Journal of Cellular Biochemistry 89:427–439 (2003)

Identification, Characterization, and Expression of a Novel α-Tropomyosin Isoform in Cardiac Tissues in Developing Chicken

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Abstract Tropomyosins are present in various muscle (skeletal, cardiac, and smooth) and non-muscle cells with different isoforms characteristic of specific cell types. We describe here a novel smooth/striated chimeric isoform that was expressed in developing chick heart in addition to the classically described TM-4 type. This novel α-Tm tropomyosin isoform, designated as α-Tm-2, contains exon 2a (in place of exon 2b). The known striated muscle isoform (α-Tm-1) was also expressed in embryonic hearts along with the striated muscle isoform of *TM-4*. In adult heart, *TM-4* was expressed, however, expression of both α-Tm-1 and α-Tm-2 isoforms was drastically reduced or downregulated. Interestingly, we were unable to detect the expression of α-Tm-2 in embryonic and adult skeletal muscle, however, the α-Tm-1 isoform is expressed in embryonic and adult skeletal muscle. Examination of other possible isoforms of the α-*TM* gene, i.e., α-smooth muscle tropomyosin (α-Sm), α-Fibroblast-1 (α-F1), and α-Fibroblast-2 (α-F2) revealed expression in embryonic hearts and a significant reduction of each of these isoforms in adult heart. In order to elucidate the role of the newly discovered tropomyosin isoform in chicken, we ectopically expressed the GFP fusion protein of α-Tm-1 and α-Tm-2 separately into cardiomyocytes isolated from neonatal rats. Each isoform was incorporated into organized myofibrils. Our results suggest that the α-*TM* gene may undergo both positive and negative transcriptional control in chicken hearts during development. J. Cell. Biochem. 89: 427–439, 2003.

Key words: heart; α-Tm-1; α-Tm-2; skeletal muscle

INTRODUCTION

The striated muscle isoform of α-TM is known to be expressed in cardiac tissues in mammals and other vertebrates, but not in avian species [Lindquester et al., 1989]. Tropomyosins are present in various muscle (skeletal, cardiac, and smooth) and non-muscle cells. However, different forms of the protein are characteristic of specific cell types. Four tropomyosin genes have been characterized in the vertebrates: α-*TM*, β-*TM*, *hTMnm*, and *TM-4*. The α-*TM* gene encodes at least ten isoforms, the β-*TM* gene is known to encode three, while the *hTMnm* and *TM-4* each encode two isoforms. In vertebrate striated muscles, including those from frog, avian, and fish, TM is encoded by exons 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a/b of the α-*TM* gene [Lin et al., 1985; Helfman et al., 1986; Wieczorek et al., 1988; Lindquester et al., 1989; Lees-Miller and Helfman, 1991]. It is now well established that vertebrate cardiac tissue produces both striated (sarcomeric) and smooth muscle type tropomyosin. In addition, smaller isoforms of tropomyosin (cytoplasmic and fibroblastic) of 248 amino acid residues are also formed in cardiac tissues. Only the striated muscle isoform derived from the *TM-4* type gene is known to be expressed in avian cardiac tissues

Robert W. Zajdel and Christopher R. Denz share the first authorship.

Grant sponsor: American Heart Association (New York Affiliate); Grant sponsor: American Heart Association (National).

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Received 12 November 2002; Accepted 23 January 2003
DOI 10.1002/jcb.10504

[Fleenor et al., 1992]. In amphibian systems (both salamander and frog), striated muscle isoforms of *α-TM* and *TM-4* genes have been reported to be expressed [Luque et al., 1994, 1997; Hardy et al., 1995; Spinner et al., 2002]. In mammalian cardiac tissues, both *α-TM* and *β-TM* express the corresponding striated muscle isoforms.

Using Northern blot analysis and RNase protection assay, the *α-TM* gene has been characterized as generating at least ten isoforms via alternate splicing of 15 exons [Nadal-Ginard, 1990; Lees-Miller and Helfman, 1991; Pittenger et al., 1994]. One of the ten comprised exons 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a/b and is thought to be the only striated muscle specific tropomyosin. We have previously described another striated muscle isoform that contains exon 2a in place of exon 2b (ATmC-2 or α-Tm-2) from the cardiac tissues of the Mexican axolotl, *Ambystoma mexicanum* [Luque et al., 1997]. Recently, Cooley and Bergstrom [2001] confirmed our earlier results with the description of another isoform of α-TM containing exons 1a, 2a (instead of 2b), 3, 4, 5, 6b, 7, 8, 9a/b found in rat tissues. In this study, the novel tropomyosin isoform α-Tm-2 was found to be expressed in chicken embryonic heart along with the α-Tm-1 isoform and the striated muscle isoform of *TM-4*. Both of the isoforms from the *α-TM* gene were not expressed in adult heart, only the *TM-4* isoform was. We were unable to detect the expression of α-Tm-2 in adult heart, skeletal muscle, and adult skeletal muscles. In addition, we have evaluated the expression of α-smooth muscle (α-Sm), α-fibroblast-1 (α-F1), and α-fibroblast-2 (α-F2) isoforms in hearts from embryonic and adult chickens. Structural and functional use of the chicken α-Tm-2 isoform was examined in two models with incorporation of exogenous GFP α-Tm-2 fusion protein into striated cardiac myofibrils in neonatal rat cardiomyocytes in culture and also in axolotl whole hearts.

MATERIALS AND METHODS

Isolation of RNA From Adult Chicken Tissues

Various organs of adult chicken (Cornish Cross), for example, heart, liver, gizzard, and breast skeletal muscle, were procured from a local farm (Cobblestone Valley Farm, Preble, NY 13141). The desired organs were surgically removed immediately after the sacrifice of the adult birds and rinsed with ice-cold saline. The washed organs were immediately put in liquid nitrogen. The frozen tissues were ground in a mortar pestle in the presence of liquid nitrogen. RNA was extracted using Ambion's RNeasy kit following manufacturer's specification. The concentration of the isolated RNA was determined by measuring the absorbance in a spectrophotometer at 260 and 280 nm.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR with RNA from embryonic and adult chicken tissues were carried out with gene and isoform specific primer-pairs (as shown in the legends of Fig. 1 or under the corresponding figure) was performed to evaluate the expression of various isoforms of tropomyosin. As a control, we also performed RT-PCR for the *GAPDH* gene with gene specific primer-pair. After PCR amplification, Southern blot analysis was performed with gene or isoform specific detector oligonucleotides (Fig. 1) as described earlier [Luque et al., 1994, 1997; Spinner et al., 2002].

DNA Sequencing

DNA sequences were determined at the Cornell University DNA sequencing facility, Ithaca, NY.

Preparation of the pEGFP.α-Tm-1 and pEGFP.α-Tm-2 Expression Constructs

We used our published strategy to make these expression constructs [Zajdel et al., 2002]. α-Tm-1 and α-Tm-2 were amplified by using 5′-TCGGAATTCATGGATGCCATCAAGAAG-3′ (+ve) and 5′-ACGAATTCAAATTATATGGA-AGTCATATCG-3′ (−ve) primer-pair. The GFP was driven by a CMV promoter and the TM was linked in frame to the carboxy terminal end of the GFP. We added an *Eco*RI site at the 5′-end of both positive and negative primers. The amplified DNA was digested with *Eco*RI and the digested DNA was then gel purified. (Quiagen, Inc.). At the same time, we digested the pEGFP plasmid with *Eco*RI also. The digested plasmid was further digested with BAP. Ligation was carried out with T4 DNA ligase. The mix was then used to transform competent *E. coli* cells (Invitrogen). Subsequent colony hybridization was carried out using isoform specific oligonucleotide end-labeled with ^{32}P.

