Journal of Cellular Biochemistry 99:952–965 (2006)

Differential Expression of Tropomyosin During Segmental Heart Development in Mexican Axolotl

Robert W. Zajdel,[1] Matthew D. McLean,[1] Christopher R. Denz,[1] Syamalima Dube,[2] Harold L. Thurston,[2] Bernard J. Poiesz,[2] and Dipak K. Dube[1,2]*

[1]Department of Cell and Developmental Biology, SUNY Upstate Medical University, 750 East Adams St., Syracuse, New York 13210
[2]Department of Medicine, SUNY Upstate Medical University, 750 East Adams St., Syracuse, New York 13210

Abstract The Mexican axolotl, *Ambystoma mexicanum*, serves as an intriguing model to investigate myofibril organization and heart development in vertebrates. The axolotl has a homozygous recessive cardiac lethal gene ''*c*'' which causes a failure of ventricular myofibril formation and contraction. However, the conus of the heart beats, and has organized myofibrils. Tropomyosin (TM), an essential component of the thin filament, has three known striated muscle isoforms (TPM1α, TPM1κ, and TPM4α) in axolotl hearts. However, it is not known whether there are differential expression patterns of these tropomyosin isoforms in various segments of the heart. Also, it is not understood whether these isoforms contribute to myofibril formation in a segment-specific manner. In this study, we have utilized anti-sense oligonucleotides to separately knockdown post-transcriptional expression of TPM1α and TPM4α. We then evaluated the organization of myofibrils in the conus and ventricle of normal and *cardiac* mutant hearts using immunohistochemical techniques. We determined that the TPM1α isoform, a product of the *TPM1* gene, was essential for myofibrillogenesis in the conus, whereas TPM4α, the striated muscle isoform of the *TPM4* gene, was essential for myofibrillogenesis in the ventricle. Our results support the segmental theory of vertebrate heart development. J. Cell. Biochem. 99: 952–965, 2006.

Key words: conus; ventricle; embryonic; anti-sense oligonucleotides; myofibrillar disarray

The development of the vertebrate heart is a highly ordered, complex process involving numerous signaling molecules, transcription factors, and tissue–tissue interactions. The process, though continuous, appears to involve a number of discrete steps. For example, cardiac lineage formation [Sugi and Lough, 1994; Gannon and Bader, 1995] and looping can both be separated from the assembly of the contractile apparatus [Manasek and Monroe, 1972; Chen et al., 1996]. Additionally, the heart forms in segments with one segment, such as the atria, forming independently of others, such as the ventricle.

There is growing evidence that the heart forms via the addition of segments and that the conus (outflow tract (OFT)) may be a distinct anatomical and transcriptional compartment [Markwald et al., 1998]. Supporting this model are a number of transgene studies in mice showing anterior–posterior differences in expression of reporter constructs driven by promoters for various cardiac proteins. For example, the MLC3F-*nlacZ*-9 construct (containing a 9-kb fragment of DNA upstream of the myosin light chain 3F transcription initiation site which promotes the expression of the *nlacZ* reporter gene) is expressed throughout the entire heart but is excluded in the outflow (conus) and inflow (sinus venosus) tracts [Franco et al., 1997]. A Troponin-I_{slow}-CAT transgene is also expressed in the entire mouse heart but not the OFT even though endogenously it is expressed in all regions including the

Grant sponsor: American Heart Association.

*Correspondence to: Dipak K. Dube, Department of Medicine, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY, 13210.
E-mail: dubed@upstate.edu

Received 27 December 2005; Accepted 17 March 2006
DOI 10.1002/jcb.20954

OFT [Zhu et al., 1995]. Cono-truncal-specific expression is also reported with the SM22α-lacZ construct; the construct directs the synthesis of a putative calcium-binding protein that is expressed in cardiac, smooth, and skeletal muscle lineages during mouse embryogenesis and in adult smooth muscle cells (SMC) [Li et al., 1996; Moessler et al., 1996]. Similar cono-truncal-specific expression was reported with the *hdf*-lacZ gene [gene locus, *hdf* (heart defect), on mouse chromosome 13 that may be required for mechanisms that initially establish and/or maintain continued development of the anterior limb of the developing heart] [Yamamura et al., 1997]. Additional evidence is derived from the differential expression of proteins across the ventricle and OFT. MLC2A is expressed in the atria and OFT but not the ventricle [Franco et al., 1998], while chick SM α-actin is initially present throughout the heart but is later restricted to the OFT after looping [Ruzicka and Schwartz, 1988].

The presence of chamber-specific defects in zebrafish where the ventricle fails to form [*lonely atrium*, Chen et al., 1996; *pandora*; Stainier et al., 1996] and the *hdf*-mouse where the right ventricle and OFT fail to develop [Yamamura et al., 1997] also support a segmental model of heart development. The affect of gene "*c*" on the ventricle of the *cardiac* mutant axolotl has been the focus for the majority of studies in this vertebrate system. As such, the affect the mutation may have on other segments of the heart and the expression of tropomyosin (TM) in these segments, particularly the conus, has not been adequately addressed.

The *cardiac lethal* mutant of the Mexican axolotl, *Ambystoma mexicanum*, is a very useful Urodele model to study the process of heart development. This strain carries a simple recessive mutation in gene "*c*" that leads to a described failure of the heart to beat [Humphrey, 1968]. The predisposing event leading to an absence of contraction is a failure in mature myofibril formation [Lemanski, 1973]. The exact defect remains elusive, but it has been ascribed to either abnormal inductive influences from the anterior endoderm [Humphrey, 1972; Lemanski et al., 1979] or the presence of an overproduced or aberrantly degraded inhibitor in the mesoderm [Humphrey, 1972; Smith and Armstrong, 1991].

Closer examination of the mutant hearts has revealed that the conus of mutant hearts can have regular contractions even though the ventricle is not beating. Although, the hearts of mutant embryos have been fairly well studied, this is the first study that directly examines the differences in myofibril formation and beating of the different heart segments. Previous studies, including those examining the effect of ectopic expression of tropomyosin in mutant ventricles [Zajdel et al., 1998] have demonstrated a lack of tropomyosin as the cause of impaired myofibril formation and contractility in mutant ventricles. Differences in contractility as well as differences in the expression of sarcomeric tropomyosin protein in the conus and ventricles of the axolotl hearts support the segmental heart development theory.

Three striated muscle isoforms of tropomyosin [Lees-Miller and Helfman, 1991] have been characterized from axolotl hearts and are designated as TPM1α, TPM1κ, and TPM4α. When ectopically expressed as a fusion protein, each of these isoforms can be incorporated into organized myofibrils in normal axolotl hearts and cardiomyocytes from chicken and rat without affecting contractility [Spinner et al., 2002; Zajdel et al., 2002; Denz et al., 2004]. Each of these isoforms appears to have some role in the development of myofibrils, possibly in specific segments. The unavailability of a technology for amphibians in particular, by which one can poison or delete a particular exon, makes it harder to delineate the role that particular exon may play in vivo. Hence, we are using other available options like anti-sense technology to knock down the expression of each of the three isoforms of tropomyosin separately and then evaluate the formation of organized myofibrils in different segments of axolotl hearts using confocal microscopy.

MATERIALS AND METHODS

Procurement and Maintenance of Axolotls

Adult axolotls were maintained in aquaria in diluted Holtfreter's solution (100% solution per liter: 3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.02 g $NaHCO_3$) and fed commercial salmon fish pellets. Normal axolotls (+/+) were obtained from mating normal adult animals while mutant embryos are obtained by crossing two heterozygous *cardiac* mutant (c) carriers (+/c). Embryos were obtained from mating animals in our colony at the SUNY Health Science Center

as well as the Indiana University Axolotl Colony.

Antibodies

The following primary antibodies and dilutions/concentrations were used in this study and were obtained from the Developmental Studies Hybridoma Bank, (Iowa City, IA) unless otherwise noted: (1) monoclonal antibody to myosin-binding-protein-C (clone MF1, 1:30); (2) monoclonal antibody to tropomyosin (clone CH1, 1:30); (3) monoclonal antibodies to TnT (CT3, 1:30); and (4) polyclonal antibody, R1749 against human RBC tropomodulin was a generous gift from Velia M. Fowler, Scripps Research Institute, (La Jolla, CA). FITC and Rhodamine-conjugated Donkey anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson Immunological and were used at 1:120 and 1:200, respectively. Hoescht dye number 33324 (Sigma Immunochemicals, St. Louis MO) was used at 1 μg/ml to detect cell nuclei.

