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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 (TnI). The amino acid sequence of rat cardiac TnI 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 TnI isoforms. In contrast, a lower degree of similarity is present in the cardiac TnI-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 TnI mRNA is weakly expressed in the 18-day fetal heart and accumulates in neonatal and postnatal stages. No difference can be demonstrated between TnI 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 TnI transcripts, detected by oligonucleotide probes specific for the 5′- and 3′-untranslated regions of slow skeletal TnI mRNA. *In situ* hybridization studies show that cardiac and slow skeletal TnI 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 Ca^{2+}, is composed of three subunits, troponin C (TnC), troponin I (TnI), 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 TnI gene family, comprising fast skeletal, slow skeletal and cardiac TnI 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 TnI 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, 1989*a*). Using electrophoretic, immunoblotting and affinity chromatography procedures, we have recently shown that distinct TnI variants are also expressed in the fetal and adult rat heart. The TnI isoform present in the fetal heart is apparently similar, with respect to electrophoretic mobility, to the predominant TnI isoform expressed in slow skeletal muscle (Saggin *et al.* 1989). Similar results were reported by others (Sabry and Dhoot, 1989*b*). Toyota and Shimada (1981) had previously reported that embryonic chicken heart is stained by antibodies to both skeletal TnI and cardiac TnI, whereas adult heart reacts exclusively with anti-cardiac TnI antibodies. However, the identity of the TnI expressed in the fetal heart and the molecular genetic basis for cardiac TnI switching remain to be established.

Here we report the isolation and sequence of a cDNA clone corresponding to the rat cardiac TnI. RNA blot analysis reveals that cardiac TnI 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 immuno-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 *Eco*RI 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 ^{32}P 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 dideoxy 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 ^{32}P-labelled probes. The full-length cDNA insert and two *Sau*3A fragments derived from this clone, corresponding to the 5′-untranslated and the 5′ coding sequences (nucleotides 1–98 and 99–227, respectively), were labelled by the random priming method and used as hybridization probes. RNA blot analysis was also performed with synthetic oligonucleotide probes specific for the slow skeletal and the fast skeletal muscle TnI mRNAs, which were end-labelled by terminal deoxynucleotidyl transferase. The probes for the slow skeletal TnI mRNA were two 40-mer oligonucleotides complementary to the 5′-untranslated (5′-GGTAACAAGGCAGCACCTGTTGGTTCCTGTGCA-GATGAGA-3′) and the 3′-untranslated (5′-GTAGGCT-TGAACCCAAGAGAGCTGACTCCTAAGAGTTCTG-3′) regions of the rat slow skeletal TnI cDNA (Koppe *et al.* 1989). The probe for the fast skeletal TnI mRNA was a 51-mer oligonucleotide (5′-GTCAAATAGCTTCTGGTTCATGTC-TTCCAGCTCCTTGCTGCTCTTCTGCAC-3′) complementary to the coding region of mouse fast skeletal muscle TnI cDNA (codons 87–102, see Fig. 1 in Koppe *et al.* 1989). This corresponds to a region conserved between fast skeletal TnIs, the amino acid sequence being completely identical in the rabbit fast skeletal TnI sequence (Wilkinson and Grand, 1978), but highly divergent in rat slow skeletal (58.3% identity, Koppe *et al.* 1989) and rat cardiac TnI genes (66.7% identity, this paper).

Hybridization experiments were performed at 42°C in 50% deionized formamide, 5×Denhardt's solution, 5×SSPE, 0.1% SDS and 100 $\mu g\,ml^{-1}$ salmon sperm DNA (see Maniatis *et al.* 1989 for the composition of SSPE and Denhardt's solution). Hybridization washes were performed at 42°C in 0.1×SSC (1×SSC=0.15 M NaCl, 0.015 sodium citrate), 0.1% SDS at 55°C for the RCTI-5 cDNA, and 1×SSC at 55°C for the other probes. RNAase protection analysis was performed as described (Maniatis *et al.* 1982) using RNA transcripts complementary to the full-length cDNA insert or to a 490 bp segment produced by cutting at a unique *Mlu*I site within the cDNA insert.

