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# A Novel Myogenic Regulatory Circuit Controls Slow/Cardiac Troponin C Gene Transcription in Skeletal Muscle

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**The slow/cardiac troponin C (cTnC) gene is expressed in three distinct striated muscle lineages: cardiac myocytes, embryonic fast skeletal myotubes, and adult slow skeletal myocytes. We have reported previously that cTnC gene expression in cardiac muscle is regulated by a cardiac-specific promoter/enhancer located in the 5′ flanking region of the gene (bp −124 to +1). In this report, we demonstrate that the cTnC gene contains a second distinct and independent transcriptional enhancer which is located in the first intron. This second enhancer is skeletal myotube specific and is developmentally up-regulated during the differentiation of myoblasts to myotubes. This enhancer contains three functionally important nuclear protein binding sites: a CACCC box, a MEF-2 binding site, and a previously undescribed nuclear protein binding site, designated MEF-3, which is also present in a large number of skeletal muscle-specific transcriptional enhancers. Unlike most skeletal muscle-specific transcriptional regulatory elements, the cTnC enhancer does not contain a consensus binding site (CANNTG) for the basic helix-loop-helix (bHLH) family of transcription factors and does not directly bind MyoD-E12 protein complexes. Despite these findings, the cTnC enhancer can be transactivated by overexpression of the myogenic bHLH proteins, MyoD and myogenin, in C3H10T1/2 (10T1/2) cells. Electrophoretic mobility shift assays demonstrated changes in the patterns of MEF-2, CACCC, and MEF-3 DNA binding activities following the conversion of 10T1/2 cells into myoblasts and myotubes by stable transfection with a MyoD expression vector. In particular, MEF-2 binding activity was up-regulated in 10T1/2 cells stably transfected with a MyoD expression vector only after these cells fused and differentiated into skeletal myotubes. Taken together, these results demonstrated that distinct lineage-specific transcriptional regulatory elements control the expression of a single myofibrillar protein gene in fast skeletal and cardiac muscle. In addition, they show that bHLH transcription factors can indirectly transactivate the expression of some muscle-specific genes.**

The expression of many muscle-specific proteins is developmentally regulated at the level of transcription (7, 34, 39, 66, 85). Thus, one approach to understanding the molecular basis of mammalian myogenesis is to elucidate the transcriptional mechanisms that regulate the expression of muscle-specific genes. The identification and characterization of the basic helix-loop-helix (bHLH) family of muscle-determining transcription factors, including MyoD, myogenin, myf-5, and MRF4/herculin/myf-6, has added significantly to our understanding of skeletal myogenesis (1, 3, 10, 17, 49, 61, 80). Expression of each of these transcription factors appears to be sufficient to activate the skeletal muscle phenotype in many types of cultured cells (77), and hexanucleotide binding sites for these factors (CANNTG), termed E boxes, have been identified in most but not all skeletal muscle-specific transcriptional regulatory elements studied to date (reviewed in reference 71). bHLH proteins each contain a basic domain which is required for DNA binding and an HLH region which is involved in the formation of homo- and heterodimers (6, 9, 74). The basic domain of each myogenic bHLH family member contains conserved alanine and threonine residues that distinguish them from the nonmyogenic bHLH transcription factors (5, 9).

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Several skeletal muscle-specific transcriptional regulatory regions that lack functional bHLH binding sites have been identified (16, 45, 72). In addition, previous studies have suggested that additional transcription factors, including MEF-2 (23, 47, 48, 59, 82), M-CAT/TEF-1 (44, 81), CArG/SRF (26), and CACCC/Sp1 (11, 65) are also important in regulating skeletal muscle-specific transcription. Thus, although myogenic bHLH proteins play a pivotal role in skeletal myocyte differentiation, it is unclear whether the direct binding of bHLH transcription factors to skeletal muscle-specific transcriptional regulatory regions is required to activate the coordinate expression of all of the contractile protein genes during skeletal myogenesis, or alternatively whether skeletal muscle-specific transcription can be activated either by bHLH-independent regulatory pathways or, indirectly, by the bHLH-dependent activation of other myogenic transcription factors.

The troponin C (TnC) genes have been used as a model system with which to study transcriptional regulation during striated muscle development (20, 54, 55, 57, 67). TnC is the calcium-binding subunit of the myofibrillar thin filament that regulates excitation-contraction coupling in both skeletal and cardiac muscle (29, 38, 56). In contrast to most other myofibrillar proteins, there are only two mammalian isoforms of TnC, which are encoded by distinct single-copy genes (54, 55, 79, 86). In the adult mammal, the slow/cardiac TnC (cTnC) gene is expressed exclusively in cardiac and slow skeletal muscle (55). However, in the embryo, the cTnC gene is also expressed transiently in fast skeletal muscle (12). In contrast,

fast skeletal TnC (sTnC) is expressed exclusively in fast skeletal muscle throughout the life of the organism (20, 54). These complex patterns of expression suggest that precise molecular mechanisms have evolved to regulate TnC gene expression with respect to both its tissue and developmental specificity.

In the studies described in this report, we have used transient transfection assays, as well as nuclear protein binding assays, to define the *cis*-acting regulatory sequences and *trans*-acting factors that control cTnC gene expression during embryonic skeletal muscle development. Particular emphasis was placed on defining the role of the myogenic bHLH family members in regulating cTnC expression in skeletal muscle. Our results demonstrated that cTnC gene expression in embryonic skeletal myocytes is regulated by an evolutionarily conserved 145-bp transcriptional enhancer located within the first intron of the gene. This enhancer is skeletal muscle specific and is activated following the differentiation of skeletal myoblasts to myotubes. The enhancer contains three nuclear protein binding sites, i.e., a CACCC box, a MEF-2 binding site, and a novel nuclear protein binding site designated MEF-3, but lacks a consensus bHLH binding site. Despite the absence of a bHLH binding site, the cTnC skeletal muscle-specific enhancer can be transactivated by overexpression of MyoD or myogenin in C3H10T1/2 (10T1/2) cells. Taken together, these data demonstrate the existence of a novel indirect bHLH transcriptional transactivation pathway during skeletal myogenesis.

## 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 (55). HeLa, NIH 3T3 cells, human Jurkat T cells, clone 13 Epstein-Barr virus-transformed B cells, and K562 chronic myelogenous leukemia cells were grown as described previously (24). 10T1/2 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM)–20% fetal bovine serum–1% chicken embryo extract. Primary cultures of neonatal rat cardiac myocytes and fibroblasts were isolated and grown as described previously (57).

**Plasmids.** The promoterless plasmids pSV0CAT (21) and pCAT-Basic (Promega, Madison, Wis.) as well as plasmid pRSVCAT (21), containing the Rous sarcoma virus long terminal repeat (LTR), plasmid pSPCAT (41), containing the minimal simian virus 40 promoter, plasmid pUTKAT1 (60), containing the herpes simplex virus thymidine kinase promoter, and plasmid pcDNAIneo (Invitrogen, San Diego, Calif.), which expresses the neomycin resistance gene, have been described previously. The pEMSVMyoD eukaryotic expression vector (10), containing the Moloney sarcoma virus (MSV) LTR linked to the MyoD cDNA, was generously provided by Andrew Lassar and Harold Weintraub (Fred Hutchinson Cancer Center, Seattle, Wash.). The pEMSVmyogenin eukaryotic expression vector, containing the MSV LTR-linked myogenin cDNA, and the myogenin-E12 basic, myogenin BS2, and myogenin T-$D_{87}$ expression plasmids, containing mutations within the myogenin basic region, have been described previously (5). The MEF-2C expression vector pCMVMEF-2C was prepared by cloning the 1.6-kb coding and untranslated regions of the murine MEF-2C cDNA (46) into *Hin*dIII-*Xba*I-digested pCDNA I/A (Invitrogen). The pE102CAT-MEF 2x2 MEF-2 reporter plasmid was the generous gift of Yie-Teh Yu and Bernardo Nadal-Ginard and has been described previously (82). The pMSVβgal (15) and pRSVβgal (42) reference plasmids contain the β-galactosidase gene under the control of the MSV and Rous sarcoma virus LTRs, respectively.

p-2.2SV0CAT and p-124SV0CAT were constructed by subcloning the 2.2-kb *Bam*HI-*Alu*I (bp −2200 to +32) and the 156-bp *Bal*I-*Alu*I (bp −124 to +32) murine cTnC genomic subfragments (55) into the *Hin*dIII site of pSV0CAT in a 5′-to-3′ orientation with respect to the CAT gene. p-2.2SV0 CAT5.5, p-2.2SV0CAT2.3, p-2.2SV0CAT1.3Ba, p-2.2SV0 CAT1.3H, p-2.2SV0CAT650H, p-2.2SV0CAT650Ba, p-2.2S V0CAT1.0, p-2.2SV0CAT600, p-2.2SV0CAT308ENH, and p-2.2SV0CAT311 were constructed by subcloning the 5.5-kb *Sph*I-*Sal*I (containing the entire cTnC gene), the 2.3-kb *Sph*I-*Eco*RV (containing the entire first intron, bp −588 to +1765), the 1,238-bp *Bal*I (bp −124 to +1114), the 1,303-bp *Hin*dIII-*Eco*RV (bp 463 to 1765), the 652-bp *Hin*dIII-*Bal*I (bp 463 to 1114), the 651-bp *Bal*I-*Eco*RV (bp 1115 to 1765), the 1,037-bp *Hin*dIII-*Hin*cII (bp 463 to 1499), the 620-bp *Pst*I-*Hin*cII (bp 880 to 1499), the 308-bp *Pvu*II (bp 881 to 1188), and the 311-bp *Pvu*II-*Hin*cII (bp 1189 to 1499) fragments into the *Bam*HI site of p-2.2SV0CAT (3′ of the chloramphenicol acetyltransferase [CAT] gene) in a 5′-to-3′ orientation with respect to the CAT gene. p-2.2SV0CAT308ENHR was constructed by subcloning the 308-bp *Pvu*II fragment into the *Bam*HI site of p-2.2SV0CAT in a 3′-to-5′ orientation with respect to the CAT gene. pSPCAT308ENH and pUTKAT308ENH were constructed by subcloning the 308-bp *Pvu*II cTnC subfragment into the *Bam*HI sites of pSPCAT and pUTKAT, respectively (see Fig. 1A for schematic diagrams of these reporter plasmids). Plasmid p-124SV0CATMCKENH was constructed by subcloning the 291-bp *Sma*I-*Bst*EII human muscle creatine kinase (MCK) genomic subfragment (73), containing the MCK upstream enhancer (kindly provided by Joe Billadello, Washington University, St. Louis, Mo.), into the *Bam*HI site of plasmid p-124SV0CAT.