Whole Mount Immunostaining

Confocal laser scanning microscopic Materials and Methods have been previously described [Mangiacapra et al., 1995; Ward et al., 1996; Zajdel et al., 1998]. Briefly, embryos were mechanically released from their jelly coats with watchmaker forceps (#5) and serially washed in filter sterilized, modified Steinberg's solution (0.34 mM $CaCl_2$, 0.83 mM $MgSO_4$, 4.6 mM HEPES, pH 7.4 [Zackson and Steinberg, 1986], containing 1× antibiotic:antimycotic (Gibco, Life Sciences: stock is composed of penicillin G sodium 10,000 Units/ml, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B). Embryonic tissues were dissected using forceps, sharpened tungsten needles and hair loops, as needed. The tissues were processed en bloc according to our adaptation of a method described by Bell et al. [1987]. All steps were carried out at room temperature with agitation, and, except for the cross-linker stock solution, all reagents were prepared in Steinberg's solution. A 100 mM stock solution of the cross-linker dithiobis (succinimidyl) propionate (DSP, Pierce Biochemical, Inc.) in DMSO was freshly prepared (4.04 mg DSP/100 μl DMSO) and subsequently diluted 1:100 in Steinberg's solution. Hearts were simultaneously crosslinked and permeablilized in DSP/DMSO solution for 15–30 min followed by an optional additional extraction with 0.5% NP-40 for a maximum of 15 min prior to immunostaining.

After treatment with a primary antibody and a fluorescent secondary antibody, hearts were post-fixed with 2% paraformaldehyde before mounting on microscope slides with an antifade solution (2% n-propyl gallate (Sigma), 50% glycerol, 1 M sodium bicarbonate) or fluorogurad (BioRad). The slides were then coverslipped with spacers (nail polish) to avoid compressing the hearts and examined as whole mounts on a BioRad MRC-1024 confocal laser scanning microscope attached to a Nikon Eclipse E600 microscope. Optical sections were collected (z-series) and further analyzed using Bio-Rad's LaserSharp software including measurements of sarcomeres, pixel intensity measurements, pixel co-localization, and 3D stereoimaging.

Lipofectin-Mediated Delivery of Anti-Sense and Sense Oligonucleotides into Myocardium in Whole-Heart Organ Culture

Sense and anti-sense oligonucleotides were designed for TPM1α and TPM4α tropomyosin isoforms. The sequence of TPM1α exon 2-specific anti-sense chimeric oligonucleotide is as follows: 5′-T*G*G*CTTTCTTGTCTGCAAG-C*T*C*C-3′ where N* represents phosphotioate-blocked nucleotide (IDT, Inc.). The sequence of TPM4α exon 9-specific anti-sense and sense chimeric oligonucleotides are as follows: 5′-G*C*G*CATGGTCAAGCTCCTC*A*C*T-3′ for the anti-sense construct and 5′-^{f}A*G*T*GAG-A*G*T*GAGGAGCTTGACCATG*C*G*C-3′ for sense where N* represents the phosphotioate-blocked nucleotides and ^{f}A represents an adenosine tagged with fluorescein at the 5′ end (IDT, Inc.).

The sense oligonucleotide was tagged with fluorescein to use as a positive control and confirm localization within the myocytes. The cationic liposome solution (Lipofectin, Gibco-BRL) (0.28 mg · ml) was mixed with Steinberg's solution as described previously [Zajdel et al., 2000] for 30–45 min. This mixture was mixed with 10 μl of oligonucleotide solution (final concentration of 0.1 μM) for 15 min. The hearts were placed into a volume of 10 μl of transfecting solution and monitored daily. The hearts were also examined for introduction of fluorescein oligonucleotides with the confocal microscope as the experiment proceeded. These hearts were

examined after fixing with 2% paraformaldehyde for 1 h. Hoescht dye (Sigma) was used at 1 mg/ml to visualize cell nuclei.

Determination of Specificity of Anti-Sense Oligonucleotides In Vitro

We determined the specificity for each of these oligonucleotides in vitro [Spinner et al., 2002; Zajdel et al., 2005]. In this study, we have presented another set of data that shows the specificity of TPM4α and TPM1α anti-sense oligonucleotides that have been used in vivo for knocking down the corresponding transcripts. In vitro specificity was determined using our published protocol [Zajdel et al., 2005]. First, total RNA from axolotl heart (5 μg) was annealed with 10 pmoles of isoform-specific sense or anti-sense oligonucleotide followed by the treatment of the mixture with RNase H (Clontech) at 37°C for 20 min as described before. The RNase H treatment destroyed the mRNA that formed hybrids with oligonucleotides. RNase H prefers the hydrolysis of an mRNA annealed to its corresponding anti-sense oligonucleotides, but not an mRNA mixed with its sense counterpart [Zajdel et al., 2005].

Reverse transcriptase polymerase chain reaction with oligo dT priming was performed to evaluate the expression of TPM1α and TPM4α isoforms using our previously published protocol [Spinner et al., 2002; Zajdel et al., 2005]. The sequences of PCR primers used are as follows: TPM1α: (+) 5′-ATG GAC GCC ATC AAG AAG AAG-3′ and (−) 5′-ACG CTC CTG AGC ACG ATC CA-3′; TPM4α: (+) 5′-CCG CTC CCT GAC ACC GGT TCC CG-3′ and (−) 5′-GCT CTC CCT CCA GAA TAA CAA GTT-3′. Southern blot hybridization was carried out using [^{32}P]-labeled isoform-specific detector oligonucleotides, the sequences of which are as follows: TPM1α: 5′-AGT ACT CGG AGT CCT TGA-3′; and TPM4α: 5′-TGG CGC GCG GAC GGG GTG TTG CT-3′.

Electron Microscopy

The ventricle and conus of normal and mutant hearts were dissected in Steinberg's and incubated overnight at 4°C in fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.01% picric acid buffered to pH 7.4 with 0.1 M cacodylate buffer) [Ito and Karnovski, 1968]. Hearts were then rinsed in cacodylate buffer and post fixed in 1% osmium tetroxide in the same buffer for 2 h, rinsed in buffer again and dehydrated in a graded series of ethanol solutions followed by two changes in propylene oxide. The ventricle and conus were embedded in Epon in flat molds and allowed to polymerize for 2 days at 60°C. Ultra-thin 90-nm sections were cut, stained with Reynolds' lead citrate and 4% uranyl acetate and viewed in a JEOL 100 CSII electron microscope at an acceleration voltage of 80 kV.

Total RNA Extraction

Tissue samples are dissected and frozen immediately in liquid nitrogen, then stored at −70°C for later processing. Total RNA was extracted from extirpated heart tissues or separated coni and ventricles using (1) Ambion Total RNA extraction kit, according to manufacturer's instructions utilizing a guanidine isothiocyanate:chlorform:isoamyl alcohol extraction method [Chomczynski and Sacchi, 1987] or (2) a Qiagen mini-RNA extraction kit utilizing a proprietary spin column method. The total RNA was treated with RNase-free DNase (Gibco BRL) for 15 min at room temperature, then phenol:chloroform extracted and precipitated with ethanol at −70°C for 1 h or overnight at −20°C. RNA purity and concentration were estimated on a Pharmacia Biotech GeneQuant II RNA/DNA calculator.