RNAase protection assays

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 *Bam*H1 to produce a full-length 830 bp probe or with *Mlu*I 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 $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each ATP, GTP, CTP, 90 μCi ^{35}S-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 DNAase (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 M NaCl, 80% formamide and 5×10^5 counts min^{-1} probe. After an overnight incubation at 50°C, RNAase digestion was performed in 300 mM NaCl, 10 mM Tris, 5 mM EDTA, 40 $\mu g\,ml^{-1}$ RNAase A, 2 $\mu g\,ml^{-1}$ RNAase T1 for 30 min at 4°C. RNAase resistant fragments were analyzed on 4% polyacrylamide sequencing gels.

In situ *hybridization*

The following probes were used for *in situ* hybridization. For detection of slow skeletal TnI mRNA, the 40-mer oligonucleotide complementary to the 5′-untranslated region of the slow skeletal TnI cDNA was labelled with ^{35}S-dATP by terminal transferase (Maniatis *et al.* 1989). For detection of cardiac TnI mRNA, the RCTI-5 clone was cut at the *Mlu*I site within the cDNA insert and antisense transcripts were synthesized from the T7 polymerase promoter according to manufacturer's conditions (Stratagene) and labelled with ^{35}S-UTP (>1000 Ci $mmol^{-1}$; New England Nuclear). Sense transcripts were synthesized from the T3 promoter after cutting the RCTI-5 clone at the *Nco*I 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-D5) 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 M 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

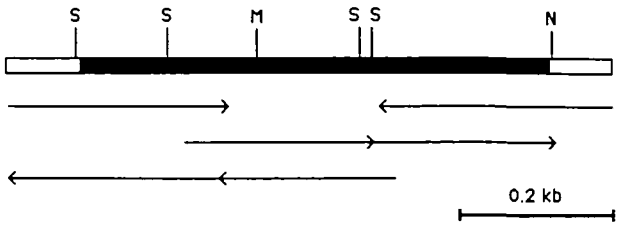

Fig. 1. Partial restriction endonuclease map and sequencing strategy for rat cardiac cDNA clone RCTI-5. The coding region is indicated by the filled box, the 5′ and 3′ untranslated regions by open boxes. The direction and extent of sequencing is indicated by arrows. Restriction sites: S, *Sau*3A; M,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

cardiac cDNA expression library with a monoclonal antibody specific for cardiac TnI. 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 (AATAAA) 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 M_r of 24 027. Fig. 3 shows a comparison of the derived amino acid sequence of rat cardiac TnI with

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                                                                CGCTTGAGCAGCACAGAGGGGACCCA

