A novel striated tropomyosin incorporated into organized myofibrils of cardiomyocytes in cell and organ culture

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Abstract Striated muscle tropomyosin is classically described as consisting of 10 exons, 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a/b, in both skeletal and cardiac muscle. A novel isoform found in embryonic axolotl heart maintains exon 9a/b of striated muscle but also has a smooth muscle exon 2a instead of exon 2b. Translation and subsequent incorporation into organized myofibrils, with both isoforms, was demonstrated with green fluorescent protein fusion protein construct. Mutant axolotl hearts lack sufficient tropomyosin in the ventricle and this smooth/straited chimeric tropomyosin was sufficient to replace the missing tropomyosin and form organized myofibrils. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Smooth muscle tropomyosin; Green fluorescent protein; Transfection; Cardiac myofibril

1. Introduction

Alternative RNA splicing is a fundamental process in eukaryotes that contributes to tissue-specific and developmentally regulated patterns of gene expression [1–5]. It is now well established that vertebrate cardiac tissues produce both striated (sarcomeric) and smooth muscle type tropomyosin, each of which is 284 amino acids in length. In addition, smaller isoforms of tropomyosin (cytoplasmic and fibroblastic) of 248 amino acid residues are also produced in cardiac tissues [2,6,7]. Only striated muscle isoforms contain troponin-binding domains (exon 9a/b). There are four tropomyosin genes (α, β, TPM3, and TM4), which exhibit a high degree of conservation in species ranging from *Drosophila* to human. Complex and diverse expression of tropomyosin isoforms has made it difficult to understand tropomyosin function in cardiac muscle. There also may be differing roles of specific tropomyosin isoforms in development and maintenance of the myofibril. Tropomyosin is among five major proteins (actin, tropomyosin, troponin-T, troponin-C, and troponin-I) that comprise the thin filament in striated muscle.

Previous studies of the α-TM gene, using Northern blot analysis and RNase protection assay, identified the expression of 10 isoforms of TM generated by alternate splicing of exons. One isoform of the 10, comprised of exons 1a, 2b, 3, 4, 5, 6b, 7, 8, and 9a/b, is known to be specific for striated, skeletal and cardiac muscles. Several other isoforms were characterized as tissue-specific and/or developmentally specific [3,5,8]. Recently, Cooley and Bergstrom [9] used a highly sensitive RT-PCR based strategy to report the expression of these and many other new isoforms in a variety of adult rat tissues. Previously, we reported the expression of a novel isoform of α-TM, containing exons 1a, 2a (instead of 2b), 3, 4, 5, 6b, 7, 8, 9a/b in the Mexican axolotl [10]. The new TM isoform was designated ATmC-2 (axolotl tropomyosin cardiac-2). Nucleotide sequence analysis of ATmC-1 (striated muscle type isoform) and ATmC-2 strongly suggests that these two isoforms are alternatively spliced isoforms of the α type TM gene in Mexican axolotl. Expression of ATmC-2 was higher than ATmC-1 in cardiac tissues [10,11].

Vertebrate hearts of the Mexican axolotl express the unique tropomyosin isoform, which also maintains exon 9a/b found in vertebrate striated muscle and is involved with troponin binding. However, this novel isoform (ATmC-2) has a smooth muscle type exon 2a. Currently there is a paucity of isoform-specific antibodies that may be used to study the diverse tropomyosin isoforms, particularly one containing both smooth and striated muscle exons. Due to the lack of a specific antibody for ATmC-2, it is impossible to establish whether the newly discovered ATmC-2 transcript is translatable in vivo. Furthermore, it cannot be determined by immunolocalization whether this isoform is incorporated into organized myofibrils like normal striated muscle isoforms (ATmC-1). We approached these issues by creating green fluorescent protein (GFP) fusion protein expression constructs with ATmC-2 or ATmC-1. These expression constructs when transfected into whole hearts or cultured cells would express GFP.ATmC-2 or GFP.ATmC-1 fusion protein. Embryonic whole hearts of the Mexican axolotl can be transfected with specific constructs while maintaining intercellular structure and communications. Normal hearts were used to demonstrate translatability and incorporation into organized myofibrils. This animal model also has a cardiac mutant that has reduced expression of tropomyosin in the ventricle. This provided an interesting perspective to determine if the naturally occurring smooth/striated chimera TM isoform (ATmC-2) could promote the formation of organized myofibrils and thus prove functional. Strong support for the incorporation of ATmC-2 isoform in cardiac cells was demonstrated in isolated embryonic chick cardiomyocytes. This report is the first to suggest that the

