

## Structure and Regulation of the Mouse Cardiac Troponin I Gene\*

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The gene coding for mouse cardiac troponin I (TnI) has been cloned and sequenced. The cardiac TnI gene contains 8 exons and has an exon-intron organization similar to the quail fast skeletal TnI gene except for the region of exons 1–3, which is highly divergent. Comparative analysis suggests that cardiac TnI exon 1 corresponds to fast TnI exons 1 and 2 and that cardiac exon 3, which codes for most of the cardiac-specific amino-terminal extension and has no counterpart in the fast gene, evolved by exon insertion/deletion. The amino acid sequence of cardiac TnI exon 4 shows limited homology (36% identity) with fast TnI exon 4 but is remarkably similar (79% identity) to the corresponding sequence of slow TnI, possibly reflecting an isoform-specific TnC-binding site. The cardiac TnI gene is one of the very few contractile protein genes expressed exclusively in cardiac muscle. To identify the regulatory sequences responsible for the cardiac-specific expression of this gene we transfected cultured cardiac and skeletal muscle cells with fragments up to 4.0 kilobases of the 5'-flanking region linked to a reporter gene. Deletion analysis reveals four major regions in the 5'-flanking sequence, a minimal promoter region, which directs expression at low level in cardiac and skeletal muscle cells, and two upstream cardiac-specific positive regions separated by a negative region.

Troponin I (TnI)<sup>1</sup> is the inhibitory subunit of the troponin complex that is responsible, in association with troponin C (TnC) and troponin T (TnT), for the Ca<sup>2+</sup>-dependent regulation of contraction in cardiac and skeletal muscle (Ebashi and Endo, 1968). Three TnI isoforms have been identified in fast skeletal, slow skeletal, and cardiac muscle (Wilkinson and Grand, 1978). These isoforms retain significant homology in their carboxyl-terminal halves, whereas the amino-terminal regions are highly divergent. A distinctive feature of cardiac TnI is the presence of a long amino-terminal extension containing a specific target site for cyclic AMP-dependent protein

kinase, which has been implicated in the response of cardiac muscle to  $\beta$ -adrenergic stimulation (see Perry (1986)).

Cardiac TnI is coded by a developmentally regulated gene that is poorly expressed in the fetal heart (for review, see Schiaffino *et al.* (1993)). We have recently found that cardiac TnI transcripts are not detected in the rat embryonic cardiac tube, which contains only slow skeletal TnI transcripts and are first detected by embryonic day 11 (Gorza *et al.*, 1993). In addition, post-transcriptional mechanisms are involved in the differential expression of cardiac TnI in atrial and ventricular embryonic myocardium (Gorza *et al.*, 1993). At variance with most other sarcomeric protein genes, the cardiac TnI gene is not expressed in avian and mammalian skeletal muscle at any developmental stage (Ausoni *et al.*, 1991; Hastings *et al.*, 1991; Gorza *et al.*, 1993). It is therefore of interest to compare the regulation of this gene with that of other genes, such as  $\beta$  slow myosin heavy chain, slow ventricular myosin light chain 2, skeletal and cardiac  $\alpha$ -actin, slow cardiac TnC, and cardiac TnT genes, which are expressed in both cardiac and skeletal muscle.

We report here the complete nucleotide sequence of the mouse cardiac TnI gene and compare this sequence with that of the quail fast skeletal TnI gene (Baldwin *et al.*, 1985). We also present a functional analysis of the regulation of cardiac TnI gene expression by transient transfection assays into cultured cardiac and skeletal muscle cells and in a nonmuscle stable cell line. The results indicate that the cardiac-specific expression of the cardiac troponin I gene is regulated by at least four main regions in the 5'-flanking sequence, a minimal promoter region, which directs expression at a low level in cardiac and skeletal muscle cells, and two upstream cardiac-specific positive regions separated by an inhibitory region.

### EXPERIMENTAL PROCEDURES

**Materials**—Restriction enzymes, T4 DNA ligase, and avian myeloblastosis virus reverse transcriptase were purchased from Boehringer Mannheim and New England Biolabs and used according to the manufacturers' instructions. Nitrocellulose filters were from Schleicher & Schuell and Gene Screen nylon membranes from DuPont NEN. Oligonucleotides used for sequences were synthesized by MedProbe (Oslo, Norway) and by TIB Molbion (Berlin, Germany). Acetyl-CoA and *o*-nitrophenyl  $\beta$ -D-galactopyranoside were purchased from Sigma. Labeled nucleotides and [<sup>14</sup>C]chloramphenicol were from DuPont NEN. M199 and Dulbecco's modified Eagle's medium culture media were obtained from Sigma; fetal calf serum and horse serum were from ICN-Flow (ICN Biomedical).

