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MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF AN ISOFORM OF CARDIAC ALPHA-TROPOMYOSIN FROM THE MEXICAN AXOLOTL (AMBYSTOMA MEXICANUM)[1]

Eileen A. Luque, Larry F. Lemanski, and Dipak K. Dube[2]

Department of Anatomy and Cell Biology, SUNY Health Science Center at Syracuse, Syracuse, New York 13210

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The cDNA for alpha-tropomyosin (TM) was cloned by the polymerase chain reaction (PCR) from a lambda gt11 library constructed with mRNA from juvenile axolotl heart tissues. Subsequently, the nucleotide sequence of the cDNA was determined. This is the first reported cDNA for axolotl alpha-tropomyosin. Comparative analyses of the deduced amino acid sequence of this cDNA with Xenopus skeletal muscle alpha-tropomyosin sequences indicate that the axolotl heart cDNA has 93% and 96% homology in the regions of amino acids 39-80 (exon 2b) and 258-284 (exon 9a), respectively. However, there is about 86.55% homology at the nucleic acid level (coding region) and 97.5% homology at the amino acid level with that of Xenopus skeletal muscle alpha-tropomyosin cDNA. Northern blot analyses with polyA+ RNA from juvenile heart suggest the presence of at least two different transcripts for alpha-tropomyosin in axolotl heart. The results of 3'-RACE concur with those of northern blot analyses.

Tropomyosins, a family of actin-binding proteins, are present in both muscle and non-muscle cells. Tropomyosins play an important role in muscle cells in regulating the interaction between actin and myosin filaments during contraction. In fact, tropomyosins are a major component of the thin filaments of organized myofibrils and in association with troponin T (TnT), troponin I (TnI) and troponin C (TnC), are involved in the calcium-dependent regulation of actin-myosin interactions in vertebrate-striated muscle (1,2,3). There are three classes of tropomyosins-cytoplasmic, smooth muscle and striated muscle-specific (4,5). Two subunits of tropomyosins viz. alpha and beta are known. In skeletal muscle, the alpha and beta chains can polymerize to form all three viz. alpha-beta, alpha-alpha and beta-beta isoforms. However, the

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[2]Correspondence should be addressed to Dr. Dipak K. Dube, Department of Anatomy and Cell Biology, SUNY HSC at Syracuse, 750 East Adams Street, Syracuse, New York 13210. FAX: (315) 464-8535.

ABBREVIATIONS: TM - alpha-tropomyosin; TnT - troponin T; TnI - troponin I; TnC - troponin C; UTR - untranslated region; PCR - polymerase chain reaction; AP - adapter primer; RACE - Rapid Amplification of the cDNA Ends; TnI, TnT and TnC - troponin complex.

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molar ratios of dimeric species may vary in different skeletal muscles. In adult cardiac muscle of small mammals, only the alpha chain is known to be expressed (6,7).

In mammalian systems, four different tropomyosin (TM) genes have been characterized in detail. The α and β genes are named because they encode alpha and beta TM in striated muscle respectively (8-11). The TM-4 and hTMnm genes are named after the rat fibroblast TM-4 and human fibroblast TM30nm isoforms respectively. Through alternate splicing, the α gene expresses at least nine different isoforms of tropomyosin whereas the β and htMnm genes express two distinct isoforms. On the contrary, the TM-4 gene encodes only one isoform. There is a paucity of knowledge regarding the functional significance of different isoforms of tropomyosin. The expression of diverse isoforms of tropomyosin are highly tissue specific suggesting that each of the isoforms may be involved in performing specific functions in the actin-based filaments of various muscle and non-muscle cells.

An excellent model for studying heart development is the cardiac mutant (gene c) axolotl, *Ambystoma mexicanum*. The hearts of homozygous (c/c) mutant embryos do not contain myofibrils and as a result, the hearts fail to beat (12). Electron microscopic, immunohistochemical and biochemical analyses suggest that mutant axolotl hearts are deficient in tropomyosin even though the concentrations of actin and myosin are comparable to those of wild type (13-14).