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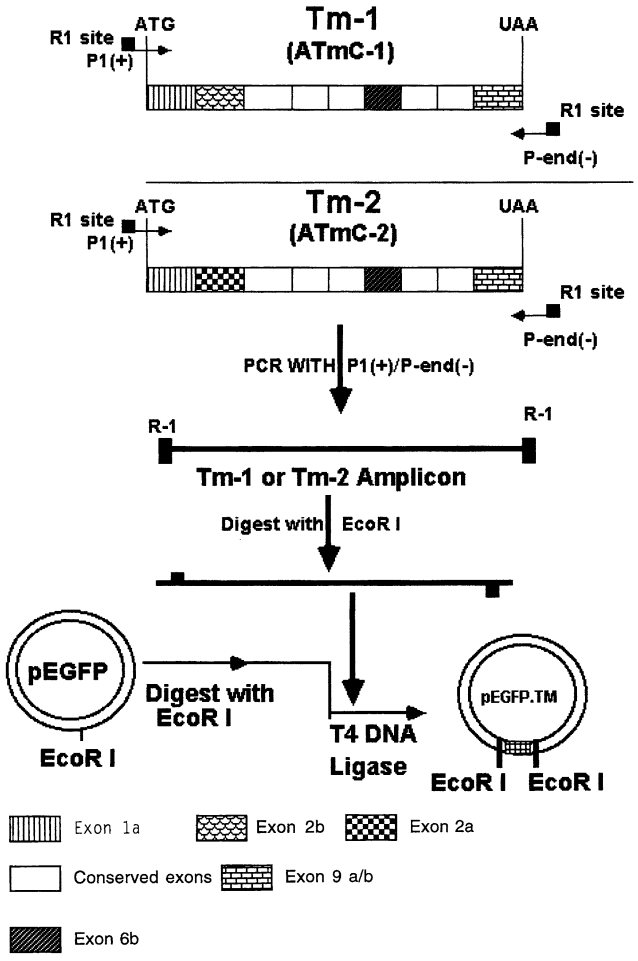

Fig. 1. Strategy for making ATmC-1 and ATmC-2 GFP constructs.

newly discovered TM transcript from a smooth muscle type exon 2 produces a protein that can incorporate into organized myofibrils.

2. Materials and methods

2.1. Embryo care

Normal and cardiac mutant axolotl embryos were obtained from matings between heterozygous (+/c×+/c) animals from the Indiana University axolotl colony and the axolotl colony at SUNY Upstate Medical University. Animals were maintained in aquaria in 50% Holfreter's solution (29 mM NaCl, 0.45 mM $CaCl_2$, 0.33 mM KCl, 0.1 mM $MgSO_4$ and 4.76 mM $NaHCO_3$) and fed commercial salmon pellets. The embryos were staged according to the standard staging system [12].

