Developmental expression of rat cardiac troponin I mRNA

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Summary

We have isolated and sequenced a full-length cDNA clone of rat cardiac troponin I (Tnl). The amino acid sequence of rat cardiac Tnl is highly similar to that of other mammalian species in the portion of the molecule (residues 33–210) that is also homologous to skeletal muscle Tnl isoforms. In contrast, a lower degree of similarity is present in the cardiac Tnl-specific amino terminal extension (residues 1–32). This region contains a conserved serine residue that has been shown to be selectively phosphorylated by cAMP-dependent protein kinase. Cardiac Tnl mRNA is weakly expressed in the 18-day fetal heart and accumulates in neonatal and postnatal stages. No difference can be demonstrated between Tnl mRNAs present in fetal and postnatal heart by RNAase protection assays. The fetal and neonatal, but not the adult heart, contain significant amounts of slow skeletal Tnl transcripts, detected by oligonucleotide probes specific for the 5'- and 3'-untranslated regions of slow skeletal Tnl mRNA. In situ hybridization studies show that cardiac and slow skeletal Tnl mRNAs are coexpressed in the rat heart from embryonic day 11 throughout fetal and perinatal stages. Changes in troponin isoform expression during development may be responsible for the difference in calcium sensitivity and in the response to β-adrenergic stimulation between fetal and adult heart.

Key words: heart, cardiac development, cardiac troponin I cDNA, slow skeletal troponin I, rat embryo.

Introduction

The troponin complex, which controls the interaction of thick and thin filaments in sarcomeric muscles in response to changes of intracellular Ca²⁺, is composed of three subunits, troponin C (TnC), troponin I (Tnl), and troponin T (TnT) (Ebashi and Endo, 1968). Each subunit exists as multiple isoforms, which exhibit tissue-specific and developmental stage-specific regulation (Perry, 1986). Troponin isoforms are the product of at least 8 genes belonging to 3 distinct gene families: the TnC gene family, comprising fast skeletal and slow skeletal/cardiac TnC genes (Wilkinson, 1980; Gahlmann et al. 1988), the Tnl gene family, comprising fast skeletal, slow skeletal and cardiac Tnl genes (Wilkinson and Grand, 1978; Baldwin et al. 1985; Nikovits et al. 1986; Koppe et al. 1989; and this paper) and the TnT gene family, comprising fast skeletal, slow skeletal and cardiac TnT genes (Perry, 1986; Pearlstone et al. 1986; Cooper and Ordhal, 1985; Breitbart and Nadal-Ginard, 1986; Gahlmann et al. 1987; Jin and Lin, 1989). Whereas each of the TnC and Tnl genes appears to code for a single protein isoform, a number of TnT variants are generated from each of the three TnT genes by alternative pre-mRNA splicing (Cooper and Ordhal, 1985; Gahlmann et al. 1987; Medford et al. 1984; Breitbart et al. 1985).

Troponin isoform transitions have recently been described during cardiac development. Developmental switching of cardiac TnT has been well characterized at the protein and mRNA level both in the avian and mammalian heart. Alternative splicing of the cardiac TnT gene (Cooper and Ordhal, 1985; Jin and Lin, 1989) has been shown to produce distinct fetal and adult cardiac TnT isoforms (Ogasawara et al. 1987; Jin and Lin, 1988; Saggin et al. 1988; Sabry and Dhoot, 1989a). Using electrophoretic, immunoblotting and affinity chromatography procedures, we have recently shown that distinct Tnl variants are also expressed in the fetal and adult rat heart. The Tnl isoform present in the fetal heart is apparently similar, with respect to electrophoretic mobility, to the predominant Tnl isoform expressed in slow skeletal muscle (Saggin et al. 1989). Similar results were reported by others (Sabry and Dhoot, 1989b). Toyota and Shimada (1981) had previously reported that embryonic chicken heart is stained by antibodies to both skeletal Tnl and cardiac Tnl, whereas adult heart reacts exclusively with anti-cardiac Tnl antibodies. However, the identity of the Tnl expressed in the fetal heart and the molecular genetic basis for cardiac Tnl switching remain to be established.