**Cloning and Sequencing Analysis**—We used a mouse genomic library (provided by G. Bressan) that was constructed by partial digestion of DNA with MboII and cloning in  $\lambda$ EMBL3 BamHI sites after removing the stuffer sequence. Approximately  $1 \times 10^5$  recombinant phages were screened using a rat cardiac troponin I cDNA (Ausoni *et al.*, 1991) as probe. The library was plated at about  $10^4$  plaque-forming units/13-cm dish, and plates were lifted with nitrocellulose filters. After denaturation, neutralization, and baking at 80 °C for 2 h, the filters were hybridized with the cDNA probe, which

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) Z22784.

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<sup>1</sup> The abbreviations used are: TnI, troponin I; CAT, chloramphenicol acetyltransferase; TnC, troponin C; TnT, troponin T; kb, kilobase(s); bp, base pair(s).

was labeled with [ $\alpha$ - $^{32}$ P]dCTP by random priming (Feinberg and Vogelstein, 1983). Hybridization was carried out overnight at 42 °C in 50% deionized formamide, 5  $\times$  Denhardt's, 5  $\times$  SSPE, 0.1% SDS, and 250  $\mu$ g/ml salmon sperm DNA (see Sambrook *et al.* (1989) for the composition of SSPE and Denhardt's solution). Final wash conditions were 1  $\times$  SSC (0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 45 °C. One positive clone was recovered from the agar plates and purified to homogeneity by repeated platings and rescreening. The clone was mapped, and appropriate restriction enzyme fragments were subcloned into pBluescript+ (Stratagene) or M13 mp18/mp19. DNA sequencing was performed on single-stranded M13 or double-stranded plasmid DNA by the dideoxy chain termination method using standard or specific oligonucleotide primers (Sanger *et al.*, 1977). The entire sequence was confirmed on both strands. DNA sequence was analyzed with MacMolly (Soft Gene, Berlin) and PC Gene (IntelliGenetics, CA) programs. Published sequences were retrieved from the European Molecular Biology Laboratory data library for sequence comparisons.

**Primer Extension**—Total RNA was extracted from adult mouse ventricles according to Chomczynski and Sacchi (1987). Poly(A)<sup>+</sup> RNA was purified on spun-packed columns containing oligo(dT)-cellulose according to the manufacturer's instructions (Pharmacia LKB Biotechnology Inc.). Primer extension analysis was performed as previously described (Winegrad *et al.*, 1990). Briefly, a 19-bp oligonucleotide complementary to the mouse genomic sequence in the region immediately across the ATG translational site of exon 1 (see Fig. 2), purified by electrophoresis on a 20% polyacrylamide native gel, was labeled with  $\gamma$ - $^{32}$ P at the 3' end and diluted to a final concentration of 1 pmol/ $\mu$ l. Annealing was performed by dissolving 1  $\mu$ g of poly(A)<sup>+</sup> RNA in 14  $\mu$ l of buffer (11 mM Tris-HCl, pH 8.0, and 110 mM NaCl) and adding 1 pmol of labeled oligonucleotide. The mix was incubated at 37 °C for 30 min, and then extension was carried out at 37 °C for 60 min by addition of 10  $\mu$ l of reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 50 mM dithiothreitol, 15 mM MgCl<sub>2</sub>), 2.5  $\mu$ l of nucleotide mixture (15 mM each), 21.5  $\mu$ l of H<sub>2</sub>O, and 1  $\mu$ l of reverse transcriptase. The reaction products were separated on a 6% polyacrylamide-urea sequencing gel, and sequencing ladders were used as size markers.

**RNAse Protection Assay**—A 211-nucleotide HindII-Sau3AI fragment of the  $\lambda$ TnI-1 genomic clone extending from position -108 to +103 relative to the transcriptional start site assigned by primer extension was subcloned into the BamHI and HindII sites of the pBluescript+. The plasmid was linearized at the XhoI site of the polylinker, and a  $^{32}$ P-labeled antisense probe was generated with T3 RNA polymerase as previously described (Ausoni *et al.*, 1991). After DNase digestion to destroy the plasmid template, the labeled RNA was ethanol-precipitated, and the 270-nucleotide transcript, including 59 bases of plasmid sequence, was gel-purified on a denaturing 5% polyacrylamide gel. The labeled probe (2  $\times$  10<sup>6</sup> cpm) was hybridized overnight at 50 °C with 20  $\mu$ g of RNA from adult mouse heart as described (Ausoni *et al.*, 1991). The same amount of the probe was hybridized with 20  $\mu$ g of RNA from adult rat liver as control. The annealed RNAs were digested with RNase A (40  $\mu$ g/ml) and RNase T1 (2  $\mu$ g/ml) for 30 min at 37 °C, and RNase-resistant fragments were analyzed on a 6.8% sequencing gel.