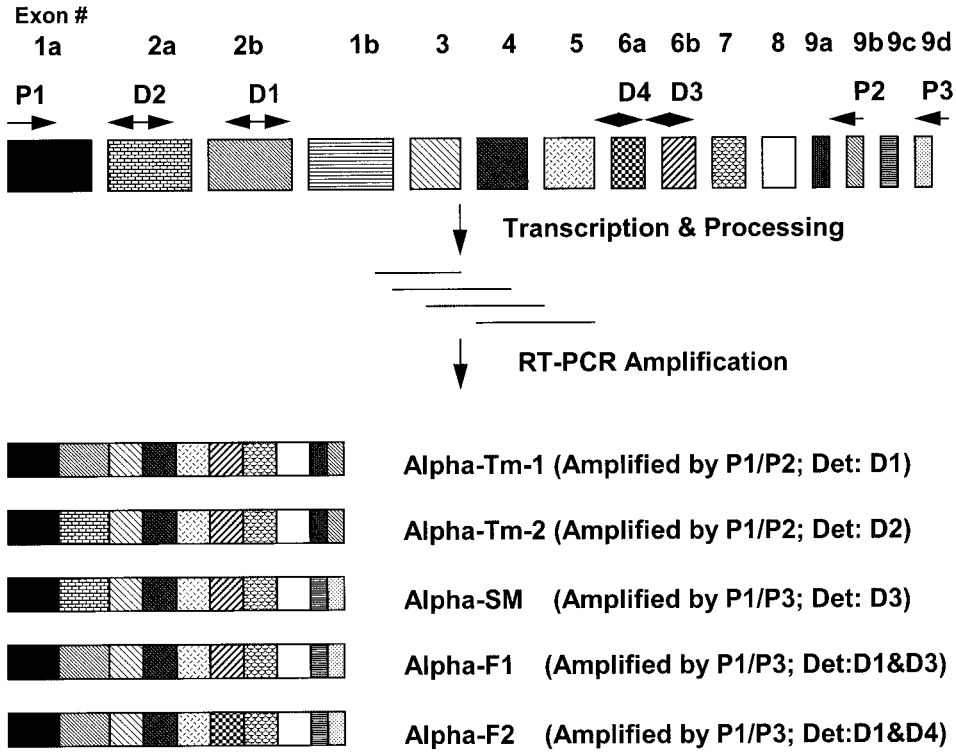

Fig. 1. Splice variants of α-*TM* gene leading to α-Tm-1, α-Tm-2, α-SM, α-Fb-1, and α-Fb-2 isoforms and scheme for RT-PCR amplification. Sequences of the primer-pairs and detector oligonucleotides for the amplification and detection of various chicken α-Tm isoforms used in this study are: P1(+): 5′-ATG GAT GCC ATC AAG AAA AAG ATG-3′; P2(−): 5′-AAA TTA TAT GGA AGT CAT ATC G-3′; P3(−): 5′-AAG GTT TTC ACA TGT TGT TTA A-3′; D1(+): 5′-TCC GAG TCC CTT AAA GAT GCA-3′; D2(+): 5′-CTG GAA GAG CTG CAC AAG-3′; D3(+)3: 5′-TGT GCT GAG CTT GAA GAG GAG-3′; D4(−): 5′-TAT TCT TAA CTG TTC TTC-3′.

Isolation of RNA From Embryonic Chicken Hearts and Skeletal Muscles

The fertile eggs (Leghorn) were incubated at 37°C for 10–15 days. Specific tissues were dissected free and placed in liquid nitrogen. The frozen tissues were ground in a mortar pestle in the presence of liquid nitrogen. The RNA was extracted using Ambion's RNeasy kit following the manufacturer's specification. The concentration of the isolated RNA was determined by measuring the absorbance in a spectrophotometer at 260 and 280 nm.

Culture of Neonatal Rat Cardiomyocytes, Transfection, and Confocal Microscopy

Primary cultures of neonatal rat cardiomyocytes were isolated and cultured as described previously [Auerbach et al., 1999]. One day after plating, cells were transiently transfected using Escort III (Sigma) according to the manufacturer's specification and then cultured in the Maintenance medium (20% medium M199, 75% DBSS-K [6.8 g/L NaCl, 0.14 mM NaH_2PO_4, 0.2 mM $CaCl_2$, 0.2 mM Mg_2SO_4, 1 mM dextrose, 2.7 mM $NaHCO_3$]) 4% horse serum, 4 mM glutamine, 1% penicillin/streptomycin, and 0.1 mM phenylephrine for 48 h. After fixing with 4% paraformaldehyde in PBS for 10 min at room temperature, immunofluorescence experiments were carried out as described previously [Auerbach et al., 1999].

The specimens were analyzed on a confocal microscope system using an inverted microscope DM IRB/E equipped with a true confocal scanner TCS NT, a PL APO 63 ×/1.32 oil immersion objective (Leica) as well as an argon-helium neon laser. Image processing was done on a Silicon Graphics workstation using Imaris (Bitplane AG), a three-dimensional multichannel image processing software specialized for confocal microscopy data sets [Messerli et al., 1993].

The monoclonal antibody against myomesin was characterized in Dr. Perriard's laboratory [Grove et al., 1984]. Secondary antibodies

Cy5-conjugated anti-mouse IGs (Jackson Immunochemicals) were used together with TRITC-phalloidin to visualize actin (Molecular Probes).

RESULTS

The avian α-*TM* gene has been found to encode for skeletal muscle, smooth muscle, and cytoskeletal isoforms using both isoform specific and common exons [Fleenor et al., 1992]. Figure 1 depicts the organization of the α-*TM* gene and also the various isoforms that are generated via alternate splicing. It also shows the strategy we have used for amplification of various isoforms by RT-PCR using isoform specific primer-pairs and detector oligonucleotides. It was believed that the avian α-*TM* gene is not expressed in cardiac muscle, however, we have identified a novel splice variant of the gene with smooth muscle type exon 2a (designated α-Tm-2) from embryonic chicken cardiac tissue (Fig. 1) as well as the striated (exon 2b) isoform α-Tm-1. Using a highly sensitive RT-PCR assay with isoform specific primer-pairs and detector oligonucleotides, we have found transient expression of α-*TM* in the developing hearts of 10- and 15-day-old chicken embryos (Fig. 2). Figure 2a shows an agarose gel of the PCR product obtained with the primer-pair [(P1(+)/P2(−)] that can amplify both α-Tm-1 and α-Tm-2. The α-Tm-1 isoform was the only striated muscle specific isoform from the α-*TM* gene known so far; α-Tm-2 is the novel isoform. Interestingly, both α-Tm-1 and α-Tm-2 are expressed only in embryonic hearts (lane 1 in Fig 2a: 10-day-old embryonic heart; lane 2: 15-day-old embryonic heart; lane 3: adult heart). This is also evident from the hybridization data with α-Tm-1 specific probe (Fig. 2b) and α-Tm-2 specific probe (Fig. 2c). No expression was observed in adult heart (lane 3). Results of RT-PCR using *GAPDH*, a house-keeping gene, suggested that the quality as well as the quantity of the total RNA we used in these experiments was sufficient.

