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Role of myofibril-inducing RNA in cardiac TnT expression in developing Mexican axolotl

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Abstract

The Mexican axolotl, *Ambystoma mexicanum*, has been a useful animal model to study heart development and cardiac myofibrillogenesis. A naturally-occurring recessive mutant, gene "*c*", for cardiac non-function in the Mexican axolotl causes a failure of myofibrillogenesis due to a lack of tropomyosin expression in homozygous mutant (*c/c*) embryonic hearts. Myofibril-inducing RNA (MIR) rescues mutant hearts *in vitro* by promoting tropomyosin expression and myofibril formation thereafter. We have studied the effect of MIR on the expression of various isoforms of cardiac troponin T (cTnT), a component of the thin filament that binds with tropomyosin. Four alternatively spliced cTnT isoforms have been characterized from developing axolotl heart. The expression of various cTnT isoforms in normal, mutant, and mutant hearts corrected with MIR, is evaluated by real-time RT-PCR using isoform specific primer pairs; MIR affects the total transcription as well as the splicing of the cTnT in axolotl heart.

Keywords: Cardiac troponin T; Axolotl; Myofibril; Alternative splicing

Troponin T (TnT) is a component of the troponin complex (troponin T, troponin I, and troponin C) that anchors the complex to the thin filaments in vertebrate striated muscle. The troponin complex functions as a regulatory system for muscle contraction, responding to the cytosolic calcium concentration changes and other signalling [1]. Three muscle type-specific TnT isoform genes, cardiac TnT (cTnT or TNNT2), slow skeletal TnT (ssTnT or TNNT1) and fast skeletal TnT (fsTnT or TNNT3) have been cloned from higher vertebrates [2,3]. In addition, alternative RNA splicing adds another dimension of generating more protein isoforms for TnT [3,4]. The human cardiac troponin T gene contains 17 exons, some of which can be alternatively spliced, leading to the creation of different isoforms [5–7].

The differential splicing for some cTnT exons is developmentally regulated. Thus, some isoforms are present only in specific stages of development [3,6,7]. Different cTnT isoforms resulting from alternative splicing respond differently to cytosolic Ca^{2+} concentrations within the cell. The different isoforms also show differing actomyosin ATPase activity within the sarcomeres. Exclusion of the 10 amino acids in adult cTnT, encoded by exon 5, results in significant changes in activation of the actomyosin ATPase when compared to the embryonic isoforms [8,9]; not surprisingly, much smaller functional differences are observed from alternate splicing of exon 4 in bovine cardiac troponin T [10].

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Our current studies are on a salamander model, the Mexican axolotl, *Ambystoma mexicanum*, in which a naturally occurring genetic recessive cardiac lethal mutation—gene *c*—was discovered [11]. Homozygous mutant (c/c) embryos are first distinguishable from their normal siblings (+/+ or +/c) at stage 34, when the mutant embryos develop hearts that fail to beat and eventually die from lack of circulation. Subsequent studies have demonstrated the defects in myofibrillogenesis and significantly decreased tropomyosin expression in the mutant embryonic hearts [12–17]. As a tropomyosin-interacting protein, cardiac troponin T has also been studied in this system in our laboratory. It has been demonstrated that the expression of TnT protein is significantly decreased in the mutant hearts [18]. One possible explanation for this observation is that splicing abnormalities could exist in the mutant embryonic hearts. To test this hypothesis, we cloned four different isoforms of the cTnT cDNA (TNNT2-1, -2, -3, and -4), resulting from an alternative splicing process, from the developing axolotl heart. Similar to some, but not all the mammalian systems [19], the embryonic isoforms of cTnT (TNNT2-1) expression persists into adulthood in the axolotl system. We conclude that the aberrant splicing of cTnT occurs in mutant embryonic axolotl hearts in addition to the significantly decreased total RNA level compared to normal hearts. Interestingly, the aberrant splicing pattern of the cTnT in mutant embryonic hearts could be compensated by supplying a small RNA molecule exogenously, the myofibril-inducing RNA (MIR). This observation is in good agreement with our previous studies that MIR is able to rescue the mutant hearts by promoting myofibrillogenesis and cardiomyocyte differentiation [15].

Materials and methods

Procurement and maintenance of axolotls. A colony of homozygous normal (+/+) and heterozygous (+/c) adult animals were maintained at Florida Atlantic University for these studies. In a temperature and light controlled room, they were kept within individual aquaria in Holtfreter's solution and fed salmon fish pellets, and brine shrimp. Gene *c* is maintained in the colony for production of mutant spawnings with occasional supplementation of embryos from the Indiana University Axolotl Colony/AGSC. The cardiac lethal mutation designated by gene *c* is a simple recessive mutation. With each mating, 25% of the embryos are *c/c* mutants.