**Plasmid Constructs**—A 6-kb EcoRI/EcoRI fragment of the  $\lambda$ TnI-1 clone was truncated at position +5 downstream of the transcriptional start site of the gene by cleavage with EcoNI restriction enzyme. The recovered fragment of about 4000 bp was blunt-ended with the Klenow fragment of DNA polymerase I and cloned into the SmaI site of a pSPT-CAT vector (a gift of G. Bressan), prepared by inserting 1603 bp of the CAT gene into the EcoRI site of pSPT vector (Pharmacia LKB). This construct was designated cTnI-4000. Another construct, cTnI-2200, was prepared by cutting the original 6-kb EcoRI/EcoRI fragment with SpeI and EcoNI, blunt-ending, and ligating into the SmaI site of pSPT-CAT. Other constructs, named cTnI-3400, cTnI-3000, cTnI-1700, cTnI-1529, cTnI-307, and cTnI-229 were obtained from cTnI-4000 by cutting with BamHI, SmaI, BglII, HindIII, PstI, and XbaI, respectively, and religating either using the same restriction site of the pSPT-CAT polylinker or after blunt ending. To construct cTnI-127 the Sau3AI/EcoNI -127/+5 fragment was blunt end ligated into the SmaI site of the pSPT-CAT polylinker. To construct cTnI-127(3000-2200) the SmaI/XbaI -3000/-2200 fragment was blunt end ligated into the blunt-ended XbaI site of the polylinker upstream of cTnI-127. Clones showing correct orientation were identified by restriction mapping and sequencing.

**Cell Cultures**—Primary cultures of rat myocardial cells were prepared from 20–21-day-old fetal hearts essentially as described by Sen

*et al.* (1988) with minor modifications. Ventricles were dissected and minced, and the cells were dispersed by repeated treatments with 80 units/ml collagenase (Worthington) and 0.25 mg/ml trypsin (Sigma) at 37 °C. The cells were resuspended in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 5% horse serum, L-glutamine, and antibiotics. Cell suspensions were preplated for 30 min to remove nonmuscle cells, and unattached muscle cells were replated at a density of 2.3  $\times$  10<sup>6</sup> cells/100-mm dish. The proportion of cardiomyocytes, as determined by immunocytochemical staining with an antibody specific for sarcomeric myosin heavy chain, was >90%. Skeletal muscle cells were obtained from hindlimb muscles of 20–21-day-old fetuses essentially using the same procedure and were plated at a density of 1.5  $\times$  10<sup>6</sup> cells/100-mm dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. 3T3NIH cells were cultured in the same medium and plated at appropriate density to have about 1.5  $\times$  10<sup>6</sup> cells/100-mm dish at the time of transfection.

**Transfections and CAT Assays**—Transfection was performed by the calcium phosphate precipitation method described by Chen and Okayama (1987). Time of transfection (48 h after plating) and time of recovering (65 h after transfection) were selected as optimal conditions to obtain high levels of CAT activity under cardiac TnI promoter control. The cells were washed with balanced salt solution 3–4 h before transfection and refed with fresh medium. Each culture dish was cotransfected with 20  $\mu$ g of CAT construct and with 250 ng of cytomegalovirus- $\beta$ Gal vector to normalize for transfection efficiency. Higher amounts of cytomegalovirus- $\beta$ Gal were found to interfere with the expression of the CAT constructs. The transfection/mixture medium was removed after 18 h, and cells were harvested after 65 h. Within each experiment parallel cultures were transfected with the promoterless pSPT-CAT, as a negative control, and with pSV2CAT (Gorman *et al.*, 1982), as a positive control.

Cell extracts were prepared by submitting the cells to three consecutive cycles of freezing and thawing. After centrifuging the pellet was resuspended in 150  $\mu$ l of 250 mM Tris-HCl, pH 8.0. CAT assays were performed using [<sup>14</sup>C]chloramphenicol as previously described (Gorman *et al.*, 1982). Reaction products were separated by ascending chromatography on silica gel plates and quantified by liquid scintillation counting. CAT values from each cell extract were normalized to  $\beta$ -galactosidase activity, to correct for transfection efficiency.

**Immunocytochemistry**—Cardiac and skeletal primary cells were stained with the monoclonal antibody TI-1, specific for cardiac TnI (Saggin *et al.*, 1989), according to previously described procedures (Corza *et al.*, 1993). A monoclonal anti-desmin antibody (Amersham Corp.) was also used to identify muscle cells. Bound antibodies were revealed using rhodamine isothiocyanate-conjugated anti-mouse immunoglobulins (Dakopatts) and using a Zeiss epifluorescence microscope.