We have undertaken the present study of axolotl cardiac tropomyosin to assess the level of expression as well as functional roles of the different tropomyosin isoforms in normal and cardiac mutant axolotls. Recently, it has been reported that the 3'-untranslated region (UTR) of α-tropomyosin can function as a regulator (riboregulator), presumably through a trans-acting mechanism, that augment muscle differentiation (15). In order to understand the roles of various tropomyosin isoforms during myofibrillogenesis in axolotl heart under normal and pathological conditions, detailed sequence information on the coding as well as non-coding regions, including 3'-UTR, is essential. We now report the presence of two isoforms of α tropomyosin in axolotl heart. The sequence analyses of the cDNAs suggest that the 3'-UTRs of the two isoforms are different.

MATERIALS AND METHODS

Molecular cloning and DNA sequence analyses: An expression cDNA library of juvenile axolotl heart RNA was constructed using oligo dT and the random priming technique in λ gt11 (16). The library was screened by polymerase chain reaction (PCR) using primer pairs following the strategy outlined in Fig. 1. The primer-pairs were designed from the conserved regions of various cDNA sequences of tropomyosin available in the data bases. The oligonucleotides were procured from Integrated DNA Technologies, Inc., Coralville, IA; and the sequences of various oligonucleotides are given in Table 1. Prior to amplification by PCR with different primer-pairs, an aliquot of the λ gt11 library was heated in a boiling water bath for at least 3 minutes and then chilled rapidly on ice. The PCR reaction was carried out according to the published procedure using the Amplitaq PCR kit with Taq polymerase from Perkin-Elmer/Cetus Corporation. Amplification was accomplished in a total volume of 100μl assay containing 10mM Tris-HCl, pH 8.3; 2.5 mM $MgCl_2$, 50 mM KCl, 270 μM of each of dATP, dGTP, dCTP and dTTP, 3 units of Taq polymerase, 20 pmol of each of two primer-pairs. The amplification was carried out for either 45 or 60 cycles in a thermocycler. If necessary, the extension time was increased up to 2 minutes (depending on the length of the amplified DNA) at 78°C. All pre and post PCR analyses were done in separate rooms with different sets of equipment (17).

Southern blot analyses of amplified DNA: All amplified DNAs were subjected to Southern blot hybridization with [^{32}P]-end labelled detector oligonucleotides designed from the conserved region of the target DNA flanked by the primer-pair used for amplification (Table 1).
Cloning and sequencing of the amplified DNA: The PCR amplified DNA or the amplified DNA of 3' or 5'- RACE reactions were cloned into a PCR™1000 vector (Invitrogen) following the manufacturer's specifications. The ligation was carried out with T_4 DNA ligase according to the instructions of the manufacturer. Subsequent transformations, colony hybridizations to pick up the desired clones and preparation of DNA templates were performed as described earlier (18). DNA sequencing was carried out with double-stranded DNA using Sanger's di-deoxy chain termination method (19) employing the Sequenase Version 2.0 kit (USB) as described earlier.
Isolation of total RNA from juvenile axolotl heart: Total RNA from juvenile heart was isolated using a Strategene RNA isolation kit according to the manufacturer's specifications. Poly A+ RNA was isolated from 10 juvenile hearts using the Invitrogen Fast Track Kit following the protocol supplied by Invitrogen Inc.
Northern blot analysis: Poly A+RNA (2-5μg) was electrophoresed on denaturing agarose gel (19) with the appropriate RNA size marker (rat liver rRNA from Gibco-BRL) and blotted onto Gene Screen Plus nylon membrane. The blot was hybridized with [^{32}P]-labelled probe prepared by PCR amplification of an axolotl tropomyosin cDNA clone using primer-pair 3(+) and 2(-) as shown in Table 1. The amplification was carried out as described above except the dATP used here was [α-^{32}P]dATP (NEN-Dupont; 6000 Ci/mol). Hybridization and subsequent washings were carried out following standard protocols. The final washing of the blot was performed twice with 0.2 x SSPE and 1% SDS at 65°C for 15 minutes each.
3'-RACE system for rapid amplification of cDNA ends: First strand DNA synthesis was initiated at the poly A+ tail of mRNA using the adapter primer (AP) provided with the kit. After first strand DNA synthesis, the original mRNA template was destroyed with RNase H. Amplification was then performed using two primers, according to diagram 1B; one was a tropomyosin specific primer, Exon-IX-P-1, and the other was a universal amplification primer (UAP) supplied by the manufacturer. Following amplification the PCR products were subjected to Southern blot analyses and also were cloned directly into a T/A cloning vector (Invitrogen) as described above.
5'-RACE system for rapid amplification of cDNA ends: First strand cDNA was synthesized from poly A+ RNA using a tropomyosin specific primer 11(-) as outlined in Fig. 1B with SuperScript II. After first strand cDNA synthesis, the original mRNA template is destroyed with RNase H. An anchor sequence was then added to the 3' end of the cDNA using TdT and dCTP. PCR amplification was accomplished using Taq DNA polymerase, a tropomyosin specific primer 9(-) that anneals to a site located within the cDNA molecule, and a novel deoxyinosine-containing anchor primer provided by the manufacturer. The PCR amplified DNA was used for southern blot analyses using the tropomyosin specific probe [^{32}P]-labelled oligonucleotide; 9(+) and another aliquot was cloned into T/A cloning vector as described earlier (18).