**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 (22, 54). Primary cultures of rat neonatal cardiac myocytes were transfected by using the Lipofectin reagent (Gibco/BRL, Gaithersburg, Md.) as described previously (57). For the MyoD and myogenin cotransfection experiments, $10^6$ 10T1/2 cells were cotransfected with 10 μg of the appropriate CAT reporter plasmid, 1 μg of the pRSVβgal reference plasmid, and either 2 μg of the pEMSVMyoD or pEMSV myogenin eukaryotic expression vector, respectively, or 2 μg of pUC18 control DNA as DNA-calcium phosphate precipitates as described above. Sixteen hours following transfection, the culture medium was changed to DMEM–10% equine serum, and cells were harvested 48 h later. Similarly, for the MEF-2C cotransfection experiments, $10^6$ 10T1/2 cells were cotransfected with 4 μg of the appropriate CAT reporter plasmid, 1 μg of the pRSVβgal reference plasmid, and either 16 μg of the pCMVMEF-2C (47) eukaryotic expression vector or 16 μg of pUC18 control DNA. Following transfection, cell lysates were prepared and normalized for protein content, using a commercially available kit (Bio-Rad, Richmond, Calif.). CAT and β-galactosidase assays were performed as described previously (35). 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 addition, 10T1/2 cells were converted to a myogenic phenotype by transfection with a MyoD and neomycin resistance genes. Stable transfectants of 10T1/2 cells that overexpress MyoD were produced by cotransfecting $5 \times 10^5$ 10T1/2 cells with 20 μg of plasmid pEMSVMyoD and 1 μg of plasmid

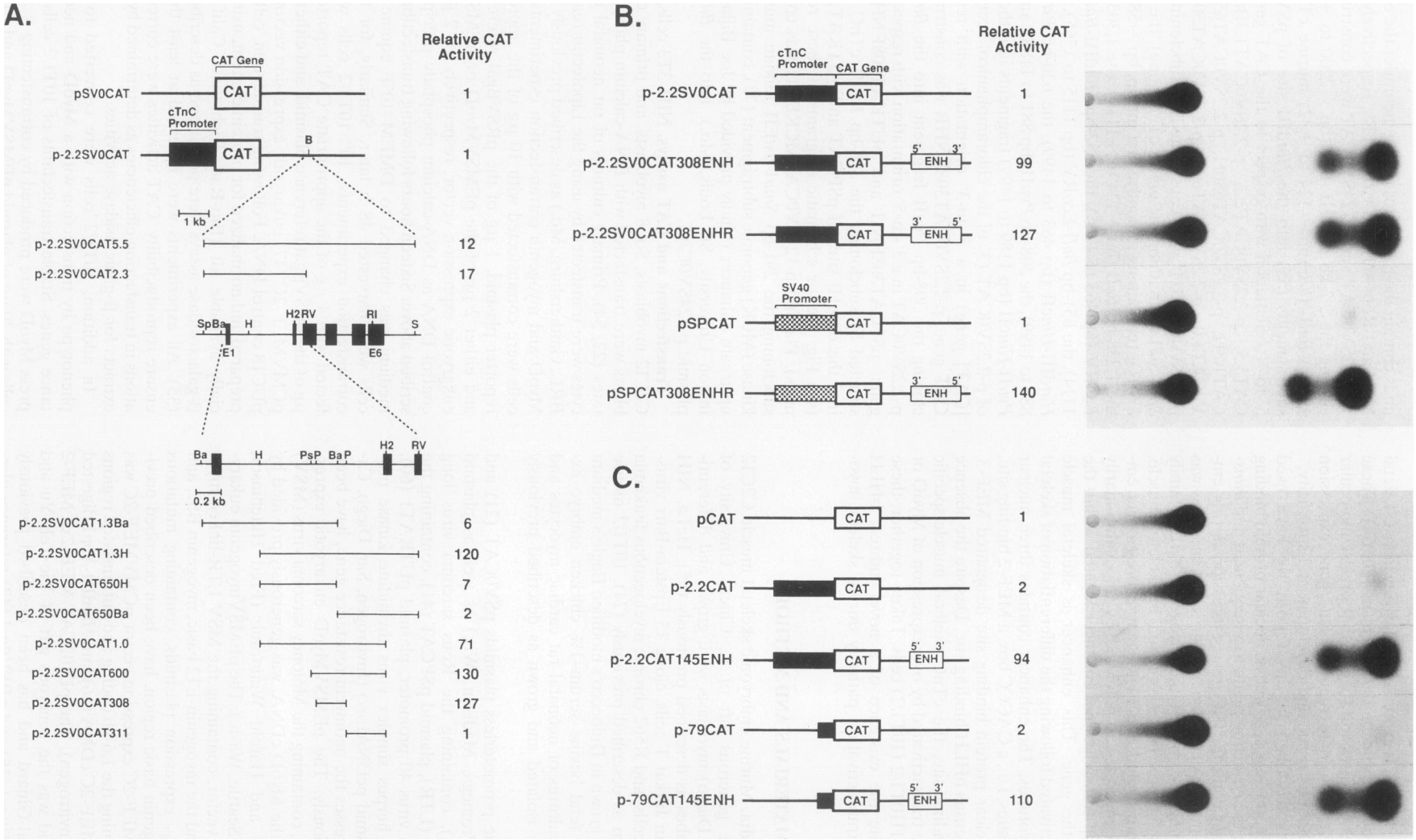
A.
Relative CAT Activity
CAT Gene
pSV0CAT
CAT
1
cTnC Promoter
p-2.2SV0CAT
CAT
B
1
1 kb
p-2.2SV0CAT5.5
12
p-2.2SV0CAT2.3
17
SpBa
H
H2
RV
RI
S
E1
E6
Ba
H
PsP
BaP
H2
RV
0.2 kb
p-2.2SV0CAT1.3Ba
6
p-2.2SV0CAT1.3H
120
p-2.2SV0CAT650H
7
p-2.2SV0CAT650Ba
2
p-2.2SV0CAT1.0
71
p-2.2SV0CAT600
130
p-2.2SV0CAT308
127
p-2.2SV0CAT311
1
B.
Relative CAT Activity
cTnC Promoter
CAT Gene
p-2.2SV0CAT
CAT
1
p-2.2SV0CAT308ENH
CAT
5' 3'
ENH
99
p-2.2SV0CAT308ENHR
CAT
3' 5'
ENH
127
SV40 Promoter
pSPCAT
CAT
1
pSPCAT308ENHR
CAT
3' 5'
ENH
140
C.
pCAT
CAT
1
p-2.2CAT
CAT
2
p-2.2CAT145ENH
CAT
5' 3'
ENH
94
p-79CAT
CAT
2
p-79CAT145ENH
CAT
5' 3'
ENH
110

FIG. 1. Identification and localization of a transcriptional enhancer element in the first intron of the murine cTnC gene. (A) Schematic representations of the promoterless plasmid pSV0CAT and plasmid p-2.2SV0CAT containing the murine cTnC promoter linked to the CAT gene are shown at the top. *Bam*HI (B), *Sph*I (Sp), *Bal*I (Ba), *Hin*dIII (H), *Eco*RV (RV), *Eco*RI (RI), *Sal*I (S), *Pst*I (Ps), *Pvu*II (P), and *Hin*cII (H2) restriction endonuclease sites are indicated. Filled boxes represent the six exons of the murine cTnC gene (E1 to E6). Overlapping restriction enzyme fragments from this clone (solid bars) were subcloned into the *Bam*HI site 3′ of the CAT gene in plasmid p-2.2SV0CAT. Fifteen micrograms of each cTnC-CAT reporter plasmid and 5 μg of the pMSVβgal reference plasmid were transfected into C2C12 myotubes, and CAT and β-galactosidase activities were determined as described in Materials and Methods. Relative CAT activities corrected for differences in transfection efficiencies are shown at right and represent the CAT activity produced by each plasmid normalized to the CAT activity produced by the control plasmid, pSV0CAT. All transfections were repeated at least three times. (B) Promoter independence of the cTnC first-intron transcriptional enhancer. The enhancerless control plasmids p-2.2SV0CAT and pSPCAT and the enhancer (ENH)-containing plasmids p-2.2SV0CAT308ENH, p-2.2SV0CAT308ENHR, and pSPCAT308ENHR, along with the pMSVβgal reference plasmid, were transfected into C2C12 myotubes as described above. Relative CAT activities corrected for differences in transfection efficiencies are shown at the right. (C) Deletion analysis of the cTnC promoter and first-intron enhancer. The promoterless control plasmid pCAT-Basic, as well as the promoter-containing plasmids p-2.2CAT and p-79CAT and the promoter/enhancer-containing plasmids p-2.2CAT145ENH and p-79CAT145ENH were transfected into C2C12 myotubes as described above. Relative CAT activities corrected for differences in transfection efficiency are shown at the right.

pcDNAINEO as calcium phosphate precipitates. Two days following transfection, cells were selected in growth medium supplemented with 520 μg of G418 (Gibco/BRL) per ml. G418-resistant colonies were split into replicate plates and assayed for myogenic differentiation by the ability to fuse into multinucleated myotubes (data not shown). One clone, designated clone A16, that differentiated into skeletal myotubes when grown at high density in differentiation medium (10% equine serum) was used to prepare nuclear extracts as described below.

**DNase I footprint analysis.** Nuclear extracts were prepared from C2C12 myoblasts and myotubes as described by Dignam et al. (14). The 145-bp (bp 997 to 1141) cTnC intron enhancer was labeled with $\alpha$-$^{32}$P-deoxynucleotides by using the Klenow fragment of DNA polymerase I and purified by polyacrylamide gel electrophoresis. DNase I footprint analyses were performed as described previously (35), using 75 to 100 μg of C2C12 nuclear extract. Reaction products were fractionated on 8% sequencing gels. Standard Maxam-Gilbert (G+A) sequencing reactions were run in parallel to identify protected sequences.

