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Identification and Characterization of a Cardiac-Specific Transcriptional Regulatory Element in the Slow/Cardiac Troponin C Gene

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The slow/cardiac troponin C (cTnC) gene has been used as a model system for defining the molecular mechanisms that regulate cardiac and skeletal muscle-specific gene expression during mammalian development. cTnC is expressed continuously in both embryonic and adult cardiac myocytes but is expressed only transiently in embryonic fast skeletal myotubes. We have reported previously that cTnC gene expression in skeletal myotubes is controlled by a developmentally regulated, skeletal muscle-specific transcriptional enhancer located within the first intron of the gene (bp 997 to 1141). In this report, we show that cTnC gene expression in cardiac myocytes both in vitro and in vivo is regulated by a distinct and independent transcriptional promoter and enhancer located within the immediate 5′ flanking region of the gene (bp −124 to +32). DNase I footprint and electrophoretic mobility shift assay analyses demonstrated that this cardiac-specific promoter/enhancer contains five nuclear protein binding sites (designated CEF1, CEF-2, and CPF1-3), four of which bind novel cardiac-specific nuclear protein complexes. Functional analysis of the cardiac-specific cTnC enhancer revealed that mutation of either the CEF-1 or CEF-2 nuclear protein binding site abolished the activity of the cTnC enhancer in cardiac myocytes. Taken together, these results define a novel mechanism for developmentally regulating a single gene in multiple muscle cell lineages. In addition, they identify previously undefined cardiac-specific transcriptional regulatory motifs and *trans*-acting factors. Finally, they demonstrate distinct transcriptional regulatory pathways in cardiac and skeletal muscle.

The striated muscle lineages cardiac, slow skeletal, and fast skeletal can be distinguished functionally by their expression of sets of distinct tissue-specific protein isoforms which include both myofibrillar proteins and intracellular enzymes. The expression of many muscle-specific proteins is developmentally regulated at the level of transcription (4, 20, 25, 39). Thus, one approach to understanding striated muscle development is to elucidate the molecular mechanisms that regulate the expression of muscle-specific genes. By using this approach, a great deal has recently been learned about skeletal myogenesis. However, relatively little is currently understood about the *cis*-acting sequences and *trans*-acting factors that regulate cardiac-specific gene expression. In particular, it remains unclear whether gene expression in these two muscle lineages is regulated by common, overlapping, or distinct sets of *cis*-acting regulatory sequences and *trans*-acting factors.

The troponin C genes have been used as a model system to study transcriptional regulation during both cardiac and skeletal muscle development (32, 33). Troponin C is the calcium-binding subunit of the myofibrillar thin filament that regulates excitation-contraction coupling in the heart and skeletal muscle. In contrast to most other contractile proteins, there are only two isoforms of troponin C, which are encoded by distinct single-copy genes (32, 38, 41). In adult mammals, the slow/cardiac troponin C (cTnC) gene is expressed exclusively in cardiac and slow skeletal muscle (33). However, the gene is also transiently expressed in embryonic fast skeletal myocytes (7). In contrast, the fast skeletal troponin C (sTnC) gene is expressed exclusively in embryonic and adult fast skeletal muscle throughout the life of the organism (12, 32). Thus, precise molecular mechanisms have evolved to regulate troponin C gene expression with respect to both its tissue and developmental specificity.

Previous studies have shown that cTnC gene expression in embryonic skeletal muscle is regulated by a skeletal muscle-specific, developmentally regulated transcriptional enhancer located within the first intron of the cTnC gene (34a). In the studies described in this report, we have used both in vitro and in vivo transient transfection assays, as well as nuclear protein binding assays, to define the *cis*-acting sequences and *trans*-acting factors that regulate cTnC gene expression in cardiac myocytes. These studies have demonstrated that cTnC gene expression in cardiac myocytes is regulated by a distinct and independent transcriptional enhancer located within the immediate 5′ flanking region of the cTnC gene (bp −124 to −56). This enhancer functions only in concert with the endogenous cTnC promoter to produce high-level cardiac-specific expression of the cTnC gene. The cTnC cardiac-specific promoter/enhancer contains five previously undescribed nuclear protein binding sites, four of which bind cardiac-specific nuclear protein complexes. Taken together, these data define novel transcriptional regulatory circuits in cardiac muscle.

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MATERIALS AND METHODS

Cells and media. Murine embryonic skeletal muscle C2C12 and Sol 8 (the generous gift of K. Chien, University of California, San Diego) myoblasts were grown and differentiated into myotubes as described previously (33). HeLa, NIH 3T3, and human Jurkat T cells were grown as described previously (15). Primary neonatal rat cardiac myocytes and cardiac fibroblasts were isolated from 1- to 2-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) by collagenase digestion (10, 27). Cultures of primary cardiocytes isolated by this method contained greater than 90% cardiac myocytes that contract spontaneously in culture.

Plasmids. The promoterless pSV0CAT (13) and pCAT-Basic (Promega Biotec, Madison, Wis.) plasmids as well as the pSPCAT plasmid (26) containing the minimal simian virus 40 (SV40) promoter and the pUTKAT plasmid (35) containing the herpes simplex virus (HSV) thymidine kinase (TK) promoter have been described elsewhere. The pRSVL reference plasmid (6) contains the luciferase gene under the control of the Rous sarcoma virus long terminal repeat, and the pMSVβgal (9) reference plasmid contains the β-galactosidase gene under the control of the Moloney murine sarcoma virus long terminal repeat.

p-2.2CAT was constructed by subcloning the 2.2-kb *Bam*HI-*Alu*I cTnC genomic fragment (bp −2200 to +32) into the *Hin*dIII site of pCAT-Basic in a 5′-to-3′ orientation with respect to the chloramphenicol acetyltransferase (CAT) gene. p-124SV0CAT was constructed by subcloning the 156-bp *Bal*I-*Alu*I cTnC genomic fragment (bp −124 to +32) into the *Hin*dIII site of pSV0CAT in a 5′-to-3′ orientation with respect to the CAT gene. p-124CAT was constructed by using the polymerase chain reaction (PCR) to generate a *Hin*dIII-*Xba*I-linkered 156-bp *Bal*I-*Alu*I cTnC genomic subfragment (bp −124 to +32) which was subcloned into *Hin*dIII-*Xba*I-digested pCAT-Basic. p-79CAT was constructed by using PCR to generate a *Hin*dIII-*Xba*I-linkered cTnC subfragment (bp −79 to +32) that was subcloned into *Hin*dIII-*Xba*I-digested pCAT-Basic. p-2.2CAT145ENH and p-124CAT145ENH were constructed by subcloning the 145-bp cTnC skeletal muscle-specific first-intron transcriptional enhancer (bp 997 to 1141) into the *Bam*HI site of the p-2.2CAT and p-124CAT vectors, respectively. pSPCAT 308ENH was constructed by subcloning the 308-bp cTnC *Pvu*II genomic subfragment (bp 881 to 1188), containing the cTnC skeletal muscle-specific enhancer, into the *Bam*HI site of the pSPCAT vector. The p-79CAT69ENH, pSPCAT69 ENH, and pUTKAT69ENH plasmids were constructed by subcloning the 69-bp PCR-generated cTnC genomic subfragment (bp −124 to −56) into the *Bam*HI site of the p-79CAT, pSPCAT, and pUTKAT vectors, respectively. All PCR-derived genomic subfragment sequences were confirmed by dideoxy DNA sequence analysis.

