Leszyk, J., Dumaswala, R., Potter, J. D., & Collins, J. H. (1988) *Biochemistry 27*. 2821-2827.
Lowry, O. H., Rosebrough, N. H., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Martell, A. E., & Smith, R. M. (1974) *Critical Stability Constants*, Plenum Press, New York.
Minta, A., Harootvian, A. T., Kao, J. P. Y., & Tsien, R. Y. (1987) *J. Cell Biol. 105*, 89a.
Minta, A., Kao, J. P. Y., & Tsien, R. Y. (1989) *J. Biol. Chem. 264*, 8171-8178.
Potter, J. D. (1982) *Methods Enzymol. 85*, 241-263.
Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L., & Means, A. R. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Carradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., & Siegel, F. L., Eds.) pp 239-250, North Holland, New York, Amsterdam, Oxford.
Potter, J. D., Strang, P. F., & Ashley, C. C. (1986) *Biophys. J. 49*, 249a.
Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., & Potter, J. D. (1983) *J. Biol. Chem. 258*, 7441-7444.
Putkey, J. A., Carrol, S. L., & Means, A. R. (1987) *Mol. Cell. Biol. 7*, 1549-1553.
Putkey, J. A., Sweeney, H. L., & Campbell, S. T. (1989) *J. Biol. Chem. 264*, 12370-12378.
Reinach, F. C., & Karlsson, R. (1988) *J. Biol. Chem. 263*, 2371-2376.
Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628-1647.
Sheng, Z., Francois, J.-M., Hitchock-DeGregori, S. E., & Potter, J. D. (1990) *Biophys. J. 57*, 144a.
Strydnaka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951-998.
Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science 227*, 945-948.
Timmerman, M. P., & Ashley, C. C. (1986) *FEBS Lett. 209*, 1-8.
Trayer, I. P., Trayer, H. R., & Levine, B. A. (1987) *Eur. J. Biochem. 164*, 259-266.
Tsien, R. Y. (1980) *Biochemistry 19*, 2396-2404.
van Eerd, J.-P. & Takahashi, K. (1976) *Biochemistry 15*, 1171-1180.
Wilkinson, J. M. (1976) *FEBS Lett. 70*, 254-256.
Wnuk, W. (1984) *J. Biol. Chem. 259*, 9017-9023.
Wnuk, W. (1988) in *Calcium and Calcium Binding Proteins* (Gerday, Ch., Bolis, L., & Gilles, R., Eds.) pp 44-68, Springer-Verlag, Berlin, Heidelberg, Germany.
Wnuk, W. (1989) *J. Biol. Chem. 264*, 18240-18246.
Xu, G.-Q., & Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem. 263*, 13962-13969.
Zot, A. S., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535-559.

Molecular Cloning of Rat Cardiac Troponin I and Analysis of Troponin I Isoform Expression in Developing Rat Heart[†,‡]

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ABSTRACT: We have isolated and sequenced a cDNA encoding rat cardiac troponin I. The predicted amino acid sequence was highly identical with previously reported chemically derived amino acid sequences for rabbit and bovine cardiac troponin I. Clones for slow skeletal muscle troponin I were also obtained from neonatal rat cardiac ventricle by the polymerase chain reaction. The nucleotide sequences of these clones were determined to be more than 99% identical with a previously reported rat slow skeletal troponin I cDNA [Koppe et al. (1989) *J. Biol. Chem. 264*, 14327-14333]. The troponin I clones hybridized to RNA from the appropriate muscle from adult animals. However, RNA from fetal and neonatal rat heart also hybridized with the slow skeletal troponin I cDNA, demonstrating its expression in fetal and neonatal rat heart. Slow skeletal troponin I steady-state mRNA levels decreased with increasing age, but cardiac troponin I mRNA levels increased through fetal and early neonatal cardiac development. Thus, during fetal and neonatal development, slow skeletal and cardiac troponin I isoforms are coexpressed in the rat heart and regulated in opposite directions. The degree of primary sequence differences in these isoforms, especially at phosphorylation sites, may result in important functional differences in the neonatal myocardium.

Troponin I (TnI),[1] the inhibitory component of the troponin complex, has an important role in the calcium-dependent regulation of striated muscle contraction [see El-Saleh et al. (1986) and Zot and Potter (1987) for reviews]. As calcium binds to troponin C (TnC), protein-protein interactions occur among TnC, TnT, tropomyosin, and TnI which remove the inhibition of TnI on actin-myosin cross bridging. Chemically determined amino acid sequences for TnI isoforms have been reported from slow skeletal, fast skeletal, and cardiac muscle (Grand et al., 1976; Wilkinson & Grand, 1978; Leszyk et al.,

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[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05304.
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[1] Abbreviations: TnI, troponin I; ssTnI, slow skeletal troponin I; cTnI, cardiac troponin I.