2.2. Preparation of the pEGFP.ATmC-1 and pEGFP.ATmC-2 expression constructs

ATmC-1 and ATmC-2 cDNA were amplified by PCR using 5′-TCG GAA TTC ATG GAC GTC ATC AAG AAG-3′ (+ve) and 5′-CTG AAT TCT TAC ATT GAA GTC ATA TCG TTG TTG AG-3′ (−ve) primer-pair. The strategy for amplification and preparation of pEGFP constructs is shown in Fig. 1. The GFP was driven by a CMV promoter and the TM was linked to the GFP at the carboxy end in frame. We added an *Eco*RI restriction site at the 5′ end of both positive and negative primers. The amplified products were digested with *Eco*RI and subsequently gel purified (Qiagen Inc.) following the manufacturer's protocol. The digested DNA was then ligated to the *Eco*RI digested pEGFP vector (Clontech) with T4 DNA ligase (Gibco BRL) and subsequently transfected into the competent *Escherichia coli* cells (Invitrogen). After colony hybridization, the positive clones were picked up and the DNA was prepared for sequence analysis (Cornell University DNA sequencing facility, Ithaca, NY, USA). The plasmid with correct orientation of insert cDNA was identified for large scale DNA preparation and subsequent transfection assays in axolotl hearts or in chicken cardiomyocytes.

2.3. Cationic liposome mediated transfection in whole hearts

Transfection of cDNA was performed according to previously published methods [13]. Transfected whole hearts were maintained in culture for five days.

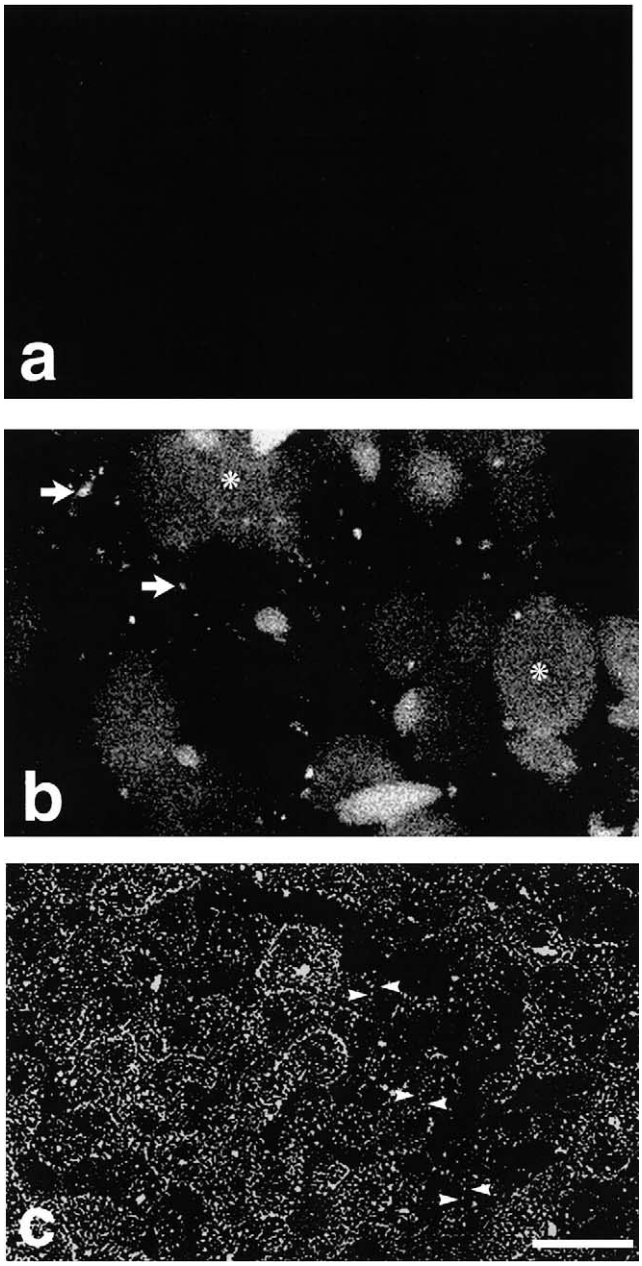

Fig. 2. Transfection of pEGFP.ATmC-2 into live hearts of normal axolotl. a: Detection of GFP fusion protein in a live normal heart at zero time. No GFP was visible. b: GFP fusion protein in a live normal heart at 48 h. Green fluorescence was found in or around the nucleus (asterisk) and within the cytoplasm (arrows). c: Transfected live normal heart at five days examined with a GFP filter on confocal laser scanning microscope. GFP protein was expressed throughout the heart with incorporation into striated myofibrils (arrowheads). Bar, 20 μm.