**In vitro transcription and translation.** The pMyoD/Bluescribe and pE12/Bluescribe vectors (51) were the generous gift of Case Murre and David Baltimore (Whitehead Institute, Cambridge, Mass.). In vitro transcription was carried out with 2 μg of linearized DNA and T3 RNA polymerase (Promega). In vitro translation was performed with a commercially available kit as instructed by the manufacturer (Promega).

**EMSAs.** The following complementary oligonucleotides were synthesized with *Bam*HI and *Bgl*II overhanging ends on an Applied Biosystems model 380B DNA synthesizer and used in electrophoretic mobility shift assay (EMSA) analyses as described previously (35): cTnC CACCC box (5′ TAACACT GCCCCACCCCCTGCAG 3′), cTnC mCACCC box (5′ TAA CACTGCCGATATCCCTGCAG 3′), myoglobin CACCC box (5′ CGCACAACCACCCCACCCCCTGTGG 3′), MCK CA CCC box (5′ TCACCCCCACCCCGGTGCA 3′), β-globin CACCC box (5′ TAGAGCCACACCCTGGTAA 3′), Sp1 (5′ CTAAAGGGGCGGGGCTTGGCCA 3′), cTnC MEF-2 (5′ TTAAAAATAGCTCAG 3′), cTnC mMEF-2 (5′ TTAAG ATATCCTCAG 3′), cTnC MEF-3 (5′ TGCCACCCTGGT CAGGTTACAGTGGGTGGCTTTG 3′), cTnC mMEF-3 CACCC box (5′ TGCTGCCCTGGTCAGGTTACAGTGGG CTGCTTTG 3′), cTnC mMEF-3 core (5′ TGCCACCCT GGATATCTATGAGTGGGTGGCTTTG 3′), and cTnC MEF-3 core (5′ CCTGGTCAGGTTACAGTGG). EMSAs were performed by using a Tris-glycine buffer system as described previously (35). All nuclear extracts were prepared as described by Dignam et al. (14).

EMSAs using the 145-bp cTnC intron enhancer fragment (bp 997 to 1141), the 167-bp human MCK *Sma*I-*Bgl*I restriction fragment (bp −932 to −766) containing both the right and left bHLH binding sites, or a double-stranded synthetic oligonucleotide corresponding to the murine $MCK_R$ high-affinity MyoD binding site (5′ CCCCCCAACACCTGCTGC CTGA 3′) (37) were performed as follows. Binding reactions were carried out for 30 min at room temperature and contained $2 \times 10^4$ dpm of radiolabeled DNA probe, 5 μl of in vitro-transcribed and translated protein, 500 ng of poly(dI-dC), 10 mM Tris, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. For cold competition experiments, 10 to 100 ng of unlabeled specific and nonspecific competitor DNA was included in the binding reactions. The complexes were separated on 5% nondenaturing polyacrylamide gels.

**In vitro mutagenesis.** In vitro mutagenesis was performed by site-directed oligonucleotide-mediated gapped heteroduplex

mutagenesis (35), using the following synthetic oligonucleotides (mutations are underlined): CACCC box (5′ GCAGATA ACACTGCCGATATCCCTGCATACCAAAGT 3′), MEF-2 (5′ AACTTTGTCCCCCTTAAGATATCTCAGTGGCCAC CCT 3′), and MEF-3 (5′ CAGTGGCCACCCTGGATATC TATGAGTGGGTGGCTTTGC 3′). A deletion mutant spanning bp 881 to 1114 of the 308-bp skeletal muscle-specific enhancer and lacking the MEF-3 binding site was prepared by digestion of the 308-bp enhancer fragment with *Bal*I.

## RESULTS

**cTnC gene expression in embryonic skeletal myotubes is regulated by a transcriptional enhancer located in the first intron of the gene.** Previous studies have demonstrated that cTnC gene expression is induced during the differentiation of embryonic C2C12 and Sol 8 skeletal myoblasts to myotubes in vitro (56). To functionally identify the *cis*-acting elements that regulate cTnC gene expression during embryonic skeletal muscle differentiation, C2C12 and Sol 8 cells were transiently transfected with a series of CAT reporter constructs containing various subfragments of the murine cTnC gene. As shown in Fig. 1A, plasmid p-2.2SV0CAT, containing 2.2 kb of cTnC 5′ flanking sequence (bp −2200 to +32) linked to the CAT reporter gene, was inactive in these cells, failing to increase transcription of the CAT reporter above levels obtained with the promoterless pSV0CAT control plasmid. Thus, the 5′ flanking region of the cTnC gene, which controls expression of the cTnC gene in cardiac muscle (57), does not contain the regulatory elements that are required to promote high-level transcription of the cTnC gene in skeletal myotubes. To identify the additional transcriptional regulatory elements that are necessary for high-level cTnC gene expression in skeletal myotubes, genomic subfragments of the cTnC gene were subcloned into the *Bam*HI site 3′ of the CAT gene in plasmid p-2.2SV0CAT and transfected into C2C12 myotubes. As shown in Fig. 1A, plasmid p-2.2SV0CAT308ENH, containing a 308-bp *Pvu*II fragment (bp 881 to 1188) from the first intron of the gene, consistently increased CAT transcription to levels 100-fold above those obtained with plasmid p-2.2SV0CAT alone. This 308-bp fragment was the only positive transcriptional regulatory element detected in these studies and functioned in either orientation when positioned 3′ of the cTnC promoter/CAT reporter gene cassette (Fig. 1B). Thus, it represents a bona fide transcriptional enhancer. This enhancer functioned equally well with 2.2- and 111-bp (bp −79 to +32) cTnC promoter fragments (Fig. 1C). Further deletion analysis of this element revealed that an internal 145-bp (bp 997 to 1141) subfragment contained full transcriptional enhancer activity (Fig. 1C).

To determine whether the first-intron cTnC enhancer required the cTnC promoter or, alternatively, could function with heterologous promoters, CAT reporter constructs containing the 308-bp cTnC transcriptional enhancer and the minimal simian virus 40 promoter (pSPCAT308ENHR) or herpes simplex virus thymidine kinase promoter (pUTKAT308 ENHR) were transfected into C2C12 and Sol 8 myotubes. In both cases, the cTnC enhancer increased CAT transcription by at least 100-fold (Fig. 1B and data not shown). Thus, the first intron of the murine cTnC gene contains a potent transcriptional enhancer that can function in concert with both the cTnC promoter and heterologous viral promoters in C2C12 skeletal myotubes.

**Developmental regulation and cell lineage specificity of the cTnC first-intron enhancer.** To determine whether the 145-bp cTnC first-intron enhancer mediates the developmentally regulated, skeletal muscle-specific pattern of cTnC gene expression, the transcriptional activities of the enhancer-containing plasmid p-2.2SV0CAT308ENH (Fig. 2) and the enhancerless plasmid p-2.2SV0CAT were compared following transfection into C2C12 and Sol 8 skeletal myoblasts and myotubes, primary neonatal cardiac myocytes, primary cardiac fibroblasts, and NIH 3T3, HeLa, and 10T1/2 cells (Fig. 2). Consistent with the results shown in Fig. 1, the enhancer was active in C2C12 and Sol 8 myotubes, increasing transcription by 50- to 100-fold. In contrast, no enhancer activity was detected in primary neonatal cardiac myocytes, skeletal myoblasts, or any of the nonmuscle cell lines studied. Similar results were obtained with CAT reporter constructs containing the minimal simian virus 40 promoter and the cTnC first-intron enhancer (57). Thus, the 145-bp cTnC first-intron enhancer element is skeletal muscle specific and is developmentally activated during the differentiation of embryonic skeletal myoblasts into myotubes.

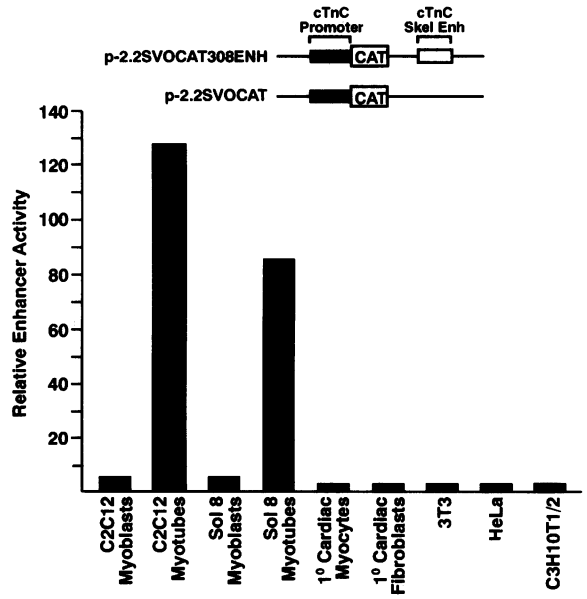


FIG. 2. Cell lineage specificity of activity of the cTnC first-intron enhancer. Fifteen micrograms of the enhancerless plasmid p-2.2SV0 CAT or the enhancer-containing plasmid p-2.2SV0CAT308ENHR along with 5 μg of the pMSVβgal reference plasmid were transfected into murine C2C12 myoblasts and myotubes, murine Sol 8 myoblasts and myotubes, primary neonatal rat cardiac myocytes, primary rat cardiac fibroblasts, 3T3 fibroblasts, HeLa cells, and murine 10T1/2 cells, and cell lysates were assayed for CAT and β-galactosidase activities. Relative CAT activity was calculated by comparing the activity of the enhancer-containing plasmid p-2.2SV0CAT308ENHR with that of the enhancerless plasmid p-2.2SV0CAT following correction for transfection efficiencies. The results were confirmed by three independent transfections into each cell line.