ACCCTCCAAGGTCACCAGGGACACCCTTCTAAGACCCTCCAGGAATCTGCAATCCCATTCTCTACCTCTGGAGATCAGC
  1                                    30                                      60
ATG GCG GAT GAG AGC AGC GAT GCG GCT GGG GAA CCC CAG CCG GCG CCT GCT CCT GTC CGA
Met Ala Asp Glu Ser Ser Asp Ala Ala Gly Glu Pro Gln Pro Ala Pro Ala Pro Val Arg
                                     90                                     120
CGT CGC TCC TCG GCC AAC TAC CGA GCC TAT GCC ACC GAG CCA CAT GCC AAG AAA AAG TCT
Arg Arg Ser Ser Ala Asn Tyr Arg Ala Tyr Ala Thr Glu Pro His Ala Lys Lys Lys Ser
                                    150                                     180
AAG ATC TCC GCC TCC AGA AAA CTT CAG TTG AAG ACT CTG ATG CTG CAG ATT GCG AAG CAG
Lys Ile Ser Ala Ser Arg Lys Leu Gln Leu Lys Thr Leu Met Leu Gln Ile Ala Lys Gln
                                    210                                     240
GAG ATG GAG CGT GAG GCA GAG GAG CGA CGT GGA GAG AAG GGC CGC GTT CTG AGC ACG CGT
Glu Met Glu Arg Glu Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Val Leu Ser Thr Arg
                                    270                                     300
TGC CAG CCC TTG GTG TTG GAT GGG CTG GGC TTT GAA GAG CTT CAG GAC CTA TGC CGG CAG
Cys Gln Pro Leu Val Leu Asp Gly Leu Gly Phe Glu Glu Leu Gln Asp Leu Cys Arg Gln
                                    330                                     360
CTT CAC GCT CGT GTG GAC AAA GTG GAT GAA GAG AGA TAC GAC GTG GAA GCA AAA GTC ACC
Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr Asp Val Glu Ala Lys Val Thr
                                    390                                     420
AAG AAC ATC ACT GAG ATT GCA GAT CTG ACC CAG AAG ATC TAT GAC CTG CGT GGC AAG TTT
Lys Asn Ile Thr Glu Ile Ala Asp Leu Thr Gln Lys Ile Tyr Asp Leu Arg Gly Lys Phe
                                    450                                     480
AAG CGG CCC ACT CTC CGA AGA GTG AGA ATC TCA GCA GAT GCC ATG ATG CAG GCA CTA CTG
Lys Arg Pro Thr Leu Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu
                                    510                                     540
GGG ACC CGG GCC AAG GAA TCC TTG GAC CTG AGG GCC CAC CTC AAG CAG GTG AAG AAG GAG
Gly Thr Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala Hys Leu Lys Gln Val Lys, Lys Glu
                                    570                                     600
GAC ATT GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGC AAG AAT ATC GAT GCA CTA AGT
Asp Ile Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg Lys Asn Ile Asp Ala Leu Ser
                                    630
GGA ATG GAA GGC CGA AAG AAA AAG TTT GAG GGC TGA GCCCATGGCTCTCACACTGTGCTCTGAAGGG
Gly Met Glu Gly Arg Lys Lys Lys Phe Glu Gly ***

CATCTCTGAGGAATAAATTTCTTTAAACTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

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.

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Rat cardiac           A D E S S D A * A G E P Q P A P A P V R R R S S A N Y R A Y A T E    32
Human cardiac         - - G - - - - * - R - - R - - - - - - I - - - - - * - - - - - - - -
Bovine cardiac        - - R - G G S T - - D T V - - - P - - - - - - - - - - - - - - - - -
Rabbit cardiac        - - - - R - - * - - E A R - - - - * - - - * - * * D * - - - - - - -
Rat slow skeletal

Rat cardiac           P H A K K K S K I S A S R K L Q L K T L M L Q I A K Q E M E R E A    65
Human cardiac         - - - - - - - - - - - - - - - - - - - - L - - - - - - - L - - - -
Bovine cardiac        - - - - - - - - - - - - - - - - - - - - - - - - - - - - L - - - -
Rabbit cardiac        - - - - S - K - - - - - - - - - - - - - - - - - - - - - L - - - -
Rat slow skeletal     - E V E R - - - - T - - - - - M - - S - - - A K - - E C W - Q - H

Rat cardiac           E E R R G E K G R V L S T R C Q P L V L D G L G F E E L Q D L C R    98
Human cardiac         - - - - - - - - - A - - - - - - - - - E - T - - - - A - - - - - - -
Bovine cardiac        - - - - - - - - - A - - - - - - - - - E - A - - - - A - - - - - - -
Rabbit cardiac        - - - - - - - - - A - - - - - - - - - E - A - - - - A - - - - - - -
Rat slow skeletal     - - - E A - - V - Y - - E - I P T - Q T R - - S L S A - - - - - - -