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1988). These isoforms are likely transcribed from different genes, as suggested by the degree of primary sequence difference among the isoforms in a single species. In addition, nucleotides encoding a cardiac-specific NH_2-terminus were not found in the upstream sequences of the fast skeletal muscle genomic clones of Baldwin et al. (1985). Finally, Koppe et al. (1989) recently examined RNA blots using slow and fast skeletal cDNA probes for TnI and found no evidence for hybridization with cardiac tissue, as would be expected for products which share exons from the same gene.

Cardiac TnI amino acid sequences, as chemically determined from purified rabbit and bovine heart protein, differ from skeletal sequences because they contain NH_2-terminal sequences which include one or two serines which may be phosphorylated by protein kinase A in vitro or β-adrenergic stimulation in situ (Moir & Perry, 1977; Moir et al., 1980; Swiderek et al., 1988). Phosphorylation of cardiac TnI by protein kinase A decreases the myofibrillar Mg-ATPase calcium sensitivity and thus may be functionally significant in cardiac muscle (Holroyde et al., 1979).

Troponin I isoforms may also be developmentally regulated. Toyota and Shimada (1981) presented evidence that a polyclonal antibody to fast skeletal TnI reacted with myofibrils from early embryonic chick heart, although Nikovits et al. (1986) could not find evidence for expression of fast skeletal TnI mRNA at a later embryonic stage in the chick. Recent evidence from protein gels and immunoblots indicates that the cardiac isoform of TnI may be regulated during fetal and postnatal development in the rat and dog (Solaro et al., 1986; Dieckman & Solaro, 1990) and that slow skeletal-like TnI may be expressed in fetal and neonatal rat and embryonic and neonatal chicken heart (Sabry & Dhoot, 1989; Saggin et al., 1989). None of these studies have definitively identified the TnI isoform expressed in embryonic or fetal heart.

We report here the characterization of the first cardiac-specific TnI clone. Slow skeletal TnI (ssTnI) isoform expression in the fetal and neonatal heart was also examined by using the polymerase chain reaction and blotting techniques. These data provide definitive evidence of the expression and regulation of slow skeletal and cardiac isoforms in the immature rat heart.

Experimental Procedures

cDNA Library Screening, Subcloning, and Sequencing. A λgt10 adult rat cardiac cDNA library available in our laboratory was screened (10^5 plaques) with a 54-mer synthetic oligonucleotide (5′-AAGCAGGTGAAGAAGGAGGACACCGAGAAGGAGAACCGCGAGGTCGGGGACTGG-3′) derived from the published rabbit cardiac TnI amino acid sequence (Grand et al., 1976). The oligonucleotide was labeled to high specific activity with the random primer method (Feinberg & Vogelstein, 1984). The filters were washed to a stringency of 15 mM NaCl, 1.5 mM sodium citrate, 1 mM EDTA, and 0.1% SDS at ambient temperature. Five positive clones were isolated from the rat library. Only a single clone contained the entire coding region. The isolates were subcloned into the plasmid vectors PGEM 3z or 4z (Promega). Sequencing was by the dideoxy method of Sanger et al. (1977), using the Sequenase kit (U.S. Biochemical). Both the universal primers SP6 and T7 and synthetic oligonucleotides derived from the nucleotide sequence were employed to generate the sequence of both strands.

RNA and DNA Blot Analysis. Total cellular RNA of rat tissues was prepared by lysis in guanidinium isothiocyante buffer and cesium gradient centrifugation (Chirgwin et al., 1979). Five micrograms of RNA per lane was separated

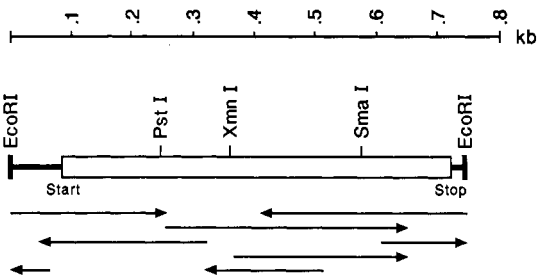

FIGURE 1: Map of cDNA for rat cardiac TnI. This clone is a 745 bp clone which encompasses the entire coding region, as well as parts of the 5′- and 3′-untranslated regions. The nucleotide sequencing strategy is shown by the small arrows. This clone was 100% sequenced from both strands.

electrophoretically on 1.2% agarose/formaldehyde gels and transferred onto Genescreen membranes (New England Nuclear). Hybridization conditions were according to the manufacturer's instructions. Probes were labeled by the random primer technique with [α-^{32}P]dCTP (Feinberg & Vogelstein, 1984). Final washing was 15 mM NaCl, 1.5 mM sodium citrate, 1 mM EDTA, and 0.1% SDS at 55–65 °C.

