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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)[1] is the inhibitory subunit of the troponin complex that is responsible, in association with troponin C (TnC) and troponin T (TnT), for the Ca^{2+}-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 β-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 β slow myosin heavy chain, slow ventricular myosin light chain 2, skeletal and cardiac α-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 β-D-galactopyranoside were purchased from Sigma. Labeled nucleotides and [^{14}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 *Mbo*II and cloning in λEMBL3 *Bam*HI sites after removing the stuffer sequence. Approximately 1×10^5 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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[1] 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 [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). Hybridization was carried out overnight at 42 °C in 50% deionized formamide, 5 × Denhardt's, 5 × SSPE, 0.1% SDS, and 250 μg/ml salmon sperm DNA (see Sambrook *et al.* (1989) for the composition of SSPE and Denhardt's solution). Final wash conditions were 1 × 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)$^+$ 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 immediatly across the ATG translational site of exon 1 (see Fig. 2), purified by elecrophoresis on a 20% polyacrylamide native gel, was labeled with γ-^{32}P at the 3′ end and diluted to a final concentration of 1 pmol/μl. Annealing was performed by dissolving 1 μg of poly(A)$^+$ RNA in 14 μl 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 μl of reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 50 mM dithiothreitol, 15 mM $MgCl_2$), 2.5 μl of nucleotide mixture (15 mM each), 21.5 μl of H_2O, and 1 μl 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 *Hin*dII-*Sau*3AI fragment of the λTnI-1 genomic clone extending from position −108 to +103 relative to the transcriptional start site assigned by primer extension was subcloned into the *Bam*HI and *Hin*dII sites of pBluescript+. The plasmid was linearized at the *Xho*I 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 × 10^5 cpm) was hybridized overnight at 50 °C with 20 μg of RNA from adult mouse heart as described (Ausoni *et al.*, 1991). The same amount of the probe was hybridized with 20 μg of RNA from adult rat liver as control. The annealed RNAs were digested with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 30 min at 37 °C, and RNase-resistant fragments were analyzed on a 6.8% sequencing gel.

Plasmid Constructs—A 6-kb *Eco*RI/*Eco*RI fragment of the λTnI-1 clone was truncated at position +5 downstream of the transcriptional start site of the gene by cleavage with *Eco*NI restriction enzyme. The recovered fragment of about 4000 bp was blunt-ended with the Klenow fragment of DNA polymerase I and cloned into the *Sma*I site of a pSPT-CAT vector (a gift of G. Bressan), prepared by inserting 1603 bp of the CAT gene into the *Eco*RI site of pSPT vector (Pharmacia LKB). This construct was designated cTnI-4000. Another construct, cTnI-2200, was prepared by cutting the original 6-kb *Eco*RI/*Eco*RI fragment with *Spe*I and *Eco*NI, blunt-ending, and ligating into the *Sma*I 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 *Bam*HI, *Sma*I, *Bgl*II, *Hin*dIII, *Pst*I, and *Xba*I, respectively, and religating either using the same restriction site of the pSPT-CAT polylinker or after blunt ending. To construct cTnI-127 the *Sau*3AI/*Eco*NI −127/+5 fragment was blunt end ligated into the *Sma*I site of the pSPT-CAT polylinker. To construct cTnI-127(3000–2200) the *Sma*I/*Xba*I −3000/−2200 fragment was blunt end ligated into the blunt-ended *Xba*I 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 × 10^6 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 × 10^6 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 × 10^6 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 μg of CAT construct and with 250 ng of cytomegalovirus-βGal vector to normalize for transfection efficiency. Higher amounts of cytomegalovirus-βGal 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.

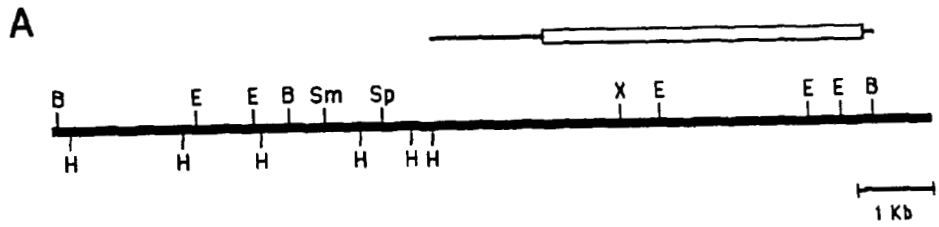
Cell extracts were prepared by submitting the cells to three consecutive cycles of freezing and thawing. After centrifuging the pellet was resuspended in 150 μl of 250 mM Tris-HCl, pH 8.0. CAT assays were performed using [^{14}C]chloramphenicol as previously described (Gorman *et al.*, 1982). Reaction 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 β-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 (Gorza *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 λTnI-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. 2*A*). 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



