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Molecular and Immunohistochemical Analyses of Cardiac Troponin T During Cardiac Development in the Mexican Axolotl, *Ambystoma mexicanum*

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Abstract The Mexican axolotl, *Ambystoma mexicanum*, is an excellent animal model for studying heart development because it carries a naturally occurring recessive genetic mutation, designated gene c, for cardiac nonfunction. The double recessive mutants (*c*/*c*) fail to form organized myofibrils in the cardiac myoblasts resulting in hearts that fail to beat. Tropomyosin expression patterns have been studied in detail and show dramatically decreased expression in the hearts of homozygous mutant embryos. Because of the direct interaction between tropomyosin and troponin T (TnT), and the crucial functions of TnT in the regulation of striated muscle contraction, we have expanded our studies on this animal model to characterize the expression of the TnT gene in cardiac muscle throughout normal axolotl development as well as in mutant axolotls. In addition, we have succeeded in cloning the full-length cardiac troponin T (cTnT) cDNA from axolotl hearts. Confocal microscopy has shown a substantial, but reduced, expression of TnT protein in the mutant hearts when compared to normal during embryonic development. J. Cell. Biochem. 100: 1–15, 2007.

Key words: cardiac Troponin T; axolotl; myofibril; striated muscle contraction

The Mexican axolotl, *Ambystoma mexicanum*, has proved to be a useful tool in the study of cardiac development. The axolotl carries a recessive mutation in gene *c*, first reported by Humphrey [1968], which results in abnormal cardiac development in the homozygous recessive condition, *c*/*c*. These mutant embryos are distinguishable from normal, first at stage 35, when mutant hearts fail to beat. The mutants do not survive beyond embryonic stage 42, which is approximately 20 days later due to lack of circulation. As a consequence of the homozygous mutation, the mutant myocytes fail to form organized myofibrils [Davis and Lemanski, 1987; Fransen and Lemanski, 1991].

Among myofibril structural proteins, tropomyosin has been shown by SDS–Page [Lemanski et al., 1979], radio-immunoassay [Moore and Lemanski, 1982], 2-D gel electrophoresis [Starr et al., 1989] and whole heart confocal microscopic analysis to be drastically reduced in mutant hearts [LaFrance and Lemanski, 1994; Zajdel et al., 1998; Zhang et al., 2003]. Interestingly, actin, myosin, titin, and troponin proteins were found to be at near normal levels with SDS gel electrophoresis analyses [Lemanski, 1976b; Fuldner et al., 1984; Erginel-Unaltuna and Lemanski, 1994]. In vivo protein synthesis studies using ^{35}S-methionine also confirmed that the changes of actin, myosin, C-protein and other myofibrillar structural proteins in the mutant heart were not significant while both tropomyosin and troponin T (TnT) were dramatically decreased [Erginel-Unaltuna et al., 1995]. In additional earlier studies from our laboratory, TnT expression in the heart was characterized using an

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antibody that recognized both skeletal and cardiac TnT and this antibody detected cardiac troponin T (cTnT) expression in all stages of embryonic hearts in both normal and mutant embryos [Fuldner et al., 1984; Lim et al., 1984]. However, these results were not quantitative. Due to the possible existence of skeletal TnT isoforms in developing heart [Wang et al., 2001], we decided for the present study to examine cardiac specific isoforms of TnT expression in normal and mutant embryonic hearts with a very specific antibody now available which detects cardiac specific TnT and does not react with skeletal muscle TnT at all, unlike the original antibody used. This antibody, Mab13-11, prepared against a rabbit heart epitope for cardiac TnT by Dr. Page Anderson of Duke University, has helped us to determine more specifically the expression of cardiac TnT in the developing axolotl hearts. This monoclonal antibody recognizes the epitope FMPNLVPPKI (5′ region of the cardiac gene exon 9) which is a highly conserved epitope of cardiac TnT [Anderson et al., 1991, 1995].

TnT protein is localized in the I-bands of striated muscle fibers, associated with the thin filaments. That TnT is necessary for myofibrillogenesis was first demonstrated in *Drosophila* (fruit fly) by Fyrberg et al. [1990]. The function of TnT is to attach the troponin complex, which consists of troponin I, the inhibitory subunit, troponin C, the calcium binding subunit and TnT, the tropomyosin binding subunit; this influences Mg^{2+} ATPase activity and ultimately muscle contraction [Reviewed by Perry, 1998]. The C-terminal half of the TnT molecule has been shown to bind to tropomyosin in a Ca^{2+} independent manner [Pearlstone and Smillie, 1982] interacting with the head to tail overlap region of tropomyosin [Brisson et al., 1986; White et al., 1987; Solaro and Rarick, 1998]. Three isoforms of TnT have been identified in vertebrates, encoded by different genes located at different loci [Barton et al., 2000; Wang et al., 2001].

cTnT has been proven to be essential in sarcomere assembly and muscle contraction in the heart by mutation studies in the zebrafish system [Sehnert et al., 2002]. Patients with point mutations or deletion mutations on their cTnT genes show familial hypertrophic or dilated cardiomyopathies [Thierfelder et al., 1994; Morimoto et al., 1999, 2002; Li et al., 2001; Fujino et al., 2002; Lu et al., 2003; Harada and Potter, 2004; Shimizu et al., 2003]. In order to determine whether TnT is involved in the diseased situations of our cardiac mutant axolotls, baseline data such as sequence analyses are needed.

In the present studies, we have cloned and sequenced the full-length cDNA sequence of the cardiac isoform of TnT from axolotl. Also we have studied the expression of cTnT in axolotl heart tissue using RT-PCR, Western blotting and confocal immunofluorescence microscopy.

MATERIALS AND METHODS

Procurement and Maintenance of Axolotls

A colony of homozygous normal (+/+) and heterozygous (+/c) adult animals were maintained at Florida Atlantic University. In a temperature and light controlled room, they were kept within individual aquaria in a dilute Holtfreter's solution and fed commercial fish pellets and brine shrimp. A typical spawning produces 200–400 embryos. The cardiac lethal mutation designated by gene *c* is a simple recessive mutation. With each mating of heterozygous (+/−) adults, 25% of the embryos are *c*/*c* mutants.

Western Blots

Axolotl heart, skeletal and smooth muscle was dissected from adult neotenous animals, quickly frozen in liquid nitrogen and stored in sealed tubes. For the Western blotting experiment, samples of approximately 1 g were crushed by mortar and pestle and solubilized in a homogenization buffer containing 2% SDS, 15% glycerol, 100 mM DTT, 80 mM Tris-HCl at pH 6.8 and a protease inhibitor cocktail. These samples were then passed through 19 gauge needles, boiled and centrifuged at 5,000*g*. The concentration of each sample was calculated by using BCA assays and equal amounts of proteins in the supernatants, along with molecular weight standards. These samples were electrophoresed on 15% polyacrylamide gels containing 0.1% SDS and transfered. These gels were subsequently transferred to 0.20 μm nitrocellulose membranes, then probed with the aforementioned monoclonal antibodies according to the methods of Towbin et al. [1979]. Visualization of all blots was performed by the chemiluminescent methods of Super-Signal Ultra by Pierce Chemical Company

(Rockford, IL). Wash buffers all contained 0.1 % tween-20 and primary antibody concentrations used were 1:250 for Mab13-11. Reacted blots were visualized on X- Omat film by Kodak.