Taq Polymerase Amplification of Slow Skeletal TnI in Neonatal Cardiac Muscle. Two cDNAs encoding slow skeletal TnI were obtained by Taq polymerase amplification (Saiki et al., 1988). The template was first-strand cDNA synthesized with reverse transcriptase from immature (7 day old) rat cardiac ventricular muscle total cellular RNA. Oligo(dT) was used as the annealing primer for the first-strand synthesis. For amplification, the primer sequences were selected from the 5′- and 3′-untranslated regions of the published cDNA sequence of slow skeletal TnI (Koppe et al., 1989). A restriction enzyme site was included in the primer sequence to facilitate subcloning. An annealing temperature of 55 °C was used in the reaction. A single product, of the predicted length for ssTnI, was evident on agarose gel electrophoresis. After being cloned into plasmid vectors, the amplified DNA product was subjected to sequence analysis. Two separate clones were analyzed. RNA blots were probed with the product after purification from an agarose gel.

Primer Extension Analysis. Synthetic oligonucleotide primers were prepared as corresponding to sequences approximately 100 bp downstream from the transcriptional start site. The oligonucleotides were end-labeled with γ-^{32}P in a kinase reaction. Approximately (0.5–1.0) × 10^6 cpm per reaction was hybridized with 5–7 μg of total RNA at 42 °C for 1 h. The primers were extended with unlabeled nucleotides and reverse transcriptase for 1.5 h at 42 °C. The products were precipitated with ethanol, resuspended in sample buffer, and analyzed on a DNA sequencing gel with a sequencing reaction of known length.

Results

Primary Sequence of Cardiac TnI. The restriction enzyme map and sequencing strategy for the longest rat cardiac TnI clone are as indicated in Figure 1. The clone contains 745 bp which span the coding region, 87 bp of 5′-untranslated sequence, and 25 bp of 3′-untranslated sequence. The nucleotide sequence of TnI clone 4C and its derived amnio acid sequence are as shown in Figure 2. The rat TnI clone lacks a polyadenylation site but contains the entire coding region. Primer extension analysis was performed as illustrated in Figure 3. This primer was designed to anneal with bp −27 to +3 of the cDNA sequence. The results indicated that the 5′-untranslated region of the cardiac TnI mRNA extends to −120 nucleotides, and thus there are an additional 33 nu-

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      -78       -68       -58       -48       -38       -28       -18        -8         3        13        23        33
gggacccaaccctccaaggtcaccagggacacccttctaagacccctccaggaatctgcaatcccattctctacctctggagatcagcATGGCGGATGAGAGCAGCGATGCGGCTGGGGAA
                                                                                        M  A  D  E  S  S  D  A  A  G  E

      43        53        63        73        83        93       103       113       123       133       143       153
CCCCAGCCGGCGCCTGCTCCTGTCCGACGTCGCTCCTCGGCCAACTACCGAGCCTATGCCACCGAGCCACATGCCAAGAAAAAGTCTAAGATCTCCGCCTCCAGAAAACTTCAGTTGAAG
 P  Q  P  A  P  A  P  V  R  R  R  S  S  A  N  Y  R  A  Y  A  T  E  P  H  A  K  K  K  S  K  I  S  A  S  R  K  L  Q  L  K

      163       173       183       193       203       213       223       233       243       253       263       273
ACTCTGATGCTGCAGATTGCGAAGCAGGAGATGGAGCGTGAGGCAGAGGAGCGACGTGGAGAGAAGGGCCGCGTTCTGAGCACGCGTTGCCAGCCCTTGGTGTTGGATGGGCTGGGCTTT
 T  L  M  L  Q  I  A  K  Q  E  M  E  R  E  A  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

      283       293       303       313       323       333       343       353       363       373       383       393
GAAGAGCTTCAGGACCTATGCCGGCAGCTTCACGCTCGTGTGGACAAAGTGGATGAAGAGAGATACGACGTGGAAGCAAAAGTCACCAAGAACATCACTGAGATTGCAGATCTGACCCAG
 E  E  L  Q  D  L  C  R  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