RESULTS AND DISCUSSION

α-tropomyosin cDNA from a juvenile axolotl heart specific cDNA library was amplified using the polymerase chain reaction (PCR) technique. Primers were designed from conserved sequences of various tropomyosin cDNA sequences available from the data bases. The strategy for PCR amplification is outlined in Fig. 1. The sequences of different primers used are given in Table 1. The sequences of the two ends of the cDNAs were determined by Rapid Amplification of the cDNA Ends (RACE) which is a procedure for amplification of nucleic acid sequences from the 3' or 5' end of the mRNA template (Fig. 1B).

The nucleotide and deduced amino acid sequences are shown in Fig. 2. The nucleotide sequence surrounding the translational initiation codon agreed with the consensus sequence for the eukaryotic initiation codon by Kozak (20). A termination codon, UAA, was found after the 284th codon. Accordingly, the open reading frame is nucleotide residues coding for 284 amino acid residues which is very similar to that of Xenopus α-tropomyosin (21). The 3'-untranslated region

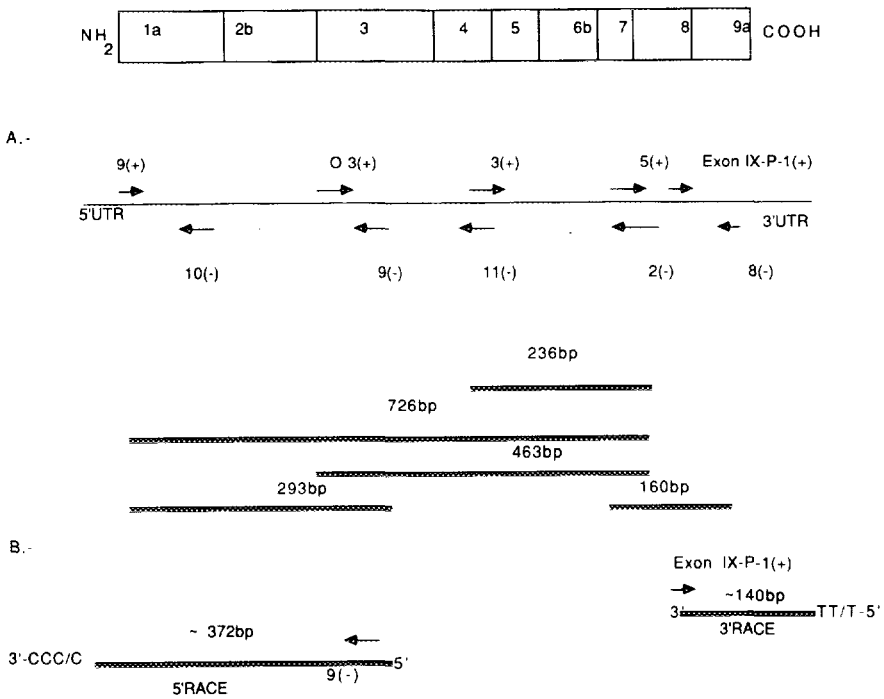

Fig. 1.
Strategies for amplification of alpha-tropomyosin cDNA by polymerase chain reaction from juvenile axolotl heart cDNA library.
Top: Organization of different exons for alpha-tropomyosin mRNA.
A. Positions of different primer-pairs used for PCR amplification. The sequences of the oligonucleotides used for the primer-pairs are listed in Table 1.
B. Positions of the oligonucleotides used for the amplification of the 5'- and 3'-ends of alpha-tropomyosin cDNA from Poly A+ RNA from juvenile axolotl heart. The sequences of the oligonucletides used in 5'- and 3'-RACE systems are given in Table 1.

Table 1

Primer	Length	Sequence
TM3 (+)	26MER	5'-AGG TCG CTC GTA AGC TGG TGA TCA TT-3'
TM5 (+)	23MER	5'-AAC TGA AGG AGG CTG AAA CAC GT-3'
TM9 (+)	21MER	5'-ATG GAC GCC ATC AAG AAG AAG-3'
TM2 (-)	36MER	5'-CTG CAA ACT CAG CAC GTG TTT CAG CCT CCT TCA GTT-3'
TM8 (-)	26MER	5'-TAT TGA AGT CAT GTC ATT GAG AGC GT-3'
TM9 (-)	20MER	5'-ACG CTC CTG AGC ACG ATC CA-3'
TM10 (-)	20MER	5'-TTG TCC TC(GI) GCT CCC TTC TT-3' (INOSINE)
TM11 (-)	20MER	5'-TAC G(AT)G C(AG)A CCT CTT CAT AT-3'
RT-PCR Specific Primer 3'RACE		
Exon-9-P-1(+)	19MER	5'-GAT GAG TTG TAT GCT CAG A-3'