Transfections and CAT assays. NIH 3T3 cells, HeLa cells, C2C12 myoblasts, Sol 8 myoblasts, and primary cardiac fibroblasts were transfected with DNA-calcium phosphate precipitates (32). Primary neonatal rat cardiac myocytes were transfected with Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, Md.) (27). All transfections contained 5 μg of the pMSVβgal reference plasmid. Following transfection, cell lysates were prepared and normalized for protein content by using a commercially available kit (Bio-Rad, Richmond, Calif.). CAT and β-galactosidase assays were performed as described previously (22). All experiments were repeated at least three times to ensure reproducibility. CAT activities were corrected for variations in transfection efficiencies as determined by assaying cell extracts for β-galactosidase activities.

In vivo transfections; CAT and luciferase assays. Six-week-old 250-g Sprague-Dawley rats were housed and cared for according to National Institutes of Health guidelines in the University Laboratory Animal Medicine facility of the University of Michigan Medical Center. Rats were anesthetized with pentobarbital (20 mg/kg of body weight intraperitoneally) and ketamine (60 mg/kg intramuscularly), intubated, and ventilated (27). A left lateral thoracotomy was performed to expose the beating heart, and 100 μg of CAT reporter plasmid and 25 μg of the pRSVL reference plasmid in 100 μl of phosphate-buffered saline (PBS) was injected with a 30-gauge needle into the apical portion of the beating left ventricle. The animals were sacrificed 5 days after injection, and hearts were removed, rinsed free of blood in ice-cold PBS, and immediately homogenized in 1 ml of ice-cold 25 mM glycylglycine (pH 7.8)–15 mM $MgSO_4$–4 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (pH 8.0)–1 mM dithiothreitol. The homogenate was centrifuged, and the supernatant was processed for CAT and luciferase activities as described previously (23). Relative CAT activities were corrected for variations in transfection efficiencies as determined by assaying the tissue extracts for luciferase activity.

DNase I footprint analysis. Nuclear extracts were prepared from neonatal Sprague-Dawley rat hearts, C2C12 myoblasts, C2C12 myotubes, and Jurkat T cells as described by Dignam et al. (8). DNase I footprint analyses were performed with 75 to 100 μg of neonatal rat cardiac, C2C12 myotube, or Jurkat nuclear extract (22). Reaction products were fractionated on 8% sequencing gels. Standard Maxam and Gilbert (G+A) sequencing reactions were run in parallel in order to identify protected sequences.

Electrophoretic mobility shift assays (EMSAs). The following complementary oligonucleotides were synthesized with *Bam*HI and *Bgl*II overhanging ends on an Applied Biosystems model 380B DNA synthesizer:

CEF-1,	5′-CCAGCCTGAGATTACAGGGA-3′
mCEF-1,	5′-CCAGCCAGAGATCTCAGGGA-3′
CEF-2,	5′-CCAGGGAGGGGTGGAGGATATTCCAGG-3′
CEF-2 core,	5′-GGTGGAGGATATTCCAGG-3′
mCEF-2 core,	5′-GGTGCAGATCTTTCCAGG-3′

EMSAs were performed in 0.25× TBE (1× TBE is 100 mM Tris, 100 mM boric acid, and 2 mM EDTA) at 4°C as described previously (15).

Preparation of deleted and mutated cTnC enhancer elements. Mutations were introduced into the 156-bp cTnC cardiac-specific promoter/enhancer by site-directed oligonucleotide-mediated gapped heteroduplex mutagenesis (22), using the following synthetic oligonucleotides (mutated nucleotides are underlined):

CEF-1,	5′-CCAGCC<u>A</u>GAGAT<u>CT</u>CAGGGACCAGGGAGGG-3′
CEF-2 CACCC,	5′-CCTGAGATTACAGGGACCAG<u>ATCT</u>GG<u>C</u>GTGGAGGATATTCC-3′
CEF-2 core,	5′-CAGGGACCAGGGAGGGGGTG<u>C</u>AG<u>ATCT</u>TTCCAGGCAGGCAGTGGTGGGCTG-3′