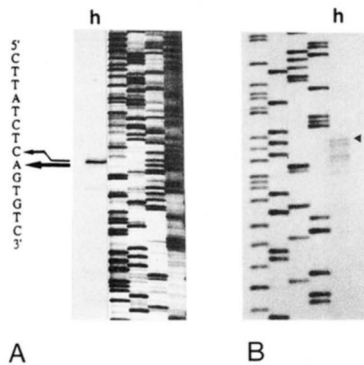
## RESULTS

**Structure of the Mouse Cardiac TnI Gene and Its 5'-Flanking Region**—The  $\lambda$ TnI-1 clone, which contains a 12-kb insert that includes the complete cardiac TnI gene, was isolated from a mouse genomic library using a rat cardiac TnI probe. Fig. 1 shows a partial restriction map of this clone with the position of the gene, determined by Southern blot hybridization with cDNA fragments, and the complete DNA sequence of the gene and its immediate 5'- and 3'-flanking regions. The mouse cardiac TnI gene is composed of 8 exons ranging in size from 13 (exon 2) to 177 bp (exon 7) and spanning approximately 4.2 kb of genomic DNA. Exons 1 and 8 each contain both translated and untranslated sequences. All of the flanking intron borders show the consensus 5'- and 3'-splice sequences (Mount, 1982). Exon 1 ends with a split codon, whereas all other exons begin and end with intact codons.

The transcriptional start site of the cardiac TnI gene was determined by primer extension analysis using a 19-mer oligonucleotide complementary to a portion of the 5'-untranslated region and the first five coding nucleotides (Fig. 2A). Primer extension resulted in a major reaction product of 133 bases and a minor reaction product of 134 bases. The 133-nucleotide product was used to map the transcription start





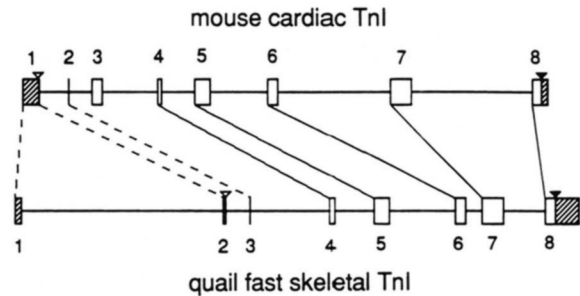


**FIG. 2. Localization of the transcription start site of the mouse cardiac TnI gene.** A, primer extension analysis. The oligonucleotide 5'-GCCATGATGATCTCCAGAG-3', complementary to the portion of the 5'-untranslated region and the first five nucleotides of the coding region, was annealed to poly(A)<sup>+</sup> from adult mouse heart, and the products of the reverse transcription reaction were analyzed on analytical gel (lane h). The sequencing ladder from a standard sequencing reaction was used as a size marker. The genomic sequence of the region containing the start site is indicated on the left; the large and small arrow indicate the positions corresponding to the major and minor reaction products, respectively. B, RNase protection assay. An antisense riboprobe that spans the region from -108 to +103 of the cardiac TnI gene was allowed to hybridize with 20  $\mu$ g of RNA isolated from adult mouse heart (lane h), and the RNase-resistant fragments were analyzed on a 6.8% sequencing gel. The longest (104 nucleotides) protected fragment is indicated by an arrowhead. A sequencing ladder is shown as a size standard.

consideration the fact that one additional nucleotide of the polylinker flanking the *Sau*3AI site is identical to nucleotide +104 of the first exon. The A residue corresponding to the start site is located 33 residues downstream of the first T of the sequence TATATTTA, which is similar to the consensus TATA sequence. No canonical CAAT motif was identified within -200 bp of the 5'-flanking sequence. It is possible that the CAACT sequence, about 100-bp upstream of the TATA box, may fulfill the role of a CAAT box, as previously suggested for the CAACT sequence of the *MLC*<sub>1/3F</sub> gene (Daubas *et al.*, 1985).

The 5'-upstream region of the cardiac TnI gene was scanned up to nucleotide -1529 for the presence of *cis*-acting regulatory elements that have been implicated in the regulation of sarcomeric muscle genes. This region was found to contain seven E-boxes with the consensus sequence CANNTG (Lassar *et al.*, 1989) at the following positions: -104/-99, -429/-424, -802/-797, -962/-957, -1135/-1130, -1345/-1340, and -1490/-1485. On the other hand, other elements, including CARG box (Minty and Kedes, 1986), M-CAT-binding site (Mar and Ordahl, 1988), MEF-2-binding site (Gossett *et al.*, 1989), CEF1 and CEF2 sites (Parmacek *et al.*, 1992), and GAR motif (Mably *et al.*, 1993), were not identified in this region of the cardiac TnI gene. We also found no evidence for the presence of sequences similar to the "cardiac elements" required for transcription of the cardiac TnT gene (Iannello *et al.*, 1991), the slow ventricular *MLC*2 gene (Zhu *et al.*, 1991; Qasba *et al.*, 1992), and the human  $\beta$ -MHC gene (Thompson *et al.*, 1992; Flink *et al.*, 1992) in myocardial cells.