B

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ctacaagttaggggactccactccgggtggcggagactcagctgtggaatctggacccgcgccctccaaagtggaatctacccgcgccctctccc
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caccttggttgaccccaactggagctttgcacacggctcccctcacaccctgttatcgcttatcctgggcaggggaggagacagcagtatatttagt
                                    Exon 1 →
ctttgtcctcgccccttatctc AGTGTCCTCAGTGAGGCTTGAGCAGCCCAGAGGAAACCCAACCTCTAGAGACCTCCAAGGTCACCAGGGACAC
CCTTCCAGGACCCTCCAGGAATCTCCGATCCTGTTCTCTGCCTCTGGAGATCATC ATG GCT GAT GA gtatgtgaatcttcgtagccttggg

gtcggagacctcaacagggagaggtgcggtgggtcttgagggtgcaaccaggttggatacttgtcctgagagagtaggaggcaagggtggttggatt
tctgtagaaatgaggtaagaatttgggaatcagactcctgggggttcaagagtcaggggaccacccatcagtgatttcctcttcacggtttctctc
        Exon 2 →
ttcag  G AGC AGC GAT GCG  gtgagctgtgagctaagaagtgggtagggaaccttcggtccagagtgggtgctgcagggagaggactgg
ggaccagtgggcagggaacctgccgctagaggtgaggtatccaggaagggctggatgggagggtttgggtagtgatgtagcctgcttcaattccag
Exon 3 →
GCT GGG GAA CCG CAG CCT GCG CCT GCT CCT GTC CGA CGC CGC TCC TCT GCC AAC TAC CGA GCC TAT GCC ACC
GAG CCA CAC GCC AAG gtgggcgggggtccaagaggatgggcttctgggttcaagaatcctctgagacccagaaggggcaggaccaaggcc

ctggaaaggggtggtagggtgatgggcagagccctatgggtgggcagaatttcagaaaggttcaggttgggcttggtgacagcggcctgtggttg
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agatgggaccgaggagcgtggcttccagtggactccgcgttctggttgaagaatgtctgacccgaatgggtatgcacctcgagtgggtcgtgatgcc
                                                    Exon 4 →
ttgcagctccacccagtgtccacttgggacctcacactgaccttttctccatatcaccttggcag AAA AAG TCT AAG ATC TCC GCC
TCC AGA AAA CTT CAG TTG AAG gtgatgatgaggtcgcagtgggtgggctttggtgggcggagctttgggggagtggtttcgcttccgga

gtgtgtcagtccccgcctccccgccccccccccacgtgcgcgcgggcagaaacgaggcaggttttgaataaaagacacaaacttggatgttcgg
                                                                                    Exon 5 →
atattctgtctctctggaggatggagggaaatttgttattcttatacttttgggactctgttgtctgctccaaccattccgcag    ACT CTG
ACT CTG ATG CTG CAG ATT GCG AAG CAG GAG ATG GAA CGA GAG GCA GAA GAG CGA CGT GGA GAG AAG GGG CGC
GTT CTG AGG ACT CGT TGC CAG CCT TTG GAG TTG GAT GGG CTG GGC TTT GAA GAG CTT CAG gtaccagtttacaaag

caaggattccggataagcctgggacccgagattcctcatctctggcctggcatcccttgggtatcaggagcataagcaccatctcaagttctgctc
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accccagcactcctctctcaaactcgggggtccagagcctagggcctcctcctcagacccagaggtctcctctctctcagaccagggaacccaacctc
                                                Exon 6 →
cagcctccctctcagcatccacgcgttccttcaccag  GAC TTA TGC CGA CAG CTT CAC GCT CGG GTG GAC AAA GTG GAT
GAA GAG AGA TAT GAC GTG GAA GCA AAA GTC ACC AAG AAC ATC ACT GAG gtgggagcccgggggaagcctgggaccttcac