Rat cardiac           Q L H A R V D K V D E E R Y D V E A K V T K N I T E I A D L T Q K    131
Human cardiac         - - - - - - - - - - - - - - - - - I - - - - - - - - - - - - - - - -
Bovine cardiac        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - N - -
Rabbit cardiac        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Rat slow skeletal     E - - - L - E V - - - - - - - - I - - - C L - - T R - - K - - K L -

Rat cardiac           I Y D L R G K F K R P T L R R V R I S A D A M M Q A L L G T R A K    164
Human cardiac         - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - A - - -
Bovine cardiac        - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - A - - -
Rabbit cardiac        - F - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Rat slow skeletal     V L - - - - - - - - - P - - - - - V - - - - - L R - - - - S K H -

Rat cardiac           E S L D L R A H L K Q V K K E D I E K E N R * E V G D W R K N I D    196
Human cardiac         - - - - - - - - - - - - - - - - - T - - - - - * - - - - - - - - - -
Bovine cardiac        - T - - - - - - - - - - - - - - - T - - - - - * - - - - - - - - - -
Rabbit cardiac        - T - - - - - - - - - - - - - - - T - - - - - * - - - - - - - - - -
Rat slow skeletal     V - M - - - - N - - S - - - - - T - - - R P V * - - - - - - - V E

Rat cardiac           A L S G M E G R K K K F E G                                        210
Human cardiac         - - - - - - - - - - - - - - - S
Bovine cardiac        - - - - - - - - - - - - - - -
Rabbit cardiac        L - - - - - - - - - - - - - -
Rat slow skeletal     - M - - - - - - - - M - D A A K S P T L Q
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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 Leszik *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 expecially 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.

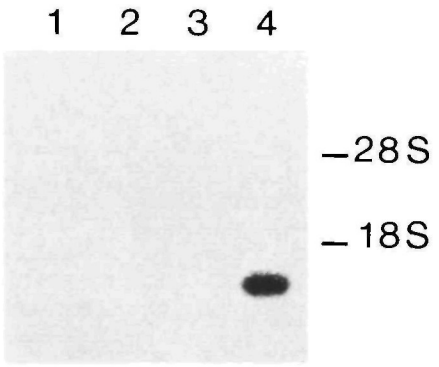

Fig. 4. Tissue specificity of the cardiac TnI cDNA. Samples containing 10 μg of total cellular RNA from rat liver (lane 1), intestine (lane 2) skeletal muscle (lane 3) and ventricular myocardium (lane 4) were electrophoresed on agarose-formaldehyde gels, blotted onto nylon filters and hybridized with ^{32}P-labelled cardiac TnI cDNA RCTI-5.

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 *Sau*3A 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 *Mlu*I 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