Immunohistochemistry of Frozen Sections

Neotenous and metamorphosed adult tissues were dissected, washed in cold PBS and fixed in a periodate-lysine-paraformaldehyde (PLP) solution according to the protocol of (MacLean and Nakane, 1974). Tissues were slowly infiltrated with increasing concentrations of sucrose and quick frozen in liquid nitrogen. Blocks were stored at −70°C, and sectioned at −22°C on a Sorvall Cryo-ultramicrotome. Sections were stored on Superfrost Plus slides at −20°C until ready for use. Before antibody labeling, at room temperature, slides were immersed in a Tris/HCl buffer for 1 h, rinsed in TBS and blocked in 3% milk for an additional hour. Approximately 100 μl of primary antibody was left on the slides overnight in a humid chamber at 4 degrees Celsius. After a 45 min secondary antibody incubation, the slides were washed and mounted in 1% n-propylgallate in 25% glycerol in 0.1 M sodium bicarbonate and viewed with a Zeiss Universal Microscope equipped with phase-contrast and epi-fluorescent optics.

Confocal Microscopy

Mutant and normal embryonic whole hearts at stages 35, 38, and 41 were dissected and fixed according to the established procedures in our laboratory [LaFrance and Lemanski, 1994; Mangiacapra et al., 1995; Ward et al., 1995a,b]. Briefly the hearts were removed from the thoracic cavity, immersed in 1 mM dithiobis (succinimidly) propionate in DMSO/Steinberg's solution to both cross link and permeablize the tissue. Hearts were rinsed several times in sterile Steinberg's solution after which primary antibody (Mab13-11) and a lissamine–rhodamine conjugated fluorescent secondary antibody were added. The hearts were then mounted, coverslipped and visualized using the BioRad MRC-600 confocal laser scanning microscope system attached to a Zeiss IM-205 inverted compound microscope. Optical sections covering equal thicknesses (same number of z-series) of each sample were collected, merged and photographed using BioRad's confocal software package, COMOS. Laser strength was adjusted so that imaging intensity was not saturated in reference to a spectrum bar. Three-dimensional images of the whole hearts were obtained and analyzed for myofibril formation.

Fluorescence intensity of the digitalized pictures was analyzed by using Adobe Photoshop software (7.0). Mean luminosities of randomly selected areas in the ventricles were calculated by software and averaged for each experimental group. Areas were selected from ventricles of different samples and from different parts of the ventricle.

Cloning of Axolotl cTnT

The cDNA for cTnT was cloned by the polymerase chain reaction (PCR) from a lambda gt11 library constructed with mRNA from juvenile axolotl heart tissues [Erginel-Unaltuna et al., 1995]. The cTnT cDNA clone was isolated from this library using a primer pair (P1: 5′-CTT CAT GCC CAA CYT GGT GCC, P2: 5′-TTC TGC YGC TTG AAC TTY TCC TGC A). The oligonucleotides were obtained from Integrated Technologies, Inc., Coralville, IA. Southern blots confirmed the bands with amplification of cTnT. Internal oligonucleotides were end-labeled with ^{32}P via a standard kinase reaction. These hot probes were hybridized to 10 μl PCR products and dry transferred to nitrocellulose from 1.5% agarose gels. Blots were stringently washed in 2XSSPE with 5% SDS at 42°C. Positive hybridization was visualized by overnight incubation at −70°C on Kodak X-omat film. The cDNA band showing a positive hybridization signal was extracted using the Qiagen Quick gel extraction kit. Purified cDNAs were sequenced by facilities at Cornell University and BioTech Core of Palo Alto California, followed by cloning into a T-vector (Promega, WI).

RACE reactions 5′ and 3′ were performed by using a SMARTRACE kit from Ambion (Austin, Texas) following the instructions of the manufacturer.

Real-Time RT-PCR

Total RNA was extracted from normal and mutant hearts at various stages after the conus areas had been removed. cDNA was synthesized from 2 μg of total RNA using Invitrogen's Thermoscript RT-PCR System. Real-time RT-PCR experiments were performed on a Capillary Lightcycler (Roche) machine using a Roche Fast Start SYBR Green I Kit following the

manufacturer's instructions. Specific amplification of desired genes was confirmed by calculated melting temperatures (Tm) for the products from the melting peak curve (−dF/dT vs. temperature). All the amplicons were collected and confirmed again by agarose gel electrophoresis and sequencing. A standard curve of cross-point versus Log concentration (copies) was created using one of the cDNA samples with serial dilutions. Negative controls were included using cDNAs synthesized the same way as above but with no reverse transcriptase added. Each cDNA sample was run in triplicates. The data were averaged and standard deviations were calculated. The beta-actin gene was used as a standard control. The cTnT, cardiac actin and myosin heavy chain (MHC) mRNA expression levels were compared between different samples after normalization relative to beta-actin concentrations. Primer sequences used in these studies are as follows:

cardiac actin:	(amplicon 185 bp)
Forward:	5′-TCAGGCAGCTACCCACCCTC-TCC
Reverse:	5′-CCATTGATTTTCGGAGGCGC-ATT
MHC:	(amplicon 214 bp)
Forward:	5′-CGCGTGGCAAACAGACCTAC-ACC
Reverse:	5′-TTGGTCCTCCACTGTGCCAC-CTC
cTnT:	(amplicon 173 bp)
Forward:	5′-CCAAGGGCTTCACCGGGCTC-AA
Reverse:	5′-TGGCAGAGGTGGAATGGAT-CACAGG
beta-actin:	(amplicon 210 bp)
Forward:	5′-TCCATGAAGGCTGCCCAACT
Reverse:	5′-TGGCGCCACATCTGATTGAT.

RESULTS

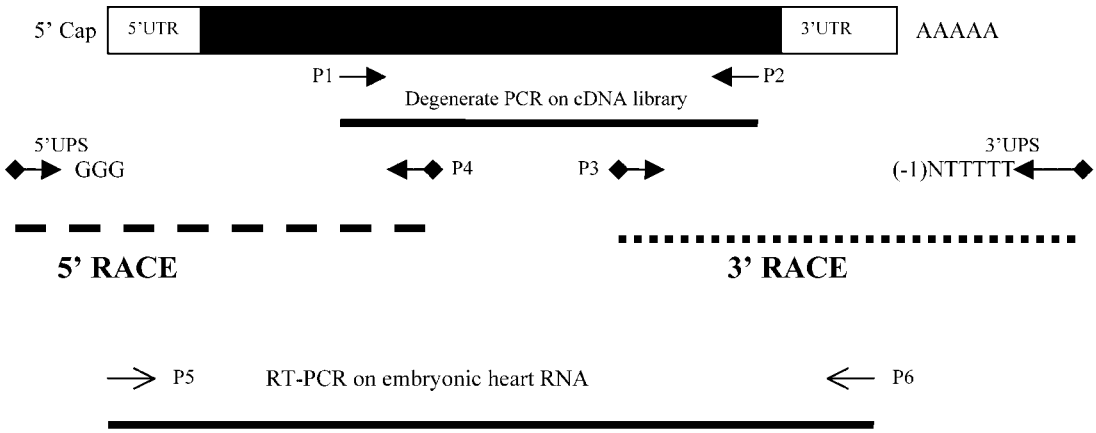
Cloning of the Full-Length Axolotl Cardiac Troponin T cDNA