attacaccaagatctccagactgtagcttcgggctgggggctatggactcttgtgcttgagataactctaagcagaaagcaggcagtggcacaggt
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ggtgcaggctggaggctttaatctgggccagagctggaagaagattagaaacagagaggcaagaaagggaaaagggagaattgtttgtttgttgg
                                                Exon 7 →
aaggaaggaaggaaggaagcagagggaagatgggggctccacaccccatatggtcctccccag  ATT GCA GAT CTG ACC CAG AAG ATC
TAT GAC CTC CGT GGC AAG TTT AAG CGG CCC ACC CTC CGA AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG
GCG CTG CTG GGG ACC CGG GCC AAG GAA TCC TTG GAC CTG AGG GCC CAC CTC AAG CAG GTG AAG AAG GAG GAC
ATT GAG AAG    gtgagtgtgggctagggtgagaaggaaggaagatgctgggggaaggcctgagtggcataaggtccagagagagagagagaga

gagagagagcagatgcacttggggtctagtgagaagctactgataaagagaattcaagggctggtgagatggctccatggttaagagcactgactg
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Exon 8 →
GAA AAC CGG GAG GTG GGA GAC TGG CGC AAG AAT ATC GAT GCA CTG AGT GGC ATG GAA GGC CGC AAG AAA AAG
TTT GAG GGC TGA GCCATGGCTCCCACACTGTGCTCTGAAGGACGTCCCTGCAGAATAAACCTCTCCAAACC——
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FIG. 1. **Structure of the mouse cardiac TnI gene.** *A*, partial restriction endonuclease map of the 12-kb cardiac TnI clone isolated from a mouse genomic library. The position of the cardiac TnI gene and the 5′-upstream region analyzed in transfection experiments are represented as an *open bar* and as a *line*, respectively. Restriction enzyme abbreviations are: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hind*III; *Sm*, *Sma*I; *Sp*, *Spe*I; and *X*, *Xho*I. *B*, nucleotide sequence of the mouse cardiac TnI gene. Exons are shown in *uppercase letters*, and introns are shown in *lowercase letters*. The putative TATA box and the AATAAA polyadenylation signal are *underlined*.

site (+1); residues upstream of this site are numbered negatively. This result was confirmed by RNase protection analysis using an antisense RNA probe, which spans the region from −108 to +103 relative to the transcription start site assigned by primer extension, corresponding to a 211-nucleotide *Hind*III-*Sau*3AI fragment of genomic sequence. The RNA probe was hybridized with 20 μg of adult mouse heart and adult rat liver; no protected band was observed in the liver sample whereas three major protected bands of 104, 103, and 101 bases were seen in the mouse heart sample (Fig. 2*B*). The 104-nucleotide band corresponds to the position of the major start site determined by primer extension, if one takes into

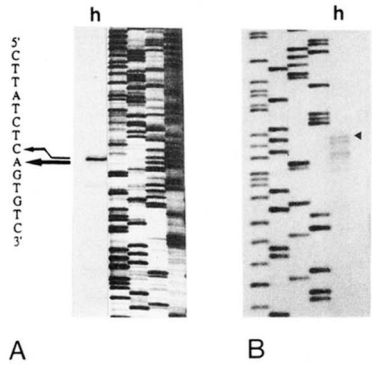

FIG. 2. **Localization of the transcription start site of the mouse cardiac TnI gene.** *A*, primer extension analysis. The oligonucleotide 5′-GCCATGATGATCTCCAGAG-3′, complementary to the portion of the 5′-untranslated region and the first five nucleotides of the coding region, was annealed to poly(A)$^+$ 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 μg 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.

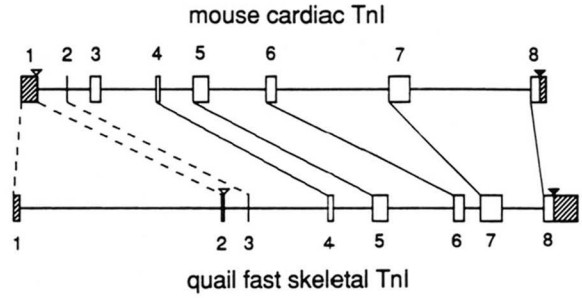

FIG. 3. **Exon-intron organization of the mouse cardiac and quail fast skeletal TnI genes.** Exons are shown as *boxes* (*open boxes*, translated sequences; *hatched boxes*, untranslated regions) and introns as *lines*. *Continuous lines* between the two genes connect exons that are clearly homologous 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.