**Comparison of the Mouse Cardiac and Quail Fast Skeletal TnI Genomic Organization**—The quail and chicken fast skeletal TnI genes, the only vertebrate sarcomeric TnI genes previously analyzed, have a similar genomic organization (Baldwin *et al.*, 1985; Nikovits *et al.*, 1986). Fig. 3 shows a schematic comparison of the mouse cardiac and quail fast skeletal TnI genes. Each gene contains 8 exons. Exons 4–8



**FIG. 3. Exon-intron organization of the mouse cardiac and quail fast skeletal TnI genes.** Exons are shown as boxes (open boxes, translated sequences; hatched boxes, untranslated regions) and introns as lines. Continuous lines between the two genes connect exons that are clearly homologous by size, sequence, and position of introns; broken lines connect exons that show no correspondence. The positions of the translation initiation codon and stop codon are marked by open and closed arrowheads, respectively.

are highly conserved in the two genes with respect to size, sequence, and positions of intron-exon junctions. In contrast, exons 1–3 are highly divergent in the cardiac and fast TnI genes, as expected from the different size and structure of the amino-terminal region of the corresponding proteins (Wilkinson and Grand, 1978).

The mouse cardiac exon 1 contains the 5'-untranslated region plus the methionine initiation codon and 8 of the 9 nucleotides coding the first three amino acids of the mature TnI protein. In contrast, the quail fast TnI exon 1 is composed entirely of an untranslated sequence, a shared feature of most sarcomeric protein genes, and exon 2 contains part of the untranslated region plus the methionine initiation codon and 5 of the 6 nucleotides encoding the first two amino acids of the protein. Thus it appears that exon 1 of the mouse cardiac gene corresponds to exon 1 and 2 of the quail fast skeletal gene. The similar split codon structure of cardiac TnI exon 1 and fast TnI exon 2 is consistent with this interpretation. Another distinctive feature of the mouse cardiac gene is the small size of the first intron (222 bp), which is probably the shortest among vertebrate sarcomeric protein genes so far analyzed. The cardiac TnI exon 2 (13 nucleotides) is similar in size to the quail fast TnI exon 3 (7 nucleotides); however, there is no sequence homology between the two exons. The cardiac exon 3 is a relatively large exon (87 nucleotides), which codes most of the cardiac-specific amino-terminal extension of the cardiac TnI gene and has no counterpart in the fast TnI gene (Fig. 4). This exon codes for a specific domain, characterized by 2 adjacent Ser residues that represent a substrate for cAMP-dependent protein kinase, which is present in all cardiac TnIs from different mammalian and avian species (see Hastings *et al.* (1991)).

The amino acid sequence of the mouse cardiac exon 4 has only 5 of 14 residues (36% identity) in common with the quail fast TnI exon 4 (Fig. 4), whereas the corresponding region of the rat slow skeletal TnI sequence (Koppe *et al.*, 1989) shares 11 of 14 residues with mouse cardiac TnI (79% identity and only one nonconservative substitution). The amino acid sequence corresponding to exon 4 is highly conserved between quail fast skeletal and mouse fast skeletal TnI (79% identity) as well as between quail cardiac and rat slow TnI (79% identity). This pattern of preferential homology between cardiac TnI and slow TnI is not seen in the 5' portion of exon 5, which is similarly conserved among the three isoforms.

The nucleotide sequences of exons 5 and 6 show a 52 and 62% identity, respectively, between the cardiac and the fast TnI genes, and exon 7 shows an even higher degree of homology (72% identity). Exon 7 contains the inhibitory do-