Cloning of TPM4α by RT-PCR

Reverse transcriptase polymerase chain reaction was used to clone TPM4α from normal and mutant axolotls using our standard protocol as described earlier [Luque et al., 1997; Spinner et al., 2002; Zajdel et al., 2005]. Each first strand cDNA synthesis reaction was completed with 1.0 μg total heart RNA using Gibco BRL Super-Script^{Tm} II Reverse Transcriptase with Oligo-$(dT)_{12\text{-}18}$ primer. The reaction mix contained 1 μg of total RNA (∼40–50 stage 39 hearts), 1 μl oligo-$(dT)_{12\text{-}18}$ primer, and dH_2O to 12 μl and was incubated at 70°C for 10 min, then placed on ice. Subsequently, 7 μl of a reaction mix (per reaction: 2 μl 10× PCR buffer, 2 μl 25 mM $MgCl_2$, 1 μl dNTP stock (10 μM each of dATP, dCTP, dGTP, and dTTP) and 2 μl 0.1 M DTT was added to the RNA-primer mixture and incubated at 42°C for 5 min. Two-hundred units of SuperScript^{Tm} II Reverse Transcriptase (1 μl) were added and the reaction incubated at 42°C for 50 min. The reaction was terminated by incubation at 70°C for 15 min and then placed on

ice. RNase H was then added to each tube and incubated at 37°C before amplification of the target cDNA. First strand synthesis reaction (2 μl) was subsequently used for amplification in a 100 μl PCR reaction. The full length coding region of TPM4α was amplified using the primer pair, 5′-TCGGAATTCCCGCTCCCTGACACC-GGT-3′ (+ve) and 5′-GGGGAATTCCGATCA-CATCAGAGTGGA-3′ (−ve). Polymerase chain reaction was performed with a PE 9600 thermocycler using AmpliTaq DNA polymerase (Perkin-Elmer).

The PCR products were transferred to GeneScreen nylon membrane following electrophoresis on 1–1.5% agarose gels. The blots were hybridized according to established protocols using specific [γ-^{32}P] (DuPont-NEN) ATP-labeled detector oligonucleotides [Luque et al., 1997; Spinner et al., 2002].

Transcript Levels of TPM1α, TPM1κ, and TPM4α in Conus and Ventricle

Reverse transcriptase polymerase chain reaction with oligo dT priming was performed to evaluate the expression of TPM1κ, TPM1α, and TPM4α isoforms using our previously published protocol [Spinner et al., 2002; Zajdel et al., 2005]. One micrograms of RNA from conus or ventricle was annealed with oligo dT and first strand cDNA synthesis was carried out as described above. Subsequent PCR amplification was performed with the following primer-pairs:TPM1κ and TPM1α are common to both isoforms: (+) 5′-ATG GAC GCC ATC AAG AAG AAG-3′ and (−) 5′-ACG CTC CTG AGC ACG ATC CA-3′; and for TPM4α: (+) 5′-CCG CTC CCT GAC ACC GGT TCC CG-3′ and (−) 5′-GCT CTC CCT CCA GAA TAA CAA GTT-3′. Southern blot hybridization was carried out using with [^{32}P]-labeled isoform-specific detector oligonucleotides, the sequence of which are as follows: TPM1κ: 5′-AGG GTG CTG GAT GAA CTG CAC AA-3′; TPM1α: 5′-AGT ACT CGG AGT CCT TGA-3′; and TPM4α: 5′-TGG CGC GCG GAC GGG GTG TTG CT-3′.

RESULTS

We have previously shown that *cardiac* mutant axolotl hearts although deficient in sarcomeric tropomyosin proteins are not deficient in tropomyosin transcripts [Spinner et al., 2002]. TPM4α is the predominant tropomyosin isoform expressed in axolotl hearts both in normal and mutant animals. In order to check whether the tropomyosin deficiency in *cardiac* mutant axolotl heart is due to a premature termination of the translation product of the TPM4α transcripts, we have cloned and sequenced the coding region of the TPM4α from mutant axolotl heart RNA. The cDNA sequence made from the mutant axolotl does not contain any premature termination codon in the coding region of TPM4α cDNA made from RNA of the mutant axolotl (Gen Bank accession # pending). Also, we cloned and sequenced the 3′-UTR, 5′-UTR, and the upstream regulatory region of the TPM4α from normal and mutant axolotl hearts and we did not find any differences between them (results not shown).

Coni but not Ventricles of Stage-Specific Mutant Hearts Contain Myofibrils

CH1, anti-sarcomeric TM antibody [Lin et al., 1985] labeled the ventricle and conus in normal stage 38/39 heart (Fig. 1A) and localized to the sarcomere in a cross-striated pattern (Figs. 2A and 3A). Stage 38/39 cardiac mutant myocardium showed significantly less staining for TM using CH1 antibody in the ventricle (Figs. 1D and 3D) but was readily detected in the conus (Figs. 1D and 2D) in a cross-striated pattern. This is further corroborated by electron microscopy studies showing abundant fibrils in mutant conus similar to those in the normal conus (Fig. 4A,C). Mutant ventricle, on the other hand, contained thick filaments and numerous z-bodies but no mature fibrils as seen in the normal ventricle (Fig. 4D). Additionally, a transition-like zone occurred in the mutant between the ventricle and the conus (Fig. 1D). This region contained CH1-positive TM that was assembled into a few sarcomeres but was largely present as an amorphous collection of protein.

Tropomodulin (Tmod), the latest marker described to date for mature sarcomeres, requires TM for assembly [Gregorio and Fowler, 1995] and was used as a marker for mature thin filament assembly. This was especially important in the mutant ventricle where CH1-positive TM was greatly reduced. Tropomodulin localized in a cross-striated pattern where TM was also observed in periodic arrays (Figs. 2B, 1E,B). However, in the mutant ventricle, where CH1-positive TM was not found, Tmod staining appeared as thin strands that