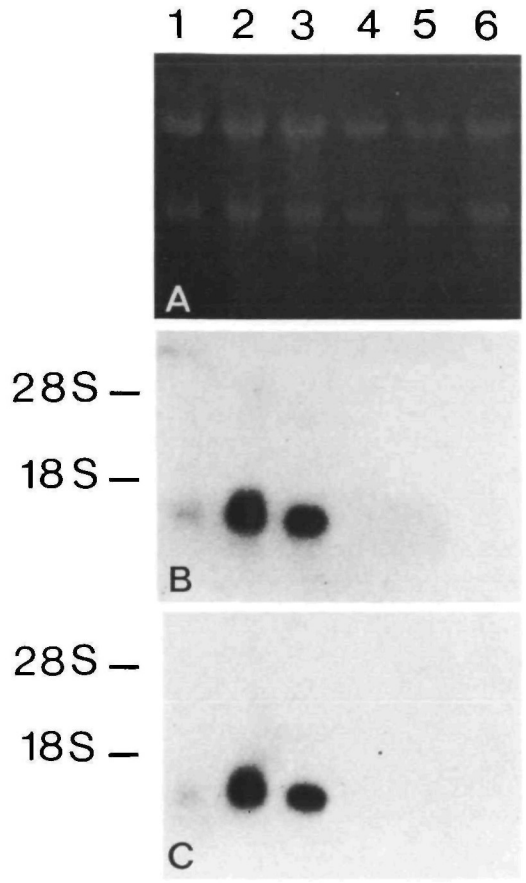

Fig. 5. Expression of cardiac TnI mRNA during development in cardiac and skeletal muscle. RNA blot analysis with ^{32}P-labelled cardiac TnI-specific probes. Samples analyzed contained 10 μg of total RNA from the following tissues: lane 1, fetal cardiac muscle; lane 2, neonatal cardiac muscle; lane 3, adult ventricles; lane 4, fetal hind limb muscle; lane 5, neonatal hind limb muscle; lane 6, adult hind limb muscle. (A) ethidium bromide stained gel; (B) blot hybridized with the complete cardiac TnI cDNA insert; (C) blot hybridized with a *Sau* 3A fragment corresponding to the 5′ coding region of the cardiac TnI mRNA.

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.

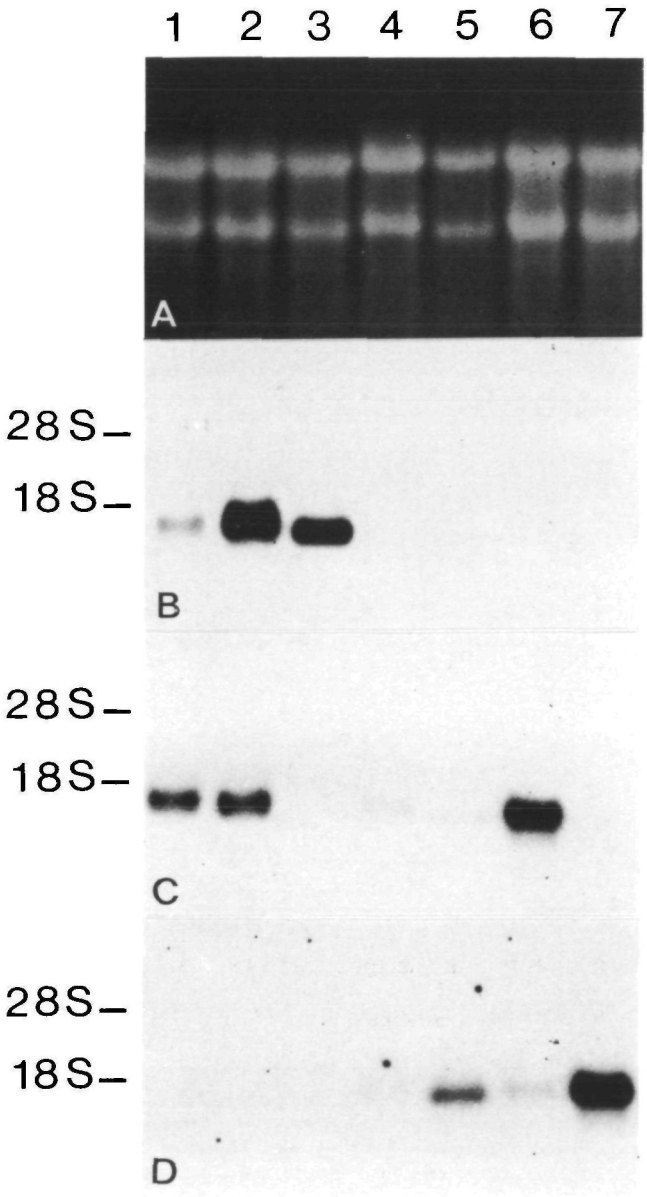

Fig. 6. Expression of cardiac, slow skeletal and fast skeletal TnI mRNAs during development in cardiac and skeletal muscle. RNA blot analysis with ^{32}P-labelled cardiac TnI-specific probes. Samples analyzed contained 30 μg of total RNA from the following tissues: lane 1, fetal cardiac muscle; lane 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.