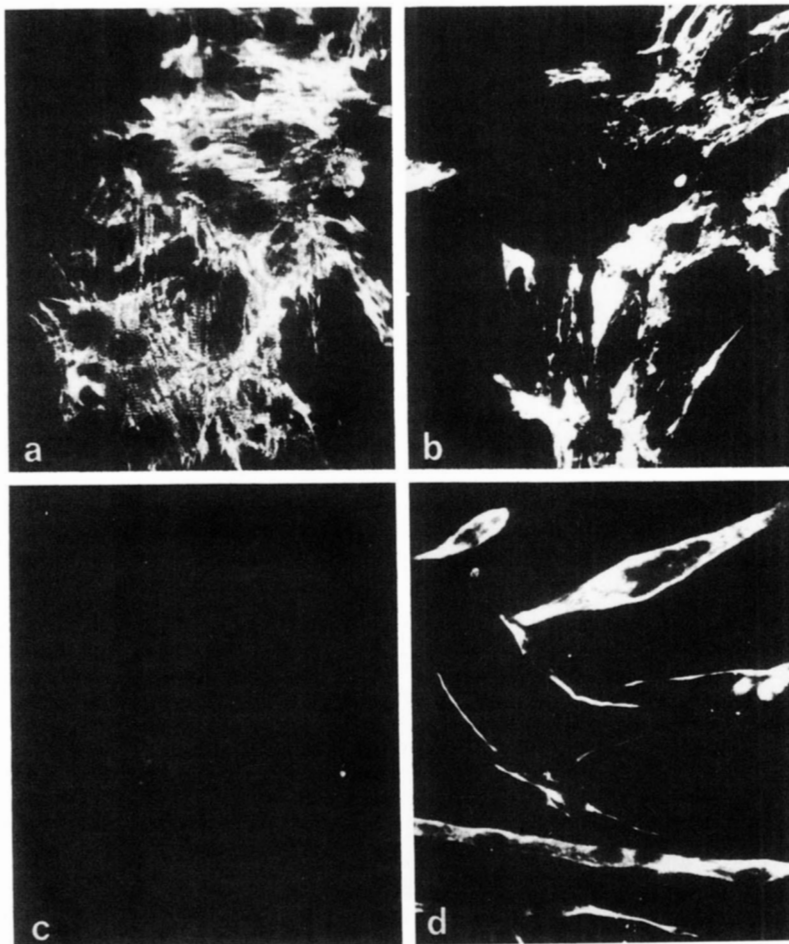


FIG. 5. Immunocytochemical detection of cardiac TnI in cultured cardiomyocytes. Cultures from fetal ventricles (a and b) and hindlimb muscles (c and d), maintained *in vitro* for 4 days, were stained with monoclonal antibodies specific for cardiac TnI (a and c) and desmin (b and d). Bound antibodies were revealed with rhodamine-labeled anti-mouse IgG. Cardiac TnI is expressed in cardiac but not in skeletal muscle cells.

terminal region. Mouse cardiac TnI is characterized by a 36-bp long amino-terminal domain coded by exons 1–3, whereas quail fast skeletal TnI has a short (4 bp) amino terminus coded by exons 2 and 3. The amino-terminal extension is a distinctive feature of cardiac TnIs from different mammalian and avian species and contains a specific protein kinase A target site implicated in the response of cardiac TnI to  $\beta$ -adrenergic stimulation (see Perry (1986)). It has been suggested that the cardiac-specific amino-terminal extension "arose as an insertion by a splice-junction-sliding mechanism that created an exon larger by 78 bp than the corresponding exon in the fast muscle gene" (Baldwin *et al.*, 1985). However, this hypothesis is not supported by our findings, because cardiac and fast TnI exon 3 share no sequence homology. An alternative interpretation is that cardiac TnI exon 1 and the corresponding fast TnI exons 1 and 2 have evolved by exon fusion/fission and that cardiac TnI exon 3 arose by exon insertion/deletion. It is possible that TnIs with long amino-terminal domains might have appeared early in evolution, as suggested by the finding that *Drosophila* and crayfish muscle TnIs have a long amino-terminal extension, similar in size though different in sequence from that of vertebrate cardiac TnI (Kobayashi *et al.*, 1989; Beall and Fyrberg, 1991). In *Drosophila* this amino-terminal region is coded by exons that have no counterpart in vertebrate TnI genes (Barbas *et al.*, 1991).

The conserved exon-intron organization of mouse cardiac and quail fast skeletal TnI genes in the exon 4–8 region indicates that these genes arose from a common ancestor by gene duplication before the avian-mammalian divergence about 300 million years ago. Comparison of amino acid se-

quences of mouse cardiac with quail fast skeletal TnI genes reveals a 58% identity in the homologous region corresponding to exon 4–8. On the other hand, mouse cardiac TnI and rat slow skeletal TnI show an overall higher degree of homology (64% identity) and an especially high similarity in exon 4 (79% identity). The homology between cardiac and slow TnI exon 4 may be functionally relevant. TnI contains two TnC-binding sites, one corresponding to the well characterized inhibitory domain in exon 7 and another less characterized binding site located at the NH<sub>2</sub> terminus, first revealed by binding studies with synthetic peptides (Syska *et al.*, 1976; Ngai and Hodges, 1992). Recent studies with a recombinant deletion mutant of fast skeletal rabbit TnI, which lacks residues 1–57, suggest that the NH<sub>2</sub> terminus of TnI may play a structural role in maintaining the stability of the troponin complex (Sheng *et al.*, 1992). The exact borders of this TnC-binding site are not known; however, some of the amino acid residues involved in the interaction between TnI and TnC have been identified. Proton magnetic resonance studies with an amino-terminal peptide derived from fast skeletal TnI indicate that different residues between positions 6 and 14 are involved in this interaction (Grand *et al.*, 1982); in addition, it has been shown that the reactivity of lysine 18 of TnI to acetimidate is changed by TnC in the presence of calcium (Moir *et al.*, 1977) and that phosphorylation of Thr 11 by phosphorylase kinase is inhibited by TnC (Cole and Perry, 1975). Interestingly, all these residues are located in exon 4, suggesting that a crucial portion of the TnC-binding region is encoded within this exon. The sequence conservation pattern of the domain coded by exon 4 may be related to an isoform-specific adaptation, because cardiac TnI and slow TnI interact

