooth muscle) and a rat clone isolated by Ruiz-Opazo *et al.* (24) are compared. Differences in nucleotide and amino acid sequence between quail and rat smooth muscle clones are underlined, as are Poly(A) addition sites of SM18. The 3' untranslated sequences of SM18 and its rat counterpart are completely divergent and not compared here.

smooth and cardiac muscle at various times during quail development to ascertain the extent of homology between skeletal, smooth, and cardiac muscle tropomyosin. Although the cDNA did not hybridize to cardiac mRNA (data not shown), it did hybridize with two smooth muscle mRNAs of 1.8 and 1.6 KB at all developmental stages tested. This data is shown in Figure 6. To determine the structure of this larger smooth muscle tropomyosin mRNA, a phage cDNA library was prepared from late embryo gizzard muscle and screened using SK74 as a probe. Three different clones were isolated and analyzed. The largest of the three, SM18, is 1.1 KB in size and contains sequences colinear with SK74 from its first base (amino acid 145) to amino acid 257. However, DNA coding for the last 27 amino acids, as well as the 3' untranslated region, was diverged from SK74. A comparison of the nucleic acid sequence and derived amino acid sequence of the last 27 amino acids of the smooth (SM18) and skeletal (SK74) muscle cDNA clones, as well as a rat smooth muscle cDNA clone (11) is shown in Figure 7. This region of SM18 is much different from corresponding sequences present in the quail skeletal muscle cDNA clones, sharing only 31% homology. In contrast the coding portion of SM18 shares a 91% homology with the rat smooth muscle cDNA clone isolated by Ruiz-Opazo *et al.* (11). The derived amino acid sequence for the final 27 amino acids of SM18 is identical to the rat smooth muscle sequence in 25 out of 27 positions. The 3' untranslated region of SM18 is 755 bases,

Figure 8. Identification of a Difference Between Skeletal and Smooth Muscle Troponin RNA in the region coding for amino acid 80.

Thirty micrograms of total RNA was isolated from various tissues and allowed to hybridize with the 340 base Pst I fragment, which was end labelled. Conditions of S1 analysis were as described in Figure 3. S = Standards, a 123 base ladder; P = Full Length Probe; M = Mock Hybridization; Li = Liver RNA; B = Breast RNA; Lg = Leg RNA; G = Gizzard RNA; H = Heart RNA.

much larger than the 3' untranslated region of the skeletal muscle cDNAs, but similar in size to the rat smooth muscle cDNA. It shares no obvious homology with the skeletal muscle sequence, and has also diverged considerably from its rat counterpart. Two poly A addition sites can be found - one 17 bases from the poly A tail, and another 190 bases upstream. If both poly A addition sites are used, this would account for the two different sized mRNAs seen on the Northern blot.

This data indicates that in smooth muscle a mRNA is present which shares precisely the same sequences as does skeletal muscle mRNA for amino acids 145-257. Sequences coding for the final 27 amino acids, as well as 3' untranslated sequences differ in the two mRNAs. The most likely interpretation of this data is that both the smooth muscle and skeletal muscle mRNAs are derived from the same gene by differential splicing of a common precursor. We now have evidence that sequences coding for the final 27 amino acids make up a separate exon in the tropomyosin gene, which would facilitate differential splicing of these sequences (manuscript in preparation).

Differential Splicing Also Occurs at the 5' End of the mRNA

Since none of the smooth muscle cDNA clones extended through the entire tropomyosin coding sequence it was not possible to use sequencing to determine if differential splicing at the 5' end of the quail mRNA also occurs, as has been reported in mammals (11). We therefore performed a series of Northern and S1 mapping experiments designed to answer this question. As shown in Figure 8, a 340 base pair Pst I fragment was excised from the skeletal muscle cDNA clone TM171, extending from amino acid 1 through 113. This fragment was 5' end-labelled and allowed to hybridize to both skeletal and smooth muscle RNA. As diagrammed in Figure 8, RNA with the same sequence as TM171 (*i.e.* skeletal type) will be fully protected and yield a band of 340 bases. A band of this size was seen in both breast and leg muscle. However, gizzard muscle RNA yielded a band of about 90 bases, indicating a discontinuity in the RNA coding for approximately amino acid 83.

In Figure 9 a second S1 analysis is shown in which a 542 base pair Nco I/Bgl I fragment was excised from a subclone of SK74. This fragment was 3' end-labelled and allowed to hybridize with either skeletal or smooth muscle RNA. A 380 base fragment was protected in breast muscle, indicating complete homology between RNA from this tissue and the probe. However, only a 110 base fragment was protected after hybridization to gizzard muscle RNA. This corresponds to a discontinuity in the sequence coding for amino acid 38.

Figure 9. Identification of a Difference Between Skeletal and Smooth Muscle Tropomyosin RNA in the region coding for amino acid 39.
RNA isolation and conditions of S1 analysis were done as described in Figure 8. P = Probe; S = Standards; L = Liver; B = Breast; G = Gizzard.