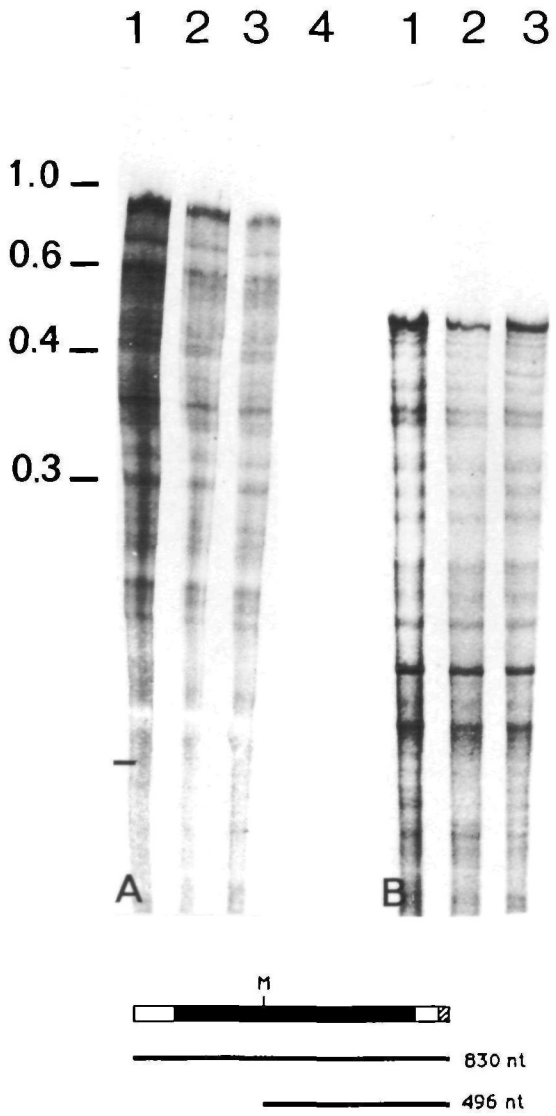

Fig. 7. RNAase protection assays. Two probes were used for these experiments: a 830 nt probe corresponding to the full-length cardiac TnI cDNA (A), and a 496 nt probe spanning from the *Mlu*I site to the poly(A) tail (B). RNAase protected fragments were analyzed on a 4% sequencing gel. Lane 1, adult ventricles; lane 2, neonatal ventricles; lane 3, 18-day-old fetal ventricles; lane 4, liver.