Combining many gene cloning techniques such as degenerate RT-PCR, 5′ and 3′ RACE and library screening, we now have cloned the full-length cDNA of the axolotl cTnT gene through the methods shown in Figure 1. Degenerate primers P1 and P2 are designed based on the conserved sequence of known cTnT from other species. PCR reactions using P1 and P2 has yielded a cTnT partial sequence from a axolotl juvenile heart cDNA library [Erginel-Unaltuna et al., 1995]. The following 5′ and 3′ RACE reactions using the total RNA extracted from embryonic axolotl hearts have provided the remaining 5′ and 3′ UTR sequences of the axolotl cTnT gene. The full sequence combined from all of these approaches has been confirmed by RT-PCR to be expressed in embryonic axolotl hearts at stages 34–42 before the embryos hatches.

The full-length cDNA is 1,360 bp (excluding the poly (A) tail) containing the entire open reading frame of 298 amino acids predicted from the sequence (Fig. 1). Sequencing results correspond very well to our Northern blotting experiments in which we have detected a ~1.3 kb band with a strong positive hybridization signal to our radioactively labeled cTnT probes (Fig. 2).

The axolotl cTnT sequence shows high sequence similarity to our cloned cTnT sequence in other species from human to zebrafish, except for the N-terminal hypervariable region (Fig. 3). The reported cardiac specific exon (exon 9) is also present in the axolotl cTnT sequence (Fig. 3), confirming this is a cardiac specific isoform of TnT and is not found in the skeletal muscle isoforms. Table I lists the identity of the sequences at the amino acid level of axolotl cTnT compared to other vertebrates. When we compare our axolotl cTnT protein sequence with the human full cTnT protein sequence of the human TnT2 gene (Genbank Accession #: P45379) [Farza et al., 1998, Gomes et al., 2002], we find that the axolotl cTnT protein sequence shows an identical length (298 aa) of amino acids to the full length human cTnT protein, indicating that all of the exons of axolotl cTnT cDNA have been successfully cloned. The identity between human and axolotl cTnT protein residues is 64.0%. Of those species examined, the *Xenopus* cTnT sequence shows the highest homology to the axolotl sequence with a 69.1% identity in total amino acids (Table I).

After analyses of the axolotl cTnT protein sequence, we have found surprisingly that many of the critical residues whose mutations are present in human patients and can cause either hypertrophic or dilated familial cardiomyopathies are conserved in the axolotl cTnT sequence (Table II). We now believe that cloning of the axolotl cTnT sequence provides us with a novel system to study cardiac muscle formation



GACTCTTTCGTTGTCCCTTCCTGGATCTGCAGGCCTCCTCTCCTCTCTCTCTCCAGCTG
CAGGTCCAGAGAAGAACATGTCTGAGTTCGAGGATGTCATGGAGGAATACGAGGGGCAG
GAGGAAGAAGAAGGAGGTGTAGAAGAGGAAGAAGGGAGAGAAGAAGAAACATTAGAAGAAGCAGC
GGCAGAGGAACACGAGGAGGCATCCGAGGCGCATGCACAAGATCGTGATGATGAGGCTCGTGAGC
GCGAAGAAGGCGGTGGCGAAGGCGAGCAAGAACAAGAAGGATCGAAACCCAAACCAAAGTTGTTT
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CAAGCGTATGGAGAAAGACTTGAATGAGCTGCAGACGCTGATCGAGGCGCACTTTGTGAACCGGA
AGAAGGAAGAAGAGGAGCTCATTGAGCTTACTGACAGAATTGAGAAGCGTCGGTCAGAGAGGGCC
GAGCAGCTGAGGATTCGGACCACGAAAGAGAAGGAGCGCCAGGCACGCATGGCAGAAGAAAAAGC
AAGAAAAGAGGACGAGGAGAACCGGAAGCGAGCTGAGGACGATGCCAAGAAGATGAAGGCTTTCT
CAAACATCTCTATGCATTTTGGTGGATACCTGCAGAAGACTGGCAGAGGCGGGAAGAAGCAGACG
GAACGGGAGAAGAAGAAGAAGATTCTGACCGAACGCCGGAAGTCTCTCAACGTCGAGGTCCTGAG
GGAGGACGAGCTGAAGGAGAAAGCCAAGGAGATGTGGCAATGGATGAAGGAGCTGCAGGCGGAGA
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AGCGACCATCAGAAGGTGAAAAGTTCCAAAACTGCACGTGGTGGAAAAGGCATTGTTGGAGGCCG
CTGGAAGTGAGGTGCCAAGGGCTTCACCGGGCTCAAGCCAGGCAGCTCTACATTATCTGGGATCT
ATGGAGATTTTCAAAGAGACCTGAGCTGCTCAAACATCAGCAAATATATTTCAATGATTTTCTGA
GTTAATAACCTGCCCAGAAATCACATCATTATCCTGTGATCCATTCCACCTCTGCCAAGAAGACC
TAGCAGCAAAGCACAAATTGTGTGCTCTTGCTCCCGGAGCATGAATGAACAGCTGCCCCCTGCTT
TTTGATCATCCCTCCCCACCATTCTGCCCCCTCAGCCTGTGCATGCTGTACTTCCTCTATTTGCT
CACTGTTATTTTGTTCCCAAATTATTTGTCGACATATTGTTCTTAATGTGATGTA**AATAAA**AACT
TGAATGCAAAAAAAAA

Fig. 1. Strategy to clone the full-length cDNA of axolotl troponin T (TnT) gene (**top**) and the full-length cDNA sequence (**bottom**). Pieces of cDNA fragments obtained from different experiments are shown to be expressed from a single cTnT gene by RT-PCR using P5 and P6 primer pairs on extracted total heart RNA. The underlined sequence is the predicted open reading frame for axolotl cardiac troponin T (cTnT). Bold sequence indicates the polyadenylation signal.

and contractibility regulation considering the existence of the unique gene *c* mutation found in this animal model.