Here we report the isolation and sequence of a cDNA clone corresponding to the rat cardiac Tnl. RNA blot analysis reveals that cardiac Tnl mRNA is poorly
expressed in the fetal heart and markedly increases at birth, whereas a distinct TnI mRNA, apparently identical to that expressed in slow skeletal muscle, is predominant in the fetal heart.

Materials and methods

Screening of cDNA library, subcloning and sequencing

A λgt11 cDNA library from adult rat heart (kindly provided by Ketty Schwartz and Anne-Marie Lompré, INSERM U127, Paris) was screened using a monoclonal antibody (TI-1) specific for cardiac TnI (Saggin et al. 1989). Growth of the plaques and immune-screening procedures were performed according to Mierendorf (1987). Two positive clones were recovered from the agar plates and purified to homogeneity by repeated plating and rescreening. The cDNA inserts, isolated by digestion with EcoRI endonuclease followed by electrophoresis on agarose gels, were about 0.8 and 0.7 kb in length. They were found to cross-hybridize when used as hybridization probes labelled with 32P by the random priming method (Feinberg and Vogelstein, 1983). The clone containing the longer insert, designated RCTI-5, was selected for further analysis. The cDNA insert was purified from a low melting point agarose gel and subcloned into Bluescribe M13 plus vector (Stratagene). Sequencing of double-stranded plasmid DNA was performed with the dye-deoxy chain termination method (Sanger et al. 1977) using Sequenase (United States Biologicals): 92% of the final sequence was determined from both strands.

RNA blot analysis

Total RNA was prepared from different rat tissues as previously described (Chomczynski and Sacchi, 1987). Samples were electrophoresed on 1% agarose, 3% formaldehyde gels, transferred to nylon membranes (Pall Biodyne A) and hybridized to 32P-labelled probes. The full-length cDNA insert and two Sau3A fragments derived from this clone, corresponding to the 5'-untranslated and the 5' coding regions of the rat slow skeletal TnI cDNA (Koppe et al. 1989). The RCTI-5 clone was selected by screening a rat heart cDNA library using a monoclonal antibody (BA-D5) specific for slow myosin heavy chain to identify slow skeletal muscle transcripts. Preliminary screening of the library was performed by hybridization with synthetic oligonucleotide probes specific for the slow skeletal and the fast skeletal muscle TnI mRNAs, which were end-labelled by terminal deoxynucleotidyltransferase. Oligonucleotide probes were synthesized from the T3 promoter after transcription from the T7 polymerase promoter. Antisense RNA probes were generated from the clone RCTI-5 by in vitro transcription from the T7 polymerase promoter. The plasmid was linearized either with BamH1 to produce a full-length 830 bp probe or with MluI to produce a 496 bp probe. After digestion, the plasmid was treated with proteinase K (30 min at 37°C), phenol-extracted and incubated with 40 mM Tris, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each ATP, GTP, CTP, 90 μC 35S-UTP (>3000 Ci mmol−1; New England Nuclear) and 10 units of T7 RNA polymerase (Boehringer) for 1 h at 37°C. The reaction was stopped with a further 15 min incubation with RNAase-free DNase (Stratagene). Hybridization was performed according to Maniatis et al. (1989). In brief, 10 μg total RNA from different tissues were denatured at 95°C for 2 min in 30 μl buffer containing 40 mM Pipes, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 mM NaCl, 80% formamide and 5×104 counts min−1 probe. After an overnight incubation at 50°C, RNase digestion was performed in 1×SSPE at 50°C. RNase resistant fragments were synthesized from the T3 promoter after cutting the RCTI-5 clone at the NcoI site within the insert. Probe length was reduced to approximately 50 nucleotides by alkali digestion. The protocol described by Sassoon et al. (1988) was used to fix and embed embryos, fetuses, neonatal and postnatal tissues, and to process paraffin sections for in situ hybridization. The same protocol was also applied to cryosections of unfixed frozen legs of 21-day-old fetuses; serial sections were stained with a monoclonal antibody (BA-DS) specific for slow myosin heavy chain to identify slow skeletal fibers (Schiaffino et al. 1989). With cRNA probes sections were hybridized at 52°C and washed at 65°C in 50% formamide, 2×SSC, 0.1 mM DTT. With oligonucleotide probes sections were hybridized at 42°C and washed with 0.1×SSC at 40°C. Slides were processed for autoradiography using Kodak NTB-2 emulsion and exposed for 3 or 7 days for cRNA probes and 15 or 21 days for oligonucleotide probes.

