J Mol Cell Cardiol **23**, 583–588 (1991)

Molecular Cloning and Developmental Expression of the Rat Cardiac-Specific Isoform of Troponin I

Anne F. Martin[1] and John Orlowski[2]

[1] *Department of Pharmacology and Cell Biophysics and* [2] *Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0575, USA*

(*Received 12 October 1990, accepted in revised form 20 December 1990*)

A. F. MARTIN AND J. ORLOWSKI. Molecular Cloning and Developmental Expression of the Rat Cardiac-Specific Isoform of Troponin I. *Journal of Molecular and Cellular Cardiology* (1991) **23**, 583–588. Troponin I is the subunit of the troponin complex in striated muscle which inhibits actomyosin ATPase activity. We have isolated a full-length cDNA clone for rat cardiac troponin I and determined its nucleic acid sequence. The amino acid sequence deduced from this clone shows 88%–92% similarity with previously reported amino acid sequences for rabbit (Wilkinson and Grand, 1978) and bovine (Leszyk *et al.*) cardiac troponin I. Examination of cardiac troponin I mRNA abundance during development revealed a 15-fold induction in its expression in the adult heart compared to that in embryonic (14 day) heart muscle. Furthermore, expression of cardiac troponin I mRNA was restricted to heart muscle and was not detected in skeletal muscle at any developmental stage.

KEY WORDS: Troponin I; Cardiac; cDNA clone; mRNA; Developmental expression.

Introduction

Troponin I is a component of the troponin complex, which with tropomyosin, plays a central role in the calcium dependent regulation of myosin and actin interaction in striated muscle. Three tissue specific isoforms of troponin I with different functional properties have been identified in fast and slow skeletal muscle and cardiac muscle by amino acid sequencing (Wilkinson and Grand, 1978). Analysis of sequence diversity indicates that these tissue specific isoforms of troponin I are the products of different genes. The amino acid sequences of rabbit (Wilkinson and Grand, 1978) and bovine (Leszyk *et al.*, 1988) cardiac troponin I differ from that of skeletal troponin I in that they contain an additional 26–33 amino acids at the amino terminus of the protein. A serine residue in the amino terminal region of rabbit cardiac troponin I (ser 20) has been shown to be phosphorylated in the intact heart by cAMP dependent protein kinase in response to β-adgrenergic stimulation (England, 1976; Moir *et al.*, 1980). An increase in the phosphorylation of troponin I at serine 20 can be correlated with the increased contractile force observed in response to inotropic agents (Moir *et al.*, 1980). This suggests that phosphorylation of troponin I at this site may be involved in the modulation of contractile function in cardiac muscle.

The gene for avian fast skeletal troponin I, from quail (Baldwin *et al.*, 1985) and chicken (Nikovits *et al.*, 1986) has been isolated and sequenced. Recently, the nucleotide sequences of cDNAs encoding mouse fast skeletal troponin I and rat slow skeletal troponin I have been reported (Koppe *et al.*, 1989). We report here the isolation and nucleotide sequence determination of a cDNA for the rodent cardiac troponin I and its mRNA expression during the development of the heart.

Materials and Methods

Screening of a cDNA library and sequencing of clones

A rat heart λgt11 library was purchased from Clontech (Palo Alto, CA, USA0. 1×10^6

[2] Present address: Department of Physiology, McGill University, 3655 Drummond Street, Montreal, P.Q. Canada, H3G 1Y6

0022-2828/91/050583 + 06 $03.00/0

plaques were screened using standard screening techniques (Maniatis *et al.*, 1982) with a 20 base oligonucleotide, containing 3 deoxyinosine substitutions (5′ C-A-(A,G)-G-T-(I,C)-A-A(A,G)-A-A-I-G-A-I-G-A-(T,C)-A-C 3′), synthesized to a conserved amino acid sequence (GlnValLysLysGluAspThr) at the carboxyl terminus of troponin I. Hybridization of the filters with the end-labeled oligonucleotide probe was performed using conditions described by Boyd *et al.* (1984). Positive clones were identified and plaque purified. One of these clones, Tn2C, encompassing the entire coding region of the TnI, was subcloned into the plasmid vector IBI30 (International Biotechnologies Inc.). Sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) utilizing the T7 polymerase (Pharmacia) reagent kit. Oligonucleotides to IBI30 sequence adjacent to the cloning site were used to obtain initial sequence in both directions. Four additional oligonucleotides, synthesized to the internal sequence of the Tn2C clone as it was obtained, were used to complete the sequence of both strands of the TnI cDNA. Sequence comparisons were performed by the DNanalyze program (Wernke and Thompson, 1989).