Expression Studies of Cardiac Troponin T Gene in Adult Tissues

Based on the cloned cTnT cDNA sequence, we have performed further RT-PCR studies on total RNA extracted from adult tissues and have confirmed that the TnT sequence we obtained is expressed only in the heart and not in adult skeletal muscle; neither is there any cTnT expression in the axolotl liver RNA used as a control (Fig. 4). With Western blotting experiments using a Mab13-11 antibody, which is reported to recognize only the cTnT isoform but not the skeletal muscle isoforms [Anderson et al., 1991], we also have verified that this

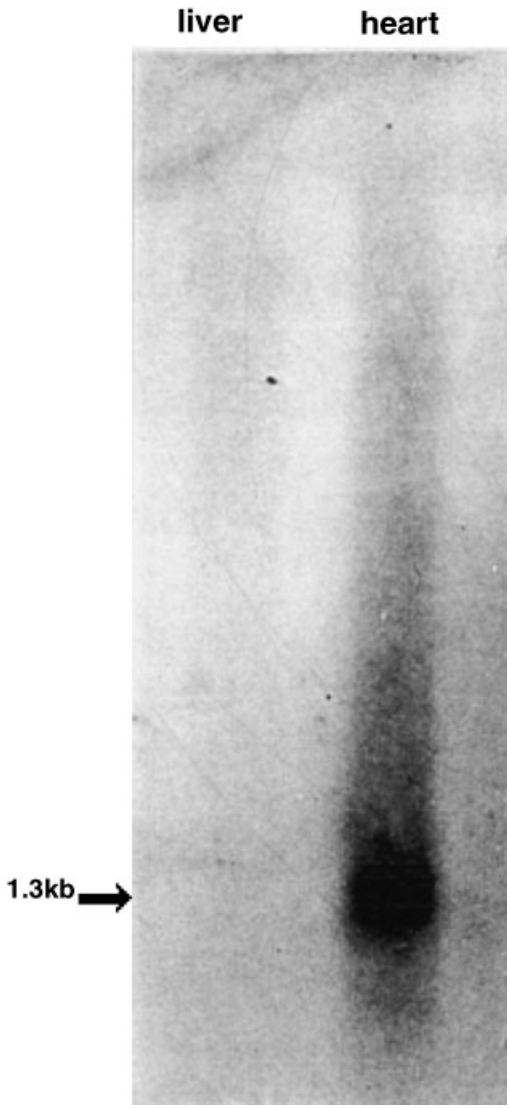

Fig. 2. Northern blotting on total RNA extractions of adult liver and heart. RNAs were separated by a 1.5% denatured agarose gel. Northern blotting was performed by hybridization with ^{32}P labeled PCR amplified DNA of the axolotl cTnT gene.

antibody specifically recognizes only cTnT but not the skeletal muscle isoforms in our axolotl system (Fig. 5). Mab13-11 antibody provides us the capability of studying TnT gene regulation in developing embryonic heart without interference from other isoforms.

Confocal Microscopy Shows the Existence but Reduced Expression of cTnT in Mutant Axolotl Embryonic Hearts Compared to Normal

Using the Mab13-11, cardiac specific antibody to TnT protein, we have compared cTnT expression by confocal immunofluorescent studies in normal and mutant embryonic hearts after the heartbeat stage. Unlike the tropomyosin protein which has been studied in our laboratory in detail and shows a dramatic decrease in the mutant embryonic hearts at all stages, cTnT shows a reduced but substantial expression pattern in the mutant embryonic hearts; moreover, the pattern of staining in the mutant hearts is drastically different than normal (Fig. 6).

Normal stage 34/35. Embryonic stage 34 represents the initial heartbeat stage in normal embryos. The beat moves from the atrium through the ventricle to the conus. Both peripheral and nascent myofibrils are present with their directionality and organization evident [Sanger et al., 2000]. In the normal embryonic hearts, we have begun to see significant amounts of cTnT protein expression beginning at stages 34–35, at the initiation of a heartbeat and when heart looping is beginning to take place. At this stage, cTnT protein exists in the peripheral areas of the cardiomyocytes in random disorganized patterns (Fig. 6A). The peripheral myofibrils have a strong and distinct staining pattern compared to nascent myofibrils.

Normal stages 37–38. Expression of cTnT protein becomes more abundant when the heart matures to stages 37–38, the post-heartbeat stages (Fig. 6C). Most of the cTnT proteins are now incorporated into the myofibrillar structures and an organized pattern of thin filament staining can be detected in the normal hearts at these stages. The striated, punctuate I band pattern in myofibrils is now evident (arrow).

Normal stage 42. When hearts continue to develop and the embryo reaches the hatching stage (stage 42), confocal microscopy of hearts stained by Mab13-11 antibody reveals that numerous well-organized sarcomeric myofibrils have formed in the hearts, especially in the ventricular area, with the characteristic banding pattern of cTnT. Now the myocytes are packed with myofibrils reaching the full perimeters of the cells forming a striated pattern throughout the cytoplasm. There is also abundant diffuse cytoplasmic staining for cTnT, which continues to be focused in myocytes in the conus regions of the heart which is attached anterior to the ventricle (Fig. 6E). At this stage, the "S" shaped heart is beating with strong, regular strokes from the contraction of multiple layers of myocardial cells.