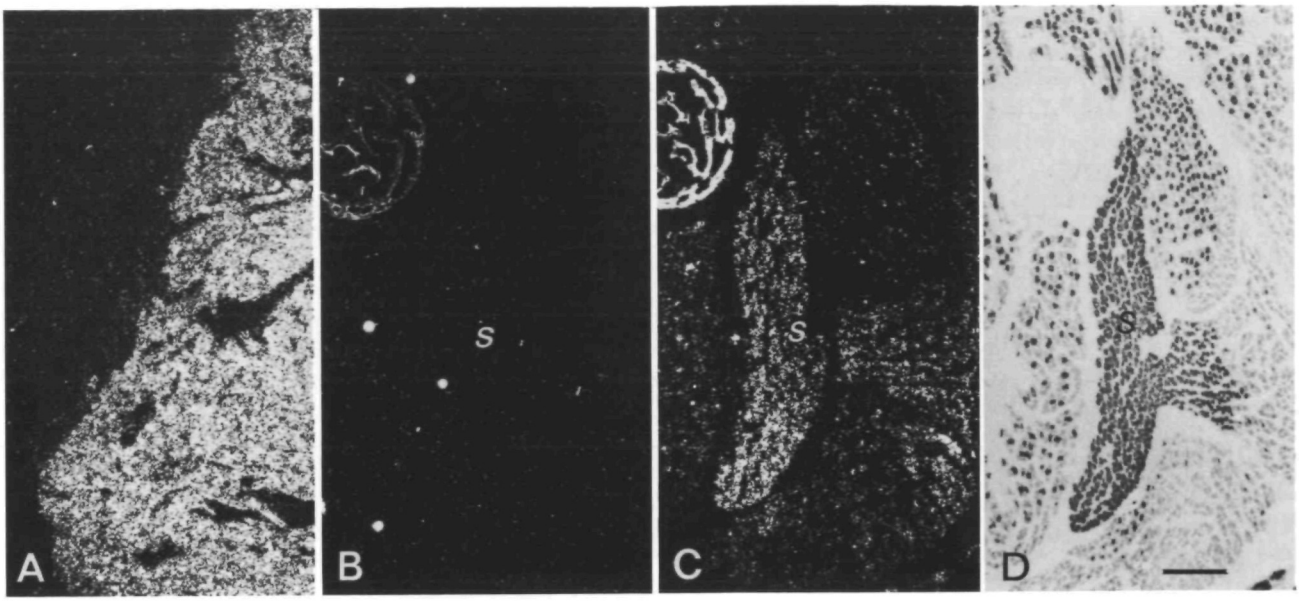

Fig. 8. Cardiac TnI transcripts are not expressed in skeletal muscle. (A) section through the adult rat ventricle (right) and soleus muscle (left) hybridized to the cardiac TnI probe. (B–D) serial sections through the leg of a 21-day-old rat fetus hybridized to cardiac (B) and slow skeletal (C) TnI probes, 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.