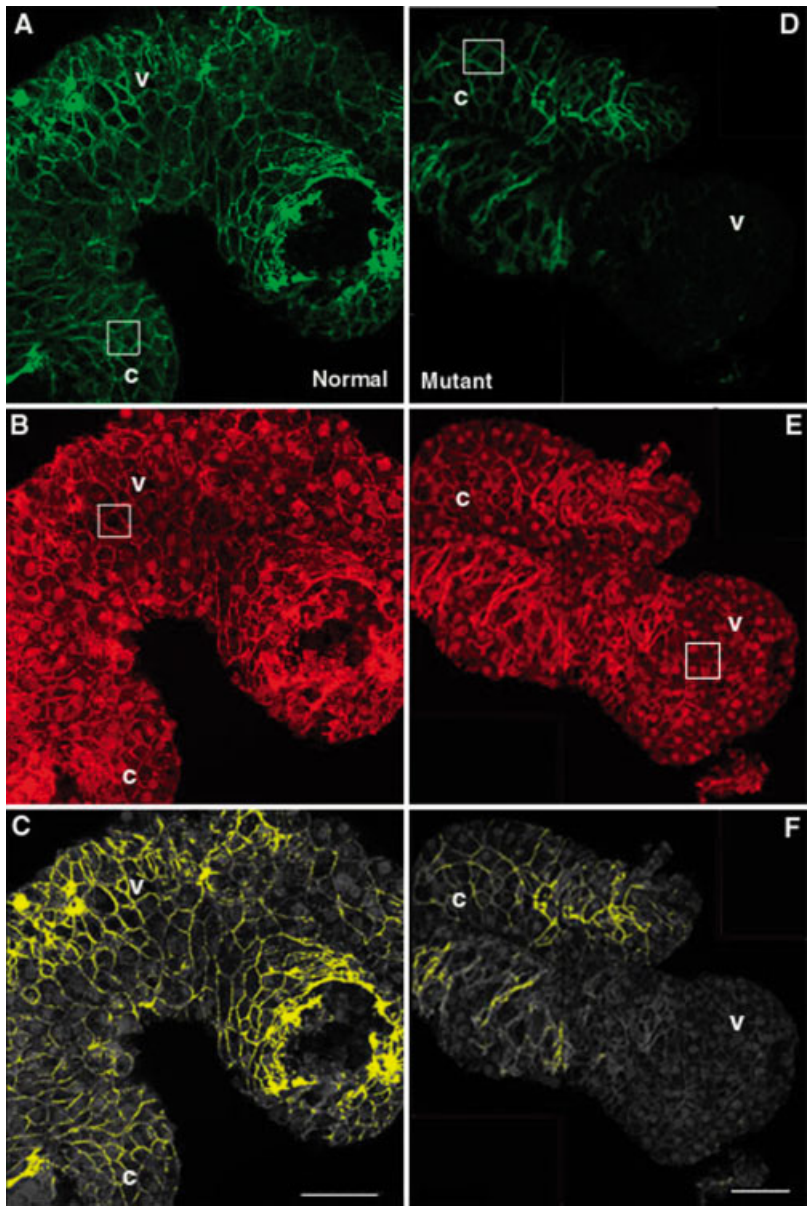

Fig. 1. CH1 anti-sarcomeric TM antibody labels the conus but not the ventricle in stage 38/39 cardiac mutant axolotl hearts. Stage 38/39 normal (**A–C**) and mutant (**D–F**) axolotl hearts were extirpated and labeled for double immunofluorescent microscopy with CH1 anti-sarcomeric TM antibody (A and D) and polyclonal anti-tropomodulin antibody, R1749 (B and E). Hearts were examined on a confocal microscope at 20× magnification. The co-localization function in the Bio-Rad confocal software was used to obtain the images in C and F. Yellow shows areas of co-localization for the two antibodies based on parameters selected with the software. White boxes in the conus (c) region (A and D) and the ventricle (v) region (B and E) of normal and mutant hearts designate the areas shown in Figures 2 and 3 for the conus and ventricle, respectively. CH1-positive TM was found throughout the conus and ventricle of normal myocardium (A) in cross-striated myofibrils capped with tropomodulin. In mutant hearts, however, only the conus region appears to contain CH1-positive TM while the ventricle does not (D). The TM-labeled in the conus is found on tropomodulin capped, cross-striated myofibrils similar to that observed in normal hearts. Tropomodulin (Tmod) is also present in mutant ventricle though CH1-positive TM is not. However, the tropomodulin is not located in mature cross-striated myofibrils as seen throughout the normal heart and mutant conus. An area located between the ventricle and the conus in the mutant hearts (T) appears to contain amorphous collections of TM and Tmod mixed with myofibrils. This appears like a transition zone between the two segments. Scale Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

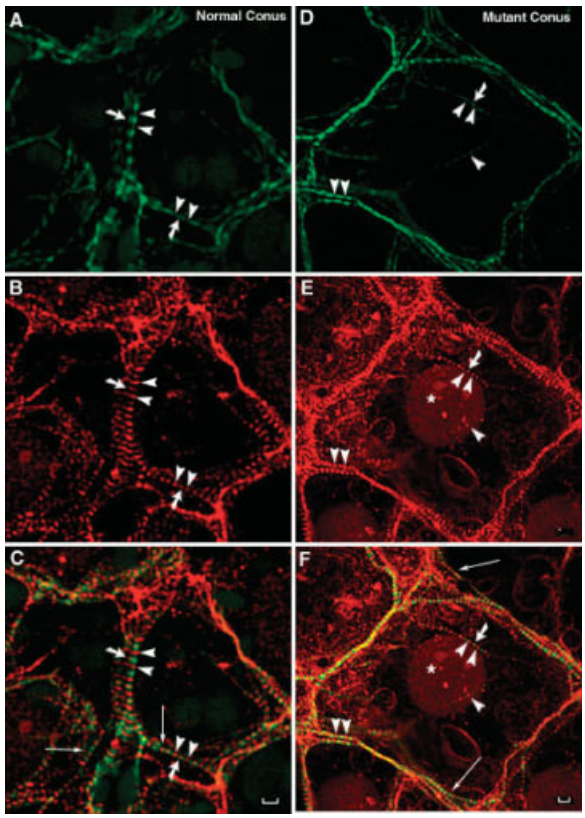

Fig. 2. Comparisons of myofibrils in the conus of stage 38/39 normal and *cardiac* mutant axolotl. The conus areas designated in Figure 1 (**A** and **D**) of normal and mutant hearts were examined under high magnification. CH1-positive TM (A and D) labeled myofibrils in a cross striated pattern in the conus of normal (A) and mutant (D) animals equally well. Thick fibril cables and very thin almost imperceptible fibrils were both labeled with CH1 antibody. Tmod staining with R1749 shows localization of Tmod in the myofibrils of normal conus (**B**) and mutant conus (**E**). TM in the thick cables was located in narrower bands along the sarcomere than the TM found in the fine fibrils (thin arrows; **C** and **F**).

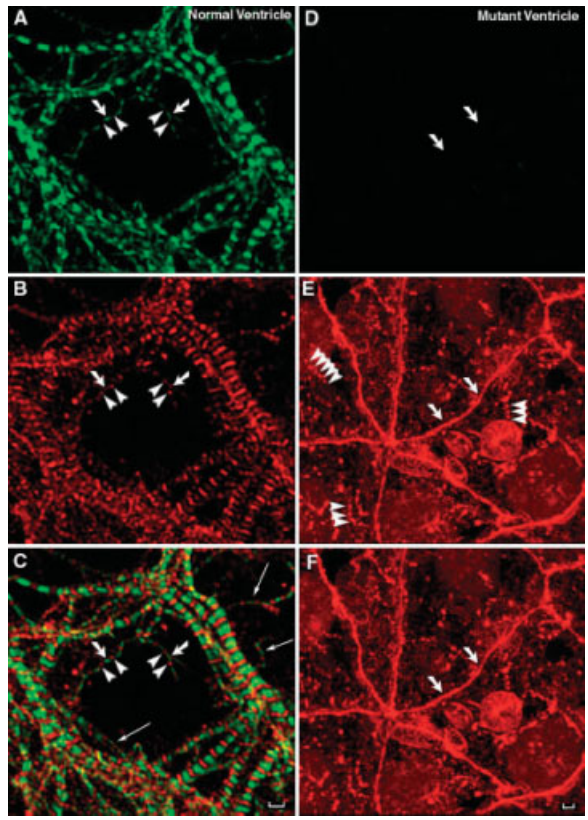

Fig. 3. TM staining in the ventricle of stage 38/39 normal and cardiac mutant axolotl. The ventricle areas designated in Figure 1 (**B** and **E**) of normal and mutant hearts were examined under high magnification. CH1-positive TM was found in the ventricle of normal hearts (**A**) but not in ventricles from mutants (**D**). All of the TM-labeled myofibrils, both large and small (thin arrows in **C**), in the normal ventricle were found to contain Tmod. Tmod (B) was discretely located in all of these fibrils in two locations, in the middle of TM positive staining (arrows) and also in the TM-negative areas on either side (arrowheads). In the mutant ventricle, where TM was not observed, Tmod was not found in thick myofibrils but rather in long continuous fibrils near cell–cell junctions (arrows, E and **F**). It was also found as discrete spots that often appeared to form linear arrays (arrowheads, E). C and F are the merged images of A/B and D/E respectively and clearly show the location of the signals for the two antibodies relative to each other. Asterisks mark cell nuclei. Scale bar = 2 μm.

outlined the junctions between cells (Fig. 3E). Also present were numerous Tmod-positive punctate spots that often aligned in linear arrays similar to those described for nascent myofibrils in cell culture (Fig. 2E). In myofibrils located in the mutant conus and throughout the normal myocardium, Tmod could be detected even on the finest of myofibrils that also co-labeled for TM (Figs. 1C,F, 2C,F). Interestingly, though tropomodulin is reported to exist predominately at the ends of the I-bands [Weber et al., 1994; Gregorio and Fowler, 1995], it is clearly observed at or near the Z-line where TM also stains (Figs. 1 and 2). This is additionally supported by the fact the spacing between Tmod bands is ~1 μm.