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 $MLC_{1/3F}$ 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 GArC 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 MLC2 gene (Zhu *et al.*, 1991; Qasba *et al.*, 1992), and the human β-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 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-

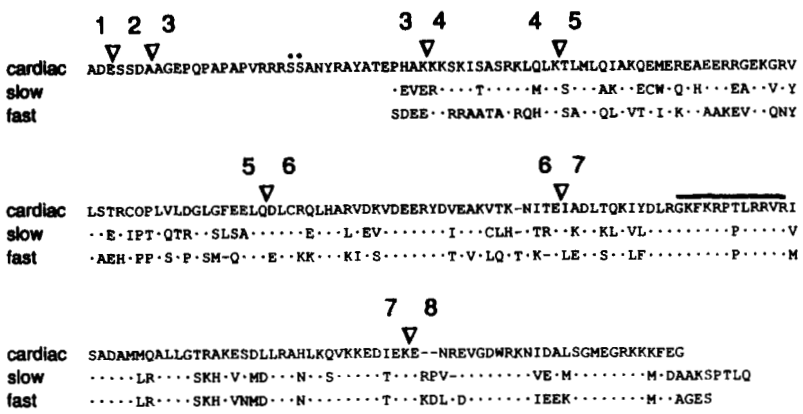

FIG. 4. **Alignment of amino acid sequences of mouse cardiac, quail fast skeletal, and rat slow skeletal TnI.** The quail fast TnI sequence is derived from a genomic clone (Baldwin *et al.*, 1985), while the rat slow TnI sequence is derived from a cDNA clone (Koppe *et al.*, 1989). *Dots* indicate identity with the cardiac sequence, and *dashes* indicate a gap introduced in a sequence to produce optimal alignment. The exon boundaries in cardiac TnI are marked by *arrowheads* with the exon number on either side. The two Ser residues in cardiac TnI exon 3 are marked by *closed circles*. The actin/TnC-binding inhibitory domain in exon 7 is *overlined*. Note the high degree of homology between the amino acid sequence of cardiac TnI exon 4 and the corresponding slow TnI sequence.

main, which is highly conserved among all TnI isoforms and corresponds to amino acids 104–115 in rabbit fast skeletal TnI (see Perry (1986)). This region, which binds both actin and TnC and inhibits actomyosin ATPase, is presumably involved in the Ca^{2+}-induced movement of TnI relative to actin in thin filaments (Tao *et al.*, 1990). Close to the inhibitory region, in the direction of the COOH terminus, there is a stretch of 8 amino acids that is highly divergent (2/8) between cardiac and fast TnI but is very similar (7/8) between fast and slow TnI. The coding region of exon 8 is also highly conserved but is longer in the fast TnI gene due to a two-triplet insertion in the sequence adjacent to intron 7, which might correspond to a loop region connecting two highly conserved sequences in exon 7 and 8, and a three-triplet insertion just before the translation termination codon.

Regulatory Elements in the 5′-Flanking Region of the Cardiac TnI Gene Confer Cell Type-specific Expression in Cultured Myocytes—Cardiac TnI transcripts are detected by Northern blotting and *in situ* hybridization in fetal and adult heart but not in fetal or adult skeletal muscle (Ausoni *et al.*, 1991; Gorza *et al.*, 1993). The tissue-specific expression of the cardiac TnI gene is also observed in cultured cells. Cardiocytes from 20-day-old fetal ventricles are strongly reactive with a monoclonal antibody specific for cardiac TnI, whereas cultured skeletal myoblasts and myotubes are completely unreactive (Fig. 5). Identical results were obtained when skeletal muscle differentiation was stimulated by switching to media containing horse serum. Primary cultures of cardiac myocytes can thus be used as a functional assay system for the transcriptional regulation of the cardiac TnI gene.

The role of the 5′ genomic sequences in the expression of the cardiac TnI gene was analyzed by transient transfection assays. A fragment containing about 4 kb of the 5′-flanking region and 5 nucleotides of exon 1 was cloned immediately upstream of the CAT reporter gene into a pSPT-CAT vector. Transfection of cardiac myocytes with this clone, designated cTnI-4000, gave a 16-fold increase of CAT activity over the level obtained with the promoterless vector and a 17- and 30-fold increase over the level obtained with the same construct in skeletal myocytes and 3T3 cells, respectively (Fig. 6 and Table I). However, the level of activity was about one order of magnitude lower than that obtained with the viral control pSV2CAT (not shown).