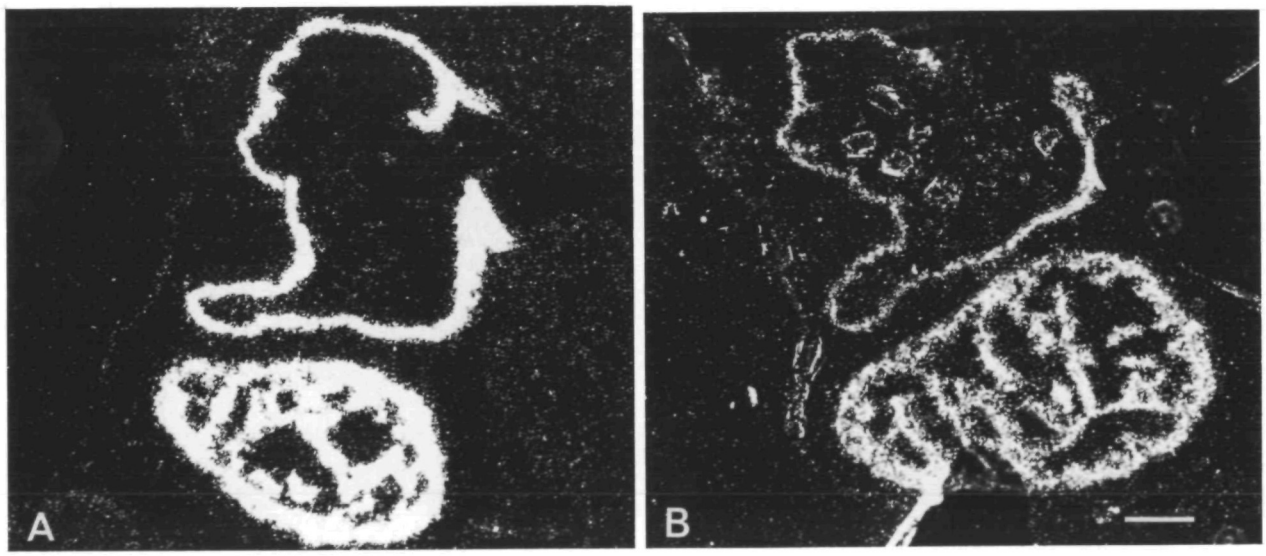

Fig. 9. Cardiac TnI and slow skeletal TnI transcripts are coexpressed in the embryonic heart. Serial sections through a 12-day-old embryonic heart hybridized with the cardiac (A) and slow skeletal (B) TnI probes. Both atrial (upper field) and ventricular (lower field) muscle cells react with the two probes. Bar: 100 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 β-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 β-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 (Leszyk *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, 1989*b*). 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 β/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 a-smooth and α-skeletal actin, β-MHC, MLC_{1A}, β-TM, slow skeletal TnI and cardiac TnT_{emb}, and up-regulation of other genes, such as α-MHC, cardiac TnI and cardiac TnT_{adult}. 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*[a]

Contractile protein genes	mRNA expressed in: Embryonic heart	mRNA expressed in: Adult heart	Refs
actin	α-cardiac actin α-skeletal actin α-smooth actin	α-cardiac actin	Mayer *et al.* (1984); Schwartz *et al.* (1986) Woodcock-Mitchell *et al.* (1988); Sassoon *et al* (1988)
MHC[b]	β-MHC α-MHC	α-MHC (β-MHC)	Lompré *et al.* (1984); Lyons *et al.* (1990)
MLC[c]	MLC_{1V} MLC_{1A}	MLC_{1V}	Lyons *et al.* (1990)
TM	α-TM β-TM	β-TM	Izumo *et al.* (1988)
TnT[d]	cardiac TnT_{emb}	cardiac TnT_{adult}	Jin and Lin (1989)
TnI	slow skeletal TnI cardiac TnI	cardiac TnI	this paper

[a] Abbreviations: MHC, myosin heavy chain; MLC, myosin light chain; TM, tropomyosin; TnT, troponin T; TnI, troponin I.

[b] α-MHC is co-expressed with β-MHC in the early embryonic mouse ventricles, then progressively disappears during fetal development to reappear before birth and become the predominant postnatal transcript (Lyons *et al.* 1990). A similar transition was found by studies at the protein level in the rat heart (DeGroot *et al.* 1989). β-MHC is identical to slow skeletal MHC and is coded by the same gene (Lompré *et al.* 1984); it disappears almost completely in the rat ventricular myocardium by 3–4 weeks after birth but is subsequently re-induced and progressively increases with aging to become again predominant in old animals (Lompré *et al.* 1984; Hoh *et al* 1978; Lakatta, 1987).

[c] A similar transition was found by studies at the protein level in the rat heart (Whalen and Sell, 1980). MLC_{1V} is identical to slow skeletal MLC (MLC_{1S}) and is coded by the same gene (Barton *et al.* 1985*a*). MLC_{1A} persists as a major isoform in the adult atrial myocardium (Hoh *et al.* 1978); it is identical to and coded by the same gene as the embryonic skeletal MLC (MLC_{1emb}) (Barton *et al.* 1985*b*; Barton *et al.* 1988).

[d] The embryonic and adult isoforms of cardiac TnT are produced by the same gene via alternative pre-mRNA splicing (Jin and Lin, 1989), as previously described in the chick (Cooper and Ordahl, 1985).

the rat (Saggin *et al.* 1990). Surprisingly, fast skeletal and slow skeletal TnI transcripts were barely detectable in fetal skeletal muscle using oligonucleotide probes specific for the corresponding mRNAs. These findings suggest that the TnI transcripts predominant in developing muscle are different from those expressed in adult muscles; it remains to be established whether they are coded by distinct gene(s). It is of interest in this respect that in the chicken a cDNA clone coding for the fast skeletal muscle TnI was found to hybridize to RNA from late embryonic skeletal muscle but not to RNA from early embryonic skeletal muscle (Nikovits *et al.* 1986).

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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.