      403       413       423       433       443       453       463       473       483       493       503       513
AAGATCTATGACCTGCGTGGCAAGTTTAAGCGGCCCACTCTCCGAAGAGTGAGAATCTCAGCAGATGCCATGATGCAGGCACTACTGGGGACCCGGGCCAAGGAATCCTTGGACCTGAGG
 K  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  E  S  L  D  L  R

      523       533       543       553       563       573       583       593       603       613       623       633
GCCCACCTCAAGCAGGTGAAGAAGGAGGACATTGAGAAGGAAAACCGGGAGGTGGGAGACTGGCGCAAGAATATCGATGCACTAAGTGGAATGGAAGGCCGAAAGAAAAAGTTTGAGGGC
 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  A  L  S  G  M  E  G  R  K  K  K  F  E  G

      643       653
TGAgccatggctctcacactgtgcg
```

FIGURE 2: Nucleotide sequence and translation of the cDNA. The protein encoded is 210 amino acids long and was identified as rat cardiac TnI by the degree of sequence identity with other chemically determined cardiac TnI amino acid sequences. The primer extension oligonucleotide (−27 to +3) is in boldface and underlined, as is the oligonucleotide used for screening (+523 to +576).

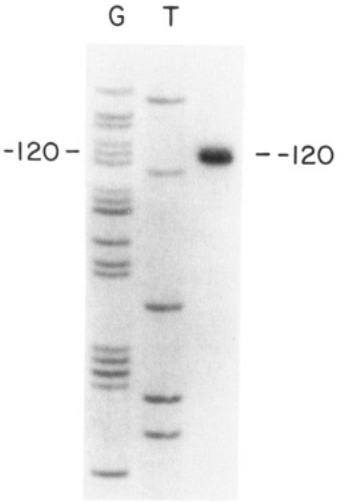

FIGURE 3: Primer extension analysis of cTnI mRNA. The 30-mer oligonucleotide complementary to nucleotides −27 to +3 of the cDNA sequence was end-labeled and hybridized with adult cardiac RNA as indicated in the text. The extended product was −120 nucleotides from the start codon as indicated by its comigration with a sequencing ladder (G and T lanes indicated).

cleotides transcribed upstream to clone 4C in rat cardiac mRNA. This was also confirmed by repeating the analysis with a second antisense oligonucleotide which was complementary to bp +4 to +33 of the cDNA sequence (data not shown).

The derived amino acid sequence of rat cardiac TnI is compared to the chemically determined amino acid sequences of rabbit and bovine cardiac TnI in Figure 4. There is 91–93% amino acid identity among these sequences. The greatest divergence is in the NH_2-terminus of TnI. This region also distinguishes the cardiac from the skeletal protein isoforms (Wilkinson & Grand, 1976).

The rat cardiac and slow skeletal TnI-derived amino acid sequences are compared in Figure 5. The 801 bp slow skeletal TnI clones obtained by Taq polymerase amplification from neonatal rat heart contained 36 bp of 5′-untranslated sequence, 561 bp of coding sequence, and 201 bp of 3′-untranslated sequence. The two clones contained only two consistent differences in nucleotide sequence to that reported by Koppe et al. (1989). These differences occurred in the coding region, and one is at the third base in a codon resulting in no change in the derived amino acid sequence. The only difference in

FIGURE 4: Comparison of sequences among rat, rabbit, and bovine cardiac TnI. Other sequences are derived from Leszyk et al. (1988) and Grand et al. (1976). We have omitted the Leu-141 from the rabbit sequence as justified by Leszyk et al. (1988). The protein kinase A phosphorylation sites upon serine residues (Ser-22 and Ser-23) are indicated by asterisks, as is the predominant protein kinase C phosphorylation site (Thr-143). The bovine and rat TnI's contain two serines, but the rabbit sequence contains a single serine in a corresponding position in the NH_2-terminus. The numbering of the amino acid residues refers to the rat sequence. Regions which interact with TnC are underlined. The cysteines which interact with TnT are overlined. Identical amino acids are included in the boxes.

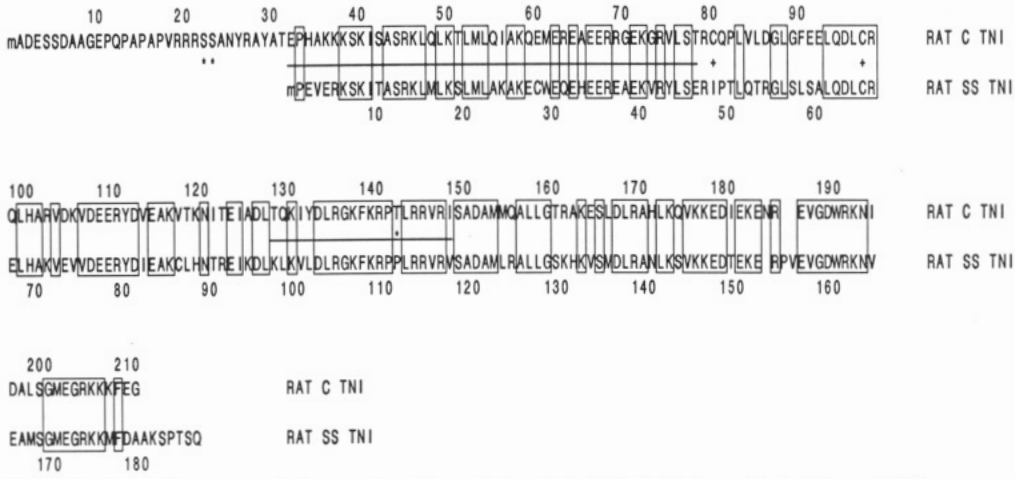

FIGURE 5: Comparison of amino acid sequences betwen rat cardiac and slow skeletal TnI. Both are derived from cDNA sequences. The underlines indicate regions which interact with troponin C. Phosphorylation sites are as indicated in the legend of Figure 3 and are marked with an asterisk. The cysteines which interact with TnT are indicated by (+). Identical amino acids are included in the boxes.