Ultrastructure Analysis of Conus and Ventricle From Stage 38/39 Normal and Mutant Hearts

The evidence for the presence of myofibrils in the conus but not in the ventricle of mutant axolotl hearts also came from electron microscopic studies. The results depicted in Figure 4 show that mutant coni contain abundant fibrils similar to those in normal coni (Fig. 4A,C). Normal ventricles (Fig. 4B) contain numerous well-defined myofibrils that cannot be distinguished from those of the conus (Fig. 4A). Mutant ventricles on the other hand, contained

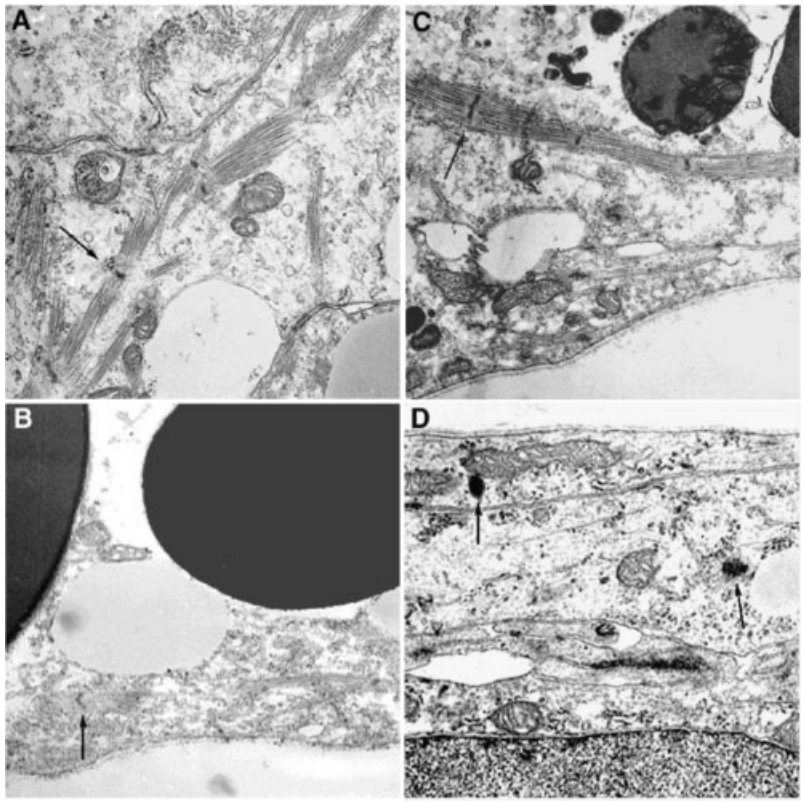

Fig. 4. Transmission electron microscopy of conus and ventricle from stage 38/39 normal and cardiac mutant hearts. Stage 38/39 hearts from normal (**A** and **B**) and cardiac mutant (**C** and **D**) animals were rapidly extirpated, the conus (A and C) and ventricle (B and D) separated and the two pieces fixed and processed separately for TEM as described in Materials and Methods. The conus from normal (A) and mutant (C) hearts both contain numerous well-formed myofibrils (small black arrows) complete with z-lines, anti-parallel thin filaments, and thick filaments. Myofibrils observed in cross section are also numerous. Normal ventricles (B) contain numerous well-defined myofibrils that cannot be distinguished from those of the conus. In contrast (D), the mutant ventricle does not contain well-formed myofibrils though numerous thick filaments are found. Of note, the z-bodies can be observed in what may be linear arrays, though at this magnification, only a few may be observed at one time (small black arrows). 10,000×.

thick filaments and numerous z-bodies but no mature fibrils as seen in normal ventricles (Fig. 4D).

Specificity of Isoform-Specific Anti-Sense Oligonucleotides

The specificity of the anti-sense oligonucleotides for TPM1α and TPM4α used in this study were determined in vitro as published earlier [Zajdel et al., 2005]. The results depicted in Figure 5 show additional data for the specificity of TPM1α-specific anti-sense oligonucleotides which do not interfere with TPM4α transcripts in vitro. In brief, total RNA from axolotl heart was annealed separately with TPM4α or TPM1α-specific oligonucleotides and then treated with RNase H followed by RT-PCR and Southern blot analyses with isoform-specific oligonucleotides as described under the figure legends. The results show that annealing with TPM1α-specific oligonucleotides specifically knocked down the concentration of the TPM1α but not the TPM4α transcripts by RNaseH treatment in vitro. Likewise, TPM4α-specific oligonucleotides specifically decreased TPM4α but not TPM1α transcripts (data not shown).

Effect of Isoform-Specific Anti-Sense Oligonucleotides in the Conus and Ventricle

Our immunohistochemical analyses strongly suggested that the ventricles of pre-hatching mutant hearts were deficient in tropomyosin protein recognized by CH1 antibody. However, we often observed the conus (OFT) to beat. Additionally, immunohistochemical and electron microscopic analyses showed the presence of well-formed organized myofibrils in the conus. The question remained whether the

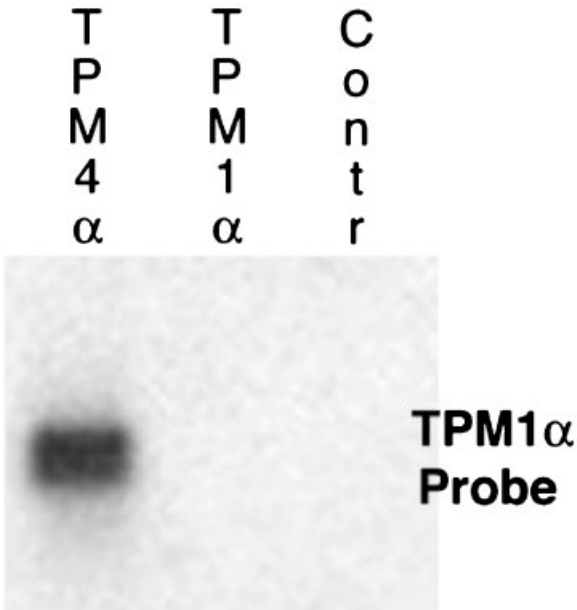

Fig. 5. In vitro analysis of the TPM1α- and TPM4α-specific anti-sense oligonucleotides using RNase H treatment followed by RT-PCR. Total RNA from axolotl heart was annealed with TPM4α- or TPM1α-specific anti-sense oligonucleotides, lanes 1 and 2 respectively, and then treated with RNase H in vitro as described under method section. Control lane represents TPM1α primer control. The results show that the annealing of TPM1α- but not TPM4α-specific oligonucleotides destroyed the TPM1α isoform. This finding is in accord with our previously published results [Spinner et al., 2002; Zajdel et al., 2005].

tropomyosin protein in the conus was derived from the TPM1α transcript or the TPM4α transcript. The introduction of TPM1α anti-sense oligonucleotide disrupted the myofibrillar organization in the conus, but not in the ventricle of normal hearts (Fig. 6A,B). TPM1α and TPM4α sense oligonucleotides were used as positive controls. The failure of sense oligonucleotides to disrupt myofibril structure was not due to a failure in uptake, since fluorescein-tagged oligonucleotides were found in the nucleus of myocardial cells (Fig. 6F). A chimeric TPM1α oligonucleotide containing a 5′-fluorescein tag showed that the myofibril structure was most disrupted in regions containing anti-sense oligonucleotide (Fig. 6A). These results indicate TPM1α was essential for myofibrillogenesis in the conus but not in the ventricle. In contrast, TPM4α-specific oligonucleotides introduced into the conus failed to disrupt the myofibril structure (Fig. 6C,E). The results indicate TPM4α was not essential for myofibril organization in the conus. However, the anti-sense TPM4α oligonucleotides disarrayed the organized myofibrils in the ventricles of the normal hearts (Fig. 6D), indicating the requirement of TPM4α in the ventricle.

Earlier studies that we have reported using whole axolotl hearts showed antisense TPM1κ, but not antisense TPM1α, caused disarray of myofibrils in the normal heart [Zajdel et al., 2005]. Although this experiment did not separate the conus from the ventricle, studies using whole-heart most closely model myofibril organization in the ventricle. The present study suggests the lack of a role of TPM1α in ventricular myofibrilogenesis. Its apparent role in the conus elucidates the limitations of whole-heart studies, while expanding the understanding of the isoform's characteristics.

Determination of the Levels of Transcripts for TPM1α, TPM1κ, and TPM4α in Conus and Ventricle Using RT-PCR

RT-PCR was carried out following our standard published methods with isolated total RNA from coni and ventricles using isoform-specific primer-pairs and detector oligonucleotides as stated under the Materials and Methods section. The results presented in Figure 7 show that the level of transcripts of each of the three isoforms are comparable in the conus and the ventricle in normal axolotl hearts. These data demonstrate that normal transcript levels were present for TPM1α and TPM4α in conus and ventricle, attributing reduced protein expression to anti-sense oligonucleotide treatment. Due to a lack of isoform-specific antibodies that will differentiate between TPM1α and TPM4α, it is not known whether these transcripts are translated into corresponding proteins in all chambers. Hence, we have employed isoform-specific anti-sense oligonucleotide to inhibit the expression of the individual isoforms in conus and ventricle.