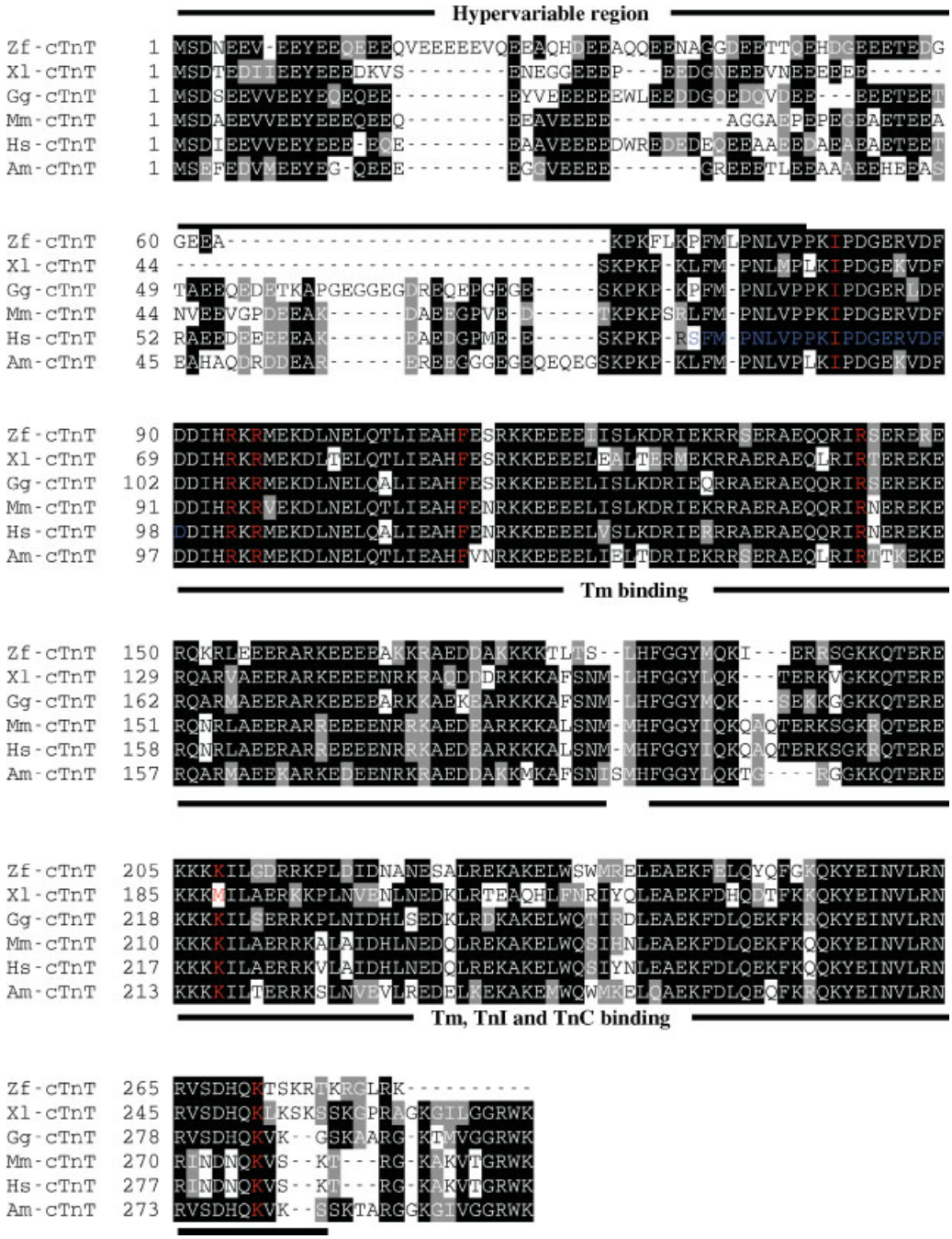

Fig. 3. Alignment of the predicted axolotl cTnT (Am-cTnT) protein sequence with zebrafish (Zf), Xenopus (Xl), Chicken (Gg), Mouse (Mm) and Human (Hs) cTnT protein sequences. Amino acids that are conserved in at least three cDNAs are shaded in black. Black bars indicate the different regions of cTnT protein that are hypervariable [Hsiao et al., 2003], the Tropomyosin (Tm)-binding region, or the Troponin I (TnI)-, Troponin C (TnC)- and weak Tm-binding region [Raggi et al., 1989]. Residues in blue indicate the cardiac specific exon, which is also the antigen for Mab13-11 antibody in human gene. Residues in red show the reported point mutations and a deletion mutation in human patients with familial cardiomyopathies.

Mutant *c/c* stage 34/35. Stage 34/35 mutant embryos are distinguishable upon inspection under a dissection microscope from normal (+/+ or +/*c*) embryos by the absence of a contracting heart with the accompanying lack of circulation through the gills. Yolk platelets remain abundant in stages 35–42 mutant hearts presumably because they are not rapidly metabolized due to the lack of heart contractions. In the stages 34–35 *c/c* mutant embryonic

TABLE I. Sequences With High Homology to Axolotl Cardiac Troponin T

Protein sequences used for comparison					
Species	Redidue (AA)	Identity	Type	Accession#	Reference
Human	298	64.0%	Cardiac	P45379	Mesnard et al. [1993, 1995]; Townsend et al. [1995]; Farza et al. [1998]
Mouse	291	63.5%	Cardiac	AAA85348	Jin et al. [1996]
Chicken	302	68.5%	Cardiac	AAA49099	Cooper and Ordahl [1985]
Chicken	265	58.9%	Slow skeletal	BAA12727	Yonemura et al. [1996]
Xenopus	272	69.1%	Cardiac	AAO33405	Hsiao et al. [2003]
Xenopus	276	54.7%	Fast skeletal	AAM55471	Hsiao et al., unpublished.
Zebrafish	282	62.5%	Cardiac	AF434187	Hsiao et al. [2003]
Zebrafish	290	53.3%	Slow skeletal	AAN32755	
Zebrafish	230	54.7%	Fast skeletal	AAF78472	Xu et al. [2000]

hearts, we cannot detect significant cTnT expression in the ventricle area (V) of the hearts (Fig. 6B) but rather find that there are large pools of cTnT protein concentrated in the conus regions. The cytoplasm of the ventricular myocytes is primarily devoid of stain, only showing yolk platelets as lightly stained ellipses (Y).

Mutant *c*/*c* stage 37/38. Ventricular staining of cTnT by Mab13-11 is most significant at stages 37–38 (Fig. 6D). cTnT is seen to localize along the peripheral myocyte membranes, similar to the cTnT staining pattern present in the normal stage 35 hearts, but in a more diffuse and random way. At the membrane, the staining is in a solid line rather than showing a striated or punctuate appearance characteristic of peripheral myofibrils in normal heart cells (arrow).

Mutant *c*/*c* stage 42. Because of the failure of a heartbeat, diffusion remains the only source for oxygenation for *c*/*c* mutants, which do not survive beyond stage 42. At this stage, the mutant embryos have visibly distended hearts possibly due to ascites in the thorax/abdomen. The mutant ventricular myocardial walls remain only one-cell-layer thick. Once again, the Mab13-11 staining reveals a greatly reduced expression of cTnT in the mutant ventricle compared to normal (Fig. 6F). Instead of well-formed myofibrils like in the normal hearts, cTnT is seen in a spotted pattern localized throughout the myocytes in the ventricle (arrow). This spotted pattern is strongest in the conus region (*). Compared to stage 34/35, the large spot pools of cTnT are no longer visible in the conus but replaced by smaller spots in larger numbers. Yolk platelets still exist in the background as lightly stained ellipses (Y). The yolk disappears in normals by stage 41; mutants retain the yolk even to stage 42 presumably because of the lowered metabolism from no heartbeat.

The expression levels of cTnT in normal and mutant hearts at various stages are compared by the intensity of fluorescence after immunostaining by Mab13-11 antibody and fluorescence-conjugated secondary antibody. The mean values of fluorescence intensity have been averaged from multiple heart samples and different areas of the ventricle and compared between normal and mutant samples (Fig. 6G). Clearly, at all stages, the mutant hearts have significantly decreased expression of cTnT in the ventricular area. Due to the uneven distribution of cTnT in the conus area and the difficulties of unequivocal identification of the conus borders, quantification of cTnT

TABLE II. cTnT Mutations in Human Patients

Mutation in human cTnT	Axolotl cTnT residue	Interaction	Syndrome	Reference
R141W	R	Tropomyosin	Dilated cardiomyopathy	Lu et al. [2003]
Delta K210	K	Troponin	Dilated cardiomyopathy	Morimoto et al. [2002]
K273E	K	Troponin	Hypertrophic to dilated cardiomyopathy	Fujino et al. [2002]
F110I	F	Tropomyosin	Hypertrophy	Lin et al. [2000]
R92Q	R	Tropomyosin	Hypertrophy	Szczesna et al. [2000]
R94L	R	Tropomyosin	Hypertrophy	Harada and Potter [2004]
I79N	I		Hypertrophy	Thierfelder et al. [1994]

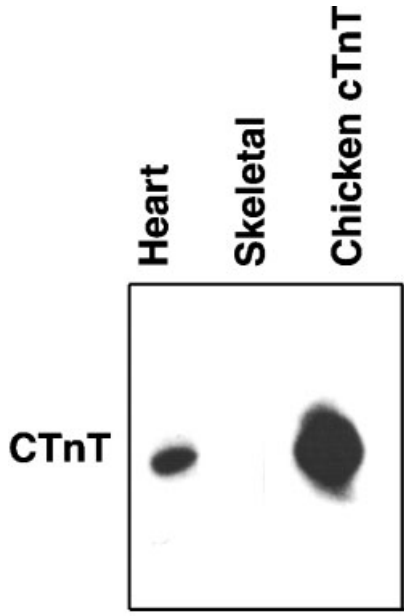

Fig. 4. RT-PCR shows a differential pattern of cTnT expression in the adult neotenous axolotl. This figure shows the tissue specific expression of the cTnT gene within striated muscle. RNA was isolated from neotenous ventricle (Heart muscle) and neotenous *dorsalis trunci* muscle (Skeletal muscle). The cTnT gene was expressed in the ventricle. Skeletal muscle failed to show any expression. As a positive control, chicken cTnT was also included in the same gel that shows a strong signal to the radioactively labeled axolotl cTnT probes.

