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The Myogenic Regulatory Circuit That Controls Cardiac/Slow Twitch Troponin C Gene Transcription in Skeletal Muscle Involves E-box, MEF-2, and MEF-3 Motifs

THORKIL H. CHRISTENSEN AND LARRY KEDES[1]

Institute for Genetic Medicine, Department of Biochemistry and Molecular Biology and Department of Medicine, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90033

We have characterized the specific DNA regulatory elements responsible for the function of the human cardiac troponin C gene (cTnC) muscle-specific enhancer in myogenic cells. We used functional transient transfection assays with deletional and site-specific mutagenesis to evaluate the role of the conserved sequence elements. Gel electrophoresis mobility shift assays (EMSA) demonstrated the ability of the functional sites to interact with nuclear proteins. We demonstrate that three distinct transcription activator binding sites commonly found in muscle-specific enhancers (a MEF-2 site, a MEF-3 site, and at least four redundant E-box sites) all contribute to full enhancer activity but a CArG box does not. Mutation of either the MEF-2 or MEF-3 sites or deletion of the E-boxes reduces expression by 70% or more. Furthermore, the MEF-2 site and the E-boxes specifically bind, respectively, to MEF-2 and myogenic determination factors derived from nuclear extracts. EMSA assays using a MEF-3 containing oligonucleotide revealed indistinguishable separation patterns with extracts from myogenic cells and nonmyogenic cells. These data suggest that expression of the cTnC gene in slow-twitch skeletal muscle is sustained through complex interactions at the 3′Ile enhancer between muscle-specific and nontissue-specific transcription factors: either a myogenic bHLH complex or MEF-2 can activate transcription but only in the presence of a third transcriptional activator that appears not to be muscle specific. We conclude from these observations that the cTnC 3′Ile element is a composite enhancer that functions through the combined interactions of at least five regulatory elements and their cognate binding factors: three or four E-boxes, a MEF-2 site, and a MEF-3 site. The data support the notion that all of these sites contribute to enhancer function in cell systems in an additive way but that none are absolutely required for enhancer activity. The data imply that the levels of transcription of cTnC in myogenic tissues in which the activities of one of the transcriptional factors is lacking would be partially but not wholly suppressed. Our data support the critical role of E-box sites in conjunction with the adjacent elements. Hence, we assign CTnC gene regulation to the "ordinary" rather than to the "novel" category of transcriptional regulation during skeletal myogenesis.

Transcription Muscle Troponin C gene E-box MEF-2 MEF-3

THE restricted and mutually exclusive expression of the two troponin C (TnC) proteins—fast-twitch TnC and slow-twitch (or cardiac) TnC—influences the contractile properties of striated muscles. The two proteins have distinctive calcium binding properties that effect their actions as subunits of the troponin complexes (15,20,64). In adult muscle the fast-twitch isoform gene (TnCf) is expressed exclusively in fast-twitch skeletal muscle while the cardiac isoform gene (cTnC) is expressed both in the myocardium

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[1]Address correspondence to Larry Kedes, Institute for Genetic Medicine, USC School of Medicine, 2050 Alcazar Street, Los Angeles, CA 90033. Tel: (323) 442-1144; Fax: (323) 442-2764; E-mail: kedes@hsc.usc.edu

and in slow-twitch skeletal muscle fibers [reviewed in (44)].

We and others have been examining the mechanisms of tissue-specific restriction of TnC genes (11–13,19,43,45–47,56). The results of previous experiments (13) revealed distinctive regulatory mechanisms responsible for the transcription of the human cTnC gene in cardiac and skeletal muscle. In primary cardiomyocytes in culture and in the myocardium of living rodents, human cTnC gene sequences upstream of the basal promoter (−13 to −61) do not appear to contribute to expression whereas an enhancer element located in the 5′-most 1028 bp of the 1523 bp first intron (5′I1e) appears to provide the major activation in cardiomyocytes. In contrast, several distinct regions in addition to the basal promoter appear to regulate skeletal muscle cell type-specific transcription: upstream regulatory sequences (from −61 to ≈−4500), and a distinct enhancer located in the 3′ 495 bp of the first intron (3′I1e). This enhancer interacts with the upstream elements to confer skeletal muscle-specific expression to the gene in a multiplicative fashion and its activity in skeletal muscle is no less than that of the entire intron (13).

Expression of a number of muscle-specific genes seems to depend on the combined actions of muscle-specific and nonmuscle-specific transcription factors acting by interactions with consensus binding sites on the promoter or enhancer regions (55). For example, the cardiac α-actin promoter, whose expression is dependent on a member of the basic helix–loop–helix (bHLH) family of muscle-specific myogenic transcription factors (e.g., MyoD, myogenin, MRF4, myf5), is inactivated in the absence of either of the ubiquitously expressed transcription factors SRF or Sp1 (54). The organization of the 3′I1e enhancer in the cTnC gene appears to fit this general paradigm. The enhancer contains DNA sequences conserved between the mouse and human genes that correspond to binding sites for both muscle-related transcription factors, including a MEF-2 site (14,22,68) and four MyoD binding sites, (E-boxes) [for reviews, see (42,60)], as well as nonmuscle-specific transcription factors, including a CArG box (3) and a MEF-3 site (23,45).