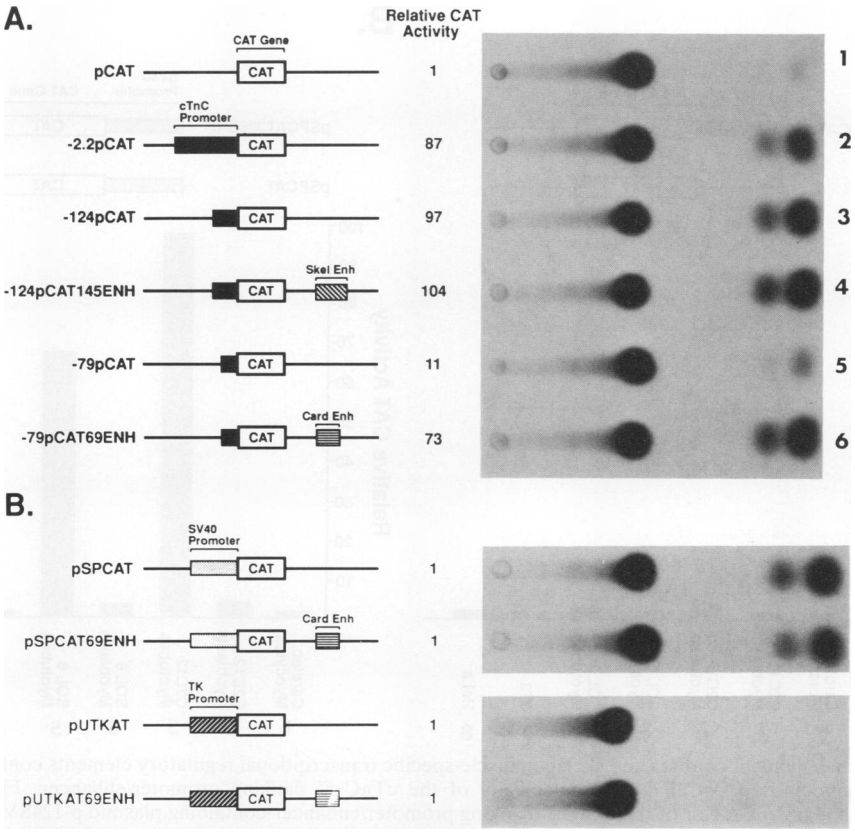

FIG. 1. Identification and localization of a cardiac-specific promoter/enhancer in the 5′ flanking region of the murine cTnC gene. (A) Localization of a cardiac-specific promoter/enhancer. Fifteen micrograms of the cTnC-CAT reporter plasmids (schematically represented at the left) and 5 μg of the pMSVβgal reference plasmid were transfected into primary cultures of neonatal rat cardiac myocytes, and CAT and β-galactosidase activities were determined. CAT activities, corrected for differences in transfection efficiencies, were normalized to the CAT activity obtained following transfection of the promoterless control plasmid pCAT-Basic, which produced 0.5% acetylation. A representative CAT assay is shown at the right. (B) Promoter specificity of the cTnC 5′ flanking transcriptional enhancer. The enhancerless control plasmids pSPCAT and pUTKAT, containing the minimal SV40 and herpes simplex virus thymidine kinase (TK) promoters, respectively, and the enhancer-containing plasmids pSPCAT69ENH and pUTKAT69ENH, along with the pMSVβgal reference plasmid, were transfected into primary cultures of neonatal rat cardiac myocytes as described above. Relative CAT activities were calculated by comparing the activities of the enhancer-containing plasmids with those of pSPCAT (which produced 30% acetylation) and pUTKAT (which produced 0.4% acetylation) following correction for transfection efficiencies.

RESULTS

cTnC expression in cardiac myocytes is regulated by a 5′ flanking promoter/enhancer. To functionally identify the *cis*-acting sequences that regulate cTnC gene expression in cardiac myocytes, transient transfections were performed by using cTnC-CAT reporter constructs and primary cultures of rat neonatal cardiac myocytes (Fig. 1). Transfection of these cells with plasmid p-2.2CAT, containing 2.2 kb of cTnC 5′ flanking sequence, increased transcription of the CAT reporter 80- to 100-fold above levels obtained with the promoterless pCAT-Basic vector (Fig. 1A, lanes 1 and 2). This 5′ flanking region of the cTnC gene appears to contain the only transcriptional regulatory elements required for high-level expression in cardiac myocytes because inclusion of additional intragenic and 3′ flanking genomic sequences failed to further increase CAT transcription (data not shown). In particular, it should be noted that the previously identified cTnC skeletal muscle-specific first-intron enhancer failed to significantly increase transcription above levels obtained with the p-124CAT vector alone in primary cardiac myocytes (Fig. 1A, lanes 3 and 4). This skeletal muscle-specific enhancer was also inactive in cardiac myocytes in conjunction with the minimal SV40 promoter (Fig. 2B).

To better delineate the cardiac-specific transcriptional regulatory sequences in the 5′ flanking region of the cTnC gene, a series of deletion mutants was transfected into primary cultures of neonatal cardiac myocytes. These experiments demonstrated that a 156-bp fragment containing 124 bp of 5′ flanking sequence (p-124CAT) displayed full transcriptional regulatory activity. However, further deletion of the 5′ flanking sequence to bp −79 resulted in a 90% reduction in CAT activity (Fig. 1A, lane 5). To determine whether the 124-bp 5′ flanking region contained a transcriptional enhancer or, alternatively, represented a cardiac-specific promoter element, a 69-bp fragment (bp −124 to −56) was subcloned into the *Bam*HI site downstream of the CAT reporter gene and the core cTnC promoter (bp −79 to +32). As shown in Fig. 1, this 69-bp fragment restored transcriptional activity to the core cTnC promoter in cardiac myocytes. Moreover, this effect was position and orientation independent (Fig. 1A, lane 6, and data not shown), demon-

