Molecular cloning and developmental expression of human cardiac troponin T

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We have isolated a full-size cDNA coding for cardiac troponin T (cTnT) from a human adult heart library, using a slow skeletal TnT probe. This cDNA detected a 1.2 kb mRNA in fetal and post-natal human heart, the amount of which increased during ontogenic development. Interestingly, a similar transcript was coexpressed in fetal skeletal muscle, together with the 0.9 kb slow skeletal muscle mRNA, and its expression was down regulated during further development.

Troponin T; Human heart; Cardiac muscle; Skeletal muscle; Development

1 INTRODUCTION

Troponins are muscle-specific proteins of the myofibrillar apparatus which are involved in the Ca^{2+}-dependent regulation of contraction in cardiac and skeletal muscles. They comprise three subunits: troponin I (TnI), which inhibits actin–myosin interaction, troponin C (TnC), which binds calcium and unblocks actin–myosin interaction, and troponin T (TnT), which links the troponin complex to tropomyosin. Multiple TnT isoforms have been identified in cardiac and skeletal muscles from the chick and a number of mammalian species [1-4]. These isoforms are generated by different genes or by alternative splicing of the same gene [5,6]. The latter mechanism is responsible for the various cardiac TnT variants, which undergo differential expression during ontogenic development [7-9]. TnT has long been considered only as the link between troponins and the thin filament of the sarcomere, but several lines of evidence have recently suggested that it may play a role, together with TnI, in modulating the sensitivity of the contractile apparatus to calcium and its changes during development [10-12].

Ontogenic development is not the only situation in which changes in cardiac gene expression have been demonstrated. Cardiac hypertrophy due to hemodynamic overload is also associated with profound changes in the expression of many contractile and non-contractile proteins, and a number of these changes have been shown to participate in the alterations of the contractile and endocrine function of the overloaded heart (review in [13]). Whereas in rodents the decreased contractility and myosin ATPase activity of the hypertrophic ventricle may be accounted for, at least in part, by the α- to β-myosin heavy chain (MHC) transition, such a transition cannot explain the corresponding changes in the adult human heart, which is almost exclusively composed of β-MHC [14]. Interestingly, whereas McAuliffe and Robbins [15] detected a single dominant TnT isoform in control and overloaded fetal sheep hearts, Anderson et al. [16] have recently reported the reexpression of a fetal TnT protein isoform in the failing human heart which could account for decrease in myofibrillar ATPase activity in this syndrome. In addition, it has recently been shown that plasma TnT is a valuable index for identifying, among patients with unstable angina, those at risk of myocardial infarction [17]. To date, the anti-TnT monoclonal antibodies used in the assay have been raised by using TnT purified from the human heart, but the epitopes involved have not been identified [18]. Our interest in the regulation of TnT gene expression in the human heart during ontogeny and hemodynamic overload and in improving the plasma TnT assay prompted us to clone human TnT cDNA from a human adult heart cDNA library, using a cDNA specific for human slow skeletal muscle TnT as probe [19]. We report the first complete amino-acid sequence of a human cardiac TnT and the differential expression of the corresponding mRNA and slow skeletal TnT mRNA in human cardiac and skeletal muscle during ontogenic development.

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2. MATERIALS AND METHODS

2.1. Screening of the human adult cardiac cDNA library and PCR analysis

cDNA clones encoding human cardiac TnT were isolated by screening a commercially available human adult heart cDNA library (Stratagene) constructed in AZAPII, with a cDNA of human slow skeletal muscle TnT isolated by Samson et al. [19]. A 639 bp XhoI–PstI restriction fragment derived from two-thirds of the 3' end of the clone MSX-2-2T was used as probe. This fragment corresponded to a part of the TnT sequence highly conserved in various TnT isoforms from different species and within a given species. The library was screened by plating 50 x 10^5 pfu per dish on a lawn of XL-1 blue cells and preparing duplicate plaque lifts with nylon membrane (Amersham) according to Benton and Davis [20]. Hybridization was carried out at 65°C for 3 h in Amersham Rapid Hybridization Buffer with the [α-^32P]dCTP-labelled probe (2 to 2.5 cpm/filter). Filters were washed 3 times for 15 min in 2 x SSC at room temperature, 15 min in 1 x SSC, 0.1% SDS at room temperature, and 10 min in 0.1 x SSC, 0.1% SDS at 50°C. They were then autoradiographed with intensifying screens.