RNA preparation, electrophoresis and hybridization

Total cellular RNA was isolated by the procedure of Chomczynski and Sacchi (1987). RNA samples (10 μg each) were denatured with formaldehyde, fractionated on 1.2% formaldehyde-agarose gels (Fig. 2) or denatured with glyoxal and fractionated on 1% agarose gels (Fig. 3) and transferred onto Nytran membranes (Schleicher and Schuell). The filters were baked, prehybridized and hybridized with the oligo-labeled (Feinberg and Vogelstein, 1983) troponin I cDNA (Tn2C, 0.72 Kb) according to the manufacturer's protocol. The filters were washed in 0.1 × SSC (SSC is 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$), 0.1% SDS at 50°C for 1–2 h. As a check to confirm that equal amounts of RNA (10 μg) were loaded in each lane, the blots were probed with a 30-mer oligonucleotide corresponding to rat 18 S ribosomal RNA. Only blots which showed approximately equal amount of RNA in all lanes were used in this study.

Results

Several positive clones were obtained from our screening of the λgt11 rat heart library with an oligonucleotide made to a conserved amino acid sequence at the carboxyl terminus of troponin I. The complete nucleotide sequence and its deduced amino acid sequence of one of these clones, Tn2C, is given in Figure 1. This cDNA consisted of 633 nts of coding sequence, 54 nts of 5′ untranslated sequence and 62 nts of 3′ untranslated sequence. Although there is no upstream stop codon in this reading frame, we selected the first ATG codon as the translation initiation site because of the extensive homology of the deduced amino acid sequence with the amino acid sequence of bovine (Leszyk *et al.*, 1988) cardiac troponin I (92%) and rabbit (Wilkinson and Grand, 1978) cardiac troponin I (88%). Comparison of the deduced amino acid sequence of rat cardiac troponin I with the amino acid sequences of fast and slow skeletal troponin I indicated a 57% similarity to fast skeletal troponin I and a 64% similarity with slow skeletal troponin I.

Analysis of the expression of mRNA for cardiac troponin I on Northern blots (Fig. 2) demonstrates that cardiac troponin I mRNA is expressed specifically in cardiac muscle as an approximately 950 bp transcript. Cross hybridization of the cardiac troponin I cDNA was not seen with RNA from fast or slow skeletal muscle on Northern blots (Fig. 2), even under conditions of relatively low stringency (2 × SSC, 37°C). We have also examined the expression of cardiac troponin I mRNA at various stages of development in cardiac and skeletal muscle (Fig. 3). Cardiac troponin I mRNA is present in 14 day embryonic hearts at low levels and shows a continuous increase in the level of expression with age. The level of expression of cardiac troponin I mRNA shows an approximately 10-fold induction by 25 days post birth and continues to increase up to 55 days at which time an approximately 15-fold increase is observed relative to levels at 14 days gestation as estimated by densitometry. Expression of cardiac troponin I mRNA was not observed in skeletal muscle at any stage of development.

FIGURE 1. The complete cDNA sequence for rat cardiac troponin I. The bottom line is the nucleic acid sequence and the top line is the deduced amino acid sequence. Numbers refer to the amino acid sequence. The star indicates the termination codon and the polyadenylation sequence is underlined. The bars indicate amino acid sequences conserved in all published amino acid sequences for cardiac (Wilkinson and Grand, 1978, Leszyk *et al.*, 1988), slow skeletal (Wilkinson and Grand, 1978, Koppe *et al.*, 1989) and fast skeletal troponin I (Wilkinson and Grand, 1978, Baldwin *et al.*, 1985, Nikovits *et al.*, 1986, Koppe *et al.*, 1989).

Discussion

This paper describes the nucleotide and amino acid sequence of rodent cardiac troponin I and its mRNA expression during the development of the heart. Several of the functional domains of troponin I have been identified by a number of groups using a variety of experimental approaches (see Leavis and Gergely, 1984 and Zot and Potter, 1987 for reviews). Using the amino acid residue numbers for the rat cardiac troponin I sequence, the region of troponin I encompassing residues 128–149 has been shown to inhibit actomyosin ATPase and to contain the actin binding site and a calcium dependent troponin C binding site (Syska *et al.*, 1976, and Van Eyk and Hodges, 1988). Leszyk *et al.* (1988) have compared the amino acid sequences of cardiac, fast and slow skeletal troponin I and demonstrated that this region is highly conserved. Several other regions of homology at the carboxyl terminus, residues 177–185, 189–195 and 200–207 in rat cardiac troponin I (Fig. 1), are completely conserved in all known sequences of troponin I although their precise function has not been defined.