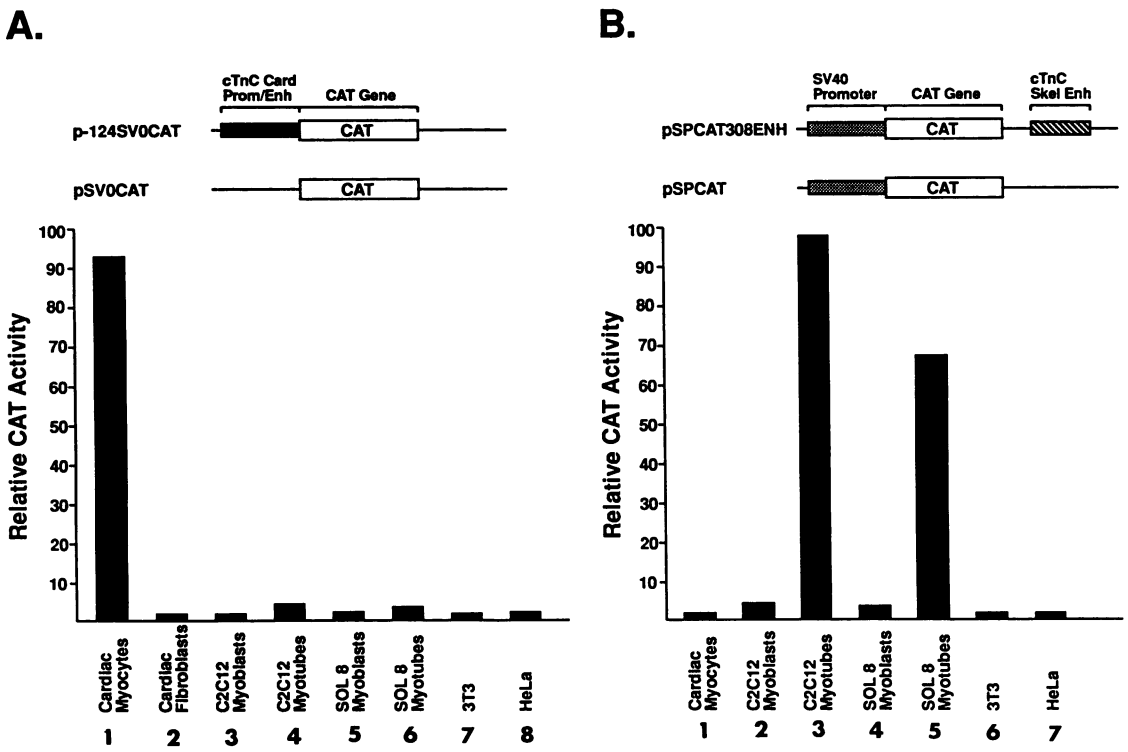

FIG. 2. Evidence that independent cardiac- and skeletal muscle-specific transcriptional regulatory elements control cTnC gene expression in cardiac and skeletal myocytes. (A) Cell lineage specificity of the cTnC 5′ flanking promoter/enhancer. Fifteen micrograms of the promoterless control plasmid pSV0CAT or of the cTnC 5′ flanking promoter/enhancer-containing plasmid p-124SV0CAT, along with 5 μg of the pMSVβgal reference plasmid, were transfected into primary cultures of neonatal cardiac myocytes and cardiac fibroblasts, murine C2C12 myoblasts and myotubes, murine Sol 8 myoblasts and myotubes, murine 3T3 fibroblasts, and human HeLa cells, and cell lysates were assayed for CAT and β-galactosidase activities. Relative CAT activity was calculated by comparing the activity of the promoter/enhancer-containing plasmid p-124SV0CAT with that of the promoterless control plasmid pSV0CAT, which produced 0.1 to 0.2% acetylation in multiple transfections, following correction for transfection efficiencies. (B) Cell lineage specificity of the cTnC first-intron transcriptional enhancer. The enhancerless control plasmid pSPCAT or the cTnC first-intron transcriptional enhancer-containing plasmid pSPCAT308ENH, along with the pMSVβgal reference plasmid, were transfected into primary cultures of cardiac myocytes, C2C12 myoblasts and myotubes, Sol 8 myoblasts and myotubes, 3T3 fibroblasts, and HeLa cells as described above. Relative CAT activity was calculated by comparing the activity of the enhancer-containing plasmid pSPCAT308ENH with that of the enhancerless plasmid pSPCAT, which produced 0.5 to 1.0% acetylation in multiple transfections, following correction for transfection efficiencies.

strating that this fragment contains a bona fide transcriptional enhancer element.

Promoter and cellular specificity of the murine cTnC 5′ flanking transcriptional enhancer. To determine whether the 69-bp 5′ flanking cardiac enhancer required the endogenous cTnC promoter or could function with heterologous promoters, it was subcloned 3′ of the CAT reporter gene in plasmids pSPCAT and pUTKAT, containing the minimal SV40 and herpes simplex virus thymidine kinase promoters, respectively. In neither case did the cardiac enhancer element increase transcription from these heterologous promoters (Fig. 1B) in cardiac myocytes. Thus, in contrast to the cTnC skeletal muscle-specific first-intron enhancer (34a), the activity of the cTnC 5′ flanking enhancer requires the endogenous cTnC promoter.

To characterize the cellular specificity of the cTnC 5′ flanking promoter/enhancer, the transcriptional activities of the cTnC promoter/enhancer-containing plasmid, p-124 SV0CAT, and the promoterless control plasmid, pSV0CAT, were compared following transfection into neonatal cardiac myocytes, skeletal myoblasts, skeletal myotubes, and a variety of nonmuscle cells (including primary cardiac fibroblasts) (Fig. 2A). The cTnC promoter/enhancer was active in primary cultures of neonatal cardiac myocytes, increasing transcription of the CAT reporter approximately 90-fold above levels obtained with the promoterless control plasmid, pSV0CAT (Fig. 2A, column 1). In contrast, the promoter/enhancer was completely inactive in C2C12 skeletal myoblasts (column 3), C2C12 skeletal myotubes (column 4), and all nonmuscle cell lines tested (columns 7 and 8). Of note, the promoter/enhancer was also inactive in Sol 8 myoblasts (column 5) and myotubes (column 6), a cell line derived from slow skeletal muscle that expresses high levels of cTnC mRNA following differentiation into myotubes (data not shown). Thus, the transcriptional regulatory elements located within the immediate 5′ flanking region of the cTnC gene are both necessary and sufficient to confer cardiac-specific gene expression.

As demonstrated in these experiments, the 5′ flanking of cTnC promoter/enhancer was completely inactive in C2C12 and Sol 8 skeletal myotubes, despite the fact that the cTnC gene is expressed at high levels in these cells. This suggested that an additional transcriptional regulatory element was necessary for high-level expression in embryonic skeletal myocytes. To test whether the previously identified transcriptional enhancer located in the first intron of the cTnC

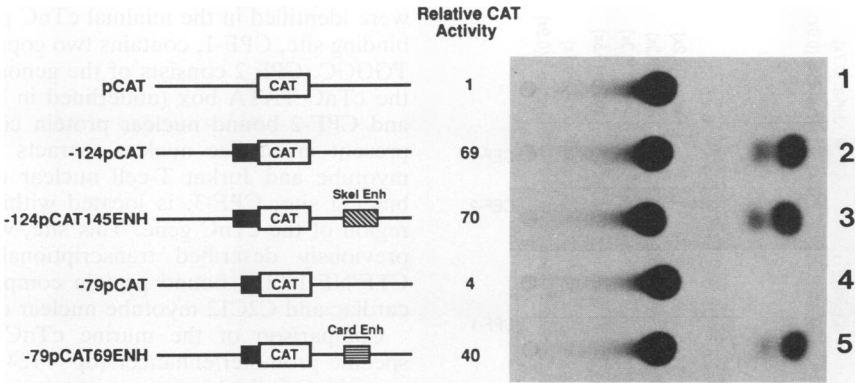

FIG. 3. Evidence that a cardiac-specific promoter/enhancer in the 5′ flanking region of the murine cTnC gene functions in vivo in cardiac myocytes. One hundred micrograms of cTnC/CAT reporter plasmid (schematically represented at the left) and 25 μg of the luciferase reference plasmid pRSVL were injected directly into the left ventricular free wall of anesthetized 6-week-old Sprague-Dawley rats as described in Materials and Methods. Five days postinjection, the animals were sacrificed, and CAT and luciferase activities were determined from cardiac homogenates. CAT activities, corrected for differences in transfection efficiencies, are expressed relative to the CAT activity obtained following injection of the promoterless plasmid pCAT-Basic which produced 0.2% acetylation. A representative CAT assay is presented at the right. All experiments were repeated in at least three animals.

gene was a distinct and independent skeletal muscle-specific transcriptional regulatory element, we compared the activity of plasmid pSPCAT, containing the minimal SV40 promoter, with that of plasmid pSPCAT308ENH, which also contains the first-intron transcriptional enhancer. The cTnC first-intron transcriptional enhancer was active in both C2C12 and Sol 8 myotubes, increasing transcription of the CAT reporter approximately 60- to 100-fold above levels obtained with the control plasmid, pSPCAT (Fig. 2B, columns 3 and 5). In contrast, the first-intron enhancer was completely inactive in C2C12 and Sol 8 myoblasts, neonatal cardiac myocytes, and all nonmuscle cell lines tested (columns 2, 4, 6, and 7). Thus, distinct and independent cardiac and skeletal muscle-specific elements regulate cTnC gene expression in heart and skeletal muscle, respectively.