In the present study, we have characterized the specific DNA regulatory elements responsible for the function of the muscle-specific HcTnC 3′I1e enhancer in myogenic cells. We used functional transient transfection assays with deletional and site-specific mutagenesis to evaluate the role of the conserved sequence elements. Gel electrophoresis mobility shift (EMSA) assays demonstrated the ability of the functional sites to interact with nuclear proteins. We demonstrate that the MEF-2, MEF-3, and the redundant E-box sites all contribute to full enhancer activity but the CArG box does not. Mutation of either the MEF-2 site or deletion of the E-boxes reduces expression dramatically. Furthermore the MEF-2 site and the E-boxes specifically bind, respectively, to MEF-2 and myogenic determination factors isolated from nuclear extracts. EMSA assays using a MEF-3-containing oligonucleotide revealed indistinguishable separation patterns with extracts from myogenic cells and nonmyogenic cells. These data suggest that expression of the cTnC gene in slow-twitch skeletal muscle is sustained at the 3′I1e compound enhancer through complex interactions involving muscle-specific and nontissue-specific transcription factors: either a myogenic bHLH complex or MEF-2 can activate transcription but only in the presence of a third transcriptional activator that does not appear to be muscle specific.

MATERIALS AND METHODS

Plasmid Constructions

The recombinant construct pHcTnC4500CAT/I1-500-S has been previously described (13). The expression of this plasmid in myogenic cells was determined by CAT assay (see below) and the expression of all other constructs was expressed as relative to wild-type (WT) at 100%. It contains the 3′-most 495 bp (bp 1029–1523) of the HcTnC first intron (3′I1e) in the sense orientation and is designated as the WT enhancer (Fig. 1). Truncated 3′I1e fragments or site directed mutants were synthesized by polymerase chain reaction (PCR) (37,38,62). These DNA segments were inserted into a *Bam*HI site downstream of the CAT gene in the plasmid pHcTnC4500CAT (13). All constructs were validated by diagnostic restriction enzyme analysis.

Synthesis of Oligonucleotides and PCR Products

HcTnC DNA fragments used for subcloning were generated by PCR using synthetic oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems model 380 A DNA synthesizer. The synthesized PCR primers are described in detail in Tables 1, 2, and 3. PCR reactions were carried out in 100 μl of PCR buffer [10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin] containing sense and antisense primers at a concentration of 0.6 μM each; 1 μg of HcTnC template DNA from plasmid pB8A (58); 0.2 mM each of dATP, dCTP, dGTP, and dTTP; and 0.5 μl of Taq polymerase (Amplitaq; Perkin-Elmer Cetus). PCR synthesis of 25 cycles on a DNA Thermal Cycler (Perkin-Elmer Cetus) was