**Identification of a novel nuclear protein binding site designated MEF-3.** To identify nuclear protein binding sites within the 145-bp skeletal muscle-specific cTnC enhancer, DNase I footprint analyses were performed with nuclear extracts prepared from C2C12 myoblasts and myotubes. Three nuclear protein binding sites were identified in these experiments (Fig. 3A). A CACCC box (bp 1036 to 1051) (13) was footprinted with both C2C12 myoblast and myotube nuclear extracts. The cTnC CACCC box contained a flanking sequence (CCCCAC CCC) that is present in the transcriptional regulatory regions of many skeletal muscle genes, including the murine sTnC enhancer (54), quail troponin I enhancer (83), mouse and rat

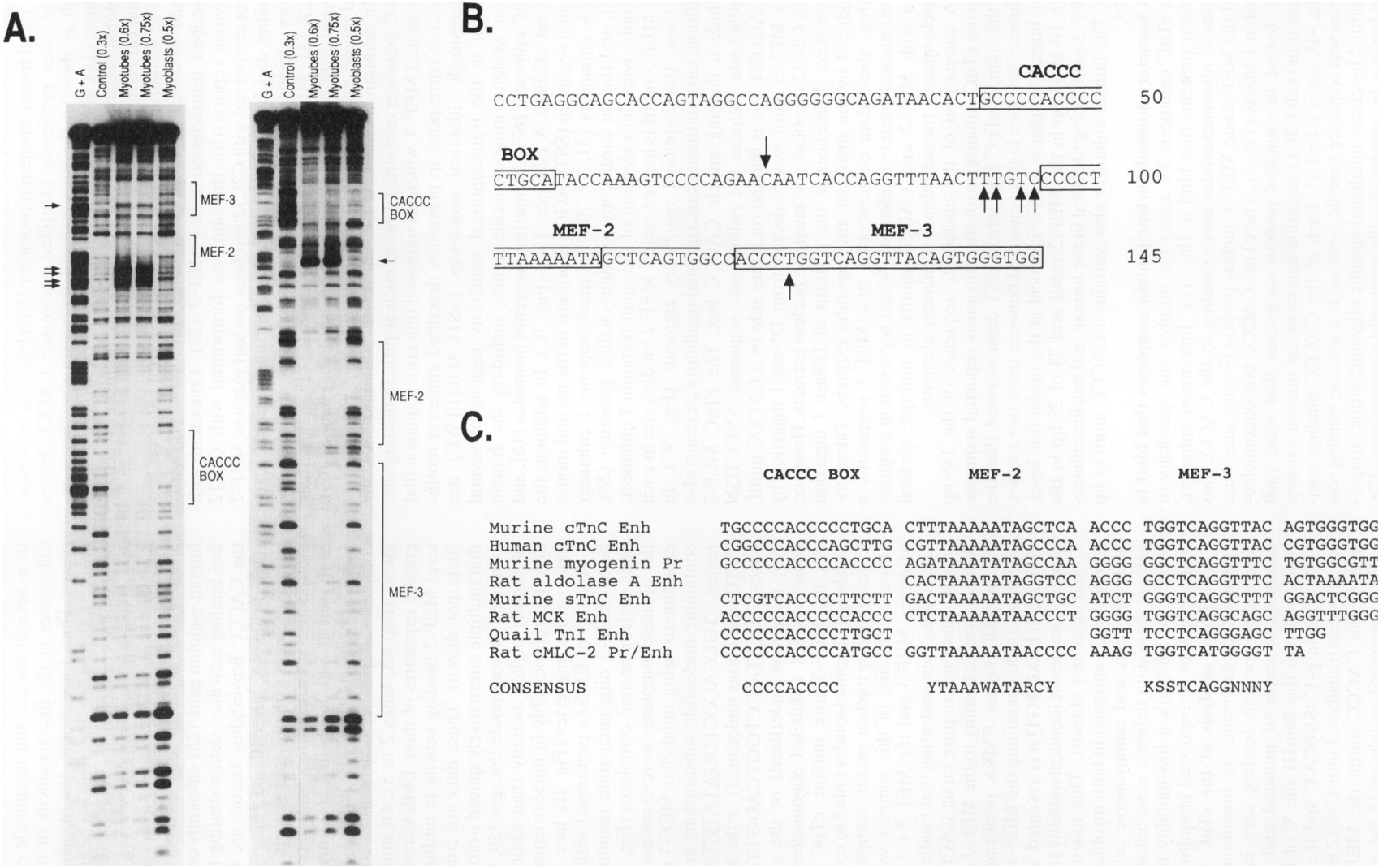


FIG. 3. DNase I footprint analysis of the murine cTnC first-intron skeletal-muscle specific enhancer. (A) DNase I footprint analyses of the cTnC skeletal muscle-specific transcriptional enhancer. The radiolabeled 145-bp cTnC enhancer fragment was incubated in the absence (control) or presence of C2C12 myoblast or C2C12 myotube nuclear extract before partial digestion with DNase I (1× DNase I = 25 U/ml); the sense (left) and antisense (right) strands are shown. Standard Maxam-Gilbert purine (G+A) sequencing reactions of the same fragments were run in parallel. Protected sequences are bracketed (CACCC box, MEF-2, and MEF-3), and DNase I-hypersensitive sites are denoted by arrows. (B) Nucleotide sequence of the cTnC skeletal muscle-specific enhancer. Nuclear protein binding sites identified by DNase I footprint analyses are overlined (sense strand) or underlined (antisense strand). DNase I-hypersensitive sites are indicated by arrows. (C) Comparison of the nucleotide sequences of the cTnC transcriptional enhancer protein binding sites with transcriptional regulatory sequences identified in previously characterized skeletal muscle genes: murine cTnC enhancer (Enh), human cTnC enhancer (67), murine myogenin promoter (Pr) (16), rat aldolase A enhancer (30), murine sTnC enhancer (54), rat MCK enhancer (31), quail troponin I (TnI) intron enhancer (83), and rat cardiac myosin light-chain 2 (cMLC-2) promoter/enhancer (28).

MCK 5′ enhancers (31, 33, 70), human myoglobin enhancer (11), and mouse myogenin promoter (16) (Fig. 3C), but absent from other previously identified nonmuscle CACCC boxes, such as those in the human porphobilinogen deaminase enhancer (18), the human β-globin enhancer (50), the human γ-globin enhancer (25), and the human tryptophan oxygenase enhancer (68).

The second footprint in the cTnC first-intron enhancer was found to contain a consensus binding site for the previously described transcription factor MEF-2 (23). Consistent with previous reports (8), this site was footprinted with C2C12 myotube but not with C2C12 myoblast nuclear extracts. MEF-2 binding sites have been identified in the transcriptional regulatory regions of many skeletal muscle genes, including the MCK (31, 33, 70, 73), myosin light-chain 1/3 (15, 62), cardiac myosin light-chain 2A (4, 28, 64), myosin light-chain 3f (69), and mouse myogenin (16) genes (Fig. 3C).

In addition to the CACCC and MEF-2 binding sites, the cTnC enhancer contained a novel nuclear protein binding site, designated MEF-3, which is composed of a core sequence that does not correspond to previously described enhancer motifs. Interestingly, identical sequences (SSTCAGGTTWC) are present in the transcriptional regulatory regions of several other skeletal muscle-specific genes, including the human cTnC enhancer (67), the murine myogenin promoter (16), and the rat aldolase A enhancer (30) (Fig. 3C). In addition, the related sequence (SSTCAGG) is present in the rat and mouse MCK enhancers (31, 33, 70), the quail troponin I enhancer (83), and the rat cardiac myosin light-chain 2 promoter (28). Of note, with the exception of the quail troponin I enhancer, the MEF-3 motif is located near a MEF-2 binding site in each of these transcriptional regulatory elements (Fig. 3C). In addition, most of these transcriptional regulatory elements also include a flanking muscle CACCC box. The cTnC MEF-3 binding site includes the palindromic sequence ANCCTGN NCAGGNT and is flanked on either side by a CACCC motif. The MEF-3 motif was protected from DNase I digestion by both C2C12 myoblast and myotube nuclear extracts. However, there were differences in the patterns of DNase I digestion produced by the myoblast and myotube extracts. Taken together, these data demonstrated that MEF-3 represents a novel nuclear protein binding site which is present in conjunction with MEF-2 and CACCC motifs in multiple skeletal muscle-specific transcriptional regulatory elements.

A comparison of the nucleotide sequence of the 145-bp murine cTnC enhancer with the previously reported sequence of the human cTnC first intron (67) demonstrated that the enhancer has been highly conserved between these two species. The human sequence is 86% identical at the nucleotide level, with 10 of 11 bp in the MEF-2 motif and 24 of 25 bp in the MEF-3 motif being identical in the human and murine enhancers. The human enhancer also contains a CACCC motif at bp 1215.

**Characterization of nuclear protein complexes that bind to the cTnC enhancer.** To assess the number and specificity of nuclear proteins that bind to the cTnC first-intron enhancer, synthetic oligonucleotides corresponding to the CACCC box and the MEF-2 and MEF-3 nuclear protein binding sites were used in EMSAs (Fig. 4). The cTnC CACCC box oligonucleotide bound five nuclear protein complexes that were each present in both C2C12 myotube and myoblast nuclear extracts (Fig. 4B, lanes 1 and 2). Each of the CACCC binding complexes was sequence specific, because in each case binding was inhibited by excess unlabeled specific oligonucleotide competitor but not by nonspecific oligonucleotide competitors (Fig. 4A, lanes 3 to 11, and data not shown). Competition experiments using unlabeled CACCC box and Sp1 oligonucleotides from several muscle and nonmuscle enhancers demonstrated differences in the fine specificity of binding of the five complexes. Thus, for example, the myoglobin and MCK CACCC boxes, which share the flanking sequence CCCCACCCCC, successfully competed for binding of four of the five complexes, while the β-globin CACCC box efficiently competed for only one of the five nuclear protein complexes (Fig. 4A, lanes 8 and 9). Of note, an unlabeled Sp1 binding site from the human γ-globin gene (25) efficiently competed for three of the five binding activities, demonstrating that some but not all of the CACCC box-binding factors are likely to be Sp1 or Sp1 related (Fig. 4A, lanes 10 and 11).

In agreement with previous reports (8, 23), the cTnC MEF-2 motif bound specifically to a protein complex (arrows) that was expressed at high levels in C2C12 myotubes but absent from C2C12 myoblasts (Fig. 4B, lanes 1 and 2). Binding of this complex was specific, as demonstrated by competition experiments (Fig. 4A, lanes 2 to 5). Of note, prolonged autoradiographic exposures of these EMSAs revealed low- to moderate-level MEF-2 binding activity in multiple nonmuscle cell lines (data not shown). These data are consistent with the finding that multiple alternatively spliced isoforms of MEF-2/rSRF are expressed in nonmuscle lineages (59, 82). In addition, a band of slightly higher mobility than skeletal muscle MEF-2 was detected reproducibly in nuclear extracts prepared from neonatal cardiac myocytes (Fig. 4B, lane 8).