The cTnC cardiac-specific promoter/enhancer functions in adult cardiac myocytes in vivo. Cultured cardiac myocytes have been reported to rapidly lose their differentiated phenotype in vitro. Thus, it was important to confirm the results obtained by using transient transfection of cardiac myocytes in vitro in an experimental system that allowed a direct assessment of transcriptional activities in vivo. Toward this end, we took advantage of a recently described property of cardiac myocytes, i.e., their ability to take up and express exogenous DNA following direct injection into the left ventricular wall in vivo (1, 23, 27). A series of cTnC-CAT reporter plasmids along with the pRSVL luciferase reference plasmid were injected directly into the left ventricular walls of 6-week-old Sprague-Dawley rats. Five days following injection, the animals were sacrificed, and cardiac homogenates were assayed for CAT and luciferase activities. Consistent with the results obtained in the in vitro transfections, the 156-bp 5′ flanking cardiac-specific promoter/enhancer increased CAT transcription by 70-fold in vivo (Fig. 3, lanes 1 and 2). Deletion of bp −124 to −79 abolished transcriptional regulatory activity in vivo. However, full transcriptional regulatory activity was restored when the 69-bp fragment (bp −124 to −56) was subcloned downstream of the core cTnC promoter CAT cassette (lanes 4 and 5). Moreover, the addition of the 145-bp skeletal muscle-specific cTnC first intron enhancer did not further increase CAT transcription in cardiac myocytes in vivo (lane 3). These results, which were reproducibly obtained in multiple animals, confirmed the identity of the cardiac-specific cTnC promoter/enhancer as determined by the in vitro transfections. In addition, they showed that direct DNA injection is a convenient and reproducible in vivo assay system for studies of cardiac-specific transcriptional regulatory elements. In summary, expression of the cTnC gene in cardiac myocytes in vitro and in vivo is regulated by the interaction of a core promoter (bp −79 to +32) and a transcriptional enhancer (bp −56 to −124) that are both contained in the immediate 5′ flanking region of the gene.

Identification of five nuclear protein binding sites in the cardiac-specific cTnC promoter/enhancer. To identify nuclear protein binding sites within the cTnC 5′ flanking, cardiac-specific promoter/enhancer, DNase I footprint analyses were performed by using nuclear extracts prepared from neonatal rat hearts, C2C12 skeletal myotubes, and the human T-cell line Jurkat (Fig. 4). Five nuclear protein binding sites were identified by using the cardiac nuclear extracts. In addition, multiple DNase I-hypersensitive sites reflected as intense bands of digestion were observed between the footprinted regions (Fig. 4A). This type of DNase I footprint has been reported previously in several enhancers containing multiple closely spaced nuclear protein binding sites and presumably reflects bending of the DNA adjacent to these binding sites (15, 19). Two footprinted regions, designated cardiac enhancer factor 1 (CEF-1) and cardiac enhancer factor 2 (CEF-2), were present in the cTnC enhancer (bp −56 to −124). CEF-1 binding activity was present in both cardiac and C2C12 myotube nuclear extracts but absent from Jurkat nuclear extracts. The pattern of binding to the CEF-2 element was more complex. A core CEF-2 sequence bound a nuclear protein complex that was present only in the cardiac nuclear extracts (Fig. 4B, shaded region). In addition, the CACCC box at the 5′ end of the CEF-2 binding site was protected by cardiac nuclear extracts but not by Jurkat T-cell nuclear extracts. The CEF-2 element was also partially protected from DNase I digestion by C2C12 myotube nuclear extracts, but the pattern of protection was clearly different from that observed with cardiac nuclear extracts (Fig. 4A). Three additional nuclear protein binding sites designated cardiac promoter factors 1 to 3 (CPF1 to CPF3)