We carried out RT-PCR using primer-pairs that can amplify the full coding sequences of various α-Tm isoforms. The next step was to clone and determine the nucleotide sequences of the various cDNAs. We cloned each of the PCR products into a T/A cloning vector (Invitrogen) and isolated the clones using filter hybridization with isoform specific probes. Once we

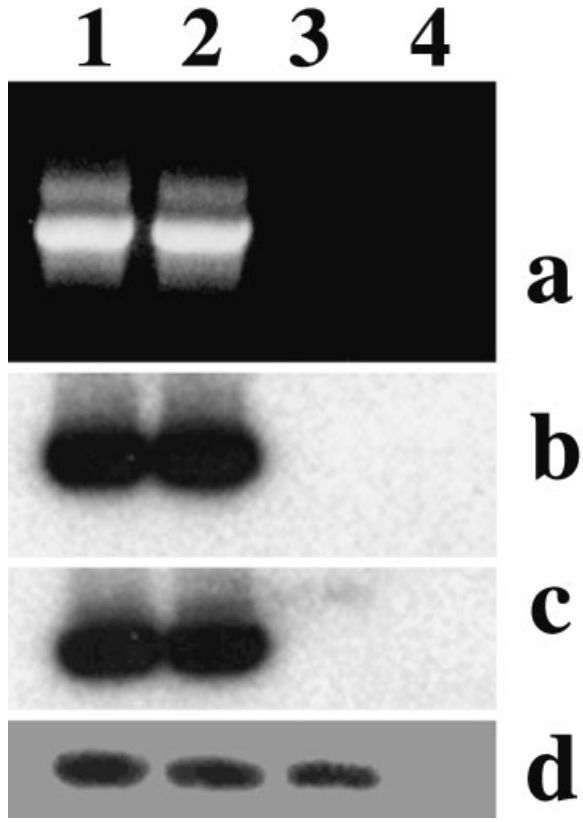

Fig. 2. Identification of a novel isoform of the α-*TM* gene that expresses in developing chicken hearts. RT-PCR was carried out with the total RNA from hearts of 10 days old, 15 days old, and adult chickens following the scheme outlined in Figure 1. P1(+) [5′-ATG GAT GCC ATC AAG AAA AAG ATG-3′] and P(−2) [5′-AAA TTA TAT GGA AGT CAT ATC G-3′] primer-pair was used to amplify both α-Tm-1 and α-Tm-2. The PCR products were subjected to Southern blot analysis following out standard protocol [Luque et al., 1997]. The blots were hybridized separately with two different oligonucleotides, viz. D1 for α-Tm-1 (5′-TCC GAG TCC CTT AAA GAT GCA-3′) and D2 (5′-CTG GAA GAG CTG CAC AAG-3′) for α-Tm-2. In this experiment, we also amplified GAPDH as a control for the normalization of the RNA used. **a**: It represents the ethidium staining of the amplified product in which one can not differentiate between α-Tm-1 and α-Tm-2. **Lane 1**: RNA from 10-day-old heart; **lane 2**: RNA from 15-day-old heart; **lane 3**: RNA from adult chicken heart; **lane 4**: primer control. **b**: It represents the hybridization with D1 oligonucleotide which is the probe for α-Tm-1 and it can not detect α-Tm-2. The results suggest that α-Tm-1 is expressed both in 10- and 15-day-old hearts. No expression of α-Tm-1 was detected in adult heart (**lane 3**). **c**: It represents the hybridization with D2 oligonucleotide which is the probe for α-Tm-2 and does not hybridize with α-Tm-1. The hybridization data strongly suggest that α-Tm-2 is expressed in embryonic hearts (**lanes 1** and **3**) but not in adult hearts (**lane 3**). **d**: It represents the Southern hybridization of GAPDH (house-keeping gene) which was amplified by GAPDH specific primer-pair [ChiGAPDH (+) 5′-GTCTCCTGTGACTTCAAT-3′ and ChiGAPDH (−) 5′-ACA-GATCAGTTTCTATCA-3′] and subsequently Southern blotted with its specific detector oligonucleotide [ChiGAPDH (+) 5′-ATGACAATGAGTTTGGAT-3′] as stated under Materials and Methods.

obtained the positive clones, DNA was isolated from multiple colonies for each isoform and the nucleotide sequence was determined from both sides of the insert.

Sequence analysis of chicken α-Tm-2 (Fig. 3) reveals 100% homology with α-Tm-1 (originally defined as the only striated muscle isoform) except at exon 2. Chicken α-Tm-2 contains smooth muscle type exon 2a instead of the striated muscle type exon 2b found in α-Tm-1. A comparison of exon 2a from chicken, axolotl (Mexican salamander), human, and rat α-Tm-2 homologs shows a high degree of homology among species (Fig. 4).

The predominant cardiac isoform of TM in the avian system has been found to be a splice variant of the *TM-4* gene with the *α-TM* gene product being predominant in striated and smooth muscle tissues. To test this supposition, RNA was extracted from adult chicken tissues followed by RT-PCR with oligo dT. Using the tissue specific cDNAs, PCR was carried out with α-Tm primer-pairs flanking the coding region. Ethidium staining (Fig. 5a) revealed high levels of amplification of α-Tm in adult skeletal muscle with levels dropping off in adult gizzard and liver. No amplification was observed in adult heart. These data are in agreement with previous findings that suggest that no α-Tm is expressed in avian cardiac tissue and that α-Tm is the predominant skeletal muscle isoform. Southern hybridization with α-Tm-1 (exon 2b) specific probe (Fig. 5b) confirmed the presence of the striated muscle isoform of α-Tm-1 in skeletal muscle, with minor expression in liver and gizzard. The expression of α-Tm-1 was totally lacking in adult heart.

Again, using an α-Tm primer-pair, PCR amplification was carried out with cDNAs from adult and 15-day embryonic heart, as well as 15-day skeletal muscle. Ethidium staining shows α-Tm expression in 15-day heart and skeletal muscle, but not in adult heart tissue (Fig. 6a). Southern hybridization with α-Tm-2 (exon 2a) specific probe (Fig. 6b) indicates expression of the α-Tm-2 isoform in 15-day heart tissue, but not in 15 skeletal muscle. This supports our hypothesis that the α-Tm-2 isoform is expressed briefly in cardiac tissues of developing chickens and then becomes down-regulated.

Restriction analysis of the cDNAs of α-Tm-1 and α-Tm-2 suggest the presence of a unique *Ava*II restriction site in exon 2a of α-Tm-2, which is absent from the α-Tm-1 (exon 2b). Our nucleic acid sequence data is in agreement with the sequence of the published *α-TM* gene [Lemonnier et al., 1991]. We exploited this *Ava*II site for differentiating α-Tm-1 and α-Tm-2 cDNA, which can be amplified by the same primer-pair. We digested the cDNA products obtained after amplification of stage 15 heart RNA by RT-PCR with P1(+) and P2 (−) primer-pair. The digested DNA (lane 2, Fig. 7a) was then subjected to agarose gel electrophoresis side by side with the uncut DNA (lane 1). *Ava*II digestion showed three bands [860 (uncut), 865, and 172 bp), which is consistent with the *Ava*II site present in α-Tm-2. Southern hybridization with a probe specific for α-Tm-1 shows one band in both cut and uncut DNA suggesting the expression of α-Tm-1 in embryonic heart (lanes 1 and 2 of Fig. 7b). However, the hybridization with the probe specific for α-Tm-2 shows two bands (Fig. 7c). The most predominant band is much lower (lane 2) than the undigested DNA (lane 1). However, there was also a minor band that is very similar to the uncut DNA suggesting the incomplete digestion of the amplified DNA (compare lane 1 and the upper minor band in lane 2).