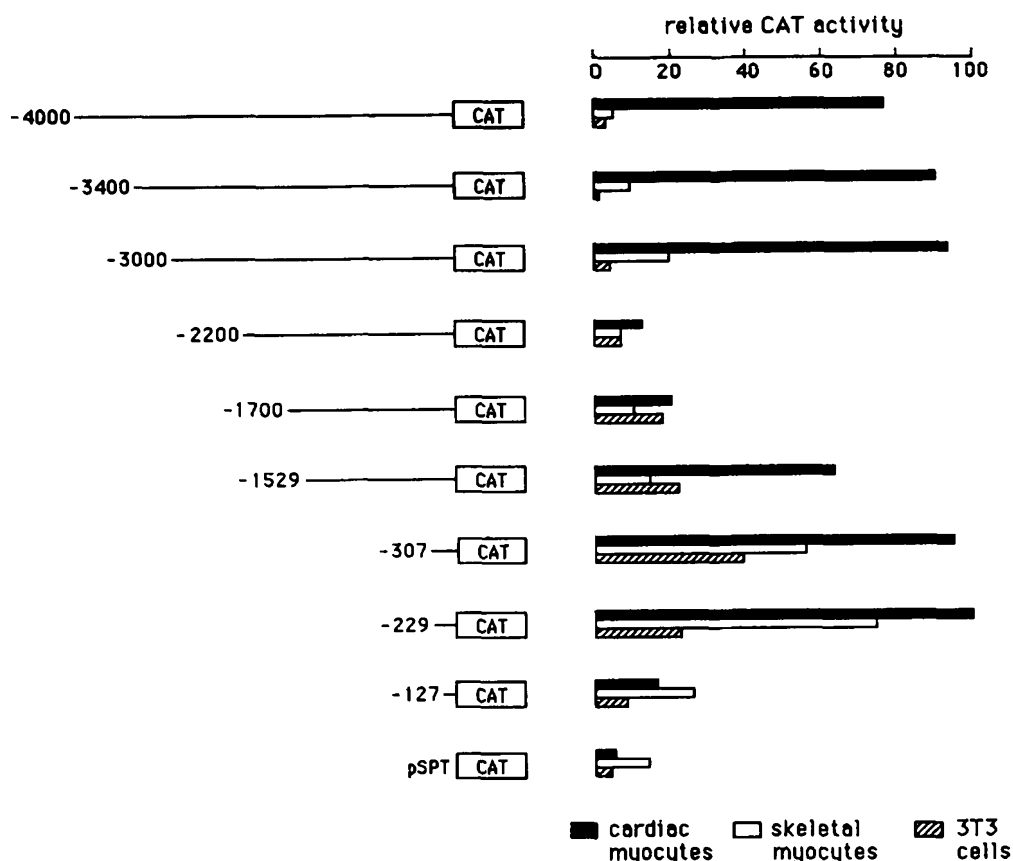


FIG. 6. Expression of cardiac TnI 5' upstream constructs transiently transfected into cardiac and skeletal myocytes and fibroblasts. *Left*, schematic representation of the expression constructs containing segments (from -4000 to -127 bp) of the 5' upstream portion of the cardiac TnI gene joined to the CAT gene. *Right*, schematic representation of the CAT activity obtained by transient transfection into cardiac and skeletal muscle cells and 3T3 cells. The results are expressed as a percentage of the maximal level of activity obtained with the -229 construct in cardiac muscle cells. The CAT activities were standardized for variation in transfection efficiency using  $\beta$ -galactosidase activity as an internal control. Each value represents the average of three or more independent transfection experiments.

TABLE I

Relative CAT activity of constructs containing nested deletions of the cardiac TnI 5'-flanking region after transfection in primary cardiac and skeletal myocytes and in 3T3 cells

Construct	Relative CAT activity <sup>a</sup>		
	Cardiac myocytes	Skeletal myocytes	3T3 cells
cTnI-4000	16.2 ± 1.6	0.3 ± 0.1	0.7 ± 0.4
cTnI-3400	19.1 ± 4.4	0.7 ± 0.5	0.2 ± 0.2
cTnI-3000	19.8 ± 3.1	1.4 ± 0.6	1.1 ± 0.5
cTnI-2200	2.6 ± 0.5	0.5 ± 0.2	1.8 ± 1.3
cTnI-1700	4.2 ± 0.7	0.7 ± 0.1	5.0 ± 1.4
cTnI-1529	13.4 ± 4.5	1.0 ± 0.3	6.3 ± 2.2
cTnI-307	20.2 ± 4.2	4.1 ± 1.1	11.1 ± 3.3
cTnI-229	21.2 ± 3.0	5.5 ± 1.1	6.4 ± 3.7
cTnI-127	3.5 ± 1.1	1.9 ± 0.6	2.3 ± 1.0