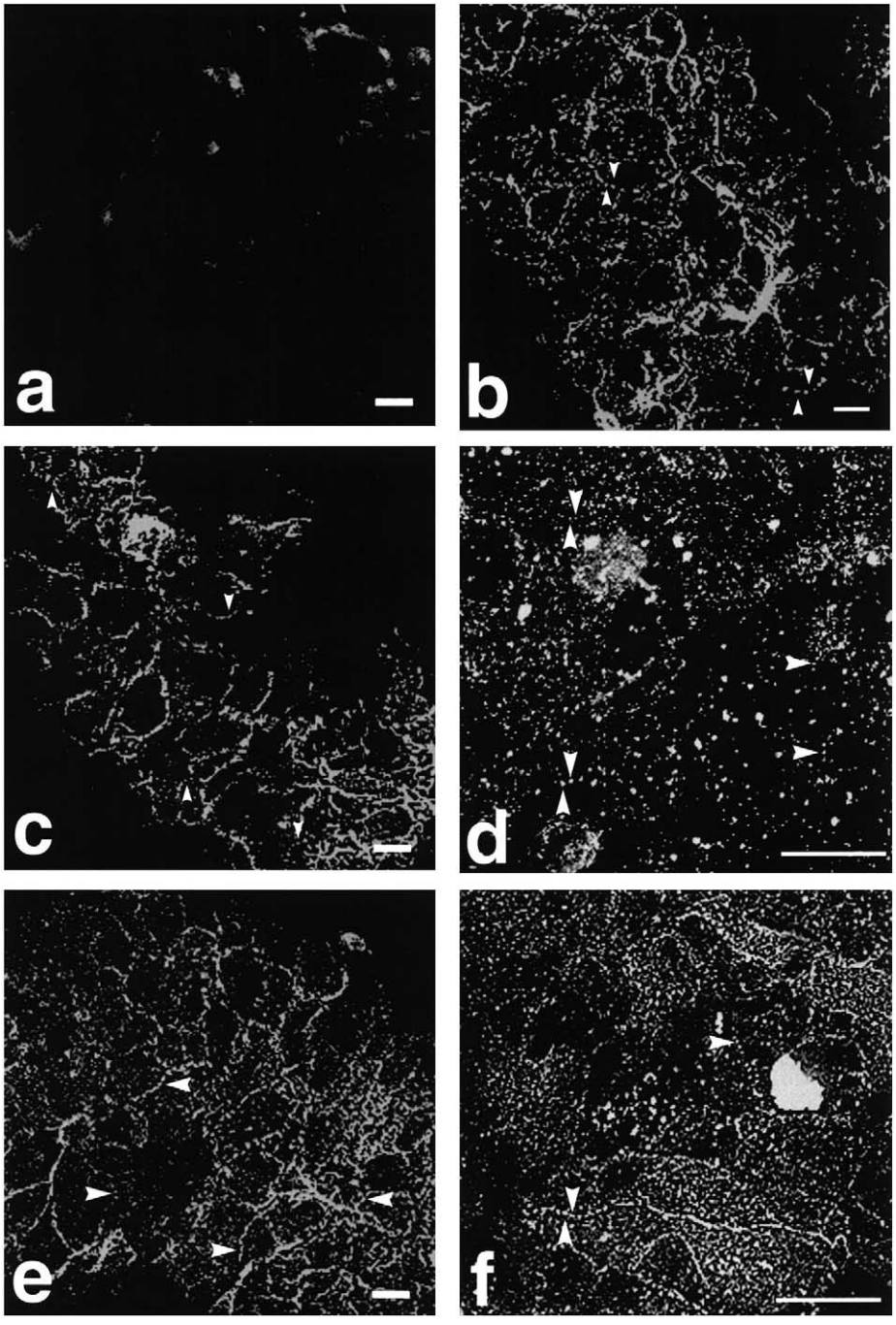

Fig. 3. Formation of striated myofibrils in whole mutant hearts. a: Mutant control heart stained with CH1 tropomyosin antibody. Tropomyosin levels are low and no organized myofibrils were found in ventricle. Bar, 20 μm. b: Normal control heart stained with CH1 antibody. Sarcomeric myofibrils were visible throughout the heart. Bar, 20 μm. c: Mutant heart transfected with pEGFP.ATmC-2 and stained with CH1 antibody. Sections of branching sarcomeric myofibrils were visible (arrowheads). Bar, 20 μm. d: Mutant heart transfected with pEGFP.ATmC-2 and stained with anti-GFP polyclonal antibody. Higher magnification demonstrates expression in the whole heart and incorporation of the fusion protein into periodic branching myofibrils (arrowheads). Bar, 20 μm. e: Mutant heart transfected with pEGFP.ATmC-1. Sections of sarcomeric myofibrils (arrowheads) were seen throughout the heart and tropomyosin levels were increased. Bar, 20 μm. f: Higher magnification of a mutant heart transfected with pEGFP. ATmC-1 and stained with polyclonal anti-GFP antibody. Sarcomeric myofibrils were throughout the heart (arrowheads). Bar, 20 μm.

2.4. Confocal microscopy

Whole mount immunostaining and confocal microscopy were performed according to our published procedures [13]. GFP protein was detected on the confocal laser scanning microscope using a specific GFP filter while examining the live non-fixed hearts. Monoclonal tropomyosin antibody [14] was obtained from the Developmental Studies Hybridoma Bank. Anti-GFP monoclonal and polyclonal antibodies (Clontech) were used for double staining of GFP and contractile proteins after fixation. Anti-mouse rhodamine red secondary antibody and anti-rabbit FITC (Sigma, St. Louis, MO, USA) at a dilution of 1:50 were used for detection of the primary antibodies in addition to direct GFP detection. Whole hearts were mounted on slides in BioRad Fluorogard antifade reagent. Specimens were viewed on a BioRad MRC 1024ES confocal laser system mounted on a Nikon

Eclipse E600 microscope. Control hearts and treated hearts were examined using identical confocal settings. A simultaneous or sequential (double staining) Z-series was made for each. Digital image processing was performed using Adobe Photoshop.

2.5. Transfection of chick cardiomyocytes

Chick cardiomyocytes were prepared from eight-day-old embryonated eggs as described by Dabiri et al. [15]. The cells were transfected after one day in culture using methods reported by Ayoob et al. [16]. The plasmids containing the two different isoforms of axolotl tropomyosin were constructed as described. The cardiomyocytes began to express the GFP-tropomyosin isoforms of tropomyosin after 1 to 2 days post-transfection (GFP.ATmC-1, GFP.ATmC-2). Images of live contracting myocytes were taken on a Nikon Diaphot 200 inverted fluorescence microscope with a phase 100X Planapochromat objective. Images were acquired with a liquid-cooled CCD (C 4742-95 Hamamatsu, Bridgewater, NJ, USA). Photographic images were assembled using Image Pro Plus (Media Cybernectics, Silver Spring, MD, USA) and Adobe Photoshop (Adobe, Mountain View, CA, USA).