2.2. Sequence analysis

In vivo excision and rescue of the double-stranded pBluescript (SK–) plasmids containing the cDNA inserts of interest from the AZAPII phagemid was performed as recommended by the manufacturer (Stratagene). DNA sequencing was performed by the dideoxy chain-termination method of Sanger et al. [21], using Sequenase (United States Biochemical) and [α-^32P]dATP (Amersham). cDNA inserts were first sequenced using primers complementary to the T3 and T7 promoter sequences and primers derived from the published sheep cTNT cDNA sequence [15]. The sequence of the full-length cDNA was obtained from two overlapping clones (HCTNT1 and HCTNT2). The full-length cDNA was sequenced on both strands and the cTNT sequence was completed by sequencing the 5' and 3' ends of the HCTNT2 clone on both strands. The sequencing strategy for each clone is shown in Fig. 1.

2.3. RNA preparation and Northern blot analysis

Total RNA was prepared according to the method of Chomczynski and Sacchi [22]. Northern blotting was performed using 10 μg of total RNA electrophoresed on a 0.8% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N*, Amersham). The cDNA probes were labelled with [α-^32P]dCTP by random priming (Klenow, Amersham). The blot was successively hybridized with the same cDNA fragment of slow skeletal muscle TnT as that used to screen the cDNA library, and with a 1,087 bp PCR product from the HCTNT1 clone (nucleotides 148 to 1,031 of the HCTNT2 sequence). In both cases, hybridization to 8 x 10^6 cpm of each cDNA probe was performed for 2 h at 65°C, and the washing conditions were those used in screening the cDNA library. Finally, to determine the precise amount of RNA on the filter, the blot was hybridized to a 20-mer oligonucleotide complementary to part of the sequence of rat ribosomal RNA. After each hybridization and washing cycle, the membrane was exposed for autoradiography with intensifying screens (Amersililene) at −80°C, dehybridized according to the standard procedure and exposed again before rehybridization to check for the absence of a detectable signal.

3. RESULTS

The first screening yielded a total of 19 positive clones. Ten of these were selected for a second screening process. Five were amplified by means of PCR with primers specific for sheep cardiac TnT and gave a single PCR product of 644 bp. These clones were subjected to a third screening and gave a positive hybridization signal on duplicate plaque-lifts. The two largest clones (HCTNT1 and HCTNT2) were selected for sequencing. HCTNT1 was 923 bp long and lacked the 5' end of the cDNA. HCTNT2, a slightly larger clone, allowed us to obtain a full-length coding sequence of human cardiac TnT (Fig. 2). This cDNA covers 1,086 nucleotides and has a 866 bp coding open reading frame which, when translated, would give a protein with a molecular mass of 39,700 Da. The clone contained a short 5' untranslated sequence upstream of the initiation ATG triplet. The 706-nucleotide 3'-untranslated sequence contained a polyadenylation signal (AATAAA) located 16 bp upstream of the poly(A) tract. Alignment of the amino-acid sequence deduced from our cDNA is shown in Fig. 3, together with published cardiac TnT sequences of other mammalian species and clone M1 of the human slow skeletal TnT [6].

The expression of human cardiac and slow skeletal TnT mRNAs during ontogenic development in both cardiac and skeletal muscles was studied by Northern-blot analysis (Fig. 4). Hybridization with the slow skeletal TnT cDNA showed that this probe detected two transcripts, of different sizes, in cardiac and skeletal muscles. In skeletal muscle, a hybridization signal corresponding to a transcript approximately 0.9 kb long was detected, whereas in human and rat hearts the size of the detected transcript was approximately 1.2 kb. Hybridization with HCTNT1 revealed a single 1.2 kb transcript in all muscles studied, including fetal skeletal muscle. A faint hybridization signal of the same apparent length was also observed in adult skeletal muscle with longer autoradiography (not shown). In addition, the 1.2 kb transcript accumulated in the heart during development. In the fetal heart, the signal detected in the atrium appeared to be stronger than that in the ventricle, and vice versa in the adult heart.