Results

The nucleotide sequence of cardiac troponin I mRNA

The RCTI-5 clone was selected by screening a rat
Cardiac troponin I cDNA

Cardiac cDNA expression library with a monoclonal antibody specific for cardiac Tnl. The restriction map and sequencing strategy for clone RCTI-5 are shown in Fig. 1 and the complete nucleotide sequence and derived amino acid sequence are shown in Fig. 2. The insert is 830 nucleotides in length and contains a 105 bp 5'-untranslated region, a 636 bp coding region, and a 62 bp 3'-untranslated region ending with a 27 bp poly(A) tail. The sequence has a typical polyadenylation consensus sequence (AATAAAA) located 42 nucleotides downstream of the TGA stop codon and 20 nucleotides upstream of the poly(A) tail. The 636 bp open reading frame encodes a protein of 210 amino acids with an Mr of 24,027. Fig. 3 shows a comparison of the derived amino acid sequence of rat cardiac TnI with

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**Fig. 1.** Partial restriction endonuclease map and sequencing strategy for rat cardiac cDNA clone RCTI-5. The coding region is indicated by the filled box, the 5' and 3' untranslated regions by open boxes. The direction and extent of sequencing is indicated by arrows. Restriction sites: S, Sau3A; M, MluI; N, NcoI.

**Fig. 2.** Nucleotide and derived amino acid sequence of rat cardiac TnI cDNA. The amino acid sequence is shown below the nucleotide sequence. Numbers correspond to nucleotides; numbering begins with the first nucleotide in the initiator methionine codon. The termination codon is indicated by three asterisks. The AATAAA polyadenylation consensus sequence is underlined.
Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Rat cardiac
Human cardiac
Bovine cardiac
Rabbit cardiac
Rat slow skeletal

Fig. 3. Comparison of TnI amino acid sequences. The amino acid sequence (single-letter code) of rat cardiac TnI has been aligned with that of bovine and rabbit cardiac TnI, previously determined by protein sequencing (Wilkinson and Grand, 1978; Leszyk et al. 1988) and with that of human cardiac TnI (Vallins et al. 1990) and rat slow skeletal TnI, derived from the nucleotide sequence (Koppe et al. 1989). Gaps (*) were inserted to maximize the number of matches. Identical amino acids are indicated by dashes (—). Numbering is based upon the rat cardiac TnI sequence. The Leu residue between Arg-145 and Arg-146, originally reported in the rabbit cardiac sequence (Wilkinson and Grand, 1978) but absent in all other TnI sequences, was removed in this Figure (see also Leszyk et al. 1988).

the published sequences of rabbit and bovine cardiac TnI, determined by direct protein sequencing (Wilkinson and Grand, 1978; Leszyk et al. 1988), human cardiac TnI, derived from the nucleotide sequence of a cDNA clone (Vallins et al. 1990) and of slow skeletal TnI, derived from the nucleotide sequence of a rat cDNA clone (Koppe et al. 1989). Cardiac TnI is highly conserved in the three species and differs from slow skeletal TnI by the presence of a long amino-terminal extension. The degree of similarity between cardiac TnIs is especially high (91.0% identity) in the portion of the molecule (residues 33-210 in the rat sequence) that is homologous to slow skeletal TnI. On the other hand, the cardiac-specific amino-terminal extension (residues 1-32 in the rat sequence) shows much less similarity (only 51.5% identity) in the different species. The length of this sequence is also variable: in rat 32 residues; human, 31; rabbit, 27 and bovine heart, 33.
The expression of cardiac troponin I mRNA is developmentally regulated