<sup>a</sup>The CAT activities are expressed as -fold increase over the activity of the basal promoter/CAT construct (pSPT-CAT) in the same cell type and are corrected for transfection efficiency by assaying for  $\beta$ -galactosidase activity. Values are means ± S.E. and represent the average of at least three separate transfections.

with the same slow cardiac TnC isoform (see Hastings *et al.* (1991)). Thus it is possible that cardiac and slow TnI exon 4 maintained a similar structure as a result of coevolution of the TnC gene family and the functional constraints imposed by the interaction with a common TnC isoform (see Hastings *et al.* (1991)). However, it remains to be established whether the interaction between TnC and the NH<sub>2</sub> terminus of TnI is isoform-specific.

*Regulation of Cardiac TnI Gene Expression*—In the present study we have begun to define the *cis*-acting elements involved in the cell type-specific expression of the mouse cardiac TnI gene using transient transfection experiments. Our results show that this gene, like many other genes, is regulated by a combination of positive and negative elements located in the 5'-flanking region. At least four main regions can be identified, including a nonspecific minimal promoter and two upstream positive elements separated by a negative element. A positive cardiac-specific element has been localized by deletion analysis in the far 5'-upstream region between nucleotide -3000 and -2200. This sequence is not a typical tissue-specific enhancer, because it confers cardiac-specific expression but does not increase the activity in cardiac cells above the level obtained with more proximal regions. In addition, the positive effect of the -3000/-2200 region is apparently dependent on surrounding sequences because this segment does not potentiate CAT expression when it is placed directly upstream of the minimal cTnI-127 promoter. Gene expression decreases dramatically when this sequence is deleted, due to the presence of a negative element between nucleotide -2200 and -1529. Thus the positive distal element could simply work by suppressing the negative effect of its downstream region and thus restoring the positive regulation of the proximal regions. It is likely that the negative region between nucleotide -2200 and -1529 contains transcriptional repressor elements that prevent gene expression in skeletal muscle and nonmuscle tissues, such as the inhibitory element asso-



## REFERENCES

- ciated with the promoter of the ventricular myosin light chain 2 gene (Ruoqian-Shen *et al.*, 1991). These elements may also be involved in the regulation of the cardiac TnI gene during early stages of heart development, when cardiac TnI transcripts are not expressed (Gorza *et al.*, 1993). Deletion analysis reveals the existence of an additional positive region between nucleotide -229 and -127. However, at variance with the positive distal region, this proximal region leads to increased activity in both cardiac myocytes and, to a lower degree, in skeletal myocytes and 3T3 cells. The finding that the level of expression from our strongest cTnI-5' upstream constructs is about one order of magnitude lower than that from the viral control pSV2CAT suggests that enhancer elements located elsewhere are probably required for full expression of the cardiac TnI gene. The quail fast TnI gene is regulated by a strong tissue-specific enhancer within the large first intron (Yutzey *et al.*, 1989). However, preliminary transfection experiments indicate that regulatory regions are not present within the first exon and the small first intron of the cardiac TnI gene.<sup>2</sup>
- Further analysis is required to identify the specific elements in the proximal and distal portions of the 5'-upstream region of the cardiac TnI gene that are responsible for the positive and negative regulation of its expression. Most transfection studies in myocardial cells have so far focused on contractile protein genes that, at variance with the cardiac TnI gene, are expressed in both cardiac and skeletal muscle; this is the case of the cardiac TnT gene (Mar and Ordahl, 1988), the slow ventricular myosin light chain 2 gene (Navankasattusas *et al.*, 1992; Qasba *et al.*, 1992), the slow cardiac TnC gene (Parmacek *et al.*, 1992), and the  $\beta$ -myosin heavy chain gene (Thompson *et al.*, 1992; Flink *et al.*, 1992). The regulatory elements directing cardiac-specific expression of these genes (for review, see Ordahl (1992) and Chien *et al.* (1993)) are not present within the sequenced segment (about 1.5 kb) of the 5'-flanking region of the cardiac TnI gene. This region contains several E-boxes, which have been implicated in the regulation of  $\alpha$ -cardiac actin and  $\alpha$ -myosin heavy chain in cardiac muscle cells (Sartorelli *et al.*, 1992; Molkentin *et al.*, 1993). However, based on the deletion experiments it is not clear what role, if any, the seven E-boxes identified between nucleotide -1529 and the start site may have in the regulation of the cardiac TnI gene, because one of them is located in the minimal promoter segment -127/+5 and six are located in the segment -1529/-307, which displays no obvious positive regulation.
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- <sup>2</sup> S. Ausoni, M. Campione, A. Picard, and S. Schiaffino, unpublished results.
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