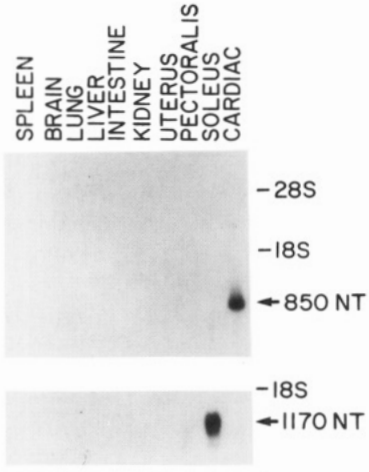

FIGURE 6: Analysis of RNA blot of rat tissues. This blot contains 5 μg per lane of total cellular RNA isolated from the tissues indicated. Integrity and transfer of RNA were confirmed by ethidium staining of 28S and 18S ribosomal RNA. The blot was washed to a stringency noted under Experimental Procedures, and to a temperature of 55 °C. The top panel was hybridized with the 745 bp cardiac cDNA probe (Figure 1). The bottom panel is the same blot which was stripped with alkali, and subsequently hybridized with the 801 bp slow skeletal TnI cDNA probe as described in the text.

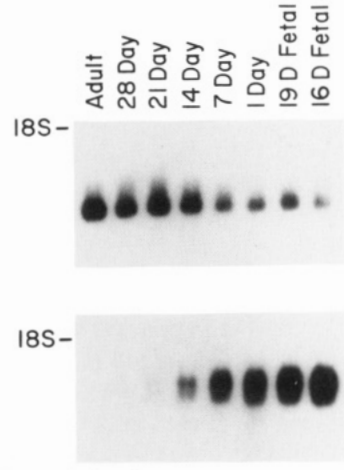

FIGURE 7: RNA analysis of rat heart TnI expression through development. The tissues probed were from 16- and 19-day fetal, 1–28-day postnatal, and adult (>70 day) rat hearts. Five micrograms of total cellular RNA was loaded on each lane. The 745 bp cardiac cDNA (Figure 1) was used as the probe in the top panel. In the bottom panel, the same blot was stripped and then hybridized with the 801 bp ssTnI cDNA. The stringency of the wash was as noted under Experimental Procedures, and to a temperature of 65 °C.

the derived amino acid sequence was due to a substitution of a C for a T at base 592, changing the amino acid residue which precedes the COOH-terminal glutamine from a leucine to a serine (Figure 5). The rabbit slow skeletal amino acid sequence also contains a serine in this position, suggesting that this sequence difference may represent a polymorphism because it was present on both slow skeletal TnI clones obtained from amplification.

Expression of TnI Isoforms in Heart. To determine the expression of cTnI mRNA among adult rat tissues, RNA blot analysis was performed. As shown in Figure 6, cTnI expression is cardiac-specific (Figure 6, top panel). This was confirmed by the lack of a signal in other tissues upon overexposure of the blot (data not shown). The estimated size of the mRNA was 850 nt. The same blot was subsequently probed with the slow skeletal TnI probe which hybridized with soleus muscle RNA (Figure 6, bottom panel). The size of the ssTnI mRNA was estimated as 1170 nt. A faint signal was detected for pectoralis muscle after prolonged exposure of the autoradiogram (data not shown). The ssTnI probe did not hybridize with adult cardiac muscle. This indicated that these two cDNA probes were specific for the appropriate muscle in the adult tissues.

Analysis of a rat heart developmental blot (Figure 7, top panel) indicated the cTnI mRNA in fetal and neonatal rat hearts was the same size as that in adult heart. The steady-state amount of cTnI mRNA increased from fetal to adult cardiac tissue, with some of this change occurring postnatally. In contrast, ssTnI was expressed exclusively in neonatal and fetal cardiac tissues (Figure 7, bottom panel) and demonstrated decreasing mRNA quantity with increasing maturity. The ssTnI mRNA in fetal heart comigrated with that in soleus muscle when these samples were run simultaneously (data not shown). These data indicate both the specificity of TnI expression in adult tissues and also the striking switch in expression patterns in the developing heart.

Discussion

These data allow comparison of the primary sequences of two troponin I isoforms in the rat. The greatest degree of identity between skeletal and cardiac isoforms is in the COOH-terminal region of the molecule where there are 66% invariant amino acid sequences (Leszyk et al., 1988). Several