The specificity of the clone RCTI-5 was tested by northern blotting using total RNA from rat liver, intestine, skeletal muscle and ventricular myocardium (Fig. 4). A strong band of about 0.85 kb was detectable only in cardiac muscle, thus showing that the isolated cDNA corresponds to a messenger specifically expressed in the heart, having the expected size of cardiac TnI mRNA. TnI expression during cardiac development was studied by northern blotting using total RNA from cardiac and skeletal muscle at three stages of development: 18-day fetuses, 1- to 2-day newborns and one-month-old rats (Fig. 5). Similar results were obtained using, as probes, the full-length cDNA insert and Sau3A fragments corresponding to the 5'-untranslated and the cardiac-specific 5' coding sequence. RNAs from neonatal and adult heart gave a strong signal with RCTI-5, while fetal heart RNA gave a very faint band. A stronger signal was observed in fetal heart by loading greater amounts of RNA (Fig. 6). No reaction was detectable with skeletal muscle RNA at any stage of development. Since in northern blots the mRNAs present in fetal and neonatal heart appeared to be slightly larger compared with the mRNA present in the one-month-old heart, RNAase protection analysis was used to compare the TnI transcripts. As shown in Fig. 7, no significant difference was detected between the three mRNAs using RNA probes complementary to the full-length cDNA insert or to the portion of the insert spanning from the MluI restriction site to the poly(A) tail.

The slow skeletal TnI gene is expressed in the fetal and neonatal heart

We have previously shown that the TnI isoform, predominantly expressed in the fetal heart, is similar with respect to electrophoretic mobility and antigenic properties to the major TnI isoform of slow skeletal muscle (Saggin et al. 1989). To examine the expression of the slow skeletal TnI gene in the fetal heart, RNA blot analyses were performed with two synthetic oligonucleotides, complementary to portions of the 5'- and 3'-untranslated regions of the rat slow skeletal TnI mRNA (Koppe et al. 1989). Total RNAs from a slow skeletal muscle, the soleus, and a fast skeletal muscle, the tibialis anterior, were used as controls. As shown in Fig. 6, the probe specific for the 5'-untranslated region of slow skeletal TnI was found to hybridize to RNA from soleus muscle and from fetal and neonatal heart; identical results were obtained using the probe specific for the 3'-untranslated region of slow skeletal TnI (not shown). With both probes no reaction was seen with the adult heart and tibialis anterior muscle, while a very weak signal was observed with fetal and neonatal hindlimb muscles. The fast skeletal TnI probe was completely unreactive with fetal, neonatal and adult heart.
Fig. 6. Expression of cardiac, slow skeletal and fast skeletal TnI mRNAs during development in cardiac and skeletal muscle. RNA blot analysis with $^{32}$P-labelled cardiac TnI-specific probes. Samples analyzed contained 30 $\mu$g of total RNA from the following tissues: lane 1, fetal cardiac muscle; lane 2, neonatal cardiac muscle; lane 3, adult ventricles; lane 4, fetal hind limb muscles; lane 5, neonatal hind limb muscles; lane 6, adult soleus muscle; lane 7, adult tibialis anterior muscle. (A) ethidium bromide stained gel; (B) blot hybridized with the cardiac TnI cDNA; (C) blot hybridized with a oligonucleotide probe complementary to the 5'-untranslated region of slow skeletal TnI mRNA; (D) blot hybridized with an oligonucleotide probe complementary to the coding region of the fast skeletal TnI mRNA.