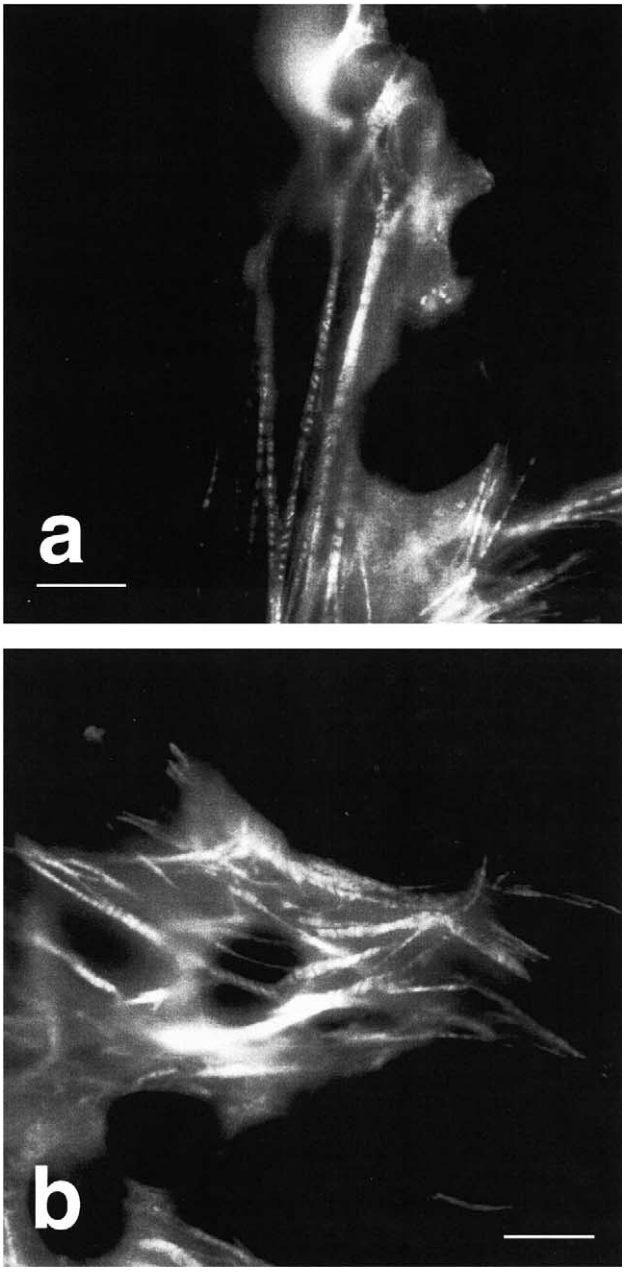

Fig. 4. Transfection of pEGFP.ATmC-1 and pEGFP.ATmC-2 into isolated chick cardiomyocytes. a: Embryonic chick cardiomyocytes transfected with GFP.ATmC-1. The sarcomeres were about 2 μm in length and the ATmC-1 isoform incorporated into the thin filaments of the chick. The dark circle inside the cell is the nucleus. Scale = 10 μm. b: Embryonic chick cardiomyocyte transfected with GFP.ATmC-2. The sarcomeres were about 2 μm in length and the axolotl tropomyosin isoform (ATmC-2) was readily incorporated into the thin filaments of the chick. The nuclei of the cardiomyocytes were detected by their round negative images. Scale = 10 μm.

3. Results and discussion

GFP fusion protein was detected in live whole hearts by using a confocal laser scanning microscope with a green fluorescence filter (laser wavelength 488 nm, filter 515). Detection of GFP, which was attached to the ATmC-2 construct, demonstrated that ATmC-2 could be translated in whole hearts. Fig. 2a shows a live whole heart at zero time and examined with the green fluorescence filter. No GFP fusion protein is detected at this time. Detection of GFP in the cytoplasm and in or near the nucleus was found at 48 h after initiation of the experiment (Fig. 2b). Traditionally most proteins are translated in the cytoplasm but recent studies [17] have shown that some proteins may have nuclear translation. Analyses of the 48 h samples suggest nuclear presence of the fusion protein but it is also possible that the newly synthesized protein actually surrounds the perimeter of the nucleus. At five days, GFP protein was found in sarcomeric myofibrils of the whole hearts (Fig. 2c). Therefore ATmC-2 can be translated and expression in the normal whole hearts indicated that the protein was incorporated into sarcomeric myofibrils.