domains have been assigned to TnI. The fast skeletal and cardiac TnI isoforms contain two cysteines (Cys-80 and Cys-97 in rat cardiac) involved in the interaction with TnT (Chong & Hodges, 1982); however, both the rabbit and rat slow skeletal sequences contain an isoleucine in a position equivalent to the first cysteine. Two regions, amino acids 32–79 and 129–149 in rat cardiac sequence, interact with TnC (Syska et al., 1976; Leavis et al., 1978; Leszyk et al., 1990). These areas demonstrate more diversity when compared between skeletal and cardiac TnI's (Figure 5). However, they are highly conserved among the cardiac sequences of different species (Figure 4). This suggests that the heterogeneity in these regions may contribute to functional differences in TnI–TnC interactions in cardiac and skeletal muscle. The residues of the skeletal TnI sequence which possess the activity inhibiting actomyosin ATPase (equivalent to residues 137–148 in the rat cardiac sequence) interact both with TnC and with tropomyosin–actin (Van Eyk & Hodges, 1988). This region is identical in the rat, bovine, and rabbit cardiac sequences. However, the slow skeletal sequence contains a proline at position 111, whereas the rat cardiac sequence contains a threonine (Thr-143) at the corresponding position (Figure 5). Recently, it has been demonstrated that bovine cTnI is phosphorylated by protein kinase C predominantly at the corresponding threonine, Thr-144 (Noland et al., 1989); however, the functional significance of protein kinase C mediated phosphorylation in the heart has yet to be determined.

The cDNA-derived amino acid sequence of rat cardiac TnI shares a high degree of identity with the chemically derived protein sequences of rabbit and bovine cardiac TnI (Figure 4). The cardiac specific NH_2-terminus (residues 1–32 in the rat), which is absent in both the slow and fast skeletal sequences, contains the greatest diversity among species. The rat cTnI clone encodes two sequential serine residues (Ser-22 and Ser-23) in the NH_2-terminal region. The residues surrounding the serines are identical with the sequence in bovine cardiac TnI (RRRSS), in which it has been demonstrated that both serines may be sites for phosphorylation by protein kinase A (Swiderek et al., 1988). This deduced sequence is identical in a human cardiac cDNA clone we have characterized,[2] suggesting that the double phosphorylation site may be the more common configuration in the mammals. We have confirmed the correct alignment of the peptide fragment 37–41 (KKKSK) in the chemically derived bovine cardiac amino acid sequence reported by Leszyk et al. (1988). This peptide was reversed by Grand et al. (1976) in the rabbit cardiac TnI sequence. Thus, comparisons between species indicate that cardiac TnI is a highly conserved protein. The sequences of rat slow skeletal and cardiac TnI suggest important functional differences between these protein isoforms.

This work establishes that the slow skeletal TnI isoform is coexpressed with the cardiac TnI isoform during fetal and neonatal cardiac development but that cardiac TnI expression is specific after the neonatal period. The possibility of skeletal TnI expression in immature heart was first suggested by data of Toyota and Shimada (1981). Their work reported staining of myocardium with antibody to fast skeletal TnI in early embryonic (3 day in ovo) chick heart. However, the antibody did not react with embryonic chick heart from a later stage (day 19–20). Both Sabry and Dhoot (1989) and Saggin et al. (1989) demonstrated the presence of antigen similar to slow skeletal TnI on immunoblots of pre- and neonatal cardiac tissue from the chick and rat. Our work has definitively established that slow skeletal and cardiac TnI's are coexpressed in fetal and neonatal rat heart and demonstrated that the mRNA levels are regulated in opposite directions during fetal and neonatal cardiac development.