DISCUSSION

The *cardiac* mutant Mexican axolotl is an intriguing model of abnormal sarcomerogenesis. Though the location and product of gene "*c*" remains unknown, it likely affects the translation of a tropomyosin protein(s) resulting in a failure to complete sarcomerogenesis in mutant ventricles. It is clear that alterations in TM expression can affect sarcomeres. This is evident in three familial forms of hypertrophic cardiomyopathy involving missense mutations

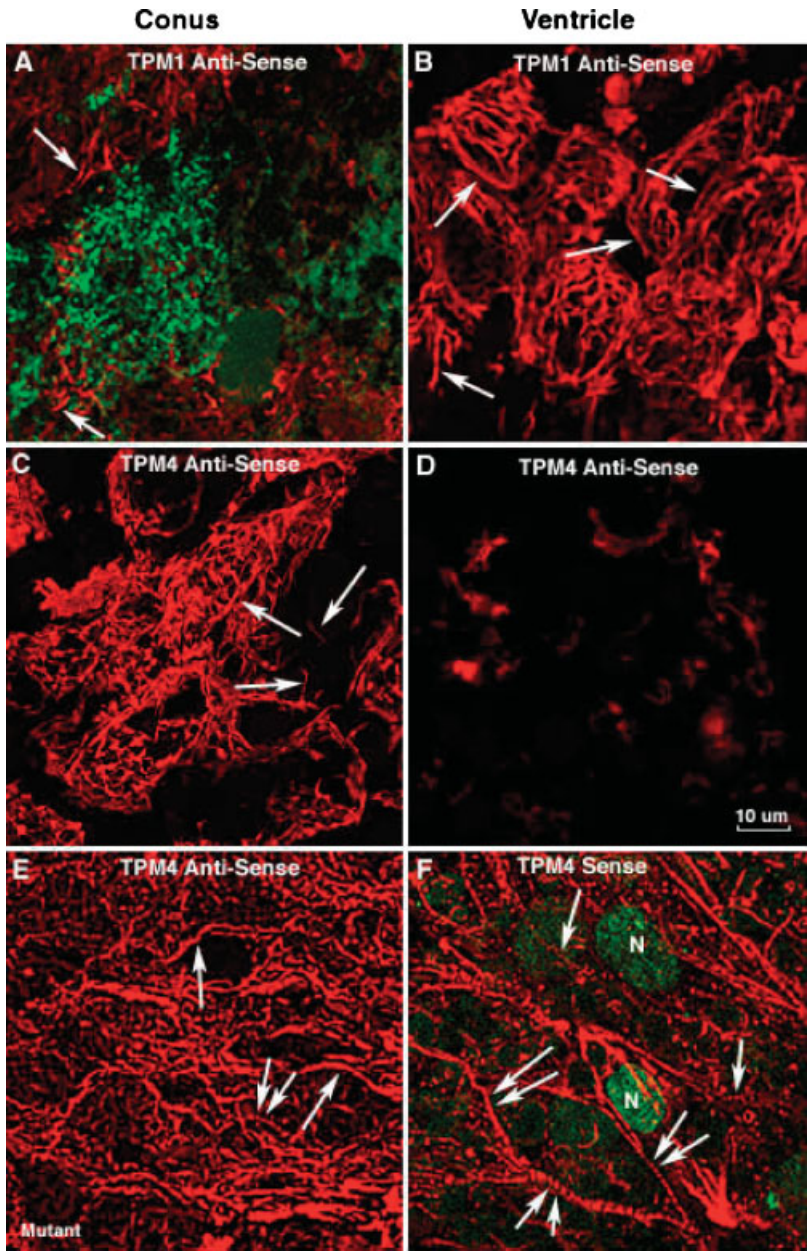

Fig. 6. Disruption of myofibrils by anti-sense oligonucleotides specific for TM isoforms. Stage 38/39 normal and mutant hearts were extirpated and anti-sense oligonucleotides (O-nt) introduced into the conus (**A**, **C**, and **E**) or ventricle (**B**, **D**, and **F**). Hearts were followed for 3–5 days and subsequently stained for TM using CH1 antibody and a rhodamine-red conjugated donkey anti-mouse secondary antibody. Green fluorescence in panels A and F are the result of chimeric oligonucleotides containing a 5′-fluorescein tag. TPM1α anti-sense O-nt (A and B) disrupted myofibril structure in the conus (A) but not the ventricle (B). TPM4α anti-sense O-nt (C, D, and E) disrupted myofibril structure in the ventricle (D) but not the conus of normal (C) or mutant (E) hearts. However, TPM4α sense O-nt had no effect on myofibril structure though they were properly localized to the nucleus (N; green fluorescence in F). Arrows in all panels indicate myofibrils though not all are resolved into a cross-striated pattern. Scale bar = 10 μm.

of the *TM* gene leading to a single amino acid change in each type, $Asp^{175}Asn$, $Glu^{180}Gly$, and $Ala^{63}Val$ [Thierfelder et al., 1994; Nakajima-Taniguchi et al., 1995; Watkins et al., 1995]. One of these mutations ($Asp^{175}Asn$) has been duplicated in transgenic mice confirming it alone can lead to cardiomyopathy [Muthuchamy et al., 1999]. The indirect flight muscles of *Drosophila* contain two different types of tropomyosin protein (3 transcripts: Ifm-TmI, TnH-33, and TnH-34) encoded by two genes, *TmI* and *TmII*. Mutations in the *TmI* gene but

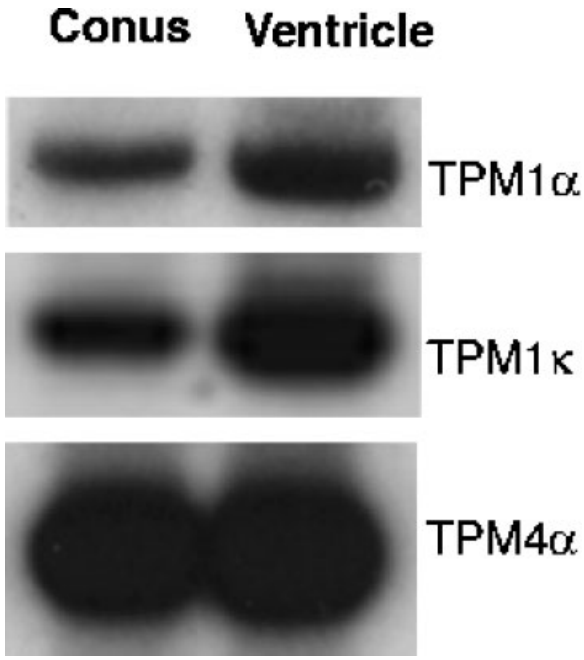

Fig. 7. RT-PCR analysis of TPM1α, TPM1κ, and TPM4α in conus and ventricle from normal axolotl hearts. RT-PCR was carried out with 1 μg of RNA from both conus and ventricle of normal axolotl [Stages (38/39)] with isoform-specific primer-pairs and detector oligonucleotides as described under Materials and Method section. We concluded that transcript levels for both TPM1α and TPM1κ were somewhat less in conus. On the other hand, the levels of TPM4α in conus and ventricle are comparable.

not the *TmII* gene have been shown to disrupt function of these muscles [Kreuz et al., 1996].

Three sarcomeric tropomyosin isoforms are found to be expressed in cardiac tissues of the Mexican axolotl viz. TPM1α [Luque et al., 1994], TPM1κ [Luque et al., 1997], and TPM4α [Spinner et al., 2002]. Of these, TPM1α and TPM1κ are alternatively spliced products of the *TPM1* gene, whereas TPM4α is the product of the *TPM4* gene. *TPM4* represents the axolotl equivalent of the *Xenopus* TM-4 cardiac [Hardy et al., 1995] and chicken cardiac genes [Forry-Schaudies et al., 1990]. We designate the striated muscle isoform as TPM4α [Narshi et al., 2005; Zajdel et al., 2005]. Although TPM4α and TPM1α are the products of two different genes, they share a high degree of homology throughout their entire coding regions. Exon 2 of these two sarcomeric tropomyosins are very similar to each other but are very different from that of TPM1κ. Interestingly, each of these isoforms ectopically expressed into mutant axolotl hearts can promote myofibril formation. However, it is not known if all three isoforms are essential for cardiac myofibril formation in vivo. In *cardiac* mutant, we propose TPM4α to be the affected TM isoform for several reasons.