A series of EMSAs performed with the full-length radiolabeled MEF-3 oligonucleotide demonstrated three predominant nuclear protein complexes (Fig. 4A, lane 1). The identities of these complexes were examined further by competition experiments using unlabeled MEF-3 mutant oligonucleotides containing nucleotide substitutions in either the MEF-3 core region (TGCCACCCTGGATATCTATGAGTGGGGTGGC TTTG; mutations underlined) or the MEF-3 flanking CACCC boxes (TGCTGCCCTGGTCAGGTTACAGTGGGCTGCTT TG; mutations underlined) or the cTnC CACCC box and consensus Sp1 unlabeled oligonucleotides. As shown in Fig. 4A, the MEF-3 core mutant, cTnC CACCC box, and Sp1 unlabeled oligonucleotides failed to compete for binding of the lowest-mobility nuclear protein complex (arrow, lanes 6 to 11). However, binding of this complex was competed for by the wild-type MEF-3 and the MEF-3 CACCC box mutant oligonucleotides, demonstrating that binding of this nuclear protein complex does not require intact CACCC elements but instead reflects specific binding to the MEF-3 core element (lanes 2 to 5). In addition, an EMSA performed with a radiolabeled oligonucleotide containing the MEF-3 core sequence (CCTG-GTCAGGTTACAGTGG) revealed a single specific nuclear protein complex of identical mobility (arrow, Fig. 4B, lanes 1 to 7, and data not shown). This low-mobility MEF-3 binding complex was expressed in both myoblasts and myotubes and in both myogenic and nonmyogenic cells (Fig. 4B). Taken together, these data define a novel nuclear protein binding site, MEF-3, which binds a low-mobility nuclear protein complex that appears to be expressed ubiquitously.

**Functional analysis of the cTnC skeletal muscle-specific enhancer.** To determine the functional significance of each of the nuclear protein binding sites in the first-intron cTnC enhancer, a series of mutated and deleted enhancers was subcloned into the *Bam*HI site 3′ of the cTnC promoter and CAT gene in p-124SV0CAT, and the resulting reporter constructs were transfected into C2C12 myotubes (Fig. 5). Mutation of the CACCC motif, the MEF-2 binding site, or the MEF-3 core motif or deletion of the entire MEF-3 binding site (including the flanking CACCC boxes) abolished the activity of

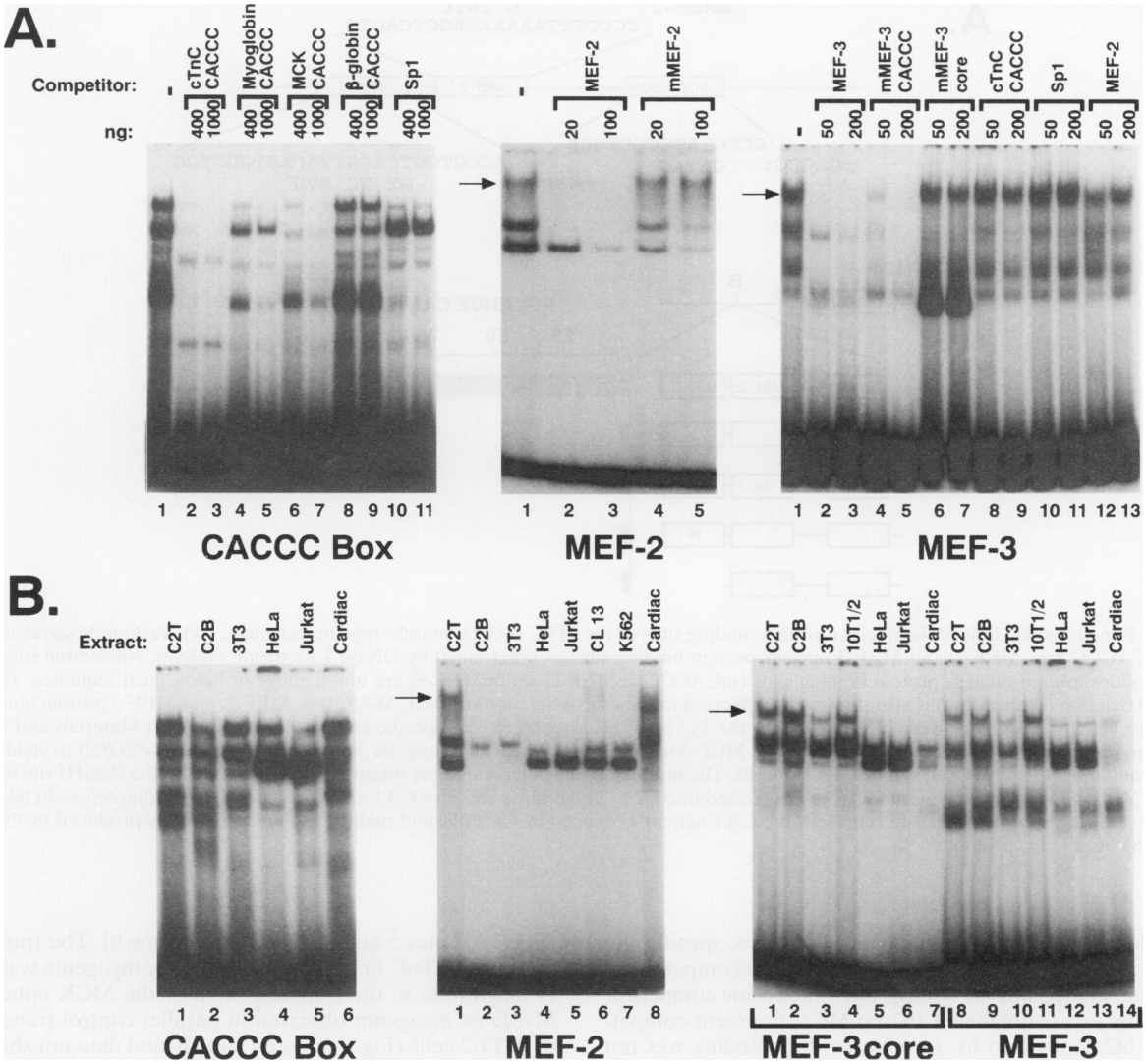


FIG. 4. EMSA analyses of the murine cTnC first-intron enhancer nuclear binding proteins. (A) Identification and specificity of nuclear protein complexes that bind to the cTnC CACCC box, MEF-2, and MEF-3 motifs. Radiolabeled, double-stranded synthetic oligonucleotides corresponding to the murine cTnC enhancer CACCC box, MEF-2, and MEF-3 binding sites (Fig. 3) were incubated with nuclear extracts prepared from C2C12 myotubes, and the resulting complexes were resolved by electrophoresis in nondenaturing 5% polyacrylamide gels; 20 to 1,000 ng of the indicated unlabeled competitor oligonucleotides was included in binding reactions containing C2C12 myotube nuclear extract and radiolabeled cTnC CACCC box, MEF-2, and MEF-3 oligonucleotides. The low-mobility MEF-2 and MEF-3 binding activities are denoted by arrows (see text). (B) Lineage specificity of nuclear protein binding. Radiolabeled, double-stranded synthetic oligonucleotides corresponding to the murine cTnC enhancer CACCC box, MEF-2, MEF-3 core, and MEF-3 binding sites were incubated with nuclear extracts prepared from C2C12 myotubes and C2C12 myoblasts, NIH 3T3 fibroblasts, HeLa cells, Jurkat T cells, clone (CL) 13 B cells, K562 chronic myelogenous leukemia cells, and neonatal rat heart cells, and EMSAs were performed as described above. The MEF-2 and MEF-3 binding activities are denoted by arrows. Autoradiograms were scanned with a Sharp JX-600 scanner into a Macintosh Quadra 700 computer, using Adobe Photoshop software and printed on a Rasterops CorrectPrint 300 printer.

the enhancer (Fig. 5). Thus, the CACCC, MEF-2, and MEF-3 core motifs are each required for the activity of the enhancer.

**The MyoD-E12 protein complex does not bind to the cTnC skeletal muscle-specific transcriptional enhancer.** Most but not all previously characterized skeletal muscle-specific transcriptional enhancers contain at least one binding site (CANNTG) for the bHLH family of transcription factors. Thus, it was somewhat surprising that the DNA sequence and DNase I footprint analyses of the cTnC first-intron enhancer failed to identify such a consensus bHLH binding site. To determine whether the cTnC enhancer can directly bind bHLH transcription factors, we performed a series of EMSAs utilizing in vitro-transcribed and translated MyoD and E12 proteins. Both a radiolabeled synthetic oligonucleotide corresponding to the murine MCK ($MCK_R$) high-affinity bHLH binding site (37) (Fig. 6A, arrow, lanes 1 and 2) and the radiolabeled 167-bp human MCK *Sma*I-*Bgl*I promoter fragment (73), containing both the high- and low-affinity bHLH binding sites, bound a single MyoD-E12 protein complex (Fig. 6B, arrow, lanes 1 to 3). In contrast, the radiolabeled 145-bp cTnC transcriptional enhancer fragment failed to bind MyoD and E12 proteins (Fig. 6B, lanes 4 to 6). Binding of MyoD and E12