TnI isoform switching has an obvious functional correlate in that ssTnI is not phosphorylated to any significant degree by protein kinase A, whereas cardiac TnI is phosphorylated at one or two serines in the NH_2-terminus (Swiderek et al., 1988). It has been postulated that TnI phosphorylation may result in the increased rate of relaxation of cardiac muscle typical of adrenergically stimulated hearts. Neonatal hearts do not demonstrate this augmentation of relaxation with β-adrenergic stimulation (Park et al., 1980), possibly because of less phosphorylatable TnI. In addition, TnI isoform variation in mammalian heart has been implicated in the relative resistance to the effects of acidosis on contractile function and myofibrillar ATPase activity in preparations from neonatal animals (Solaro et al., 1986, 1988), and in the diminished response to calmidazolium, an agent which alters the calcium sensitivity of the contractile apparatus (Murphy & Solaro, 1990). Thus, the presence of ssTnI in the fetal and neonatal myocardium results in functional differences in the contractile response of the immature myocardium.

The developmental regulation of contractile protein genes may occur by several mechanisms including sequential activation of different genes or by alternative splicing of a single gene, as recently reviewed by Wade and Kedes (1989). The degree of primary sequence difference in both translated and untranslated regions of the TnI cDNAs provides evidence that rat slow skeletal and cardiac TnI's are products of different genes. TnI regulation appears to be similar to α-actins in which there is coexpression of the skeletal and cardiac isoforms during cardiac development (Ordahl et al., 1984; Nudel et al., 1984). However, unlike α-actin, there was no evidence of cardiac TnI expression in chick embryonic skeletal muscle probed with antibody specific to cardiac TnI (Toyota & Shimada, 1981). After maturation, the fiber-type specific isoforms of TnI are specific in their expression. Further work exploring the regulatory sequences of the rat cardiac TnI gene may implicate specific regulatory elements responsible for developmental up-regulation of the cardiac gene and down-regulation of the slow skeletal gene in the heart. Analysis of these regulatory elements and comparison among different gene families will elucidate the nature of molecular signals which result in the functional transition from immature to mature myocardium.

Acknowledgments

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Registry No. DNA (rat heart troponin I messenger RNA complementary), 130726-92-0; troponin I (rat heart reduced), 130726-88-4; troponin I (rat heart clone 4C protein moiety reduced), 130726-89-5; troponin I (rat slow skeletal muscle precursor reduced), 130726-90-8; troponin I (rat slow skeletal muscle reduced), 130726-91-9.

References

Baldwin, A. S., Jr., Kittler, E. L. W., & Emerson, C. P., Jr. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 8080–8084.

Chirgwin, J. M., Przybla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chong, P. C. S., & Hodges, R. S. (1982) *J. Biol. Chem. 257*, 2549–2555.

Dieckman, L. J., & Solaro, R. J. (1990) *Circ. Res. 67*, 344–351.

[2] A. M. Murphy, unpublished data.

El-Saleh, S. C., Warber, K. D., & Potter, J. D. (1986) *J. Muscle Res. Cell Motil. 7*, 387-404.
Feinberg, A., & Vogelstein, B. (1984) *Anal. Biochem. 137*, 266-267.
Grand, R. J. A., Wilkinson, J. M., & Mole, L. E. (1976) *Biochem. J. 159*, 633-641.
Holroyde, M. J., Howe, E., & Solaro, R. J. (1979) *Biochim. Biophys. Acta 586*, 63-69.
Koppe, R. I., Hallauer, P. L., Karpati, G., & Hastings, K. E. M. (1989) *J. Biol. Chem. 264*, 14327-14333.
Leavis, P. C., Rosenfeld, S. S., Gergly, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452-5459.
Leszyk, J., Dumaswain, R., Potter, J. D., & Collins, J. H. (1988) *Biochemistry 27*, 2821-2827.
Leszyk, J., Grabarek, Z., Gergely, J., & Collins, J. H. (1990) *Biochemistry 29*, 299-304.
Moir, A. J. G., & Perry, S. V. (1977) *Biochem. J. 167*, 333-343.
Moir, A. J. G., Solaro, R. J., & Perry, S. V. (1980) *Biochem. J. 185*, 505-513.