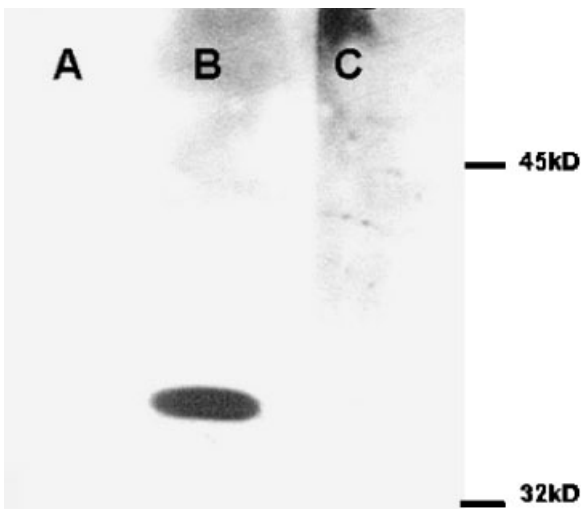

Fig. 5. Western blot analyses of axolotl cTnT. These blots were incubated with TnT anibody Mab13-11 (Labvision Corp., CA). The 10% polyacrylamide gels were loaded with adult tissue protein (**A**) skeletal muscle (*dorsalis trunci*), (**B**) heart (ventricle) and (**C**) intestine. Axolotl adult cTnT molecular weight is estimated to be ~36 kDa.

expression in the conus area has not been done. Very clearly there is a significant amount of cTnT expression in the mutant conus.

Real-Time RT-PCR Studies of cTnT Expression Between Normal and Mutant Embryonic Hearts

As shown above, confocal studies have revealed that the cTnT expression is reduced or delayed during embryonic heart development. Whether it is caused at the transcription level or translation level is still unknown. To answer this question, real-time RT-PCR studies have been performed to compare mRNA expression level differences between normal and mutant embryonic hearts. Due to the difficulty in dissection procedures to collect large quantities of small embryonic hearts at early stages such as stages 34–35 and also to avoid the problem of the progressive general decrease of gene expression level near the hatching stage (stage 42) in the mutant hearts shown by our previous studies [Erginel-Unaltuna et al., 1995], we have focused our studies on embryos at stages 37–38. Using the beta-actin gene expression level as a standard, normalized expression levels of cTnT mRNA have been compared between normal and mutant hearts. Primers used in RT-PCR are designed based on the 3′ UTR region of cTnT cDNA, which is shared by all possible isoforms of cTnT transcripts. Results show that the cTnT mRNA expression in the mutant hearts is significantly decreased to only 1/12 that of the normal heart (Fig. 7), indicating that the defects in the mutant hearts are actually at the transcription level.

Interestingly, quantitative RT-PCR experiments on the MHC gene and the alpha-actin gene show no significant changes between normal (+/+) and mutant (*c*/*c*) hearts at stage 37–38 (Fig. 8), consistent with our previous studies [Lemanski, 1976b; Erginel-Unaltuna and Lemanski, 1994; Erginel-Unaltuna et al., 1995]. Surprisingly, although a dramatic decrease of tropomyosin expression at the protein level has been observed constantly, no significant RNA level changes of tropomyosin gene expression have been detected, indicating a differential gene regulatory pathway for tropomyosin and cTnT (data not shown).

DISCUSSION

Our past and current studies form the foundation to determine the role of troponin-T

in the regulation of contraction and its own expression regulation by other tissue specific factors using the gene c mutation animal system [Lemanski, 1976a,b; Lemanski et al., 1985, 1996; LaFrance et al., 1993; Luque et al., 1994, 1997; Gaur et al., 2001]. The troponin complex along with tropomyosin are essential for the efficient use of calcium in muscle contraction. In vitro binding studies by Palm et al. [2003] have demonstrated that the complexes formed by Tropomyosin N- and C-terminal ends and only those ends in stiated muscle tropomyosins, are required for their binding to the regulatory protein, TnT. Their studies using the peptide model system also show that a peptide from human cTnT, hcTn70-170, promotes the affinity of striated muscle tropomyosin for actin up to 15 folds [Palm et al., 2003]. Tropomyosin expression has been shown before to be dramatically decreased in the gene *c* mutant animals [LaFrance and Lemanski, 1994; Zajdel et al., 1998; Zhang et al., 2003]. Decreased expression of cTnT protein in the mutant hearts could possibly be the reason for the failure of incorporation of tropomyosin protein to the myofibril structure, thus abnormally high degradation of tropomyosin protein in the mutant hearts may occur. To determine which gene deficiency is the secondary defect, we are now taking advantage of the mutant axolotl system that has virtually no sarcomeric tropomyosin and decreased cTnT by introducing different isoforms of tropomyosin and TnT isoforms (including point mutated TnT at the key residues listed in Table II) exogenously into the mutant embryonic hearts to determine the role(s) they play in myofibril formation and function.

Our first experiments dealt with cloning the axolotl cTnT sequence. We determined the expression of cTnT in neotenic cardiac and neotenic skeletal muscles through tissue-specific RT-PCR. Using troponin-T specific primers we cloned cTnT from the axolotl heart library.