Sequences of the oligonucleotides used for the PCR amplification of tropomyosin cDNA for screening the λ gt11 library and for the 5'- & 3'-RACE systems as outlined in Fig 1.

```
   1  tactcagctcccgcctaaccgcacatcagctcgcaccgctcaccgcccggccccaccaca

              M  D  V  I  K  K  K  M  Q  M  L  K  L  D  K  E  N     17
  61  cccgccgccatggacgtcatcaagaagaagatgcagatgctcaagctcgacaaggaaaat

      A  M  D  R  A  E  Q  A  E  A  D  K  K  G  A  E  D  K  S  K     37
 121  gccatggaccgggcagagcaggcggaggcagacaagaagggagccgaggacaagagcaag

      Q  L  E  D  E  L  V  A  L  Q  K  K  L  K  G  T  E  D  E  L     57
 181  cagcttgaggacgagctagtagccctgcagaagaagctgaagggtaccgaggacgagttg

      D  K  Y  S  E  S  L  K  D  A  Q  E  K  L  E  L  A  D  K  K     77
 241  gacaagtactcggagtccttgaaagatgcacaggagaagttggagcttgcagacaagaag

      A  T  K  A  E  S  D  V  A  S  L  N  R  R  I  Q  L  V  E  E     97
 301  gccactaaggctgagagtgatgtagcctccctgaacaggcgtatccagctggttgaggag

      E  L  D  R  A  Q  E  R  L  A  T  A  L  Q  K  L  E  E  A  E    117
 361  gagttggatcgtgctcaggagcgtctggccacagccctacaaaagctggaggaggctgaa

      K  A  A  D  E  S  E  R  G  M  K  V  I  E  N  R  A  L  K  D    137
 421  aaggctgctgatgagagtgagagaggaatgaaggtcattgaaaacagggccttaaaggat

      E  E  K  M  E  L  Q  E  I  Q  L  Q  E  A  K  H  I  A  E  E    157
 481  gaagaaaagatggaactgcaagaaattcaacttcaagaggcaaaacacattgctgaagag

      A  D  R  K  Y  E  E  V  A  R  K  L  V  I  I  E  G  D  L  E    177
 541  gctgacagaaaatatgaagaggttgctcgtaagctggtgatcattgagggtgatctggag

      R  A  E  E  R  A  E  L  S  E  G  K  C  A  E  L  E  E  E  L    197
 601  cgtgcggaagaaagggcagaactctctgaaggcaaatgtgctgaacttgaagaagaattg

      K  T  V  T  N  N  L  K  S  L  E  A  Q  A  E  K  Y  S  Q  K    217
 661  aaaactgttaccaacaacctgaagtcactggaggctcaggctgaaaagtactcccagaag

      E  D  K  Y  E  E  E  I  K  V  L  T  D  K  L  K  E  A  E  T    237
 721  gaagacaagtatgaggaggaaattaaggtcctgaccgacaaactgaaggaggctgaaaca

      R  A  E  F  A  E  R  T  V  A  K  L  E  K  S  I  D  D  L  E    257
 781  cgtgctgagtttgcggagaggacagtagccaaactggaaaagagcattgatgacttggaa

      D  E  L  Y  A  Q  K  L  K  Y  K  A  I  S  E  E  L  D  H  A    277
 841  gatgagttgtatgctcagaagctgaagtacaaagcaatcagcgaagaactggaccatgct

      L  N  D  M  T  S  M  *                                         284
 901  ctcaacgatatgacttcaatgtaaatgttttcttacgtgtccgtccgtcttctgtaccct

 961  cctactttgcctaataaactcacggctaccctt  993
```