This strategy was further employed to evaluate whether α-Tm-2 was expressed specifically in embryonic heart but not in embryonic skeletal muscle. Figure 8a showed the ethidium staining of 15-day heart and skeletal muscle cDNA amplified with α-Tm primer-pairs, both uncut and cut with *Ava*II. In lane 2, *Ava*II digestion produced two band of approximately 860 and 685 bp, confirming the presence of α-Tm-2 exon 2a in 15-day embryonic heart. Southern blot analysis with three different detector oligonucleotide probes was performed. Figure 8b showed hybridization with the generic probe for common exon 6b in all bands. Hybridization with α-Tm-1 (exon 2b) occurred in every band except the lower in lane 2, which is where the *Ava*II digested α-Tm-2 was localized (Fig. 8c). Figure 8d shows hybridization of the α-Tm-2 probe (exon 2a) with the uncut amplicon DNA from 15 day heart and also with the lower band of the *Ava*II cut PCR amplified DNA. A faint hybridization signal was seen in the upper band of lane 2 that may be due to an incomplete *Ava*II digestion. From these results, one can conclude that the *α-TM* gene is expressed in the cardiac tissues of developing chick embryos,

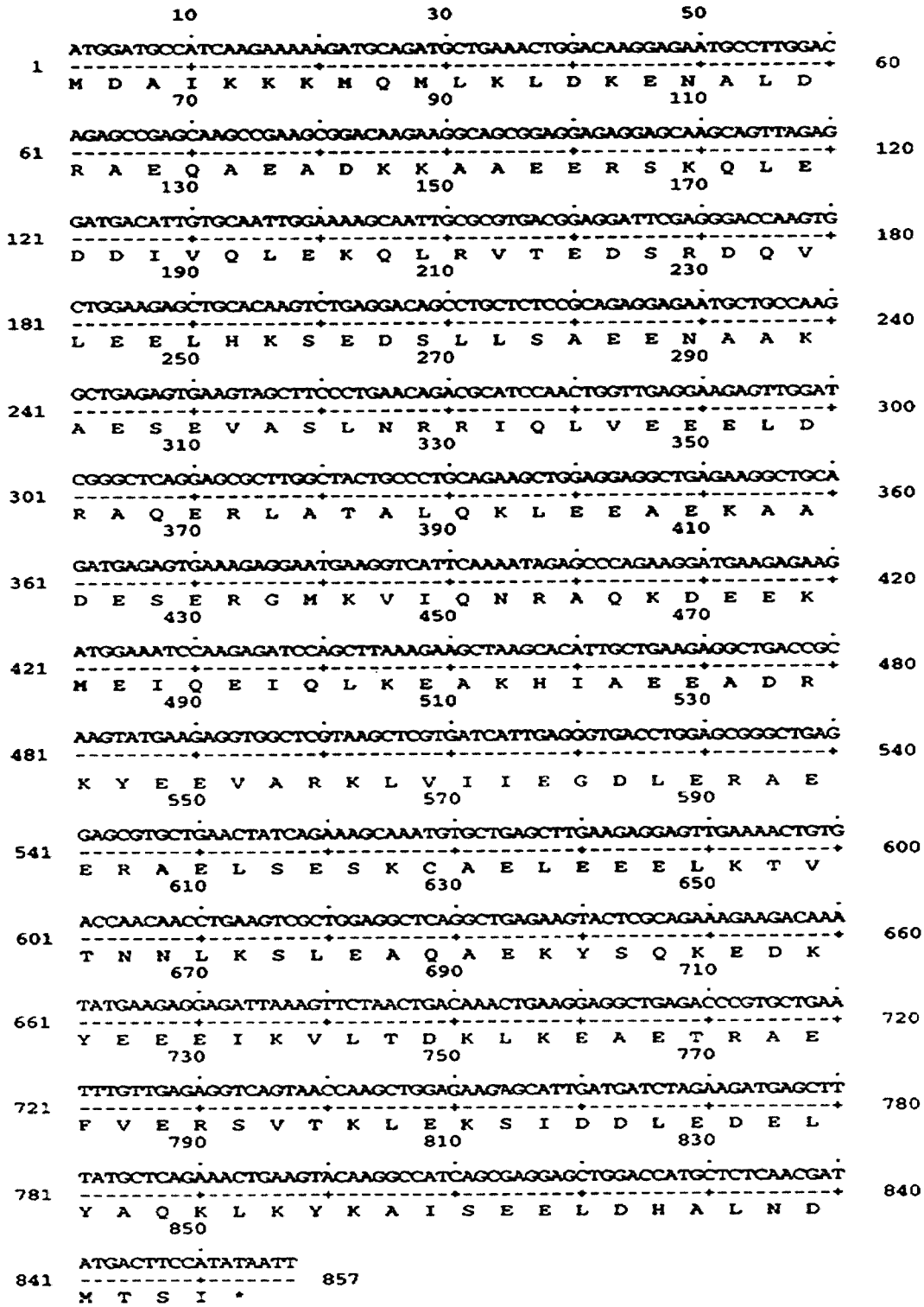

Fig. 3. Nucleotide and amino acid sequence of chicken α-Tm-2. Sequence shares 100% homology with chicken α-Tm-1 except at exon 2 (amino acid 40 through 80).

Amino Acid #	39	80
Chi.Alpha-Tm-2	LEDDIVQLEKQLRVTEDSRDQVLEELHKSEDSLLSAEENAAK	
Axo.Alpha-Tm-2	..EE.....IS..E..R..V.....E...T.D.K...	
Hum.Alpha-Tm-2	..E..AAK..L...S..E..R.....A.....A...A...	
Chi.Alpha-Tm-1	...EL.A.Q.K.KG...EL.KYS.S.KDAQEK.EL.DKK.TD	
Axo.Alpha-Tm-1	...EL.A.Q.K.KG...EL.KYS.S.KDAQEK.EL.DKK.TD	
Hum.Alpha-Tm-1	...EL.S.Q.K.KG...EL.KYS.A.KDAQEK.EL..KK.TD	

Fig. 4. Comparison of amino acid sequence of exon 2 (amino acid residues 39–80) from chicken, axolotl, and human TM isoforms. Chi, chicken; Axo, axolotl; Hum, human.

with both α-Tm-1 and the novel α-Tm-2 isoforms present.