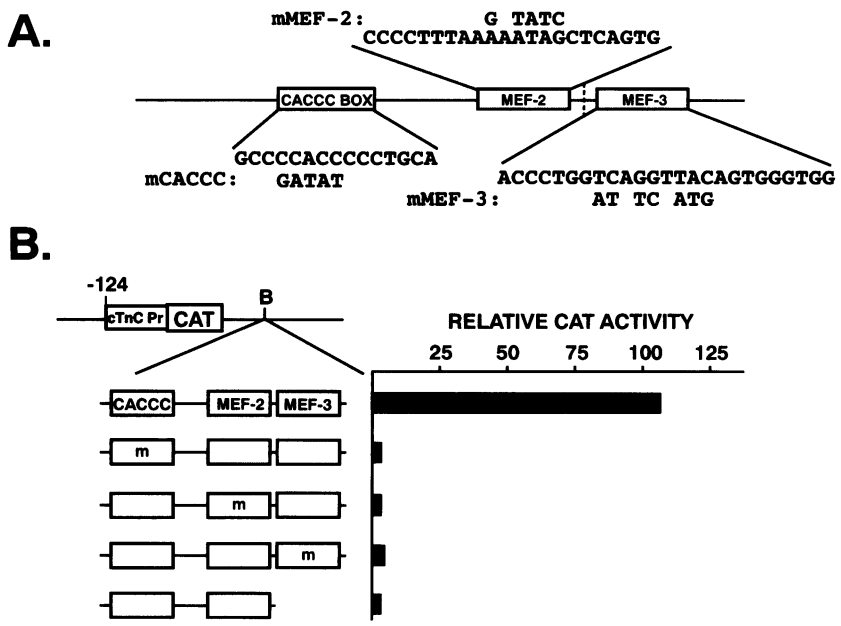


FIG. 5. Functional analysis of the nuclear protein binding sites of the cTnC skeletal muscle-specific enhancer. (A) Nucleotide sequences of the wild-type CACCC box, MEF-2, and MEF-3 nuclear protein binding sites as determined by DNase I footprint analyses. Nucleotide substitutions used to produce mutant nuclear protein binding sites (mCACCC, mMEF-2, and mMEF-3) are noted above or below each sequence. The site of the MEF-3 deletion (dashed line) is also shown. (B) Effects of mutations or deletions of the CACCC box, MEF-2, and MEF-3 protein binding sites on enhancer activity. Mutations were introduced into the 145-bp cTnC skeletal muscle-specific enhancer as described in Materials and Methods. Mutated binding sites are indicated by a boxed m. The MEF-3 motif was deleted by digesting the 308-bp cTnC enhancer with *Ball* to yield a 233-bp fragment containing the CACCC box and MEF-2 motifs. The mutated and deleted enhancer fragments were cloned into the *Bam*HI site of plasmid p-124SV0CAT, and the resulting plasmids were transfected into C2C12 myotubes. Relative CAT activities, corrected for differences in transfection efficiencies, are shown at the right and represent the CAT activity produced by each plasmid relative to the CAT activity produced by the control plasmid, pSV0CAT. Pr, promoter.

to the radiolabeled $MCK_R$ oligonucleotide was specific because the formation of this complex (arrow) was competed for by excess unlabeled murine $MCK_R$ oligonucleotide competitor as well as by excess unlabeled 167-bp MCK fragment competitor (Fig. 6C, lanes 1 to 6). In contrast, this binding was not inhibited efficiently by the unlabeled 145-bp cTnC transcriptional enhancer fragment or the 156-bp core cTnC promoter, again demonstrating the absence of a bHLH binding site in this enhancer (Fig. 6C, lanes 7 to 10). The small amount of competition observed with the unlabeled cTnC promoter and enhancer fragments represented nonspecific competition in that it was also observed with unrelated competitor DNAs of similar sizes (data not shown). In summary, unlike the MCK enhancer, the cTnC skeletal muscle-specific transcriptional enhancer does not bind MyoD-E12 protein complexes.

**The cTnC skeletal muscle-specific enhancer is transactivated by MyoD and myogenin in 10T1/2 cells.** To determine whether cTnC gene expression in skeletal muscle is activated by a bHLH-independent transcriptional pathway or, alternatively, is activated indirectly by bHLH proteins in skeletal myocytes, murine 10T1/2 fibroblasts were cotransfected with a cTnC-CAT reporter construct and either a MyoD or myogenin eukaryotic expression vector (Fig. 7). Overexpression of MyoD or myogenin failed to transactivate the promoterless plasmid pSV0CAT or the p-124SV0CAT vector, containing the cTnC promoter linked to the CAT gene (Fig. 7A, lanes 1 to 4, and data not shown). In contrast, transfection of the MyoD or myogenin expression vector with plasmid p-124SV0CAT 145ENH, containing the cTnC skeletal muscle-specific enhancer, resulted in an 8- to 10-fold induction in CAT activity (Fig. 7A, lanes 5 and 6, and data not shown). The transactivation of the cTnC first-intron enhancer by myogenin was similar in magnitude to the transactivation of the MCK enhancer by MyoD or myogenin observed in parallel control transfections of 10T1/2 cells (Fig. 7A, lanes 7 and 8, and data not shown). In addition, transfection of either the MyoD or myogenin expression vector with the plasmid pUTKAT145ENH, containing the herpes simplex virus thymidine kinase promoter and the cTnC enhancer, resulted in a 10- to 15-fold transactivation (Fig. 7B and data not shown). Thus, the cTnC skeletal muscle-specific enhancer, which lacks a MyoD binding site, can be indirectly transactivated by overexpression of MyoD or myogenin in 10T1/2 cells.

To determine whether the indirect activation of the cTnC intragenic enhancer required binding of the bHLH factors directly to DNA, the cotransfection experiments were repeated with a series of expression plasmids encoding mutant forms of myogenin that fail to bind DNA ($T\text{-}D_{87}$ and BS2 mutants) or that bind DNA but fail to activate muscle transcription (myogenin-E12 basic) (5). As shown in Fig. 8, cotransfection of the cTnC-CAT reporter construct with plasmids encoding either the myogenin-E12 basic mutant, in which the basic region of myogenin is replaced with that of E12, which binds DNA with wild-type affinity but is devoid of myogenic activity, the $T\text{-}D_{87}$ mutant, which contains an aspartic acid residue in place of threonine 87 in the center of the DNA binding domain and dimerizes normally but fails to bind DNA, or the BS2 mutant, which lacks the second cluster of residues in the myogenin basic domain and cannot bind DNA, resulted in a 90% decrease in CAT activity relative to cotransfection with the

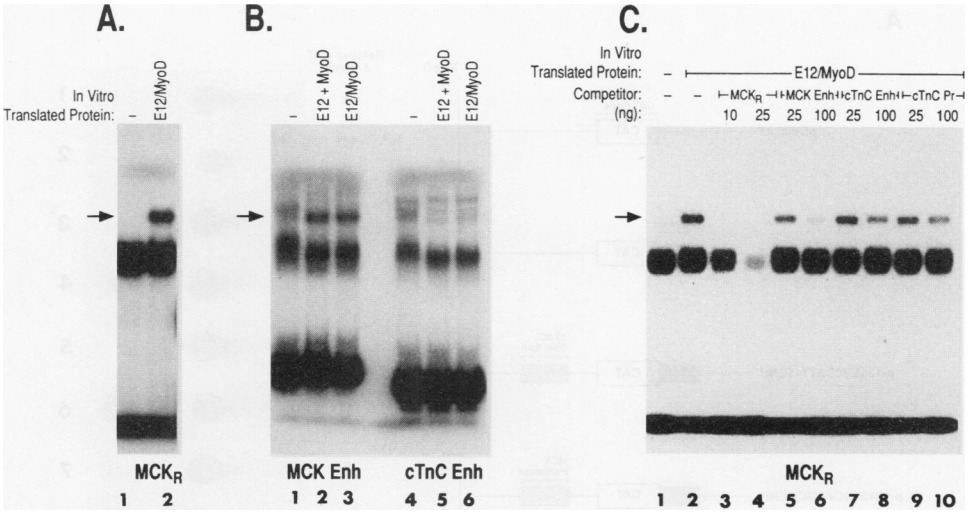


FIG. 6. EMSA analysis of bHLH binding sites in the MCK and cTnC skeletal muscle-specific enhancers. (A) Binding of in vitro-transcribed and translated E12 and MyoD proteins to a synthetic oligonucleotide containing the high-affinity MyoD binding site from the murine MCK enhancer. A radiolabeled synthetic oligonucleotide containing the high-affinity MyoD binding site from the MCK enhancer was used in EMSAs with reticulocyte lysates programmed with either water (–) or a combination of in vitro-transcribed E12 and MyoD RNAs (E12/MyoD). The band corresponding to E12-MyoD protein complexes is denoted with an arrow. (B) Binding of E12-MyoD complexes to the wild-type MCK and cTnC skeletal muscle-specific enhancer fragments. Radiolabeled enhancer fragments from the MCK and cTnC enhancers were used in EMSAs with in vitro-translated E12 and MyoD proteins. The E12 and MyoD proteins were either translated separately and mixed (E12 + MyoD) or cotranslated (E12/MyoD). Control translations lacking RNA were included in each experiment (–). (C) Cold competition experiments. EMSAs using a radiolabeled high-affinity MyoD binding site from the MCK enhancer (MCK$_R$) and in vitro-cotranslated E12 plus MyoD proteins were performed as described for panel A. Some reactions contained 10 to 100 ng of the following unlabeled competitor DNAs: (i) the synthetic oligonucleotide containing the high-affinity binding site from the murine MCK enhancer (MCK$_R$), (ii) the 167-bp MCK enhancer containing two MyoD binding sites (MCK Enh), (iii) the 145-bp first intron cTnC enhancer (cTnC Enh), or the 156-bp fragment from the cTnC promoter (bp −124 to +32) (cTnC Pr). The arrow denotes the band corresponding to E12-MyoD binding.

wild-type myogenin expression plasmid. These data demonstrated that both the DNA binding and transcriptional activation functions of myogenin are required for the indirect transactivation of the cTnC gene.

**MyoD-mediated transactivation of the cTnC enhancer correlates with changes in the patterns of CACCC, MEF-2, and MEF-3 DNA binding activities.** As discussed above, the cTnC skeletal muscle-specific transcriptional enhancer contains three nuclear protein binding sites: a CACCC box, a MEF-2 binding site, and a MEF-3 motif. Thus, it was of interest to determine whether the overexpression of MyoD in 10T1/2 cells and the resulting transactivation of the first-intron cTnC enhancer were associated with changes in the nuclear protein complexes that bind these sites. Toward this end, we performed a series of EMSAs using nuclear extracts prepared from wild-type 10T1/2 fibroblasts and from myoblasts and myotubes that had been produced by stable transfection of 10T1/2 cells with a MyoD expression vector. As shown in Fig. 9, EMSAs performed with a radiolabeled MEF-3 probe showed a down-regulation in the low-mobility MEF-3 binding activity following the overexpression of MyoD in 10T1/2 cells. EMSAs using a radiolabeled cTnC CACCC box oligonucleotide demonstrated several changes. First, we observed the induction of a high-mobility CACCC box-binding complex following MyoD transfection (lower arrow). In addition, there was a decrease in the relative abundance of the low-mobility Sp-1-related CACCC box-binding complex (upper arrow). However, it should be noted that these changes were observed in both MyoD-transfected 10T1/2 myoblasts and myotubes. Thus, it seems unlikely that they alone explain the induction of the cTnC first-intron enhancer, which was observed only in the MyoD-transfected 10T1/2 myotubes. Finally, EMSAs using the radiolabeled MEF-2 probe demonstrated that overexpression of MyoD in 10T1/2 cells was associated with the induction of the MEF-2 binding activity, which was observed only following differentiation of these cells into myotubes (dashed arrow). This pattern of MEF-2 induction correlated with the MyoD-mediated transactivation of the first-intron cTnC enhancer.