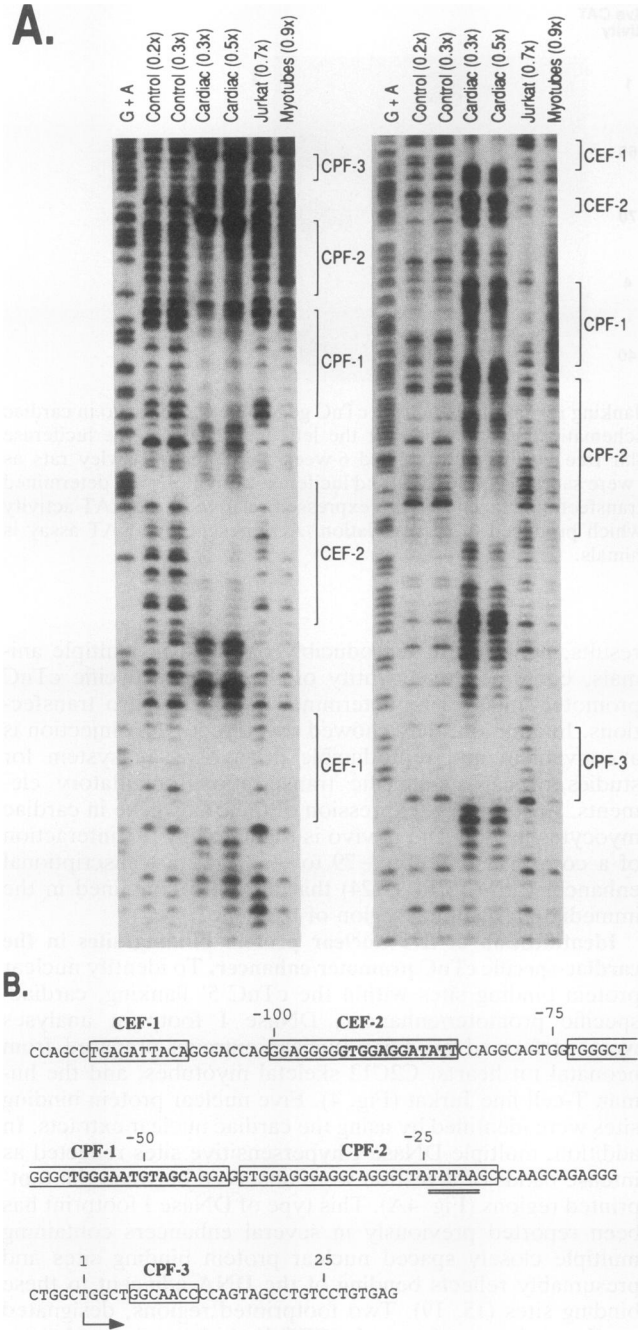

FIG. 4. DNase I footprint analysis of the cTnC cardiac-specific promoter/enhancer. (A) Footprint analysis. The 156-bp *Bal*I-*Alu*I fragment containing the cTnC cardiac-specific promoter/enhancer was subjected to DNase I footprint analysis using rat neonatal cardiac, Jurkat T-cell, and C2C12 skeletal myotube nuclear extracts. The sense (left) and antisense (right) strands of the 156-bp fragment were end labeled and incubated in the absence (control) or presence of cardiac, Jurkat, and C2C12 myotube nuclear extracts before partial digestion with DNase I (0.2× to 0.9×) (1× DNase I = 25 U/ml). Standard Maxam and Gilbert purine (G+A) sequencing reactions of the same fragments were run in parallel. Protected sequences identified with cardiac nuclear extracts (CEF-1, CEF-2, CPF-1, CPF-2, and CPF-3) are bracketed. (B) Nucleotide sequence of the cTnC cardiac-specific promoter/enhancer. Nuclear protein binding sites identified with neonatal cardiac nuclear extracts are boxed. Both the CEF-2 and CPF-1 protein binding sites include completely protected core regions (shaded) and partially protected flanking regions (unshaded) (see text). The TATA box (double underline) and transcriptional start site (arrow) are indicated. The CEF-2 binding site contains a CACCC box on the antisense strand (bp −91 to −95).

were identified in the minimal cTnC promoter. The 5′-most binding site, CPF-1, contains two copies of the direct repeat TGGGC. CPF-2 consists of the genomic sequence flanking the cTnC TATA box (underlined in Fig. 4B). Both CPF-1 and CPF-2 bound nuclear protein complexes which were present in cardiac nuclear extracts but absent in C2C12 myotube and Jurkat T-cell nuclear extracts. The 3′-most binding site, CPF-3, is located within the 5′ untranslated region of the cTnC gene. This site, which is similar to the previously described transcriptional regulatory element CTF/NF-1 (21), bound protein complexes present in both cardiac and C2C12 myotube nuclear extracts.

Comparison of the murine cTnC 5′ flanking cardiac-specific promoter/enhancer (bp −124 to +32) with the human cTnC 5′ flanking genomic sequence revealed conservation of 8 of 10 and 17 of 20 nucleotides in the CEF-1 and CEF-2 binding sites, respectively. Within the CEF-2 motif, 10 of 11 bp flanking the CACCC box and 10 of 12 bp within the CEF-2 core region were conserved. Within the cTnC promoter, 21 of 26 nucleotides within CPF-1 were identical in the mouse and human genes. However, the murine sequence contained two copies of the direct repeat TGGGC, while the human sequence contained five copies of this motif. Similarly, 21 of 26 bp of the CPF-2 protein binding site (15 of 19 bp flanking the TATA box) were conserved. Finally, all seven nucleotides within the CPF-3 binding site were identical. Moreover, the CPF-3 site is embedded within a 15-bp nucleotide sequence that is 100% conserved between murine and human species. Taken together, these experiments demonstrated that the 5′ flanking cTnC cardiac-specific promoter/enhancer contains five nuclear protein binding sites, four of which bind cardiac-specific nuclear proteins. Each of these nuclear protein binding sites is conserved between the murine and human cTnC genes. Notably, with the exception of the CACCC box in CEF-2 and the potential CTF/NF-1 binding site in CPF-3, none of the DNase I footprints corresponds to a previously described enhancer motif, suggesting that these elements reflect one or more novel cardiac-specific transcriptional regulatory pathways.

CEF-1 and CEF-2 bind novel cardiac-specific nuclear protein complexes. To assess the number and specificity of nuclear proteins that bind to the cTnC 5′ flanking transcriptional enhancer, synthetic oligonucleotides corresponding to the CEF-1, CEF-2, and CEF-2 core nuclear protein binding sites were used in EMSAs (Fig. 5A). The CEF-1 binding site bound at least four nuclear protein complexes present in neonatal cardiac nuclear extracts (Fig. 5A, lane 2). Binding of each of these complexes as determined by using unlabeled competitor oligonucleotides was specific (lanes 3 to 6). Two of these binding activities (solid arrows) were cardiac specific in that they were not present in nuclear extracts prepared from C2C12 myoblasts, C2C12 myotubes, or NIH 3T3 cells (lanes 7 to 9). In addition, a third faint low-mobility nuclear protein complex was detected only in the cardiac extracts (open arrow). Notably, none of these three complexes was detected in nuclear extracts prepared from primary cardiac fibroblasts (data not shown).