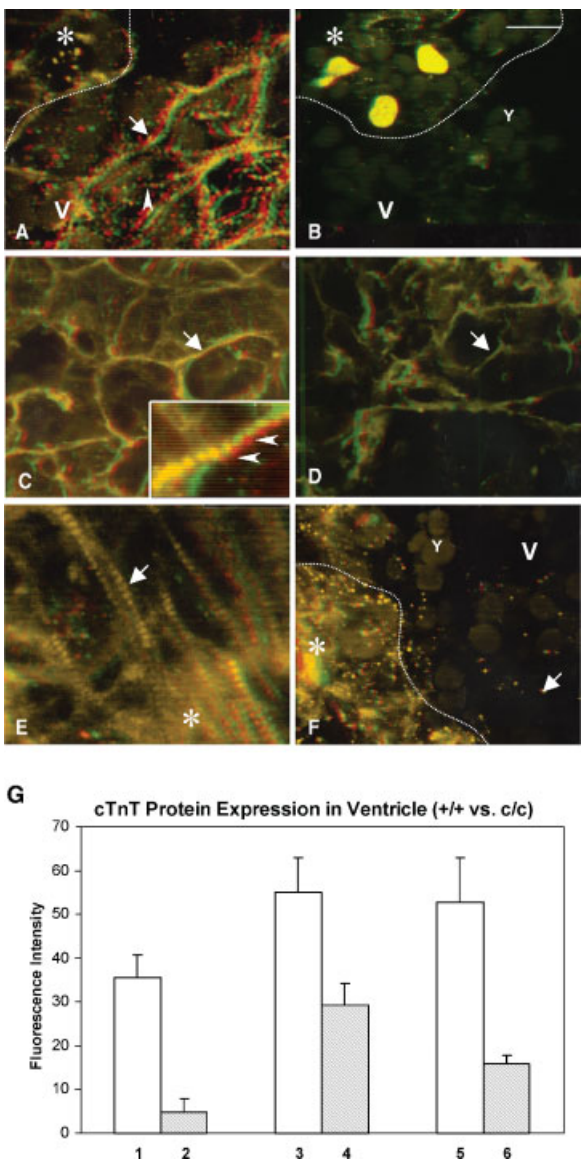

Fig. 6. Confocal images of axolotl embryonic hearts stained with Mab13-11 antibody showing cTnT expression and distribution. **A**: Normal (+/+) stages 34–35 heart shows staining of both nascent (arrow head) and peripheral fibers (arrow) in the ventricle. **B**: Mutant (*c*/*c*) stages 34–35 heart shows dramatically decreased staining compared to A in the ventricle but spotted pools of cTnT protein concentrated in the conus region. **C**: Normal (+/+) stages 37–38 heart shows striated, punctuate I-band staining (arrow) of cTnT. The bottom right panel is the enlarged picture of the area in the same heart indicated by the arrow showing an obvious striated pattern of cTnT staining (arrow heads). **D**: Mutant (*c*/*c*) stages 37–38 heart shows peripheral staining seen in solid lines rather than the punctuate appearance of the peripheral myofibrils of normal hearts. **E**: Normal (+/+) stage 42 heart shows well-organized staining of myofibrils in the whole ventricle region. There is abundant diffuse cytoplasmic staining in the conus region (*). **F**: Mutant (*c*/*c*) stage 42 heart shows bright staining with cTnT in small pools (arrowhead) localized throughout the myocytes. This spotted pattern is strongest in the conus region (bar shows 20 μm; *: conus; V: ventricle; dashed lines indicate the borders between conus and ventricle. Borders are identified from morphology). **G**: Average mean values of fluorescence intensity from multiple samples of both normal and mutant hearts (ventricle area only) at different stages show decreased but significant staining of cTnT in the mutant hearts compared to normal. (1) Normal stages 34–35 hearts; (2) mutant stages 34–35 hearts; (3) normal stages 37–38 hearts; (4) mutant stages 37–38 hearts; (5) normal stage 42 hearts; and (6) mutant stage 42 hearts.

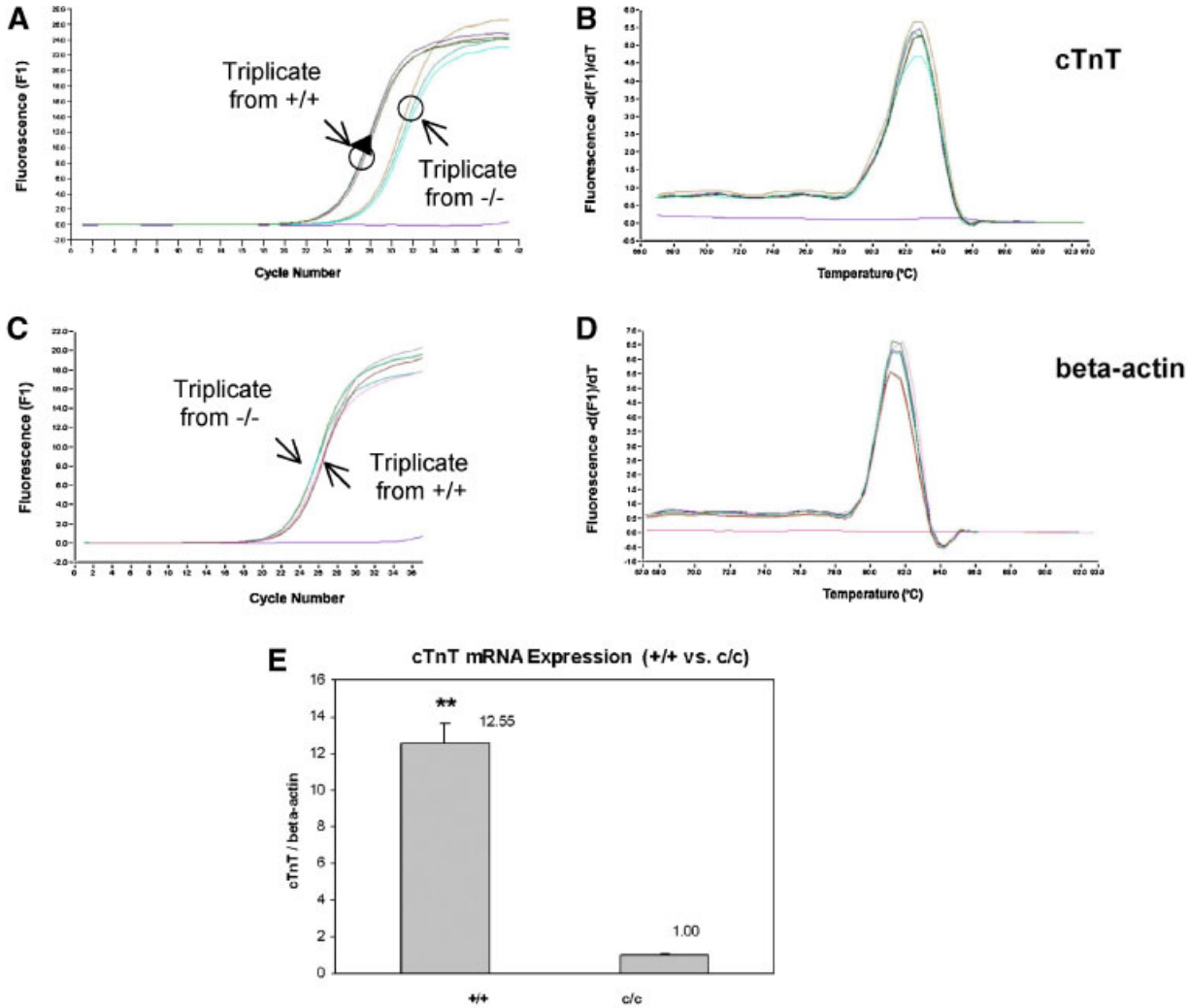

Fig. 7. Real-time RT-PCR of cTnT mRNA expression in both normal and mutant stages 37–38 embryonic hearts. **A**: Real-time amplification curve of beta-actin gene on both normal and mutant heart cDNA. The curves are drawn by plotting real-time fluorescence intensity (detected by the F1 channel for SYBR green fluorescence indicating the amount of synthesized double-stranded DNA amplicons) versus time. Triplicates from each group (+/+ or *c*/*c*) are conducted showing good consistency. Negative controls on cDNA with no reverse transcriptase during cDNA synthesis show no amplification. **B**: Melting curve analyses after cTnT RT-PCR confirm that all the amplicons are having the same melting temperature (Tm) as calculated except for negative controls with no amplicons detected, demonstrating the right amplicons from PCR reactions. The original melting curve data are not shown. Melting temperatures (Tm) are calculated by locating the corresponding temperature values at the peaks in the plots of the derivation of the real-time fluorescence intensity versus time (−d(F1)/dT) when the samples are heated up at a constant rate of temperature increase. Gel electrophoresis confirmed the same results (data not shown). **C**: Real-time amplification curve of cTnT gene. **D**: Melting curve analysis on cTnT amplicons. **E**: Normalized cTnT RNA expression level by beta-actin gene demonstrated there are only less than 1/12 of cTnT RNA transcribed in the mutant hearts compared to the normal ones ($^{**}P < 0.05$).