(1) The skeletal muscle of mutant animals does not appear to be affected and TPM1α transcript is found to be expressed predominantly in skeletal muscle.
(2) Although TPM1κ RNA is expressed predominantly in cardiac tissues, it is not known whether its cognate protein is expressed in vivo. However like TPM1α or TPM4α., when ectopically expressed, it promotes myofibril formation in mutant hearts in culture.
(3) TPM4α transcript is detected predominantly in heart tissue and the onset of transcription is around stage 35 when the heart starts beating [Luque et al., 1997].
(4) The results of experiments on the transfection of anti-sense oligonucleotides specific for TPM1α, TPM1κ, and TPM4α into embryonic heart indicate that TPM1κ and TPM4α, but not TPM1α, are necessary to maintain myofibril structure in the developing axolotl ventricle. This is in contrast to an apparent necessity of TPM1α, but not TPM4α, in the conus for myofibril integrity. The need for TPM1κ expression in the axolotl conus is currently under investigation.

Since the comparison of the coding region of TPM4α from normal and mutant hearts did not detect any changes and as no sarcomeric tropomyosin protein can be detected in mutant ventricles, we conclude that tropomyosin expression is repressed at the level of translation of TPM4α in mutant axolotl hearts due to a mutation in gene '*c*'. Translational control of tropomyosin has been proposed to play an important role in isoform expression in normal and transformed human fibroblast cells [Novy et al., 1993]. This conclusion was based on the observation that the differential expression of tropomyosin isoforms at the RNA level in normal and transformed human fibroblast cells was not in complete agreement with the differences observed in the protein amounts. More recently, Piples and Wieczorek [2000] have proposed the possibility of operating a translational mechanism that may regulate the production of various TM proteins, for example TPM3α and TPM2α in murine slow and fast-twitch muscles. In their studies, the researchers

determined both the transcript and protein levels of the striated muscle isoforms of TPM1, TPM2, and TPM3 in soleus (a representative slow twitch muscle) and fast-twitch muscle and found that transcript level for each of these isoforms does not correlate its translated protein level.

Several scenarios could account for inhibited TM translation in the mutant ventricle.

(1) Translational control of a number of mRNAs has been shown to involve stem-loop structures [Manzella and Blackshear, 1990]. Occasionally, synonymous mutations (that do not alter the coded amino acids) may cause the mRNA to assume stem-loop secondary structures that interferes with translation. However, we found no such mutations in mutant TPM4α.

(2) An inhibitory protein acting in *trans* is always expressed in the ventricle, though it does not inhibit the synthesis of tropomyosin protein TPM4α in normal tissues. However, due to a mutation in the TPM4α transcript (*cis* elements either at the 3′-UTR or at the 5′-UTR), the transcript forms a secondary structure that is recognized by the hypothetical inhibitory protein. Consequently, the protein binds with the mutant *cis* elements and inhibits TM biosynthesis. However, this is not the most likely scenario because we did not find any mutation/alteration in either 3′- or 5′-UTRs including the upstream regulatory regions of the *TPM4* gene from normal and *cardiac* mutant (c/c) animals.

(3) One can argue about the stability of tropomyosin protein in mutant ventricles. First, we have failed to detect any missense mutation in the coding region of TPM4α. Hence, we do not think that the turnover of tropomyosin in the mutant ventricle will be high enough to impede the detection of tropomyosin protein. Again, the degradation of tropomyosin may be high if some protease(s) is expressed in mutant heart, which degrades tropomyosin protein at an extremely high rate. However, we have shown that either ectopic expression of tropomyosin or lipofection of tropomyosin protein in mutant ventricle promotes myofibril formation [Zajdel et al., 1998, 2000, 2002, 2005; Denz et al., 2004; Narshi et al., 2005]. These results do not support the hypothesis that tropomyosin deficiency in mutant ventricle is due to its rapid degradation.

(4) Alternatively, a putative mutation in, or overexpression of, an inhibitor gene whose cognate protein could act as a *trans*-acting element with the UTRs of tropomyosin mRNA could explain to the inhibiton of TM biosynthesis in mutant axolotl hearts. We favor this scenario somewhat because ectopic expression of sarcomeric tropomyosins from the expression constructs with heterologous UTR regions have been found to promote myofibril formation in mutant ventricle [Zajdel et al., 1998, 2000; Spinner et al., 2002]. However, we cannot rule out the possibility that the normal axolotl heart contains a protein that stimulate TPM4α translation and which is either decreased or defective in the mutant axolotl hearts.

Studies on the *cardiac* mutant have largely focused on the affect of gene "*c*" on the heart and non-beating ventricle, while the effect it may have on the conus region has not been adequately addressed. The current model of heart development suggests the conus to be a separate transcription unit, which may be derived from extra-cardiac mesoderm [Markwald et al., 1998]. If the gene "*c*" defect resides in the cardiac mesoderm and results from some sort of inhibition rather than a failure of induction, then the ability of the conus to form myofibrils may be unaffected. We speculated this would also be true if the underlying defect was a failure of TM translation in the ventricle. Immunofluorescent analysis of TM expression using CH1 antibody shows normal myofibril formation in the conus region. This is further supported by the fact the coni are observed to beat in mutant animals. We have hypothesized based on the data presented here and elsewhere [Luque et al., 1997; Spinner et al., 2002] that the translation of CH1-positive TM in the ventricle is inhibited even though we readily observe CH1(+) TM in the conus. Indeed, myofibrillogenesis grossly appears to occur normally in the conus of mutant animals evidenced by striated sarcomeres containing tropomyosin, tropomodulin and corroborated by abundant sarcomeres present in the electron microscope. It is to be noted that CH1 mono-

clonal antibodies recognize all sarcomeric tropomyosins containing exon 9 [Lin et al., 1985], which has been implicated in binding with troponins in striated muscle [Lees-Miller and Helfman, 1991]. CH1 anti-tropomyosin antibody does not differentiate between TPM1α and TPM4α. Currently an isoform-specific antibody that will differentiate between TPM1α and TPM4α is not available. Hence, we have employed isoform-specific anti-sense technology to knock down expression of various tropomyosin isoforms in different segments of axolotl hearts. Thus, our data are in agreement with the model for the conus as a separate transcriptional unit and possibly a 5th segment of the developing heart [Markwald et al., 1998].

ACKNOWLEDGMENTS

This work was supported by a grant from American Heart Association (New York Affiliate) and was also supported in part by a grant for American Heart Association (National). A Children's Miracle Network grant also supported this work. We are grateful to Dr. Velia M. Fowler, Scripps Research Institute, La Jolla, CA for her generous gift of anti-tropomodulin antibodies and valuable suggestions whenever asked.

REFERENCES

Bell P, Rundquist I, Svensson I, Collins VP. 1987. Formaldehyde sensitivity of a GFAP epitope, removed by extraction of the cytoskeleton with high salt. J Histochem Cytochem 35:1375–1380.

Chen JN, Haffter P, Odenthal J, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Nusslein-Volhard C. 1996. Mutations affecting the cardiovascular system and other internal organs in zebrafish. Development 123:293–302.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Denz CR, Narshi A, Zajdel RW, Dube DK. 2004. Expression of a novel cardiac-specific tropomyosin isoform in humans. Biochem Biophys Res Commun 320:1291–1297.

Forry-Schaudies S, Gruber CE, Hughes SH. 1990. Chicken cardiac tropomyosin and a low-molecular-weight nonmuscle tropomyosin are related by alternative splicing. Cell Growth Differ 1:473–481.

Franco D, Kelly R, Lamers WH, Buckingham M, Moorman AF. 1997. Regionalized transcriptional domains of myosin light chain 3f transgenes in the embryonic mouse heart: Morphogenetic implications. Dev Biol 188:17–33.

Franco D, Lamers WH, Moorman AF. 1998. Patterns of expression in the developing myocardium: Towards a morphologically integrated transcriptional model. Cardiovasc Res 38:25–53.

Gannon M, Bader D. 1995. Initiation of cardiac differentiation occurs in the absence of anterior endoderm. Development 121:2439–2450.

Gregorio CC, Fowler VM. 1995. Mechanisms of thin filament assembly in embryonic chick cardiac myocytes: tropomodulin requires tropomyosin for assembly. J Cell Biol 129:683–695.