These two S1 analyses indicate that differences between smooth and skeletal muscle tropomyosin RNA exist at approximately the region coding for amino acid 38 and again at the region coding for amino acid 83. The simplest interpretation of this data is that sequences coding for approximately amino acids 39-83 and which are expressed in skeletal muscle are not expressed in

Figure 10. Hybridization of an Oligonucleotide containing 5' Coding Sequences to Skeletal and Smooth Muscle RNA.

RNA isolation and conditions of Northern analysis were done as described in Figure 6. A) Skeletal RNA was isolated from six different times during development and hybridized with an oligonucleotide spanning sequences coding for amino acids 46-57. B) RNA was isolated from smooth muscle at six times during development and hybridized with the same oligonucleotide as in A. C) Skeletal muscle RNA was isolated and hybridized with an oligonucleotide spanning those sequences coding for amino acids 46-57. D) RNA was isolated from smooth muscle and hybridized with the same probe as in C. The arrows represent the position of 28s and 18s ribosomal markers.

smooth muscle. In fact, analysis of the quail tropomyosin gene encoding these mRNAs indicates that sequences coding for amino acids 39-80 are encoded in a separate exon (manuscript in preparation). Taken together, the sequencing data and S1 analyses indicate the likelihood that an exon coding for amino acids 39-80 is expressed in skeletal muscle but not in smooth muscle.

Since the S1 data indicate only that differences exist between skeletal and smooth tropomyosin RNA at those sequences coding for approximately amino acids 39 and 83, there is a possibility that the sequences between these two discontinuities are the same. Therefore, an oligonucleotide was synthesized which spanned amino acids 46-57, end-labelled, and used as a probe in a Northern analysis. Figure 10 shows the results of this experiment. In Figure 10C, the probe was hybridized to skeletal muscle RNA taken from six different times of embryonic quail development. As expected, there was strong hybridization to a tropomyosin-sized RNA at all developmental times studied. In Figure 10D the probe was hybridized to smooth muscle RNA isolated from different times during development. There was essentially no hybridization, indicating that these sequences are not expressed in smooth muscle.

In a separate experiment an oligonucleotide was constructed which spanned amino acids 20-31. Figure 10A shows the results of this probe hybridized to skeletal muscle RNA. As expected, there is strong hybridization to a tropomyosin sized RNA. The same probe also hybridized to smooth muscle RNA (Figure 10B) indicating that these sequences are expressed in both skeletal and smooth muscle RNA. Thus, although sequences coding for amino acids 39-80 are differentially spliced in skeletal versus smooth muscle, coding sequences 5' to these are present in both muscle types. The additional bands hybridizing in Figure 10A, B, and C represent cross hybridization of the oligonucleotides with beta and cardiac tropomyosin mRNAs (manuscript in preparation).

Taken together, the Northern and S1 analyses strongly indicate that sequences coding for amino acids 39-83 are not expressed in smooth muscle, but are expressed in skeletal muscle.

DISCUSSION

We have isolated several different cDNA clones which contain sequences coding for alpha tropomyosin. The fact that all the cDNA clones we have sequenced to date have exactly the same sequence where they overlap between

amino acids 81-257 strongly suggests that they are products of the same gene. Comparison of a quail alpha skeletal muscle tropomyosin cDNA (SK74) with the published sequences of chicken and rat tropomyosins reveals an extraordinarily high degree of sequence conservation between the chicken and quail clones. There are only 5 differences out of 783 nucleotides where the coding regions of the quail and chicken cDNAs overlap, and all of these occur in the wobble position. None results in a change in the amino acid sequence of the alpha skeletal muscle protein. Furthermore, there are only 12 differences out of 205 bases where the 3' nontranslated regions overlap. A comparison of the quail and rat clones reveals somewhat more divergence. There are twelve amino acid differences and the coding regions share only 80% homology. The 3' untranslated regions share almost no homology.

Despite the strong similarities among the cDNA clones, there are also differences, indicating the likelihood that differential splicing is used to produce multiple mRNAs from a single tropomyosin gene. One difference, exemplified by TM682 and TM394, is in the length of the 3' untranslated sequences. TM682 continues 205 bases past the termination codon, ending with a stretch of 35 A's. TM394 has the same 205 bases, but continues for an additional 83 bases, ending with a stretch of 26 A's. TM394 contains two consensus poly(A) addition sites: one located 20 bases from the poly(A) stretch and one located 18 bases from where TM682 ends. It thus appears that the inclusion or exclusion of the last 27 amino acids, as well as the choice of poly A addition site used, results in the accumulation of at least four different mRNAs. These multiple RNAs which differ by a maximum of 160 bases could explain why the skeletal RNA bands seen on Northern gels, such as that shown in Figure 6, are reproducibly broad and diffuse.