A comparison of the amino acid sequence at the amino terminal cardiac specific region of troponin I of rat, bovine and rabbit cardiac troponin I is given in Figure 4. Fifty per cent of the variable amino acid residues between cardiac troponin Is occur in this region, the

functional significance of the additional amino acids present at the amino terminus of cardiac troponin I is postulated to reside in the rapid phosphorylation of serine 23,24 by cAMP dependent protein kinase in the intact heart in response to inotropic agents that increase cAMP (England, 1976 Moir *et al.*, 1980). Phosphorylation of troponin I at this site has been shown to modify the affinity of the Ca^{2+} specific site of cardiac troponin C in the troponin complex for calcium (Robertson *et al.*, 1982). Thus it is postulated that troponin I phosphorylation which occurs in response to β-adrenergic stimulation in beating hearts may be responsible for the increased rate of relaxation seen following this inotropic intervention. As shown in Figure 4 the sequence of the phosphorylation site in rat cardiac troponin I is similar to that of bovine cardiac troponin I (Leszyk *et al.*, 1988) and contains two serines (residues 23,24) whereas rabbit cardiac troponin I has only one serine (residue 20) in this region. In the serine containing peptide isolated from bovine heart (Swiderek *et al.*, 1988) both serine residues were found to be phosphorylated although the first, residue 23, was phosphorylated to a lesser extent than the second serine. The extent to which one or both serine residues are phosphorylated in response to β-adrenergic stimulation in the heart remains to be determined.

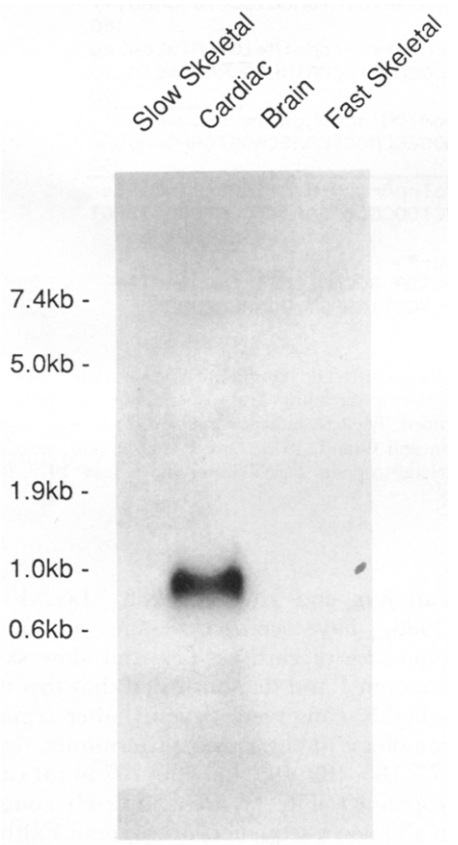

FIGURE 2. Northern blot analysis of RNA from rat slow skeletal (soleus), cardiac and fast skeletal (white vastus lateralis) muscles and rat brain. The total RNA (10 μg) was fractionated on a formaldehyde-agarose gel and transferred to a Nytran filter. The blots were hybridized with the full-length (0.72 kb) [^{32}P]-labeled troponin I cDNA (Tn2C). The autoradiogram shown was exposed to the blot for a 12 h period.

A number of developmentally regulated transitions in the expression of isoforms of the contractile proteins in cardiac tissue have been reported (Swynghedauw, 1986; Wade and Kedes, 1989). For rodent troponin I, recent studies using immunological techniques have indicated that two antigenically distinct isoforms of troponin I are expressed in a temporal manner during cardiac development (Sabry and Dhoot, 1989; Saggin *et al.*, 1989). The slow skeletal isoform of troponin I predominates in fetal heart and is replaced gradually by the cardiac isoform in the adult. These results are consistent with our findings of low mRNA levels for the cardiac troponin I in fetal heart, increasing approximately 15-fold upon reaching adulthood. This developmental induction of cardiac troponin I mRNA expression indicates that a pretranslational mechanism plays a significant role in determining the isoform composition of troponin I in the heart. However, the molecular signals responsible for altering the expression of the troponin I isoforms in heart muscle are not known. The time course of troponin I isoform switching is similar to the transitions observed for the α- and β-myosin heavy chain isoforms in rat heart (Hoh *et al.*, 1978; Lompre *et al.*, 1981). Substantial evidence from *in vivo* and *in vitro* studies has implicated thyroid hormone as the primary physiological modulator for the inverse regulation of these genes (Hoh *et al.*, 1978; Martin *et al.*, 1982; Chizzonite and Zak, 1984; Gustafson *et al.*, 1987. Izume *et al.*, 1986). Thus, it is possible that thyroid hormone may also be a modulator of cardiac troponin I isoform expression, although this remains to be tested.