As the striated muscle isoform of the *TM-4* gene is known to be expressed in avian cardiac tissues, we compared the expression of α-Tm-1 and α-Tm-2 with TM-4 transcripts in cardiac and skeletal muscles from embryonic and adult chickens. For normalization, we also performed RT-PCR for *GAPDH*, which is a house-keeping gene. The results depicted in Figure 9a show the ethidium staining of the PCR product containing both α-Tm-1 and α-Tm-2. Figure 9b represents the Southern hybridization with α-Tm-1 detector oligonucleotide. Clearly, α-Tm-1 was expressed in embryonic heart (lane 1), embryonic skeletal muscles (lane 2), and in adult skeletal muscles (lane 4). However, it was significantly downregulated in adult heart (lane 3). The hybridization data with α-Tm-2 probe strongly suggest that α-Tm-2 is expressed only in embryonic cardiac tissues (lane 1). The results are consistent with those presented earlier (Fig. 2). The levels of expression of GAPDH are very similar in all the tissues used in this study (Fig. 9f). Regarding the expression of *TM-4*, it was found that the striated muscle isoform of this gene is expressed in both embryonic and adult cardiac tissues (lanes 1 and 3, respectively, Fig. 9d). However, the expression of *TM-4* was not detected in adult skeletal muscle (lane 4, Fig. 9d,e), although low levels of expression were observed in embryonic skeletal tissues (lane 2, Fig. 9d,e). Our results are consistent with published findings, which suggest that the *TM-4* gene is expressed predominantly in avian cardiac tissues.

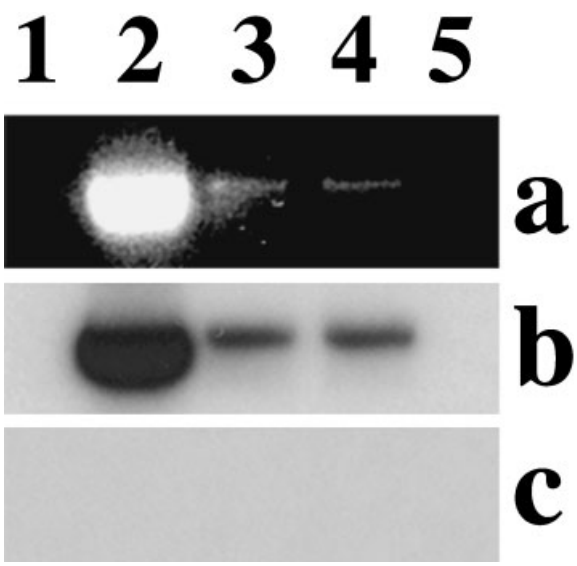

Fig. 5. Levels of α-Tm-1 and α-Tm-2 in adult chicken heart, skeletal muscle, liver, and gizzard. RT-PCR was carried out using P1(+) and P2(−) primer-pair. **Lane 1**: Adult heart; **lane 2**: adult skeletal muscle; **lane 3**: adult liver; **lane 4**: adult gizzard; **lane 5**: primer control. **a**: It shows ethidium staining of the agarose gel. No PCR amplification was observed in adult heart (**lane 1**) with slight amplification in the liver and gizzard. **b**: It represents the Southern hybridization with D1 detector oligonucleotide which is specific for α-Tm-1. Strong hybridization was observed in skeletal muscle (**lane 2**) with levels dropping in liver and gizzard. Results in (b) demonstrate the predominant expression of α-Tm-1 in skeletal muscle with no expression in cardiac tissue. **c**: Southern hybridization with D2 detector oligonucleotide specific for α-Tm-2. No hybridization was observed in any of the adult tissues.

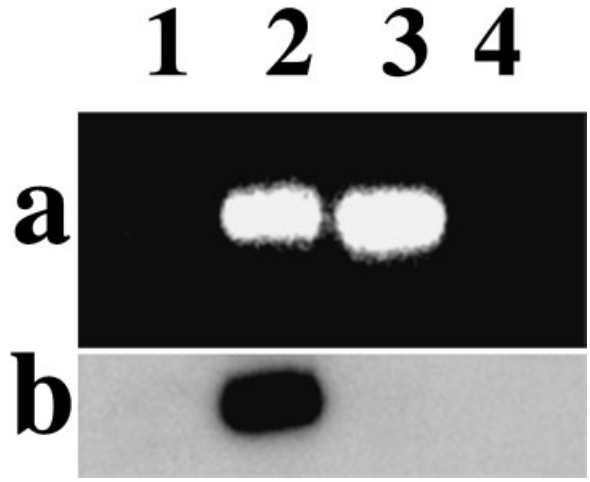

Fig. 6. Levels of α-Tm-2 in adult chicken heart, 15-day embryonic heart, and 15-day embryonic skeletal muscle. **a**: Ethidium staining of the amplified product by P1(+)/P2(−) primer-pair. **Lane 1**: Adult heart; **lane 2**: 15-day heart; **lane 3**: 15-day skeletal muscle; **lane 4**: primer control. **b**: Southern hybridization with D2 probe (sequence given in the legends of Fig. 1) specific for α-Tm-2. No amplification of the α-Tm was observed in adult heart tissue, however, 15-day embryonic heart showed a significant amount of expression as well as 15-day skeletal muscle. Southern blotting with α-Tm-2 probe [D2 (+)] shows that only the 15-day heart contains the α-Tm-2 isoform.

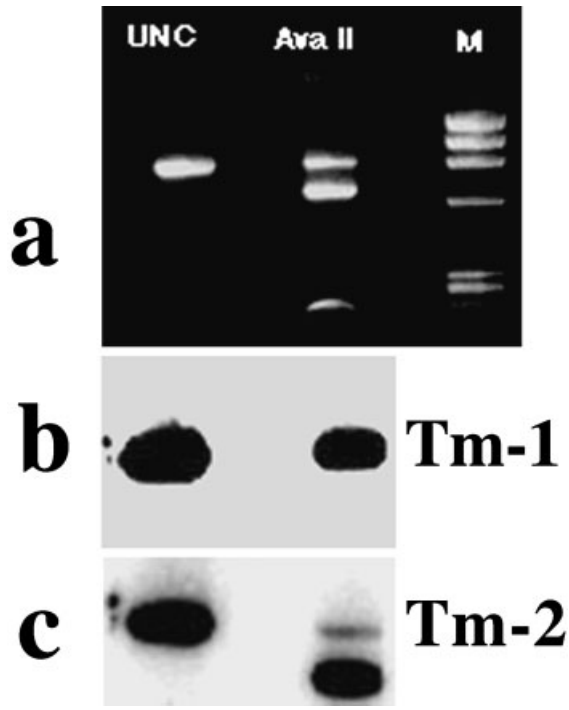

Fig. 7. A unique AvaII site cleaves α-Tm-2 but not the α-Tm-1 cDNA. Restriction map analysis using GCG program indicated the presence of an AvaII site at 171 nt of the α-Tm-2 cDNA (Fig. 3) which is absent in α-Tm-1. RT-PCR products of the 15-day-old chicken heart RNA amplified by P1(+) and P2(−) (see Fig. 2) was digested with AvaII. The digested DNA was subjected to agarose gel electrophoresis following our standard method. **a**: Ethidium staining of the agarose gel after electrophoresis. **Lane 1**: Uncut DNA from 15-day-old heart; **lane 2**: DNA digested with AvaII. Lane 1 shows a single band but lane 2 shows three bands; the uppermost band is the uncut DNA, which may contain α-Tm-1 DNA, the middle and the lowest bands are the digested product of the α-Tm-2. **b**: Southern hybridization with D1 detector/probe which recognizes the α-Tm-1 band only. Hybridization is detected both in **lanes 1** and **2** suggesting the expression of α-Tm-1 in 15-day-old heart. **c**: It represents the Southern hybridization with D2 detector oligonucleotide/probe which is specific for α-Tm-2. Hybridization was detected both in **lanes 1** and **2**. However, in lane 2, two bands were detected. The most predominant one is ~740 nt which suggests that the amplified product is predominantly α-Tm-2. A minor band is also seen in ~890 nt region (uncut). This may be explained if we assume that some α-Tm-2 DNA remained undigested.