**Overexpression of MEF-2C does not activate the cTnC enhancer.** The finding that the transactivation of the cTnC enhancer by overexpression of MyoD correlated with the induction of a MEF-2 DNA binding activity was consistent with a model in which expression of bHLH family members induces the expression of MEF-2 (and possibly CACCC box) DNA binding activities, which in turn bind to and transactivate the cTnC enhancer. To determine whether expression of MEF-2C is sufficient to transactivate the cTnC enhancer, 10T1/2 cells were cotransfected with the p-124SV0CAT145 ENH cTnC enhancer reporter plasmid and the MEF-2C expression vector pCMVMEF-2C. As shown in Fig. 10, overexpression of MEF-2C failed to transactivate the cTnC enhancer in 10T1/2 cells. In contrast, MEF-2C expression produced 11-fold transactivation of a control MEF-2 reporter plasmid (pE102CAT-MEF 2x2) containing two copies of the MEF-2 binding site 5′ of the minimal embryonic myosin heavy-chain promoter (82). These results demonstrated that the induction of MEF-2C alone is insufficient to account for the bHLH-mediated transactivation of the cTnC enhancer.

## DISCUSSION

The striated muscle lineages, cardiac and fast and slow skeletal, are each derived from mesodermal precursor cells. Myocytes from each lineage express overlapping but distinct

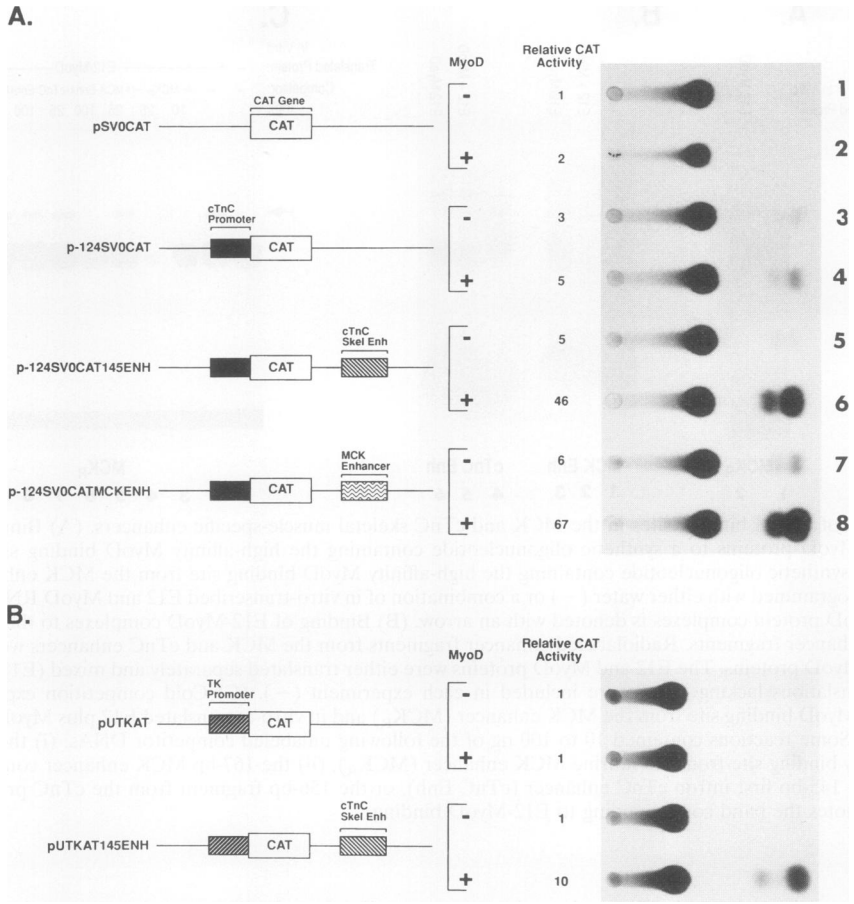


FIG. 7. Overexpression of MyoD or myogenin transactivates the murine cTnC skeletal muscle-specific transcriptional enhancer. Ten micrograms of the cTnC-CAT reporter plasmids (left) and 1 μg of the pRSVβgal reference plasmid were cotransfected with either 2 μg of the pEMSVMyoD eukaryotic expression vector (MyoD+) or 2 μg of pUC18 control DNA (MyoD−) into 10T1/2 cells; 16 h posttransfection, cells were placed into differentiation medium (DMEM–10% equine serum). Cells were harvested after 48 h, and cell lysates were analyzed for CAT and β-galactosidase activities. CAT activities, corrected for differences in transfection efficiencies, were normalized to the CAT activity produced by the promoterless control vector pSV0CAT. Autoradiograms from representative experiments are shown on the right. Each transfection was repeated at least three times.

sets of muscle-specific genes and display distinct contractile phenotypes. The molecular mechanisms regulating muscle-specific gene expression in the different muscle lineages have only recently begun to be elucidated. We have used the cTnC gene as a model system for understanding the molecular mechanisms that regulate myofibrillar gene expression during cardiac and fast skeletal muscle cell development. We have reported previously that cTnC expression in cardiac myocytes is regulated by a cardiac muscle-specific promoter/enhancer located in the 5′ flanking region of the gene (57). In this report, we have demonstrated that expression of the cTnC gene in embryonic skeletal myocytes is controlled by a distinct, developmentally regulated, skeletal muscle-specific transcriptional enhancer located within the first intron of the cTnC gene. This transcriptional enhancer contains three functionally important nuclear protein binding sites: a CACCC box (13), MEF-2 motif (23), and a novel nuclear protein binding site designated MEF-3. This skeletal muscle-specific intragenic enhancer does not contain a binding site for the myogenic family of bHLH transcription factors but can be transactivated by overexpression of MyoD or myogenin in 10T1/2 cells. Transactivation of the cTnC enhancer by MyoD is associated with the induction of a MEF-2 DNA binding activity and with changes in CACCC and MEF-3 binding activities. However, overexpression of MEF-2C alone is not sufficient to activate this downstream myogenic pathway in 10T1/2 cells. These findings have a number of implications for our understanding of the transcriptional mechanisms underlying muscle development.

**Distinct lineage-specific transcriptional enhancers control the expression of a single gene in cardiac and fast skeletal muscle.** A number of myofibrillar protein genes, including the cTnC gene, are expressed in multiple striated muscle cell lineages. Moreover, some but not all muscle transcription factors have been shown to be expressed in both fast skeletal and cardiac muscle. For example, the MEF-2, M-CAT, and CArG transcription factors are expressed in both lineages, whereas expression of the myogenic bHLH family members is restricted to skeletal myocytes. Given these findings, it remains unclear whether the coexpression of myofibrillar genes in distinct muscle lineages is regulated by common, distinct, or overlapping sets of muscle-specific transcription factors. The findings described in this report demonstrate that at least in the case of the cTnC gene, expression in fast skeletal and cardiac lineages is controlled by distinct and independent

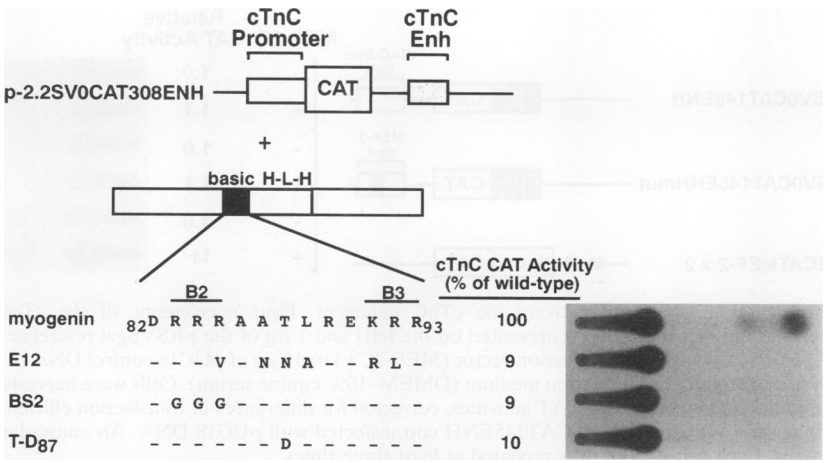


FIG. 8. Mutation of the myogenin basic domain abolishes transactivation of the cTnC skeletal muscle-specific transcriptional enhancer. Ten micrograms of the cTnC promoter/enhancer (Enh)-containing plasmid p-2.2SV0CAT308ENH and 1 μg of the pRSVβgal reference plasmid were cotransfected with either 2 μg of the myogenin expression plasmid pEMSVmyogenin or the myogenin mutant plasmids myogenin-E12 basic, myogenin BS2, and myogenin T-$D_{87}$, respectively. The myogenin basic domain amino acid substitutions are shown at the lower left. Dashes indicate conserved amino acids. CAT activities, normalized for differences in transfection efficiencies, were normalized to the CAT activity produced following cotransfection with the myogenin expression plasmid pEMSVmyogenin. An autoradiogram from a representative experiment is shown on the right. Each transfection was repeated in duplicate at least three times.