Hardy S, Nadine T, Lepetit D, Allo MR, Thiebaud P. 1995. The Xenopus laevis *TM-4* gene encodes non-muscle and cardiac tropomyosin isoforms through alternative splicing. Gene 156:265–270.

Humphrey RR. 1968. A genetically determined absence of heart function in embryos of the mexican axolotl (Ambystoma mexicanum). Anat Rec 162:475.

Humphrey RR. 1972. Genetic and experimental studies on a mutant gene (c) determining absence of heart action in embryos of the Mexican axolotl (Ambystoma mexicanum). Dev Biol 27:365–375.

Ito S, Karnovski M. 1968. Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. J Cell Biol 112: 276–283.

Kreuz AJ, Simcox A, Maughan D. 1996. Alterations in flight muscle ultrastructure and function in Drosophila tropomyosin mutants. J Cell Biol 135:673–687.

Lees-Miller JP, Helfman DM. 1991. The molecular basis for tropomyosin isoform diversity. Bioessays 13:429–437.

Lemanski LF. 1973. Morphology of developing heart in cardiac lethal mutant Mexican axolotls, Ambystoma mexicanum. Dev Biol 33:312–333.

Lemanski LF, Paulson DJ, Hill CS. 1979. Normal anterior endoderm corrects the heart defect in cardiac mutant salamanders (Ambystoma mexicanum). Science 204: 860–862.

Li L, Miano JM, Mercer B, Olson EN. 1996. Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. J Cell Biol 132:849–859.

Lin JJ, Chou CS, Lin JL. 1985. Monoclonal antibodies against chicken tropomyosin isoforms: Production, characterization, and application. Hybridoma 4:223–242.

Luque EA, Lemanski LF, Dube DK. 1994. Molecular cloning, sequencing and expression of an isoform of cardiac alpha-tropomyosin from the Mexican axolotl (Ambystoma mexicanum). Biochem Biophys Res Commun 203:319–325.

Luque EA, Spinner BJ, Dube S, Dube DK, Lemanski LF. 1997. Differential expression of a novel isoform of alpha-tropomyosin in cardiac and skeletal muscle of the Mexican axolotl (Ambystoma mexicanum). Gene 185: 175–180.

Manasek FJ, Monroe RG. 1972. Early cardiac morphogenesis is independent of function. Dev Biol 27:584–588.

Mangiacapra FJ, Fransen ME, Lemanski LF. 1995. Activin A and transforming growth factor-beta stimulate heart formation in axolotls but does not rescue cardiac lethal mutants. Cell Tissue Res 282:227–236.

Manzella JM, Blackshear PJ. 1990. Regulation of rat ornithine decarboxylase mRNA translation by its 5′-untranslated region. J Biol Chem 265:11817–11822.

Markwald RR, Trusk T, Moreno-Rodriquez R. 1998. Formation and septation of the tubular heart: Integrating

the dynamics of morphology with emerging molecular concepts. In: de la Cruz MV, Markwal RR, editors. Living morphogenesis of the heart. Vol. 3. Boston: Birkhauser. pp 43–84.

Moessler H, Mericskay M, Li Z, Nagl S, Paulin D, Small JV. 1996. The SM 22 promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. Development 122:2415–2425.

Muthuchamy M, Pieples K, Rethinasamy P, Hoit B, Grupp IL, Boivin GP, Wolska B, Evans C, Solaro RJ, Wieczorek DF. 1999. Mouse model of a familial hypertrophic cardiomyopathy mutation in alpha-tropomyosin manifests cardiac dysfunction. Circ Res 85:47–56.

Nakajima-Taniguchi C, Matsui H, Nagata S, Kishimoto T, Yamauchi-Takihara K. 1995. Novel missense mutation in alpha-tropomyosin gene found in Japanese patients with hypertrophic cardiomyopathy. J Mol Cell Cardiol 27:2053–2058.

Narshi A, Denz CR, Nakatsugawa M, Zajdel RW, Dube S, Poiesz BJ, Dube DK. 2005. Cardiac myofibril formation is not affected by modification of both N- and C- termini of sarcomeric tropomyosin. Cardiovascular Toxicology 5: 1–8.

Novy RE, Lin JL, Lin CS, Lin JJ. 1993. Human fibroblast tropomyosin isoforms: characterization of cDNA clones and analysis of tropomyosin isoform expression in human tissues and in normal and transformed cells. Cell Motil Cytoskeleton 25:267–281.

Piples K, Wieczorek DF. 2000. Tropomyosin 3 increases striated muscle isoform diversity. Biochemistry 39:8291–8297.

Ruzicka DL, Schwartz RJ. 1988. Sequential activation of alpha-actin genes during avian cardiogenesis: Vascular smooth muscle alpha-actin gene transcripts mark the onset of cardiomyocyte differentiation. J Cell Biol 107: 2575–2586.

Smith SC, Armstrong JB. 1991. Heart development in normal and cardiac-lethal mutant axolotls: A model for the control of vertebrate cardiogenesis. Differentiation 47:129–134.

Spinner BJ, Zajdel RW, McLean MD, Denz CR, Dube S, Mehta S, Choudhury A, Nakatsugawa M, Dobbins N, Lemanski LF, Dube DK. 2002. Characterization of a TM-4 type tropomyosin that is essential for myofibrillogenesis and contractile activity in enbryonic hearts of the Mexican axolotl. J Cell Biochem 85:747–761.

Stainier DY, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W, Fishman MC. 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. Development 123:285–292.

Sugi Y, Lough J. 1994. Anterior endoderm is a specific effector of terminal cardiac myocyte differentiation of cells from the embryonic heart forming region. Dev Dyn 200:155–162.

Thierfelder L, Watkins H, MacRae C, Lamas R, McKenna W, Vosberg HP, Seidman JG, Seidman CE. 1994. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: A disease of the sarcomere. Cell 77:701–712.

Ward SM, Dube DK, Fransen ME, Lemanski LF. 1996. Differential expression of C-protein isoforms in the developing heart of normal and cardiac lethal mutant axolotls (Ambystoma mexicanum). Dev Dyn 205:93–103.

Watkins H, Anan R, Coviello DA, Spirito P, Seidman JG, Seidman CE. 1995. A de novo mutation in alpha-tropomyosin that causes hypertrophic cardiomyopathy. Circulation 91:2302–2305.

Weber A, Pennise CR, Babcock GG, Fowler VM. 1994. Tropomodulin caps the pointed ends of actin filaments [see comments]. J Cell Biol 127:1627–1635.

Yamamura H, Zhang M, Markwald RR, Mjaatvedt CH. 1997. A heart segmental defect in the anterior-posterior axis of a transgenic mutant mouse. Dev Biol 186:58–72.

Zackson SL, Steinberg MS. 1986. Cranial neural crest cells exhibit directed migration on the pronephric duct pathway: Further evidence for an in vivo adhesion gradient. Dev Biol 117:342–353.

Zajdel RW, McLean MD, Lemanski SL, Muthuchamy M, Wieczorek DF, Lemanski LF, Dube DK. 1998. Ectopic expression of tropomyosin promotes myofibrillogenesis in mutant axolotl hearts. Dev Dyn 213:412–420.

Zajdel RW, McLean MD, Isitmangil G, Lemanski L, Wieczorek DF, Dube DK. 2000. Alterations of cardiac myofibrillogenesis by liposome mediated delivery of exogenous proteins and nucleic acid into whole embryonic hearts. Anat Embryol (Berl) 201:217–228.

Zajdel RW, Sanger JM, Denz CR, Lee S, Dube S, Poiesz BJ, Sanger JW, Dube DK. 2002. A novel striated tropomyosin incorporated into organized myofibrils of cardiomyocytes in cell and organ culture. FEBS Letters 520:35–39.

Zajdel RW, Denz CR, Narshi A, Dube S, Dube DK. 2005. Anti-sense-mediated inhibition of expression of the novel striated tropomyosin isoform TPM1k disrupts myofibril organization in embryonic axolotl hearts. J Cell Biochem 95:840–848.

Zhu L, Lyons GE, Juhasz O, Joya JE, Hardeman EC, Wade R. 1995. Developmental regulation of troponin I isoform genes in striated muscles of transgenic mice. Dev Biol 169:487–503.