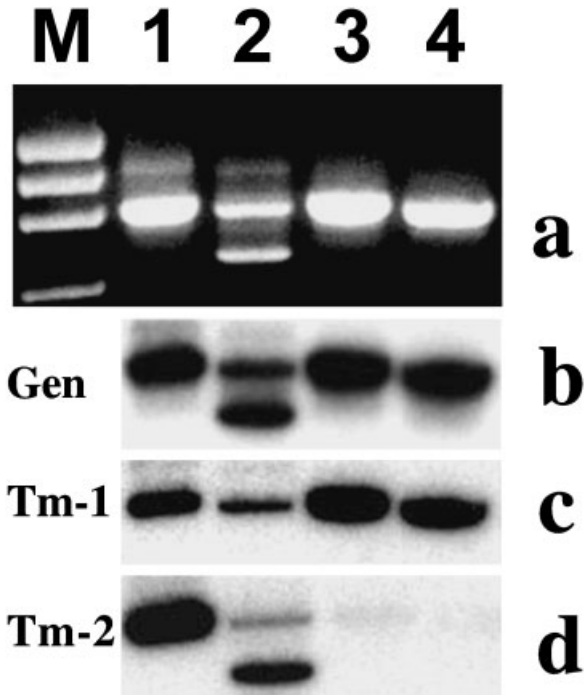

Fig. 8. AvaII digestion of the amplified DNA by the primer-pair P1(+) and P2(−) confirms the absence of the α-Tm-2 isoform in skeletal muscle. **a**: Ethidium staining of AvaII digested and uncut PCR amplified products using α-Tm primer-pairs [P1 (+) and P2 (−)]. **Lane 1**: Fifteen-day embryonic heart (uncut); **lane 2**: 15-day embryonic heart (AvaII cut); **lane 3**: 15-day embryonic skeletal muscle (uncut); **lane 4**: 15-day skeletal muscle (AvaII cut). Two bands are observed in lane 2 indicating the presence of α-Tm-2 in 15-day heart. **b**: Southern blot using ^{32}P labeled probe with generic exon 6b [detector D3: 5′-TGT GCT GAG CTT GAA GAG GAG-3′] shows hybridization in all bands. **c**: Southern blot with D1, α-Tm-1 probe. **d**: Hybridization with D2, α-Tm-2 probe (exon 2a).

The avian α-*TM* gene is known to produce at least eight isoforms through the use of alternate promoters, alternatively spliced exons, and multiple 3′-end processing [Lemonnier et al., 1991]. Of the eight, five isoforms are known to use the promoter present in the upstream region of the entire coding sequences. These isoforms are designated as α-skeletal, α-smooth muscle (α-Sm), α-fibroblast-1 (α-F1), α-fibroblast-2 (α-F2), and α-brain. We now describe another isoform expressed specifically in avian heart designated as α-Tm-2. The isoforms α-Sm, α-F1, and α-F2 contain exon 1a and exon 9d [Lemonnier et al., 1991]. The difference between the α-Sm and α-F1/α-F2 is in exon 2; α-Sm contains exon 2a, whereas both α-F1 and α-F2 contain exon 2b. The main difference between α-F1 and α-F2 is in exon 6; α-F1 contains exon 6b while α-F2 contains exon 6a. The α-F1 isoform differs from the known α-Tm-1 (striated isoform) in exon 9; the striated muscle isoform contains exon 9a/b, while α-F-1 contains exon 9d.

In order to check whether all these isoforms were downregulated in adult chicken heart, like the striated muscle isoforms α-Tm-1 and α-Tm-2, we performed RT-PCR analysis with RNA from embryonic (15 days old) and adult hearts using the same primer-pair (from exon 1a and 9d) which amplify α-Sm, α-F1, and α-F2 followed

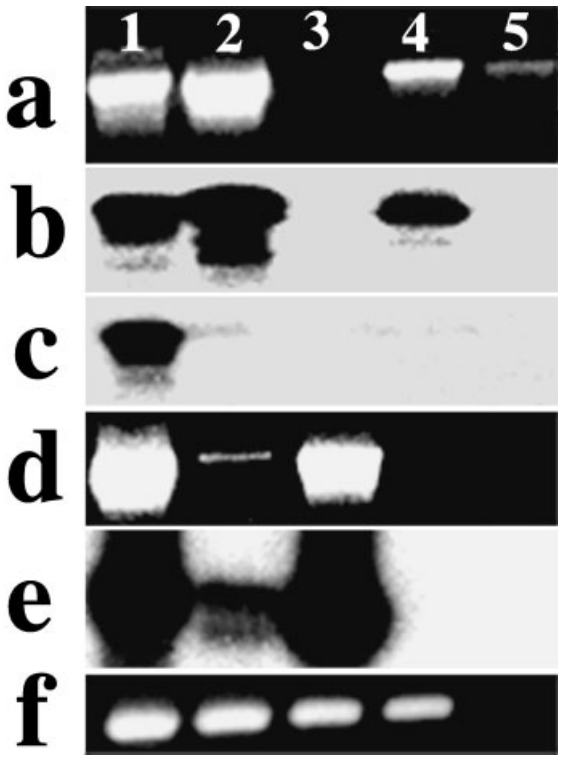

Fig. 9. Expression of α-*Tm* and *TM-4* in embryonic heart, embryonic skeletal muscle, adult heart, and adult skeletal muscle. **a**: Ethidium staining of the amplicons amplified by P1(+)/P2(−) specific for α-TM as outlined in Figure 1. Total RNA was isolated from heart and skeletal muscle and RT-PCR was carried out as stated under Materials and Methods. **Lane 1**: Fifteen-day heart; **lane 2**: 15-day skeletal muscle; **lane 3**: adult heart; **lane 4**: adult skeletal muscle; **lane 5**: primer control. **b**: Hybridization with D1 detector oligonucleotide (specific for α-Tm-1). **c**: Hybridization with α-Tm-2 specific oligonucleotide probe D2. **d**: It represents the RT-PCR amplification of ~860 nt band specific for TM-4 gene with the primer-pair [TM4-1(+) (5′-ATGGATGCCATCAAGAAAAAGATG-3′)] and [TM4-2 (−) (5′-AGACTACAGGGAGGTCATATCATT-3′)]. **Lane 1**: Fifteen-day heart; **lane 2**: 15-day skeletal muscle; **lane 3**: adult heart; **lane 4**: adult skeletal muscle; **lane 5**: primer control. **e**: It represents the Southern hybridization of the amplicons amplified by RT-PCR and probed with TM-4 specific detector oligonucleotide [TM4d(+): 5′-GATGAGCTGGTAGCTCTGCAG-3′)]. **f**: Ethidium staining of the RT-PCR product of GAPDH.

by Southern analyses using isoform specific detector oligonucleotides. We used detector oligonucleotides from exon 2a for α-Sm; exon 2b for α-F1; and exon 6a for α-F2. In order to verify whether each of these oligonucleotides detected the specific isoform as postulated, we isolated cDNAs for each isoform and subsequently determined the nucleotide sequences (results not shown). The nucleotide as well as the deduced amino acid sequences has been submitted to GenBank.