To localize more precisely the regulatory elements required to direct cardiac-specific expression of the gene we analyzed a set of promoter upstream deletion mutants that had the same 3′ end point and progressively shorter 5′-flanking sequences (Fig. 6). High level cardiac-specific expression was obtained with constructs cTnI-3400 and cTnI-3000, whereas deletion of the region between nucleotides −3000 and −2200 led to an 8-fold decrease of activity in cardiac cells. Further deletions to nucleotide −1700, −1529, −307, and −229 resulted in a progressive increase of CAT expression in cardiac cells to levels comparable with those obtained with the three longest constructs. However, a similar trend toward a progressive increase of CAT activity was also seen with the same constructs in skeletal myocytes and, to a lower degree, in 3T3 cells. With cTnI-229 the absolute level of activity in cardiac myocytes was not significantly different from that obtained with skeletal myocytes (Fig. 6), while a 4-fold difference was observed when the activity derived from the promoterless construct was subtracted in each cell type (Table I); this is due to the higher basal level of activity of pSPT-CAT in skeletal compared with cardiac myocytes. Deletion of the sequence −229/−127 led to a 6-fold reduction in activity in cardiac cells and about a 3-fold reduction in skeletal myocytes and 3T3 cells. The shorter promoter fragment (cTnI-127) was only a fewfold more active than the promoterless vector and showed no significant tissue specificity. To determine whether the distal positive segment −3000/−2200 can confer cardiac-specific expression upon the minimal promoter, this segment was excised and inserted upstream of cTnI-127 in the correct orientation. When transfected into cardiac muscle cells the new construct cTnI-127(3000–2200) showed a low level of activity (8.1% of cTnI-229), comparable with that obtained with the minimal promoter. Further studies are required to define more precisely the activity of the −3000/−2200 fragment itself and to map the regulatory sequences within this segment.

DISCUSSION

Structure of the Cardiac TnI Gene—The genomic organization of the mouse cardiac TnI gene reported here is homologous to that of the quail fast skeletal TnI gene in the region from exon 4 to exon 8 but is highly divergent in the region from exon 1 to exon 3. The divergent exon/intron organization at the 5′ of the gene is related to the different protein structure of cardiac TnI and fast skeletal TnI in the amino-