**Cardiac TnI and slow skeletal TnI mRNAs are coexpressed in the early embryonic rat heart**

Developmental changes in the expression of cardiac and slow skeletal TnI mRNAs were also examined by in situ hybridization. The specificity of the probes used in this study is illustrated in Fig. 8. Cardiac TnI transcripts were detected in cardiac but not in skeletal muscle in fetal, neonatal and adult rats. Slow skeletal TnI transcripts were selectively expressed in slow muscle fibers, identified by a monoclonal antibody to slow myosin heavy chain; in addition, they were also detected in fetal and neonatal but not in adult heart. Cardiac TnI and slow skeletal TnI transcripts were found to be coexpressed in atria and ventricles of the developing rat heart since the earliest stage examined (embryonic day 11) (Fig. 9). The hybridization signal was consistently stronger with the cardiac-specific cRNA probe, which is presumably much more sensitive than the slow skeletal-specific oligonucleotide probe.
Cardiac troponin I cDNA

Fig. 8. Cardiac TnI transcripts are not expressed in skeletal muscle. (A) section through the adult rat ventricle (right) and soleus muscle (left) hybridized to the cardiac TnI probe. (B–D) serial sections through the leg of a 21-day-old rat fetus hybridized to cardiac (B) and slow skeletal (C) TnI probes, or stained with an antibody to slow skeletal myosin heavy chain (D). The slow skeletal TnI mRNA is expressed at high levels in the soleus muscle (s) which is easily identified by the high proportion of fibers expressing slow myosin heavy chain. Bar: A, 100 μm; B–D, 200 μm.

Cardiac TnI transcripts were never expressed in skeletal muscle at any developmental stage.

Discussion

Cardiac TnI protein structure

We have isolated and sequenced a cDNA clone corresponding to the cardiac isoform of TnI. The amino acid sequence of the protein encoded by this cDNA is very similar to the bovine and rabbit cardiac TnI sequences determined by direct protein sequencing. However, substantial differences between the three species are found at the amino terminus (residues 1–32 in the rat sequence). Cardiac TnIs are characterized by a much longer amino-terminal domain compared to both fast and slow skeletal muscle forms of TnI (Wilkinson and Grand, 1978; Leszyk et al. 1988). Amino acid sequence comparisons show that the length of this extension varies in the different species and...
several amino acid substitutions have occurred. The functional role of this portion of the molecule is not known. Rabbit cardiac TnI contains a serine (Ser 20, corresponding to Ser 22 in the rat sequence) which is selectively phosphorylated when hearts are perfused with catecholamines (England, 1976; Solaro et al. 1976) and when purified troponin is treated with cyclic AMP-dependent protein kinase (Moir et al. 1977; Stull and Buss, 1977). This phosphorylation results in a decreased affinity of troponin C for Ca$^{2+}$ and a rightward shift of the Ca$^{2+}$/force curve (Mope et al. 1980). Fast and slow skeletal TnI isoforms lack the amino-terminal extension and do not display the phosphorylation-mediated regulation by $\beta$-adrenergic agents. This is also probably true for the fetal cardiac TnI, which appears to be identical to slow skeletal TnI (see below), and could account in part for the different response of the fetal and neonatal myocardium to $\beta$-adrenergic stimulation (Park et al. 1980 and 1982). It remains to be established whether TnI isoform switching, together with TnT isoform switching (Anderson et al. 1991), may also be responsible for the lower calcium sensitivity of fetal compared to adult heart.

It is possible that the major role of the extended amino terminus of cardiac TnI is to provide an appropriate target for cAMP-dependent protein kinase. Species differences in the primary structure of this region might be explained if a certain degree of variability in the context of the phosphorylation target can be tolerated. In the rat, as in the bovine heart (Leszynk et al. 1988; Swiderek et al. 1988), cardiac TnI has two adjacent serines (Ser 22 and 23) that fulfill the structural requirements of a substrate of cyclic AMP-dependent protein kinase (Shenolikar and Cohen, 1978). Both serines are phosphorylated in the intact bovine heart (Swiderek et al. 1988) but it remains to be established whether both residues are phosphorylated by cyclic AMP-dependent protein kinase. Two other adjacent serine residues are present in positions 4 and 5 of the rat sequence, but these residues are not likely to be a substrate for the cyclic AMP-dependent protein kinase.