Fig. 2.
Nucleotide and deduced amino acid sequences of Axolotl heart alpha-tropomyosin cDNA. Bases underlined indicate translation initiation site (atg at the beginning), termination codons (taa) and polyadenylation signal, aataaa. The amino acids changes when comparing with *Xenopus Laevis* skeletal alpha-tropomyosin are in bold type. The axolotl tropomyosin sequence has been submitted to the Database Genebank (accession code pending).

is 60 nucleotides long and has the polyadenylational signal AATAAA. Like many structural proteins where isoform diversity is a common feature, the expression of various isoforms of tropomyosin involves both the use of multiple genes and by alternate processing of RNA. Four different tropomyosin genes have been characterized in vertebrates. Each of the genes have been named after the proteins they encode. The α and β genes are named after striated muscle α and β-TMs respectively. The α-TM gene is the most complex one and encodes at least nine isoforms. In striated muscle mRNA (both skeletal and cardiac), ten (1a, 2b, 3, 4, 5, 6b, 7, 8, 9a and 9b) out of a total of 15 exons of α-TM genes are present. However, exon 9a is used only in striated muscle (skeletal and cardiac) and exon 9b is spliced to exon 9a and provides only the

3'-untranslated region. It should be noted that all vertebrate tropomyosins consist of highly conserved core sequences encoded by exons 3, 4, 5, 7, and 8. In the case of axolotl heart tropomyosins, there are 13 non-synonymous changes and as a consequence there are approximately 10 changes in the amino acid sequence. Major differences occur in different tropomyosin isoforms due to the alternate splicing of exons. However, sequences contained within some alternatively spliced exons are highly conserved among various species. The deduced amino acid sequences suggest that the isoforms of tropomyosin derived from exons 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a are equivalent to the rat α-tropomyosin gene. The amino acid sequences of exon 2b and exon 9a are given in Fig. 3. The amino acid sequences of exon 2b (amino acid residues 39-80) are 92.5% and 90.5% similar to those of Xenopus and rat skeletal muscle α-tropomyosin sequences.