Murphy, A. M., & Solaro, R. J. (1990) *Pediatr. Res. 28*, 46-49.
Nikovits, W., Kuncio, G., & Ordahl, C. P. (1986) *Nucleic Acids Res. 14*, 3377-3390.
Noland, T. A., Jr., Raynor, R. L., & Kuo, J. F. (1989) *J. Biol. Chem. 264*, 20778-20785.
Nudel, U., Mayer, Y., Zakut, R., Shani, M., Czosnek, H., Aloni, B., Melloul, D., & Yaffe, D. (1984) *Exp. Biol. Med. 9*, 219-227.
Ordahl, C. P., Peng, I., & Cooper, T. A. (1984) *Exp. Biol. Med. 9*, 211-218.
Park, M. K., Sheridan, P. H., Morgan, W. W., & Beck, N. (1980) *Dev. Pharmacol. Ther. 1*, 70-82.
Sabry, M. A., & Dhoot, G. K. (1989) *J. Muscle Res. Cell Motil. 10*, 85-91.
Saggin, L., Gorza, L., Ausoni, S., & Schiaffino, S. (1989) *J. Biol. Chem. 264*, 16299-16302.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487-491.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Solaro, R. J., Kumar, P., Blanchard, E. M., & Martin, A. F. (1986) *Circ. Res. 58*, 721-729.
Solaro, R. J., Lee, J. A., Kentish, J. C., & Allen, D. G. (1988) *Circ. Res. 63*, 779-787.
Swiderek, K., Jaquet, K., Meyer, H. E., & Heilmeyer, L. M. G., Jr. (1988) *Eur. J. Biochem. 176*, 335-342.
Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry S. V. (1976) *Biochem. J. 153*, 375-387.
Toyota, N., & Shimada, Y. (1981) *J. Cell Biol. 91*, 497-504.
Van Eyk, J. E., & Hodges, R. S. (1988) *J. Biol. Chem. 263*, 1726-1732.
Wilkinson, J. M., & Grand, R. J. A. (1978) *Nature 271*, 31-35.
Zot, A. S., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biochem. 16*, 535-559.

Mechanism for the Inhibition of Acto-Heavy Meromyosin ATPase by the Actin/Calmodulin Binding Domain of Caldesmon†

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ABSTRACT: Caldesmon, an actin/calmodulin binding protein, inhibits acto-heavy meromyosin (HMM) ATPase, while it increases the binding of HMM to actin, presumably mediated through an interaction between the myosin subfragment 2 region of HMM and caldesmon, which is bound to actin. In order to study the mechanism for the inhibition of acto-HMM ATPase, we utilized the chymotryptic fragment of caldesmon (38-kDa fragment), which possesses the actin/calmodulin binding region but lacks the myosin binding portion. The 38-kDa fragment inhibits the actin-activated HMM ATPase to the same extent as does the intact caldesmon molecule. In the absence of tropomyosin, the 38-kDa fragment decreased the K_{ATPase} and $K_{binding}$ without any effect on the V_{max}. However, when the actin filament contained bound tropomyosin, the caldesmon fragment caused a 2-3-fold decrease in the V_{max}, in addition to lowering the K_{ATPase} and the $K_{binding}$. The 38-kDa fragment-induced inhibition is partially reversed by calmodulin at a 10:1 molar ratio to caldesmon fragment; the reversal was more remarkable in 100 mM ionic strength at 37 °C than in 20 or 50 mM at 25 °C. Results from these experiments demonstrate that the 38-kDa domain of caldesmon inhibits the binding of myosin head to actin; however, when the actin filament contains bound tropomyosin, caldesmon fragment affects not only the binding of HMM to actin but also the catalytic step in the ATPase cycle. The interaction between the 38-kDa domain of caldesmon and tropomyosin-actin is likely to play a role in the regulation of actomyosin ATPase and contraction in smooth muscle.

Regulation of actomyosin ATPase and force development in smooth muscle are myosin-mediated, through the Ca^{2+}-calmodulin-dependent myosin light chain phosphorylation [Gorecka et al., 1976; Sobieszek & Small, 1977; Chacko et al., 1977; Dillon et al., 1981; Butler & Siegman, 1982; for a review, see Kamm and Stull (1985)]. There is also evidence for the existence of a thin filament mediated regulation through the interaction of proteins associated with the thin filament [for reviews, see Marston et al. (1985) and Chacko et al.

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