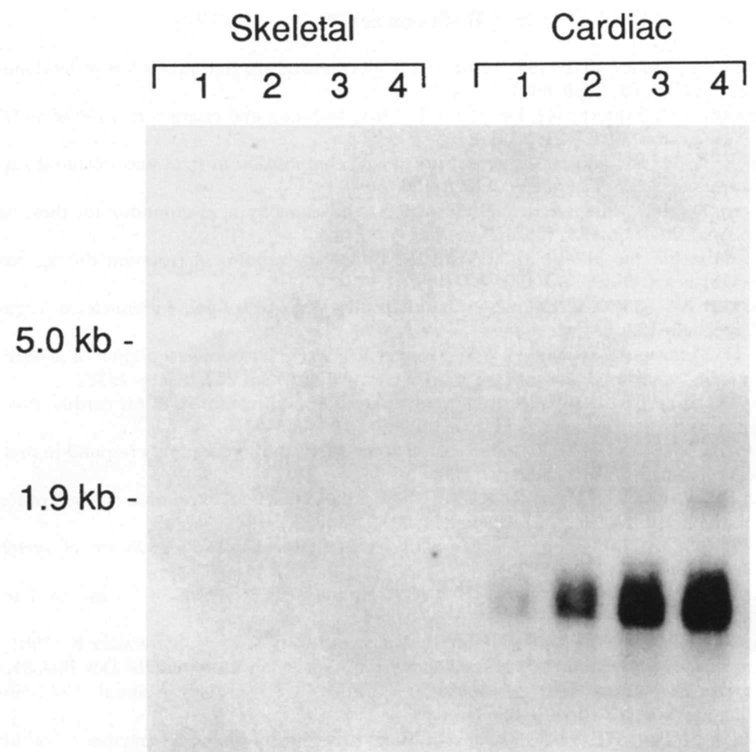

FIGURE 3. Northern blot analysis of RNA from developing rat cardiac and skeletal muscle. Each lane contained 10 μg of total cellular RNA isolated from hindleg skeletal muscle and cardiac muscle at different stages of development: lane 1, 14 days gestation; lane 2, 2 days post-natal; lane 3, 25 days of age; lane 4, 55 days of age. The RNAs were fractionated on a glyoxal gel, transferred to a Nytran filter and hybridized with the full-length (0.72 Kb) troponin I cDNA. The autoradiogram shown was exposed to the blot for a 12 h period.

Acknowledgements

The author would like to thank Drs Patrick Umeda and Radovan Zak for their help and encouragement in initiating this project and Dr Jeffrey Robbins for the generous sharing of his expertise and time and continuing support throughout this study. We also thank Ms Rita Eveleigh for the preparation of the manuscript and Ms Gwen Kraft for making the figures. This work was supported by grants from the National American Heart Association (84–102) and the American Heart Association, Ohio Affiliate (SW–88–29) (A. F. M.) and by a postdoctoral Fellowship from the Medical Research Council of Canada (J. O.).

```
                        *      *                   *   *     15
RABBIT   AlaAspGluSerArgAspAla     AlaGlyGluAlaLysProAlaProAla
RAT      AlaAspGluSerSerAspSer     AlaGlyGluProGlnProAlaProAla
BOVINE   AlaAspArgSerGlyGlySerThrAlaGlyAspThrValProAlaProPro
                  *     *  *     *        *   *   *           *

           *     *           *   *   *   *        30
RABBIT      Val   ArgArgSerAsp            ArgAlaTyrAlaThrGlu
RAT      ProValArgArgArgSerSerAlaAsnTyrArgAlaTyrAlaThrGlu
BOVINE   ProValArgArgArgSerSerAlaAsnTyrArgAlaTyrAlaThrGlu
```

FIGURE 4. Comparison of the deduced amino acid sequence of the cardiac specific amino terminal region of rat cardiac troponin I with that of rabbit (Wilkinson and Grand, 1979) and bovine (Leszyk *et al.*, 1988) cardiac troponin I. Stars indicate differences from the rat cardiac troponin I sequence. Blank spaces denote amino acid deletions.

References

Baldwin Jr AS, Kittler EL, Emerson CP (1985) Structure, evolution and regulation of a fast skeletal muscle troponin I gene. Proc Natl Acad Sci USA **82**: 8080–8084.