An initial series of EMSAs performed with the full-length

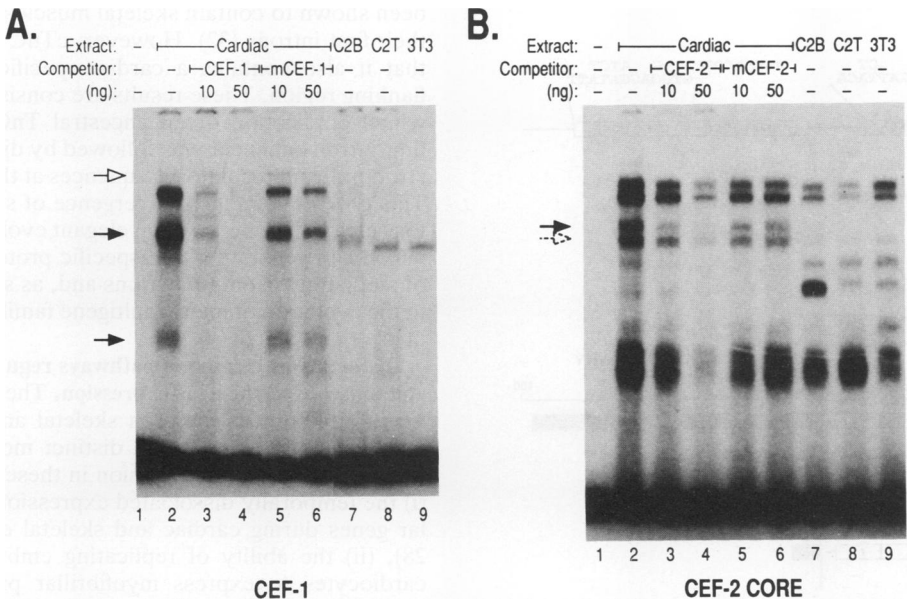

FIG. 5. EMSA analyses of the murine cTnC cardiac-specific transcriptional enhancer. (A) Identification of nuclear protein complexes which bind to the CEF-1 motif. Radiolabeled oligonucleotides corresponding to the CEF-1 binding site were subjected to EMSAs using 5 μg of nuclear extracts prepared from neonatal rat hearts (Cardiac), C2C12 myoblasts (C2B), C2C12 myotubes (C2T), and NIH 3T3 fibroblasts (3T3). Some binding reaction mixtures included 10 to 50 ng of the indicated unlabeled competitor oligonucleotides. Cardiac-specific complexes are indicated by arrows (see text). (B) Identification of nuclear protein complexes that bind to the CEF-2 core motif. The cardiac-specific complexes are indicated by arrows (see text).

radiolabeled CEF-2 oligonucleotide and cardiac nuclear extracts demonstrated multiple DNA-protein complexes with distinct electrophoretic mobilities. Several of these complexes migrated with mobilities identical to those observed with the cTnC CACCC box oligonucleotide probe. The identities of these complexes were confirmed in competition experiments with unlabeled CACCC box oligonucleotides (data not shown). To identify non-CACCC box nuclear protein complexes that bind to the CEF-2 core protein binding site as determined by DNase I footprint analyses, a series of EMSAs was performed with a radiolabeled CEF-2 core oligonucleotide (Fig. 5B). This probe bound four nuclear protein complexes present in cardiac nuclear extracts (Fig. 5B, lane 2). Binding was specific because binding of each complex was competed by excess unlabeled specific competitor oligonucleotide (lanes 3 and 4) but not by nonspecific oligonucleotide competitors (lanes 5 and 6). One of the binding activities (solid arrow) was cardiac specific, as it was not present in nuclear extracts prepared with C2C12 myoblasts or myotubes or with NIH 3T3 cells (lanes 7 to 9). A second binding activity (dotted arrow) was significantly enriched in cardiac nuclear extracts but was also seen in nuclear extracts prepared from 3T3 cells. Of note, neither of these binding activities was detectable in nuclear extracts prepared from primary cardiac fibroblasts (data not shown).

Functional analyses of the cardiac-specific cTnC enhancer. To determine the functional significance of CEF-1 and CEF-2 for cTnC enhancer function, mutant enhancer constructs containing nucleotide substitutions in CEF-1, the CEF-2 core, and the CEF-2 CACCC box were transfected into neonatal cardiac myocytes. All three mutations resulted in approximately 90% reductions in CAT transcription relative to that observed with the wild-type enhancer (Fig. 6). Therefore, CEF-1 and CEF-2 are both required for cTnC enhancer function. In summary, as is the case for many other viral and cellular enhancers, the cTnC promoter/enhancer is composed of multiple nuclear protein binding sites that function in concert to control the tissue specificity and high-level expression of this gene in cardiac muscle (Fig. 7).

DISCUSSION

The unique pattern of expression of the cTnC gene, transient expression in embryonic fast skeletal muscle and continuous expression in cardiac myocytes, suggested that studies of the transcriptional regulation of this gene might yield important insights into the molecular mechanisms underlying both cardiac and skeletal muscle development. In the studies described in this report, we have demonstrated that cTnC gene expression in cardiac myocytes in vitro and in vivo is controlled by a core promoter and transcriptional enhancer contained within the immediate 5′ flanking region of the gene. This 5′ flanking promoter/enhancer functions in a cardiac-specific fashion. In contrast, cTnC gene expression in embryonic skeletal myotubes is controlled by a second, independent transcriptional enhancer located within the first intron of the gene (Fig. 7). DNase I footprint and EMSA analyses of the cTnC 5′ flanking, cardiac-specific promoter/enhancer revealed five previously undescribed nuclear protein binding sites which bind multiple ubiquitously expressed and cardiac-specific nuclear protein complexes.

One gene, two enhancers. cTnC belongs to the troponin C supergene family of calcium-binding proteins that includes sTnC, parvalbumin, calmodulin, and myosin light-chain 1 (23). Previous studies have suggested that the TnC family members arose from early gene duplications followed by sequence divergence (3, 17, 24, 33). The cTnC and sTnC proteins are expressed in a tissue-specific fashion in adult cardiac and fast skeletal muscle and have been shown to

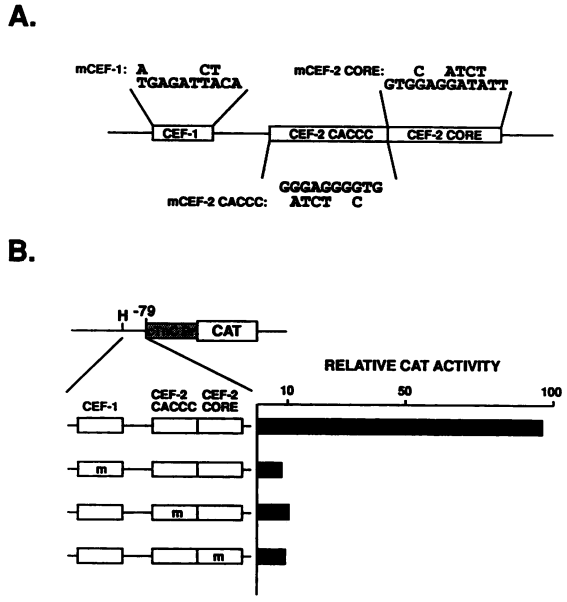

FIG. 6. Functional analysis of the nuclear protein binding sites of the cTnC cardiac-specific enhancer. (A) The nucleotide sequences of the CEF-1, CEF-2 CACCC box, and CEF-2 core nuclear protein binding sites are shown, with nucleotide substitutions used for mutations (mCEF-1, mCEF-2 CACCC, and mCEF-2 core) depicted above or below each sequence. (B) Effects of mutation of the CEF-1, CEF-2 CACCC box, and CEF-2 core nuclear protein binding sites on enhancer activity in neonatal cardiac myocytes. Mutations were introduced into the 156-bp cTnC cardiac-specific promoter/enhancer as described in Materials and Methods. The mutant enhancers were subcloned into pCAT-Basic, and the resulting plasmids were transfected into primary neonatal rat cardiomyocytes. CAT activities, corrected for differences in transfection efficiencies and normalized to the CAT activity produced by the pCAT-Basic control plasmid, are shown at the right. Each transfection was repeated at least three times.