We found the overall size of the message coding axolotl cardiac troponin-T by Northern blotting. The apparent molecular weight of troponin-T is about 1.5 Kb including the poly (A) tail. We compared the predicted full-length amino acid sequence to other species and found the axolotl cTnT protein sequence to be more than 64% homologous to other mammalian, avian and fish cardiac TnT cDNAs. It has exactly the same number of residues as human full-length cTnT protein and also contains the cardiac specific exon sequence [Anderson et al., 1991, 1995]. The axolotl cTnT protein shows striking similarity to the human cTnT sequence also in that it has many critical residues conserved with the same amino acids as human's.

The following experiments were performed to determine the cardiac specificity of the cTnT

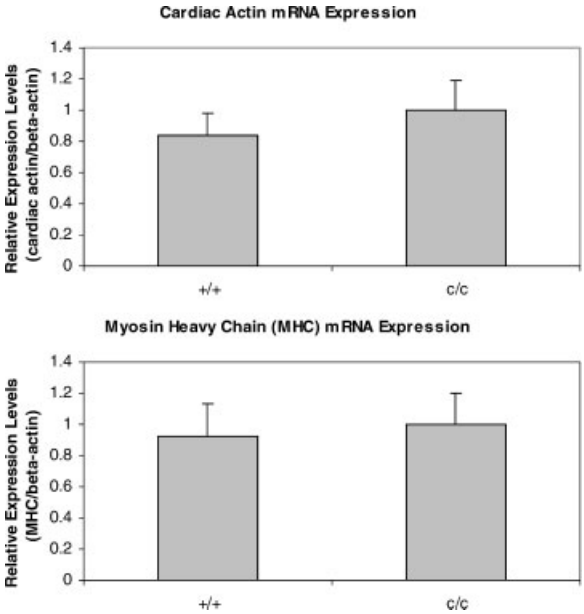

Fig. 8. Real-time RT-PCR of cardiac actin and myosin heavy chain (MHC) mRNA expression in both normal and mutant stages 37–38 embryonic hearts. No significant differences at mRNA level for the two genes are detected between normal and mutant hearts. The relative expression levels of each gene in normal and mutant hearts are shown in both panels with the ratio of the expression level of that gene to beta-actin in *c/c* mutant hearts designated as unit 1.

antibodies, Mab13-11. We tested the antibody by both immunohistochemistry of frozen sections and Western blot analysis of adult skeletal and cardiac tissues. We demonstrated that the cTnT is localized specifically in the I bands of cardiac muscle in a regular periodicity by immunocytochemistry. Western blots and frozen sections of skeletal muscle failed to react with the antibody. Thus, it was concluded that Mab13-11 specifically stains a cardiac specific TnT isoform in the axolotl heart. This antibody was then used as a tool to elucidate the normal and mutant patterns of expression.

Normal embryonic hearts were observed to have an increasingly organized myofibrillar structure with progressive development. It would be expected that TnT staining increased with the differentiation of the sarcomeres. In the unique cardiac lethal condition *c/c*, cTnT appeared to have a reduced expression in the mutant ventricles, but was still present in large collections or pools in the conus. Interestingly, a bright spotted staining pattern of cTnT was observed in the conus region of stage 34/35 mutant hearts (Fig. 6B). We do not consider stained pools to be artifacts because similar staining patterns were observed for titin in stage 32 normal hearts [Erginel-Unaltuna and Lemanski, 1994]. These results support either a cTnT protein distribution defect or a delayed expression pattern for cTnT in the mutant hearts. These results confirm that cTnT is also affected by the mutant condition. Our updated studies here with comparisons between normal and mutant embryonic hearts for a series of developmental stages have clarified our previous results, which were controversial due to lack of cardiac specific cTnT antibody and no separation of ventricle from conus [Fuldner et al., 1984; Erginel-Unaltuna et al., 1995]. The significantly decreased expression of cTnT in the ventricle region are likely taking place at the transcription level based on our Real-time RT-PCR results although the possibility of an abnormal rate of RNA processing for cTnT is not ruled out. Less than 1/12 of the mRNA is transcribed in the mutant hearts compared to the normal ones while nearly half of the protein is present in mutant hearts compared to the normal ones at stages 37–38. This result indicates that there exists some mechanism for translation level compensation in the mutant hearts. We have not ruled out the possibility that more than one isoform of TnT may be present in cardiac tissue, with isoforms being specific for the ventricle versus conus regions. After seeing the change in mutant ventricles at stage 37/38 versus stage 42, we hypothesize that the difference may result from the switching of an embryonic and adult isoforms of cTnT [Jin, 1996; Siedner et al., 2003]. The temporary existence of embryonic form of cTnT might contribute to the highest expression level of cTnT at stage 37 in the mutant ventricle (Fig. 6G). Alternatively, myocytes from the different regions could be under different transcriptional or translational control. Interestingly, it has been reported that the mutant conus region also contains significant amounts of tropomyosin although the ventricular areas are virtually devoid of this protein [Zajdel et al., 1998]. Very likely, cTnT isoforms are partially regulated by common factors shared with tropomyosin, which influences their expression or RNA processing in the mutant condition. However, our results indicate that cTnT and tropomyosin transcriptions are differentially regulated in the mutant embryonic hearts at the mRNA expression level. Whether the decreased expression level of cTnT

may cause the loss of tropomyosin protein from the heart cells is unknown. Thus the unique *c*/*c* mutant animal is providing us an excellent model to study genome level regulation of cardiac specific gene expression. Our studies to date on both sarcomeric tropomyosin and cTnT confirm the importance of continuing this research at the genomic level.

Our previous studies using electron microscopy indicate that the myocardial cells are stalled in development in the embryonic mutant hearts. This raises a question as to whether the significantly decreased levels of cTnT mRNA expression are due to the decrease of mRNA expression in individual myocardial cells or that there are fewer myocardial cells in the mutant hearts than in normals. We are addressing this question by performing in situ hybridization on both normal and mutant hearts using cTnT specific probes.

Our studies form the foundation for determining the role of TnT in the regulation of contraction and its own expression regulation by other tissue specific factors using the gene *c* mutation animal system. Our immunohistochemical as well as Western blot analyses strongly support the presence of two isoforms of TnT in axolotl hearts—one is recognized by Mab11-13 antibodies and the other by JLT12 antibodies (data not shown). The latter antibodies are known to recognize skeletal muscle specific TnT. Our confocal microscopic analyses did not show a well-defined pattern of TnT recognized by JLT12 in the ventricles of normal and mutant axolotl hearts. It seems likely that the skeletal muscle specific TnT isoform is not required for myofibrillar organization in the axolotl heart, however, the cardiac specific isoform is.

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