Cloning of cTnT isoforms. Cardiac RNA was extracted from the embryonic axolotl hearts (stages 34–42) using TRI reagent (Sigma, St. Louis). Reverse transcription was done using the Thermoscript RT system (Invitrogen, CA). The cDNA synthesized was then used to carry out PCR using Pfx DNA polymerase (Invitrogen, CA). Primers to amplify cTnT isoforms were designed in 5′UTR (forward primer) and 3′UTR (reverse primer) sequences.

Forward primer: 5′-CCTTCCTGGATCTGCAAGCCTCCTC.
Reverse primer: 5′-TGCTGCTAGGTCTTCTTGGCAGAGGTG

PCR bands with different sizes were isolated from agarose gels and cloned into a T-vector using the pGEM-T Easy Vector System (Promega, WI). The plasmids were purified after transformation and sequenced by Davis sequencing, LLC (San Diego, CA) after digestion with *Eco*RI enzyme and confirmed with insertion.

Organ cultures for embryonic hearts. Stages 37–38 embryos were anesthetized using a 1:5000 dilution of MS—222 and hearts were dissected in Steinberg's solution. Mutant hearts were separated from normal based on the presence or absence of beating activity. All dissections were carried out under a dissecting microscope. Hearts were then placed in 96 U-well shaped plates containing Steinberg's solution for liposome-mediated RNA transfection following our published methods [15–18]. Mutant hearts were divided into two groups: those treated with sense MIR and those treated with anti-sense MIR. RNA molecules were in vitro synthesized following our published methods [17]. Control hearts were treated with Lipofectamine 2000 (Invitrogen, CA) and Steinberg's solution only. Hearts were then collected and processed for total RNA extraction after RNA transfection for 36 and 72 h.

Quantitative RT-PCR. Total RNA was extracted from normal and mutant hearts at stages 37–38 after the conuses were 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 calculating melting temperatures (T_m) 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 vs. Log concentration (copies) was created using one of the cDNA samples with serial dilutions or with known concentrations of plasmid DNA with a cTnT insert. Negative controls were included using cDNAs synthesized the same way as above but with no reverse transcriptase added. Each cDNA sample was run in triplicate. The data were averaged and standard deviations were calculated. The β-actin gene was used as a standard control. The sizes of the PCR products were further confirmed by polyacrylamide gel electrophoresis. Primers for total cTnT amplification were designed based on the 3′UTR sequence that is common for all the isoforms. Primers for TNNT2-2, -3, and -4 were designed to span the neighboring exons for specific amplification of individual isoforms. All primer pairs designed to amplify shorter isoforms have been pre-tested and their specificity has been verified by not being able to amplify the full-length cTnT insert in a plasmid (data not shown). Primer sequences used in these studies are as follows:

1. TNNT2-2-for: 5′-TGGAGGAATACGAATCGTGATGATG
2. TNNT2-2-rev: 5′-CAAAGTGCGCCTCGATCAGC
3. TNNT2-3-for: 5′-ACCCAAACCAAAGAGAAGCGTCGG
4. TNNT2-3-rev: 5′-TTCTTCCCGCCTCTGCCAGTC
5. TNNT2-4-for: 5′-CCAGGCACGCATGGCAGAAG
6. TNNT2-4-rev: 5′-GGACATTGATTTCAGCTCGTCCTCC
7. cTnT-3UTR-for: 5′-CCAAGGGCTTCACCGGGCTCAA
8. cTnT-3UTR-rev: 5′-TGGCAGAGGTGGAATGGATCACAGG
9. β-Actin-for: 5′-TCCATGAAGGCTGCCCAACT
10. β-Actin-rev: 5′-TGGCGCCACATCTGATTGAT

Results and discussion

We have previously cloned the full-length sequence of the cardiac troponin T cDNA from the heartbeat initiation stages of embryonic axolotls [18]. In our current studies, we have designed PCR primers based on both 5′- and 3′-UTR sequences of the full-length cTnT and tried amplifying all possible isoforms of this gene. RT-PCR was performed by using the primer pairs on total RNA extracted from normal (+/+) stage 34–37 embryonic hearts. After ligation of the amplified PCR bands from RT-PCR into T-vectors, more than seventy clones were randomly picked from the culture plates. Plasmid DNA from the individual clones