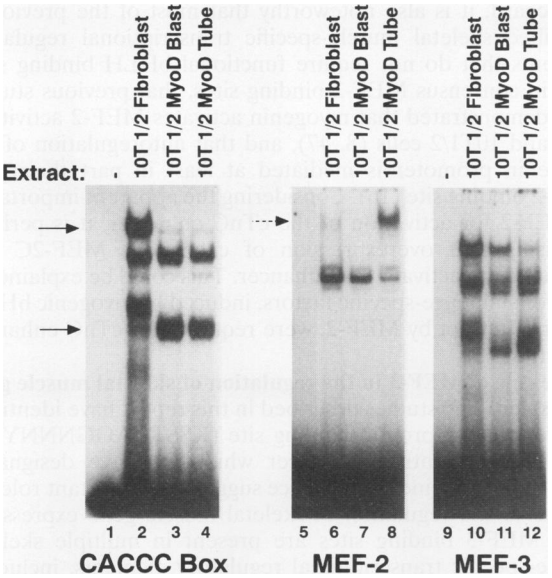


FIG. 9. EMSA analysis of the nuclear proteins that bind to the cTnC transcriptional enhancer in 10T1/2 cells that overexpress MyoD. Radiolabeled, double-stranded synthetic oligonucleotides corresponding to the murine cTnC enhancer CACCC box, MEF-2, and MEF-3 binding sites (Fig. 3) were incubated with nuclear extracts prepared from 10T1/2 cells (10T1/2 Fibroblast) and from 10T1/2 cells stably transfected with MyoD and maintained as myoblasts (10T1/2 MyoD Blast) and following differentiation into myotubes (10T1/2 MyoD Tube), and the resulting complexes were resolved by electrophoresis in nondenaturing 5% polyacrylamide gels. The high-mobility CACCC box binding activity is denoted by the lower arrow. The low-mobility CACCC box binding Sp1-related complex is denoted by the upper arrow. The MEF-2 binding activity is denoted by a dashed arrow. Autoradiograms were scanned and printed as described for Fig. 4.

transcriptional regulatory elements. Moreover, a comparison of the nuclear protein binding sites in the cardiac and skeletal muscle-specific cTnC enhancers revealed that they appear to share only one nuclear protein binding site: each contains at least one CACCC box. In contrast, the remainder of the nuclear protein binding sites in these two elements appear to be distinct, suggesting that they are regulated by different sets of lineage-specific transcription factors.

Other studies have also suggested that overlapping but distinct sets of transcription factors differentially regulate the expression of genes which are expressed in both cardiac and skeletal muscle. Iannello and coworkers (32) reported that transcription of the chicken cTnT gene in myocardial cells requires 5′ flanking sequences not essential for cTnT expression in embryonic skeletal muscle cells. Similarly, Jaynes and coworkers (33) identified a 5′ flanking region of the MCK gene that mediates a 200-fold increase in transcriptional activity in cardiac muscle but is inactive in skeletal muscle as assayed in transgenic mice. Finally, Lee et al. (40) demonstrated that the *cis*-acting elements that control expression of the rat cardiac myosin light-chain 2 gene in cardiac and slow skeletal muscle can be clearly differentiated. Taken together with our data (57), these studies suggest that overlapping but distinct sets of *cis*-acting sequences and lineage-restricted *trans*-acting factors may differentially regulate cardiac and skeletal muscle-specific gene expression.

Previous studies have also demonstrated distinct patterns of gene expression in fast- and slow-twitch skeletal muscle. Interestingly, the cTnC gene is transiently expressed in embryonic fast skeletal muscle but stably expressed in both embryonic and adult slow skeletal muscle. Of note, C2C12 cells express the embryonic fast myosin heavy-chain isoform and both the slow/cardiac and fast skeletal troponin C isoforms, suggesting that this cell line most closely resembles an embryonic fast skeletal myoblast. In contrast, Sol 8 myotubes express embryonic fast and slow skeletal muscle protein isoforms, making it difficult to characterize this cell line as either fast or slow skeletal muscle (84). Thus, our results for C2C12 cells demonstrate the importance of the cTnC first-intron enhancer in

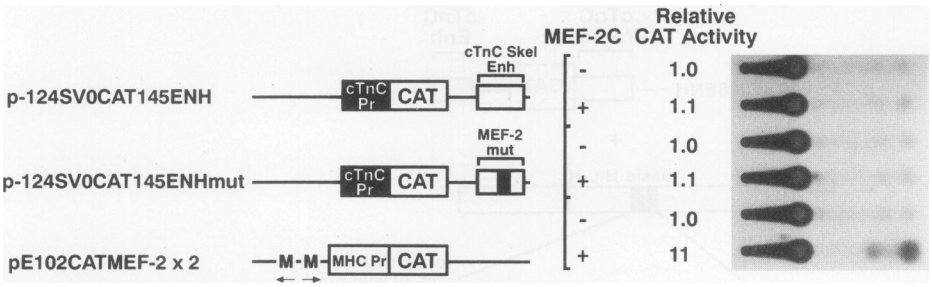


FIG. 10. Overexpression of MEF-2C does not activate the cTnC enhancer. Four micrograms of the cTnC-CAT or positive control pE102CAT-MEF 2x2 reporter plasmid (schematically represented on the left) and 1 μg of the pRSVβgal reference plasmid were cotransfected with either 16 μg of the pCMVMEF-2C eukaryotic expression vector (MEF-2C+) or 16 μg of pUC18 control DNA (MEF-2C−) into 10T1/2 cells; 16 h posttransfection, cells were placed into differentiation medium (DMEM–10% equine serum). Cells were harvested after 48 h, and cell lysates were analyzed for CAT and β-galactosidase activities. CAT activities, corrected for differences in transfection efficiencies, were normalized to the CAT activity produced by the control vector p-124SV0CAT145ENH cotransfected with pUC18 DNA. An autoradiogram from a representative experiment is shown on the right. Each transfection was repeated at least three times.

regulating the expression of this gene in fast-twitch skeletal muscle. However, we are currently unable to determine the role of this element in regulating cTnC gene expression in slow-twitch skeletal muscle. A definitive answer to this question awaits the construction of appropriate transgenic animals.

**Indirect activation of myofibrillar gene expression by myogenic bHLH transcription factors.** Several lines of evidence suggest that the myogenic family of bHLH transcription factors regulate the development of mammalian fast skeletal muscle. Each of these factors is able to convert many nonmuscle cell types to the myogenic phenotype in vitro (reviewed in references 52 and 76). Moreover, gene targeting experiments in mice have suggested that MyoD, myf-5, and myogenin each play an important role in skeletal myogenesis in vivo (2, 27, 53, 63). Binding sites for the bHLH transcription factors have been found in most but not all previously characterized skeletal muscle-specific transcriptional regulatory elements (5, 19, 36, 37, 43, 58, 65, 75, 78). Thus, it has been suggested that these proteins regulate skeletal myocyte differentiation by binding to and directly activating the transcription of a large set of muscle-specific genes. However, recent studies have identified several muscle-specific transcriptional regulatory regions which do not appear to contain binding sites for myogenic bHLH transcription factors (44, 72). In addition, mutagenesis experiments have suggested that bHLH binding sites are not required for the muscle-specific activity of some myogenic transcriptional control regions (16). These findings suggested that there may be a bHLH-independent transcriptional regulatory pathway in skeletal myotubes or, alternatively, that bHLH family members can indirectly transactivate some muscle-specific genes.

The lack of an E box within the first-intron cTnC enhancer led us to examine whether this enhancer could be transactivated by myogenic bHLH transcription factors and, if so, to examine the molecular mechanisms underlying this transactivation. The finding that the enhancer can be transactivated by overexpression of either MyoD or myogenin in 10T1/2 fibroblasts demonstrated the existence of an indirect bHLH-regulated transcriptional regulatory pathway. This pathway requires the DNA binding activity of the bHLH transcription factors because mutant myogenin proteins lacking a functional DNA binding domain were incapable of transactivating the cTnC enhancer. The transcriptional transactivation function of myogenin is also required for activation of the indirect pathway because mutant myogenin-E12 basic, which binds DNA but cannot activate transcription, fails to activate the cTnC enhancer.

Several findings suggest that MEF-2 plays an important role in this bHLH-mediated indirect transactivation pathway: (i) MyoD-mediated transactivation of the cTnC enhancer correlates with the induction of MEF-2 DNA binding activity, (ii) MEF-2 is expressed in a lineage-restricted, developmentally regulated pattern consistent with that of the cTnC gene, and (iii) mutation of the MEF-2 binding site abolishes the activity of the cTnC transcriptional enhancer in skeletal myotubes. In this regard, it is also noteworthy that most of the previously described skeletal muscle-specific transcriptional regulatory elements that do not require functional bHLH binding sites contain consensus MEF-2 binding sites, that previous studies have demonstrated that myogenin activates MEF-2 activity in CV1 and 10T1/2 cells (8, 47), and that autoregulation of the myogenin promoter is mediated at least in part through a MEF-2 binding site (16). Considering the apparent importance of MEF-2 for activation of the cTnC enhancer, it is perhaps surprising that overexpression of exogenous MEF-2C was insufficient to activate this enhancer. This could be explained if additional lineage-specific factors, induced by myogenic bHLH proteins but not by MEF-2, were required for cTnC enhancer activation.

**The role of MEF-3 in the regulation of skeletal muscle gene expression.** The studies described in this report have identified a novel nuclear protein binding site (KSSTCAGGNNNY) in the cTnC first-intron enhancer which we have designated MEF-3. Several lines of evidence suggest an important role for MEF-3 in the regulation of skeletal muscle gene expression. First, MEF-3 binding sites are present in multiple skeletal muscle-specific transcriptional regulatory elements, including the human cTnC enhancer (67), the murine myogenin promoter (16), the rat aldolase A enhancer (30), the murine sTnC enhancer (54, 69a), the rat and mouse MCK enhancers (31, 33, 70), and the quail troponin I enhancer (83). Second, mutation of the MEF-3 core sequence abolishes the activity of the cTnC enhancer. Of note, EMSAs demonstrated that the MEF-3 core site binds specifically to one major low-mobility nuclear protein complex which appears to be expressed in both muscle and nonmuscle cell lines. Interestingly, in most cases, MEF-3 sites are located near both MEF-2 and CACCC binding sites. These data are consistent with a model in which interactions between specific lineage-restricted and developmentally regulated transcription factors such as MEF-2 and specific ubiquitous tran-

scription factors such as the CACCC and MEF-3 binding activities regulate gene expression during skeletal muscle cell differentiation.

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