4. DISCUSSION

Using a cDNA probe coding for a human slow skeletal TnT, we isolated two clones which, by sequence comparison with cardiac TnTs of other mammalian species and Northern blot analysis, were found to encode human cardiac TnT. Peptide identity was 88–90% with rat and sheep heart TnT on a 288 amino-acid overlap and 96–98% when comparison started from amino acid 70. As expected, the most divergent region was in the N-terminal portion. The sequence was less strongly conserved when compared to that of human slow skeletal TnT (67%). In particular, a stretch of 105 bp was present in the human cTnT in the N-terminal region but lacked the human slow skeletal TnT cDNA clone M1 described by Gahlmann et al. [6]. More interestingly, multiple nucleotide differences were spread along the two sequences, suggesting that the two isoforms did not derive from a single primary transcript.
through tissue-specific processing, but were rather encoded by two similar genes. This remains to be confirmed by cloning and sequencing of the relevant gene(s). The expected molecular mass of the human cardiac TnT deduced from our sequence was 39,700 Da. This is in good agreement with the average size of the cardiac TnT isoforms recently identified in the human heart by means of SDS-PAGE and Western blot analysis by various groups [8,24,25]. On the basis of the work by Anderson et al. [16], at least two cardiac TnT isoforms exist in the adult human heart, and further studies are thus needed to determine if our clone encodes the major isoform in the adult heart or corresponds to the persistent expression of a fetal form.

Northern blot analysis of RNAs isolated from postnatal human hearts with HCTNT1 yielded a single transcript of approximately 1.2 kb, which was also found in the rat and chick heart (not shown). This length is consistent with that of the various cardiac TnT mRNAs described so far [4,7,15]. Moreover, this mRNA was also present in the fetal heart and accumulated during development, suggesting that it corresponded to an adult isoform. However, since the resolution of this technique is not sufficient to differentiate closely size-related transcripts, our probe may in fact have detected several cardiac TnT transcripts of approximately the same molecular weight. We have isolated other clones, the analysis of which will probably yield cDNAs of other human cardiac TnT isoforms and thus answer this question.

Interestingly, whereas the 0.9 kb transcript found in the skeletal muscle was never detected in the human heart, a 1.2 kb transcript was found both in fetal and adult skeletal muscle, although to a far lesser extent in the latter (not shown). These data strongly suggested that slow skeletal TnT is not coexpressed in the human heart, whatever the stage of development, although a very low level of expression cannot be ruled out because of the insufficient sensitivity of Northern blot analysis. By contrast, our data show that the two cardiac and skeletal TnT transcripts are coexpressed in fetal skeletal muscle, and that the former is downregulated during further development, as previously reported in the chick and rat [7,8]. In addition, our Northern blot analysis
Fig. 2. Nucleotide sequence of human cardiac TnT deduced by sequencing clones HCTNT1 and HCTNT2 and the corresponding amino-acid sequence. The asterisks indicate the termination codon (TAG), and the polyadenylation signal sequence is underlined.
Fig. 3. Alignment of human cardiac TnT amino-acid sequence, deduced from the nucleotidic sequence, with those of other species and the human slow skeletal TnT sequence deduced from clone M1 (isolated by Gahlmann et al. [6]). Amino-acid sequences from bovine and rabbit cardiac TnT were determined directly by amino-acid sequencing after protein isolation. The single-letter code is used; (*) no corresponding amino acid.
Fig. 4. Expression of human cardiac and slow skeletal TnT mRNAs analysed by Northern-blot hybridization with cDNA probes of slow skeletal TnT and cardiac TnT. Ten µg of total RNA was loaded on each lane: rat liver (RL), rat ventricle (RV), human ventricle (HV), atrium (HA), and skeletal muscle (skM). The tissues were from a 29-week-old fetus (F 29w.), a 12-year-old child (12y.), and an adult (Ad.). The blot was successively hybridized with a fragment of slow skeletal TnT cDNA (ssk TnT), with a PCR fragment of HCTNT1 (c TnT), and with the 18s oligonucleotide to check the quantity of RNA on each lane.

suggested that the cardiac transcript first accumulates in the fetal atria, whereas accumulation is greater in the adult ventricles. A similar finding has been made by others working on chick and rat hearts [36]. Isolation and characterisation of other human cardiac TnT cDNAs and the use of isoform-specific probes will enable us to determine the precise expression of human cardiac TnTs according to the chamber (atria vs. ventricles), tissue (sinus-atrial and atrio-ventricular nodes and conduction system) and developmental stage, as well as in a number of disease states.

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REFERENCES