Boyd D, Jain SK, Crampton J, Barrett KJ, Drysdale J (1984) Isolation and characterization of a cDNA clone for human ferritin. Proc Natl Acad Sci USA **81**: 4751–4755.

Chizzonite RA, Zak R (1984) Regulation of myosin isoenzyme composition in fetal and neonatal rat ventricle by endogenous thyroid hormones. J Biol Chem **259**: 12628–12632.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **163**: 156–169.

England PJ (1976) Studies on the phosphorylation of the inhibitory subunit of troponin during modification of contraction in perfused rat heart. Biochem J **160**: 295–303.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132**: 6–13.

Gustafson TA, Bahl JJ, Markham BE, Roeske WR, Morkin E (1987) Hormonal regulation of myosin heavy chain and α-actin gene expression in cultured fetal rat heart myocytes. J Biol Chem **262**: 13316–13322.

Hoh JFY, McGrath PA, Hale PT (1978) Electrophoretic analysis of multiple forms of rat cardiac myosin: effects of hypophysectomy and thyroxine replacement. J Mol Cell Cardiol **10**: 1053–1076.

Izumo S, Nadal-Ginard B, Mahdavi V (1986) All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. Science **203**: 597–600.

Koppe RI, Hallauer PL, Karpati G, Hastings KEM (1989) cDNA clone and expression analysis of rodent fast and slow skeletal muscle troponin I mRNAs. J Biol Chem **264**: 14327–14333.

Leavis PC, Gergely J (1984) Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. CRC Crit Rev Biochem **16**: 235–305.

Leszyk J, Dumaswala R, Potter JD, Collins JH (1988) Amino acid sequence of bovine cardiac troponin I. Biochemistry **27**: 2821–2827.

Lompre AM, Mercadier JJ, Wisnewsky C, Bouveret P, Pantaloni C, d'Albis A, Schwartz K (1981) Species-and age-dependent changes in the relative amounts of cardiac myosin isoenzymes in mammals. Dev Biol **84**: 286–290.

Maniatis T, Fritsch EF, Sambroof J (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp 312–328.

Martin AF, Pagani ED, Solaro RJ (1982) Thyroxine-induced redistribution of isoenzymes of rabbit ventricular myosin. Circ Res **50**: 117-124.

Moir AJG, Solaro RJ, Perry SV (1980) The site of phosphorylation of troponin I in the perfused rabbit heart. Biochem J **185**: 505–513.

Nikovits Jr W, Kuncio G, Ordahl CP (1986) The chicken fast skeletal troponin I gene: exon organization and sequence. Nucleic Acids Res **14**: 3377–3390.

Robertson SP, Johnson JD, Holroyde MJ, Kranias EG, Potter JD, Solaro RJ (1982) The effect of troponin I phosphorylation on the Ca^{2+}-binding properties of the Ca^{2+}-regulatory site of bovine cardiac troponin. J Biol Chem **257**: 260–263.

Sabry MA, Dhoot DK (1989) Identification and pattern of expression of a developmental isoform of troponin I in chicken and rat cardiac muscle. J Muscle Res Cell Motility **10**: 85–91.

Saggin L, Gorza L, Ausoni S, Schiaffino S (1989) Troponin I switching in the developing heart. J Biol Chem **264**: 16299–16302.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74**: 5463–5467.

Swiderek K, Jaquet K, Meyer HE, Heilmeyer LMG (1988) Cardiac troponin I, isolated from bovine heart, contains two adjacent phosphoserines. Eur J Biochem **176**: 335–342.

Swynghedau B (1986) Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. Physiol Rev **66**: 711–771.

Syska H, Wilkinson JM, Grand RJA, Perry SV (1976) The relationship between biological activity and primary structure of troponin I from white skeletal muscle of the rabbit. Biochem J **153**: 375–387.

Van Eyk JE, Hodges RS (1988) The biological importance of each amino acid residue of the troponin I inhibitory sequence 104–115 in the interaction with troponin C and trompmyosin-actin. J Biol Chem **263**: 1726–1732.

Wade R, Kedes L (1989) Developmental regulation of contractile protein genes. Annu Rev Physiol **51**: 179–188.

Wernke GR, Thompson RL (1989) DNANALYZE: A comprehensive nucleotide and amino acid sequence analysis system emphasizing sequence similarity comparisons. Biophys J **59**: 390a.

Wilkinson JM, Grand RJA (1978) Comparison of amino acid sequence of troponin I from different striated muscles. Nature **271**: 31–35.

Zot A, Potter JD (1987) Structural aspects of troponin-tropomyosin regulations of skeletal muscle contraction. Annu Rev Biophys Chem **16**: 535–559.