display important functional differences (2, 31). Structurally, the two genes share a high degree of amino acid sequence identity and identical intron-exon boundaries with the exception of exon 1 and the 5′ flanking regions, which are distinct (12, 32, 33, 37). Interestingly, both genes have now been shown to contain skeletal muscle-specific enhancers in their first introns (32). However, cTnC differs from sTnC in that it also contains a cardiac-specific enhancer in its 5′ flanking region. These results are consistent with a model in which duplication of an ancestral TnC gene containing a first-intron enhancer was followed by divergence of both the structural and regulatory sequences at the 5′ end of the gene. This type of coincident divergence of structural and regulatory elements represents an elegant evolutionary mechanism which can generate tissue-specific protein isoforms capable of mediating distinct functions and, as such, may play a role in the evolution of many multigene families in higher eukaryotes.

Distinct transcriptional pathways regulate cardiac and skeletal muscle-specific gene expression. There are several fundamental differences between skeletal and cardiac myocytes which have suggested that distinct molecular mechanisms might regulate gene expression in these cells. These include (i) the temporally dissociated expression of single myofibrillar genes during cardiac and skeletal embryogenesis (5, 7, 28), (ii) the ability of replicating embryonic and neonatal cardiocytes to express myofibrillar proteins, while these proteins are expressed only in terminally differentiated nondividing skeletal myotubes (18, 29), and (iii) the demonstration that known basic helix-loop-helix (bHLH) transcription factors are not expressed in the embryonic or adult heart (36). Our results concerning the cardiac-specific cTnC promoter/enhancer begin to suggest a molecular basis for these differences. The identification of two independently regulated lineage-specific transcriptional regulatory elements in the cTnC gene provides one mechanism for the temporal dissociation of gene expression in the different muscle cell lineages. The finding that the cTnC promoter/enhancer contains five evolutionarily conserved and previously undescribed nuclear protein binding sites, and that none of these sites is related to previously described skeletal muscle-specific *cis*-acting regulatory sequences or to the consensus bHLH binding site, strongly suggests the existence of novel cardiac-specific non-bHLH transcription factors. The identification of multiple cardiac-specific nuclear protein complexes that bind to the cTnC 5′ flanking promoter/enhancer demonstrates the existence of cardiac-specific transcriptional regulatory proteins. Finally, the finding that the cTnC enhancer functions only in concert with the minimal cTnC

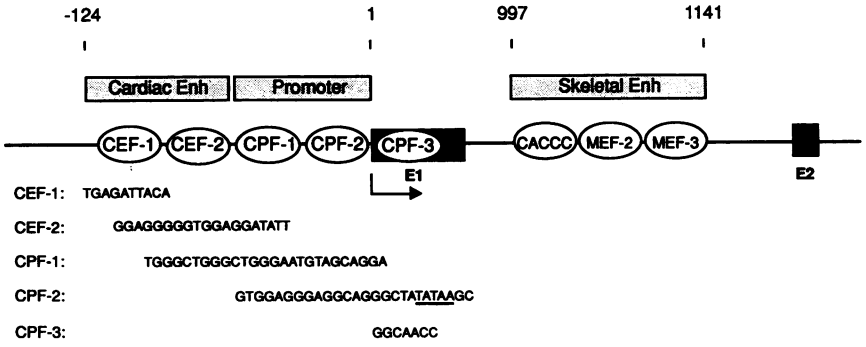

FIG. 7. Schematic representation of the transcriptional regulatory elements that control cTnC gene expression in cardiac and skeletal myocytes. Cardiac (Cardiac Enh) and skeletal muscle-specific (Skeletal Enh) enhancers are shown by shaded boxes, as is the core cTnC promoter (Promoter). Nuclear protein binding sites are shown graphically as labeled ovals. The CACCC, MEF-2, and MEF-3 nuclear protein binding sites in the skeletal muscle-specific enhancer are from Parmacek and Leiden (34). Exons 1 (E1) and 2 (E2) are shown as solid boxes. The transcriptional start site is represented by an arrow. Nucleotide positions are shown at the top, and sequences of the cardiac promoter/enhancer nuclear protein binding sites are shown at the bottom.

promoter, and that both bind cardiac-specific nuclear protein complexes, suggests that protein-protein interactions between these cardiac-specific *trans*-acting factors are important for transcriptional regulation in cardiac myocytes.

Cardiac-specific gene expression. Until recently, technical limitations caused by the lack of an established cardiac cell line and the terminally differentiated state of primary cardiac myocytes have restricted our ability to examine the molecular mechanisms that regulate cardiac-specific gene expression. Thus, it remained unclear whether cardiac and fast skeletal muscle gene expression are controlled by similar or distinct transcriptional regulatory pathways. These limitations have been overcome recently with the advent of more efficient transfection techniques (11, 27), by the use of transgenic animals (20), and by taking advantage of the unique property of cardiac (and skeletal) myocytes to take up and express recombinant DNA following direct injection (1, 23, 27). Recent reports by Zhu and coworkers (40) and Mar and Ordahl (30) have begun to functionally characterize cardiac-specific sequence elements located within the promoters of the rat cardiac myosin 2 gene and the chicken troponin T genes. However, neither of these studies demonstrated binding of cardiac-specific *trans*-acting factors to these cardiac-specific transcriptional regulatory elements. Interestingly, HF-1, a cardiac-specific element located within the rat cardiac myosin light-chain 2 promoter, includes a CArG box (16) and a MEF-2 motif (14), transcriptional regulatory elements that have previously been demonstrated to play a role in skeletal muscle-specific gene expression. In contrast, neither of the cardiac-specific *cis*-acting sequences located within the chicken troponin T promoter, or within the murine cTnC promoter/enhancer, contains previously described skeletal muscle-specific *cis*-acting motifs. Moreover, the results described in this report demonstrated the existence of novel cardiac-specific DNA-binding proteins. These results should facilitate future studies designed to elucidate these cardiac-specific transcriptional regulatory circuits.

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