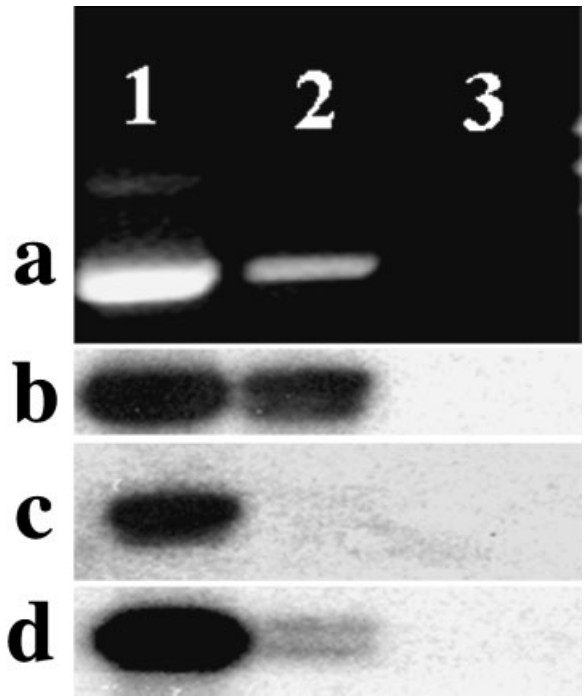

Fig. 10. Expression of smooth muscle type α-Tm (α-Sm), alpha-Tm Fibroblast-1 (α-F1), and alpha-Tm-Fibroblast-2 (α-F2) in embryonic heart (15-day old) (**lane 1**), and adult chicken heart (**lane 2**). **a**: Ethidium staining of the PCR products after agarose gel electrophoresis. The amplification was carried out with P1(+) and P3 (−) primers which can amplify all three isoforms. The results clearly show that there is overall reduction of the expression of all three isoforms. **b**: Hybridization with D2 detector oligonucleotide which specifically recognizes α-Sm. The results indicate the downregulation of α-Sm in adult hearts. **c**: Hybridization with D1 detector in exon 2a which specifically recognizes α-F1. The results suggest that α-F1 is expressed in embryonic heart (**lane 1**) but the expression is clearly reduced in adult heart. **d**: Hybridization with D4 oligonucleotide which is the probe for α-F2 (see Fig. 1). Again, the expression of α-F2 is clearly decreased in adult heart compared to the embryonic counterpart (compare **lane 2** with **lane 1**).

The results in Figure 10a represent the ethidium staining of the amplified products, which suggest that there was a significant reduction of total α-Tm (α-Sm, α-F1, and α-F2) [compare lane 1 (embryonic) and lane 2 (adult)]. The results are very similar to those from RT-PCR for α-Tm-1 and α-Tm-2. Figure 10b represents the expression analysis of α-Sm, which again shows downregulation in adult cardiac tissues. Similar results obtained for α-F1 (Fig. 10c) and α-F2 (Fig. 10d).

Next we determined whether the translation product of this unusual isoform of α-TM, designated as α-Tm-2, could be incorporated into organized myofibrils, the apparatus for the cardiac contractile activity. One of the limitations

in the field of tropomyosin as well as many other myofibrillar proteins is the unavailability of isoform specific antibodies. One approach is to introduce GFP fusion protein constructs. These expression constructs were designated as pEGFP.α-Tm-1 and pEGFP.α-Tm-2 following our published strategy [Zajdel et al., 2002a]. For this experiment, GFP fusion protein constructs were expressed in primary cultures of neonatal rat cardiomyocytes. These cells are well suited for studying the incorporation of exogenously expressed proteins into myofibrils because they spread well in culture and assemble large arrays of myofibrils. Cardiomyocytes transfected with either GFP.α-Tm-1 or GFP.α-Tm-2 incorporated the exogenously expressed protein into their myofibrils in narrow stripes. We used TRITC-phalloidin (Molecular Probe) to visualize actin. We also used monoclonal antibodies against myomesin, which is located in the M-band of striated muscle where it interacts with myosin and titin, possibly connecting thick filaments with the third filament system [Auerbach et al., 1999]. Both fusion proteins were found to be localized in a similar fashion in the cardiomyocytes; incorporated into mature myofibrils in the center of the cell as well as in the regions of myofibril formation at the cellular periphery where they are localized in a continuous fashion similar to actin (Fig. 11).

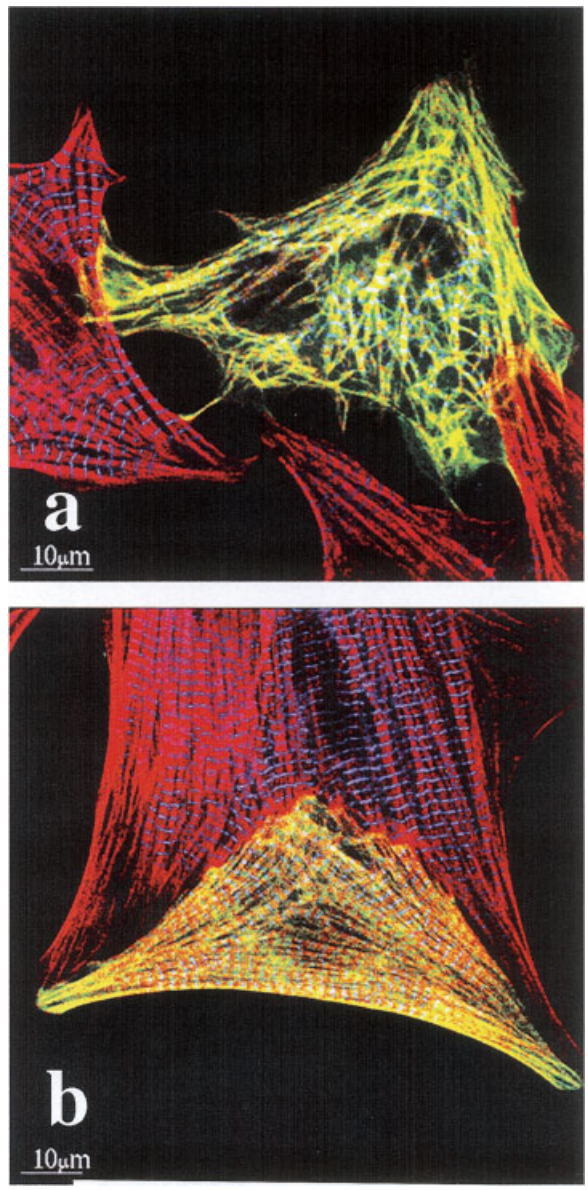

Fig. 11. Ectopic expression and subsequent incorporation of exogenous GFP.α-Tm-1 and GFP.α-Tm-2 into cardiac myofibrils. Confocal images of neonatal rat cardiomyocytes expressing GFP.α-Tm-1 and GFP.α-Tm-2. Cells were stained with the monoclonal antibody against myomesin, which was characterized in the laboratory of Dr. J-C Perriard [Grove et al., 1984]. Secondary antibody Cy5-conjugated anti-mouse Igs (Jackson Immunochemicals) was used together with TRITC-phalloidin to visualize actin (Molecular Probes). The GFP was excited with at 488-nm wavelength. The results show that both GFP.α-Tm-1 (**panel a**) and GFP.α-Tm-2 (**panel b**) were incorporated into mature myofibrils. Red: actin; blue: myomesin; yellow: GFP.α-Tm-1 or GFP.α-Tm-2.