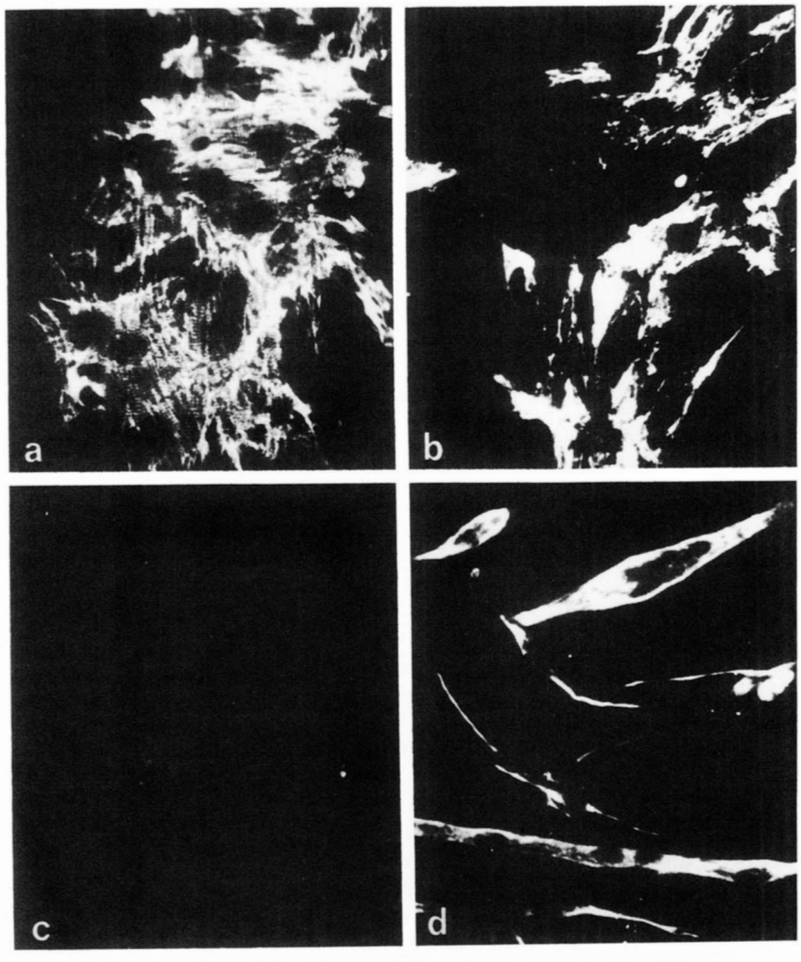

FIG. 5. **Immunocytochemical detection of cardiac TnI in cultured cardiomyocytes.** Cultures from fetal ventricles (*a* and *b*) and hindlimb muscles (*c* and *d*), maintained *in vitro* for 4 days, were stained with monoclonal antibodies specific for cardiac TnI (*a* and *c*) and desmin (*b* and *d*). 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 β-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 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 sequences 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_2 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_2 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

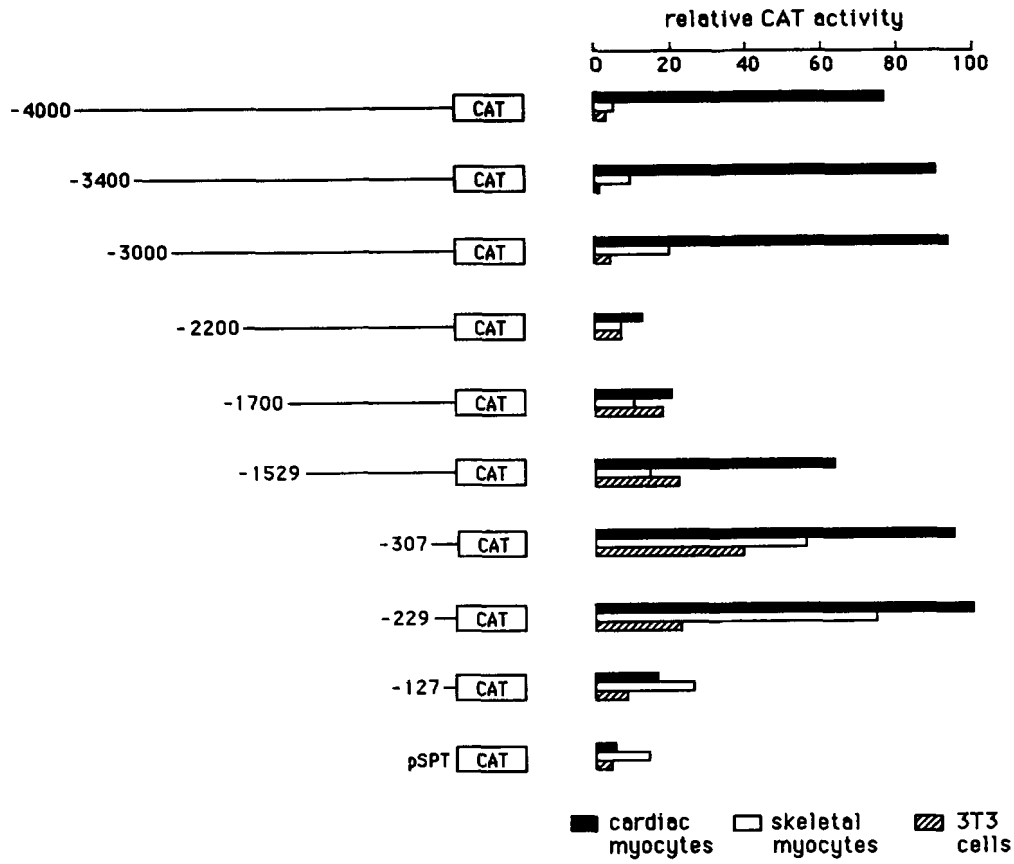

FIG. 6. **Expression of cardiac TnI 5′ upstream constructs transiently transfected into cardiac and skeletal myocytes and fibroblasts.** *Left*, schematic representation of the expression constructs containing segments (from −4000 to −127 bp) of the 5′ upstream portion of the cardiac TnI gene joined 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 β-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[a]		
	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

[a] 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 β-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_2 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-

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.[2]

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 β-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 α-cardiac actin and α-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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[2] S. Ausoni, M. Campione, A. Picard, and S. Schiaffino, unpublished results.

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