**TnI gene expression during cardiac development**

The expression of the cardiac TnI gene varies during development. The relatively weak band observed with the cDNA probe indicates that the corresponding mRNA is a minor component of TnI mRNA in the fetal heart. The intensity of this band is markedly increased at birth. The pattern of accumulation of cardiac TnI mRNA during perinatal development parallels that of the corresponding protein, which is also accumulated around birth (Saggin et al. 1989). However, there is no close correlation between mRNA and protein levels during embryonic stages: as reported here, *in situ* hybridization shows that cardiac TnI transcripts are present in the embryonic heart since embryonic day 11, whereas cardiac TnI protein is not detectable by immunoblotting in the ventricular myocardium until day 16–17 (Saggin et al. 1989; Sabry and Dhoot, 1989b). Immunohistochemical observations are in agreement with this result (L. Gorza, unpublished data). These findings appear to indicate that the expression of cardiac TnI protein during late embryonic development is not simply regulated by changes in the level of the corresponding mRNA. Muscle contractile genes can be regulated at the level of translation (see Endo and Nadal-Ginard, 1987) and we are presently exploring the possibility that translational control may also be implicated in the regulation of cardiac TnI expression in the embryonic heart.

We have previously shown that the fetal rat heart contains a distinct TnI isoform whose electrophoretic mobility is similar to that of the major TnI isoform of slow skeletal muscle (Saggin et al. 1989). We show here that slow skeletal TnI transcripts are expressed at a high level in the fetal and neonatal rat heart, as determined by RNA blot analysis with oligonucleotide probes complementary to the 5'- and 3'-untranslated regions of slow skeletal TnI. This appears to be a further example of a contractile protein isoform expressed both in cardiac and slow skeletal muscle, like the $\beta$-slow-MHC and the MLC$_{1V/1S}$ genes (Table 1). *In situ* hybridization studies and RNA blot analyses show that the slow skeletal and cardiac TnI genes are coexpressed in the rat heart throughout prenatal and neonatal development, the slow skeletal gene being down-regulated during the first weeks after birth. This developmentally regulated pattern of isoform transition based on gene switching can be contrasted with that of cardiac TnT isoform transition, based on alternative splicing of the same gene (Cooper and Ordhal, 1985; Jin and Lin, 1989).

Developmental changes of cardiac TnI in the rat ventricular myocardium are part of the reprogramming of contractile protein gene expression that occurs in the perinatal period (Table 1). This includes down-regulation of certain genes, such as $\alpha$-smooth and $\alpha$-skeletal actin, $\beta$-MHC, MLC$_{1A}$, $\beta$-TM, slow skeletal TnI and cardiac TnTemb, and up-regulation of other genes, such as $\alpha$-MHC, cardiac TnI and cardiac TnTadult. A common feature in these developmental changes is the tendency for different isogenes to be coexpressed in the embryonic heart with the subsequent repression of some of them. However, there seems to be no coordinate regulation of contractile protein gene expression (see Lyons et al. 1990). Whereas MHC gene switching is regulated by thyroid hormone and can be reversibly modulated at any developmental stage (Hoh et al. 1978; Sartore et al. 1981; Chizzonite and Zak, 1984; Lompré et al. 1984), cardiac TnT gene switching is independent of thyroid hormone (Saggin et al. 1988). Preliminary observations indicate that the TnI isogene expression is also not affected by hypothyroidism (S. Ausoni, unpublished data).

**TnI gene expression in developing skeletal muscle**

The results reported here show that the cardiac TnI gene is not expressed during skeletal muscle development. This is in contrast with the cardiac TnT gene which is transiently expressed in developing skeletal muscle both in the chick (Cooper and Ordhal, 1985) and
Table 1. Developmental transitions of contractile protein gene expression in the rat and mouse ventricular myocardium

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<tr>
<th>Contractile protein genes</th>
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*Abbreviations: MHC, myosin heavy chain; MLC, myosin light chain; TM, tropomyosin; Tnl, troponin T; TnI, troponin I.

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References


Cardiac troponin I cDNA


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Note added in proof
The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X58499.

Since the submission of this manuscript a paper describing rat cardiac and slow skeletal TnI cDNA clones and their developmental expression in the rat heart has appeared (Murphy et al., Biochemistry 30, 707-712, 1991). The results are in agreement with our data.