A fifth mRNA is exemplified by SM18, which is found in smooth muscle. SM18 contains identical sequences to alpha skeletal tropomyosin from amino acid 145 to amino acid 257. The last 27 amino acids, and the entire 3' untranslated region differ completely from its alpha skeletal counterpart. It thus appears that in quail, as in rats (11), smooth muscle tropomyosin mRNA is likely derived from the skeletal muscle gene by differential splicing at the 3' end of the gene. The presence of two poly(A) addition sites 190 bases apart in SM18 correlates well with the appearance of two smooth muscle mRNAs which differ in size by 200 bases.

Ruiz-Opazo *et al.* (11) also reported that smooth muscle tropomyosin mRNA in rats differs from its skeletal muscle counterpart at the 5' end of the RNA. Although we have been unable to obtain a full length smooth muscle

cDNA, S1 mapping studies and Northern analyses indicate that, in general agreement with Ruiz-Opazo, smooth muscle tropomyosin mRNA does not use the same sequences coding for amino acids 39-80 as does skeletal muscle.

The most unusual kind of mRNA is exemplified by TM682, which is missing nucleotides coding for the last 27 amino acids. The S1 analysis data strongly suggest that mRNA with this configuration exists in skeletal muscle. The function of TM682-like mRNA is presently unclear, although it appears to be present in roughly equal proportion to the "normal" tropomyosin, as well as at all times during development. Breitbart *et al.* (12) reported that different troponin T cDNAs missing specific exons arise by a series of complex splicing events; TM682 may arise in a similar manner.

One surprising finding was that none of the alpha cDNA clones hybridize with cardiac mRNA, even though mammalian cardiac tropomyosin is identical with alpha skeletal tropomyosin (5). Clearly in avians this is not the case. In fact, cardiac tropomyosin in quails is derived from a separate tropomyosin gene, which is expressed in both cardiac and smooth muscle by differential splicing (manuscript in preparation).

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LITERATURE CITED

1. Adelstein, R.S. and E. Eisenberg. 1980. *A. Rev. Biochem.* 49: 921-956.
2. Perry, S.V. 1979. *Biochem. Soc. Trans.* 7, 593.
3. Taylor, E.W. 1979. *CRC Crit. Rev. Biochem.* 6, 103-164.
4. Cummins, P. and S.V. Perry. 1973. *Biochem. J.* 133: 765-777.
5. Cummins, P. and S.V. Perry. 1973. *Biochem. J.* 141: 43-49.
6. Chacko, S., M.A. Conti, and R.S. Adelstein. 1977. *Proc. Nat. Acad. Sci.* 74: 129-133.
7. Sobieszek, A. & J.V. Small. 1976. *J. Mol. Biol.* 101: 75-92.
8. Basi, G.S, M. Boardman, and R.V. Storti. 1984. *Cell. Biol.* 4: 2828-2831.

9. Bautch, V. and R.V. Storti. 1983. Proc. Nat. Acad.Sci. 80: 7123-7127.
10. Karlick, C.C., J.W. Mahaffey, M.D. Coutu, and E.A. Fyrberg. 1984. Cell 37: 469-481.
11. Ruiz-Opazo, N., J. Weinberger, and B. Nadal-Ginard. 1985. Nature 315: 67-70.
12. Breibart, R.E., H.T. Nguyen, R.M. Medford, A.T. Destree, V. Mahdavi, and B. Nadal-Ginard. 1985. Cell 41: 67-82.
13. Medford, R.M., H.T. Nguyen, A.T. Destree, E. Summers, and B. Nadal-Ginard. 1984. Cell 38: 409-421.
14. Devlin, R.B. and S.G. Haskell. 1983. Develop. Biol. 95: 476-483.
15. Huynh, T.V., R. Young, and R. Davis. 1984. In: DNA Cloning Techniques: A Practical Approach (ed. D. Glover). IRL Press, Oxford.
16. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). "Molecular Cloning." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
17. Ricciardi, R.P., J.S. Miller, and B.E. Roberts. 1979. Proc. Nat. Acad. Sci. 76: 4927-4931.
18. Devlin, R.B. and C.P. Emerson, Jr. 1978. Develop. Biol. 69: 202-216.
19. Devlin, R.B. and C.P. Emerson, Jr. 1977. Cell 13: 599-611.
20. Favaloro, J.R., R. Treisman, and R. Kamen. 1980. Methods in Enzymol. 68: 718-749.
21. Sanger, F. and A.R. Coulson. 1978. FEBS Letters 87: 107- 110.
22. Covington, M., D. Fleenor, and R.B. Devlin. 1983. Nuc. Acids Res. 12: 4559-73.
23. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. Proc. Nat. Acad. Sci. 74: 5463-67.
24. Dale, R.M., McClure, B.A. and Houchins, J.P. (1985). Plasmid 13, 31-40.
25. Mak, A.S., L.B. Smillie, and G.R. Stewart. 1980. J. Biol. Chem. 255: 3647-3655.
26. MacLeod, A.R. 1981. Nuc. Acids Res. 9: 2675-89.