DISCUSSION

Although the role of TM is well defined in striated muscles, the precise mechanism of how TM functions is still unclear. This is in part due to the complexity of isoform diversity of different myofibrillar proteins, including TM. There are four TM genes (α, β, *TPM-3*, and *TPM-4*) highly conserved among vertebrates, each of which encodes for multiple isoforms.

Alternative mRNA splicing is a fundamental process in eukaryotes that contributes to tissue-specific and developmentally regulated patterns of *TM* gene expression [Helfman et al., 1986; Wieczorek et al., 1988; Lees-Miller and Helfman, 1991]. It has been shown that vertebrate cardiac tissues produce, either by alternative splicing or gene truncation, both striated (sarcomeric) and smooth muscle type TM, each 284 amino acids in length, as well as smaller isoforms of TM (cytoplasmic and fibroblastic) 248 amino acids in length. Striated muscle isoforms are the only ones that contain troponin-binding domains (exon 9a/b).

Previous studies of the α-*TM* gene using Northern blot and ribonuclease protection

assay methods identified the expression of the known striated muscle specific isoform, α-Tm-1, in all vertebrate hearts [Lees-Miller and Helfman, 1991] except for avian species [Fleenor et al., 1992]. In both amphibian and avian species, the known striated muscle isoform of the *TM-4* gene has been reported to be expressed in cardiac tissues [Forry-Schaudies et al., 1990a; Hardy et al., 1995; Spinner et al., 2002]. In addition, there is minor expression of smooth muscle and cytoskeletal tropomyosin from the *TM-4* gene [Forry-Schaudies et al., 1990b]. Unlike in amphibians and mammals, others believe that the avian α-*TM* gene is not expressed in cardiac muscle. However, the methods employed may not have been sensitive enough to detect low levels of expression during development. The data presented here clearly shows the brief expression of the α-*TM* gene in chicken hearts during embryogenesis. Interestingly, the α-*TM* gene expresses the newly discovered sarcomeric α-Tm-2 along with the already known α-Tm-1 isoform (Fig. 1) in the embryonic chicken hearts. The extent of expression of both isoforms is comparable.

In addition, we have found that GFP fusion protein constructs containing α-Tm-1 and α-Tm-2 isoforms of TM can incorporate into organized myofibrils in cardiac tissues/cells from axolotl (results not shown) and rats. Due to a lack of isoform specific antibodies against various TMs, we utilized the pEGFP construct with a CMV promoter to visualize the localization of the α-Tm-1 and α-Tm-2 cDNA constructs using laser scanning confocal microscopy.

Both α-Tm-1, the known striated muscle α-Tm isoform with exon 1a, 2b, 3, 4, 5, 6b, 7, 8. 9a/b, and the newly discovered α-*TM* isoform with exon 2a (instead of exon 2b) are expressed in embryonic chicken hearts. Also, we have found the expression of the smooth muscle isoform of α-*TM* in embryonic hearts. In addition, the striated muscle isoform of *TM-4* gene is expressed in embryonic and adult cardiac muscles. While the striated muscle isoform of the *TM-4* gene is most predominant in adult heart tissues, the known striated muscle isoform of the α-*TM* gene is predominant in adult skeletal muscles (Fig. 9).

Our findings are in agreement with those of Wang et al. [1996] who also found that the skeletal muscle α-TM is expressed in embryonic heart. While studying the differential display of mRNAs from the atrioventricular region of developing chicken hearts at stages 15 and 21, these authors found that skeletal α-*TM* expression is temporarily and spatially decreased in the developing hearts. However, the major emphasis of the work was on the expression of a novel *21C* gene in developing hearts. They performed Northern blot analysis and in situ hybridization to study the expression of the known striated muscle isoform of the skeletal α-TM both in embryonic cardiac tissues and in somites at stage 15 and stage 21 embryos. The hybridization signal was significantly stronger in the stage 15 heart and became weaker in stage 21 heart. On the other hand, the signals became much stronger in the stage 21 somites, which mostly contain skeletal muscle. However, no comparative expression study was conducted in hearts from adult chickens. Most importantly, no emphasis or explanation was given concerning the regulation of α-Tm expression in developing and adult chicken hearts.

Our results suggest that the newly discovered α-Tm-2 isoform is expressed only in cardiac tissues but not in skeletal muscles (Figs. 2 and 8). We observed similar results in the Mexican axolotl where α-Tm-2 (or ATmC-2) is expressed predominantly in cardiac tissues [Luque et al., 1997]. However, the major differences between axolotl and chicken are that both the *TM-4* and α-*TM* genes are expressed in adult axolotl hearts [Luque et al., 1997; Spinner et al., 2002], while adult chicken hearts predominantly, if not exclusively, express the *TM-4* gene. Although both α-*TM* and *TM-4* are expressed in embryonic chicken hearts (stages 10–15), the α-*TM* gene undergoes downregulation after hatching. In juvenile and adult hearts, expression of all the α-*Tm* isoforms tested is drastically reduced.

TMs are known to be house-keeping gene(s) and are thought to produce a variety of isoforms via tissue-specific and developmentally regulated alternate splicing. However, our expression analysis using RT-PCR strongly suggests that the α-*TM* gene does not behave like a typical house-keeping gene because various isoforms are expressed in a tissue specific and developmentally regulated manner. For example, α-Tm-1, α-Tm-2, α-Sm, and fibroblast α-Tm are expressed in embryonic cardiac tissues (10- and 15-day) and the expression of each of these isoforms is downregulated drastically in adult cardiac tissues, except for α-Sm (Fig. 10). Lesser inhibition of the α-Sm isoform as shown in

Figure 10 (compared with α-Tm-1, α-Tm-2, and fibroblast isoform of *α-TM*) may be explained by the fact that vertebrate hearts contain a variety of cell types, some of which express smooth muscle isoforms of a variety of myofibrillar proteins including TM. For example, smooth muscle alpha-actin has been found in specific areas of developing mouse hearts such as the right and left bundle branches [Franco and Icardo, 2001]. Also, smooth muscle α-TM may be expressed in coronary vessels when they are formed. Interestingly, α-Tm-1 is expressed in both embryonic and adult skeletal muscles. One could argue that the expression of α-Tm-1 in skeletal and cardiac tissues is from the products of two different genes. In order to clarify this point, we determined the complete nucleotide sequences of α-Tm-1 cDNAs from heart and skeletal muscle and found them to be identical. Hence, it is tempting to hypothesize that the α-TM may undergo transcriptional regulation also in a tissue specific manner in developing chicken. Meinnel et al. [1989] also suggested the tissue-specific transcriptional control of α- and β-tropomyosin in chicken muscle development. The same group subsequently isolated and characterized the promoter region of the chicken *α-TM* gene and found no TATA, CAAT, or CCArG boxes in the upstream from the CAP site [Lemonnier et al., 1991]. However, no deletion mutagenesis or other studies were carried out to find out the differences of expression between the skeletal and cardiac muscles.

ACKNOWLEDGMENTS

The work was supported by a grant from American Heart Association (New York Affiliate) and also supported in part by a grant from American Heart Association (National). We thank Dr. Roger Markwald, South Carolina for his generous gift of a chicken heart cDNA library. We thank Dr. David Helfman, Professor Cold Spring Harbor Laboratory for his suggestion to use a restriction enzyme to differentiate between the two α-TM isoforms. We thank Dr. David Packard for his help in dissecting different tissues from chicken embryos.

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