On the contrary, the amino acid sequence of exon 9a (amino acid residues 258-284) is 96% and 88% similar with those of rat and Xenopus skeletal muscle α-tropomyosins respectively. In the case of axolotl heart, the last amino acid is methionine whereas it is isoleucine in both rat and xenopus skeletal muscle α-tropomyosin. Tropomyosin in skeletal and cardiac muscles functions in association with the troponin complex (TnI, TnT and TnC) to regulate the calcium sensitive interactions of actin and myosin. In skeletal and cardiac muscles, TnT interacts with two regions on the tropomyosin molecule, one located in close proximity to Cys190 (exon 6b), and the other at the COOH-terminal of the protein (exon 9a). In the axolotl heart cDNA, a Cys190 and the amino acid residues 189-213 are well conserved. Also, the sequence of the COOH-terminal region with the exception of the last amino acid is well conserved. In axolotl heart TM-cDNA, Ile284 has been replaced by Met which is a conservative replacement. Among the other changes, Ala_3-Val,

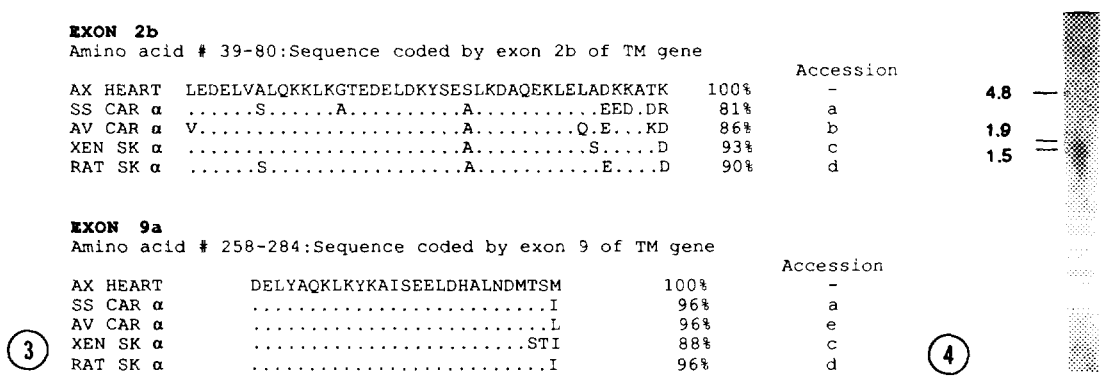

Fig. 3.
Comparison of the amino acid sequences encoded by exons 2b and 9a from different vertebrates. The sequences of the axolotl heart are listed on the top. Percentage comparison at the right of corresponding sequence comparison of exon 2b and 9a. Abbreviations include (AX) axolotl , (SS) pig, (AV) quail, (RAT) rat, (CAR) cardiac and (SK) skeletal. Accession file code a: X66274; b: X54278; c: X61273; d: M24634; and e: X54281.

Fig. 4.
Northern blot analysis of RNA from juvenile axolotl heart. The band appears at 1.5 kb.

Leu_{63}-Met in exon 1a; Gly_{83}-Ser, Ser_{107}-Ala in exon 3; Lys_{149}-Gln in exon 4; Ser_{188}-Gly in exon 5 are notable.

To study the expression of the axolotl α-TM gene, total RNA and poly A+ RNA of axolotl juvenile hearts were analyzed by northern blotting with radiolabelled PCR amplified [with primer-pairs 3(+) and 2(-)] DNA probe and using stringent washing conditions (at 65°C in 0.2 SSPE). At least two mRNA bands about 1.5 kb and above were detected in the axolotl heart tissue (Fig. 4). It is interesting to note that we have detected two different untranslated regions for the α-TM cDNA in axolotl heart by using a 3'-RACE system with poly A+ RNA from juvenile heart. This untranslated region is much longer than the one presented in Fig. 2. The polyadenylation signal AATAAA has also been detected (data not shown). The last exon of this TM-mRNA is also similar to exon 9a; with the only difference in the amino acid sequences being the replacement of Met284 by Ile. Although there are a few changes in the nucleic acid sequences in exon 9a, the amino acid sequences in general are well conserved. The 3'-RACE reaction as well as northern blotting suggest that there is more than one α-TM transcript expressed in juvenile heart. The sequence analyses of products of the 3'-RACE reaction suggest the expression of more than one α-TM-gene in axolotl heart (data not shown). Further studies using RT-PCR and in situ hybridization with transcript specific probes are currently in progress to provide more detailed information on the expression of various isoforms of α-TM in normal and mutant axolotl hearts.

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