Ectopic over-expression of the GFP fusion protein does not affect the contractile activity of the normal hearts or isolated cardiomyocytes. There has been some concern of GFP toxicity in other systems but we have not detected that with the systems used for these experiments.

Cardiac mutant axolotl hearts have reduced levels of sarcomeric tropomyosin, no well-organized myofibrils and no contractions. Fig. 3a demonstrates a control heart stained with CH1 antibody. A control normal heart with sarcomeric myofibrils can be seen in Fig. 3b. Previously we have found that classical TM-4 type tropomyosin found in embryonic axolotl can replace the reduced tropomyosin and promote myofibril formation. All three transcripts of axolotl tropomyosin, ATmC-1, ATmC-2, and ATmC-3, have been found to be present in mutant hearts. An interesting question arises then of whether the chimeric ATmC-2 smooth/striated type tropomyosin that is naturally occurring could sufficiently replace missing tropomyosin and promote the formation of organized myofibrils. We also completed the analysis by examining transfection of a classical tropomyosin, ATmC-1, into mutant hearts.

Mutant hearts transfected with pEGFP.ATmC-2 demonstrated that ATmC-2 was expressed in the mutant hearts and with evidence obtained from the CH1 tropomyosin staining we could determine that small sarcomeric myofibrils were formed (Fig. 3b,c). Anti-GFP antibody staining was used to coincide with the CH1 processing of the transfected hearts. GFP expression was fairly uniform throughout the whole heart and higher magnification of the GFP transfected mutant heart better showed the periodicity of sarcomeric myofibrils (Fig. 3d). Therefore ATmC-2, a novel TM isoform with smooth muscle type exon 2a, was determined to be incorpo-

rated into myofibrils in normal and mutant whole hearts. This incorporation was detected first using a GFP filter on live hearts and secondly by anti-GFP polyclonal antibody.

Transfection of pEGFP.ATmC-1, which is the classical form of α-tropomyosin, demonstrated that the levels of tropomyosin protein had increased compared to the control mutant and sarcomeric myofibrils had formed (CH1 staining, Fig. 3e). The presence of GFP protein, detected in this case by anti-GFP antibody, demonstrated the expression of the transfected construct and confirmed the fusion protein was within sarcomeric myofibrils (Fig. 3f).

CH1 monoclonal antibody recognizes only sarcomeric TM with exon 9a/b [14]. Although ATmC-1 and ATmC-2 differ at exon 2, both have exon 9a/b [10]. CH1 antibody most likely can recognize both of the transfected isoforms.

Since this is the first description of the incorporation of a tropomyosin with smooth muscle type exon 2 into myofibrils, we also used a different animal model and method. The constructs GFP.ATmC-1 and GFP.ATmC-2 were transfected into chicken cardiomyocytes in culture. GFP.ATmC-1 was expressed and incorporated into striated myofibrils. GFP.ATmC-2 was also incorporated into the myofibrils with detection of the fusion protein (Fig. 4). Both patterns of incorporation were similar with little differences detected. These results in cultured chick cardiomyocytes confirm the results shown with axolotl whole hearts. The novel ATmC-2 protein can be incorporated into sarcomeric myofibrils in isolated culture or whole hearts, chick or amphibian. We have isolated and characterized the ATmC-2 homologues from rat, chicken and human hearts (results not shown). Human and chicken ATmC-2 homologues, like ATmC-2, can also be incorporated into cardiac myofibrils in axolotl whole hearts.

ATmC-1 and ATmC-2 transfected into mutant hearts promoted the formation of striated myofibrils. The primary question remaining is what role the novel ATmC-2 plays in cardiac myofibrillogenesis and contractile activity. We believe smooth muscle type exon 2 in ATmC-2 may have developmental implications for the formation of myofibrils. The presence of a smooth muscle type exon may be involved in transition of thin filaments in previously proposed multi-step models of myofibrillogenesis [18].

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