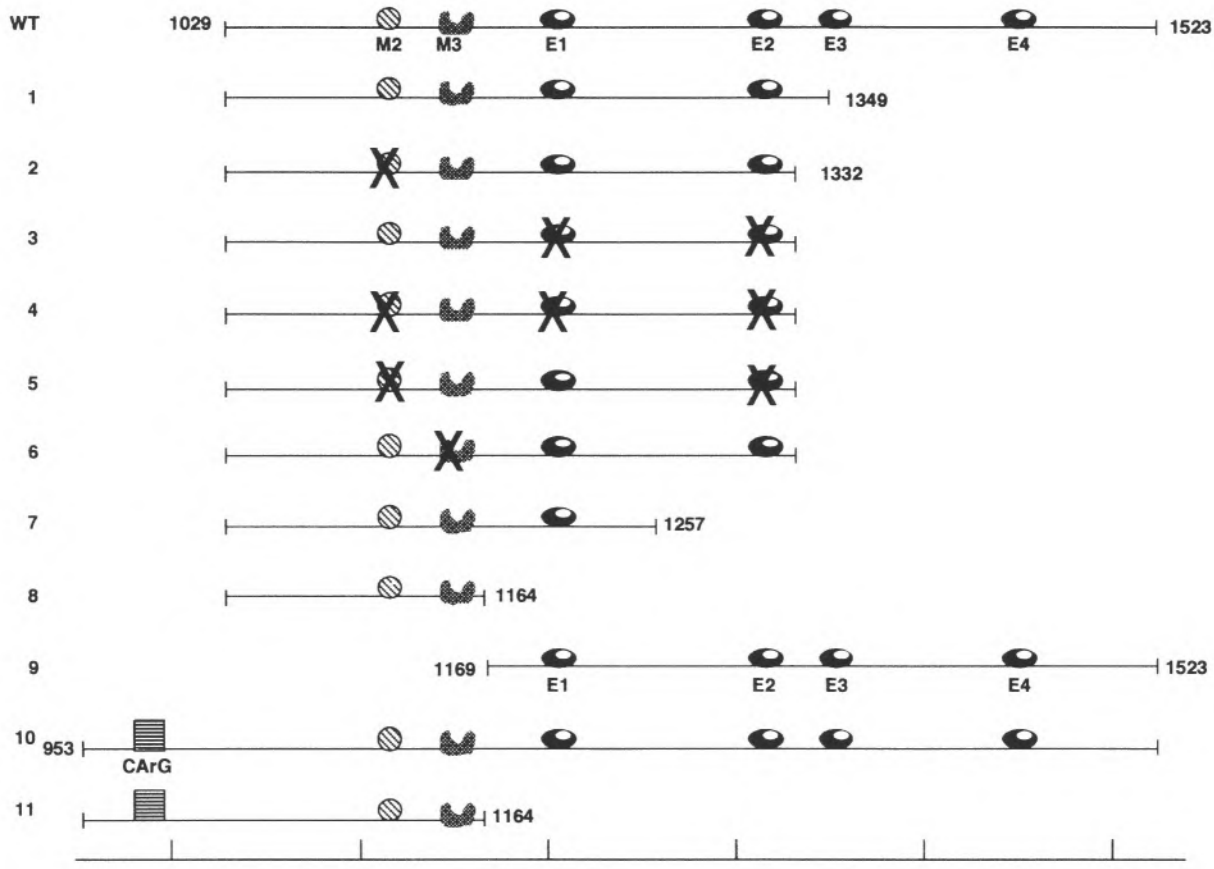

FIG. 1. Schematic maps of HcTnC intron 1 fragments. The constructs were fabricated by the polymerase chain reaction using synthetic oligonucleotides as described in Materials and Methods. The resulting PCR products were cloned downstream of the CAT gene, in sense orientation, into pHcTnC4500CAT (12), which carries 4500 bases of 5′ upstream sequences of the HcTnC gene. Fragment 1 deletes two 3′ E-boxes from the WT fragment; WT and fragment 1 appear to have equal enhancer activity when linked to the HcTnC4500 promoter. Except for site-specific mutations and an additional 18 bp at the 3′ end, fragment 1 is identical to fragments 2, 3, 4, 5, and 6. These 18 bases do not contain known transcription factor consensus binding sites. The promoter maps are drawn to scale (1 division = 100 bp). The locations of the consensus binding sequences are: CArG 977–986; MEF2 1113–1127; MEF3 1142–1151; E-box 1 1202–1207; E-box 2 1311–1316; E-box 3 1350–1355; E-box 4 1448–1453. The X symbols represent site-specific mutations created in the binding site sequences.

carried out at 94°C for 1 min, 45°C for 40 s, and 72°C for 2 min. Following phenol-chloroform extraction, DNA fragments were prepared for subcloning by *Bam*HI restriction endonuclease cleavage.

Cell Culture, DNA Transfection, and CAT Assays

C2 cells (67) were cultured in growth medium to 70% confluency, and transfected with DNA by the calcium phosphate method essentially as described previously (34,56). In each transfection, the DNA precipitate contained an equimolar amount of reporter plasmid. Twenty-two hours after transfection, the C2 cells were switched to differentiation medium and incubated for another 48 h before harvesting as myotubes. All transfection experiments were carried out in duplicate or triplicate and were repeated at least twice using plasmid DNA that had been purified by two CsCl density gradient centrifugations. Thin-layer chromatography was used for CAT assays (21); plates were analyzed on the AMBIS radioanalytic imaging system. The amount of nuclear cell extract used in the assays ensured that percent acetylation of substrate was less than 30% to maintain values in the linear range of enzyme activity. Reported values were normalized to the CAT activity of the HcTnC4500/I1-500-S construct (WT; reported as 100% expression); the promoterless pCAT-Basic plasmid (Promega) served as negative control. pMCK-CAT (1) functioned as a positive control for transfection efficiency (data not shown).

Nuclear Protein Extracts

Crude nuclear extracts from C2 cells, C3H 10T1/2 cells (50) and C3H 10T1/2 cells stably transfected with a retrovirus expressing MyoD (27) were prepared according to Dignam et al. (16); buffers were

TABLE 1
OLIGONUCLEOTIDES USED FOR GENERATION OF HcTnC INTRON FRAGMENTS

Name	Location and Orientation*	Sequence (5′ to 3′)†
TCO1–4	A(1536–1510)	GTCAGCTGCTCTGGATCCAAGGAAAGG
TCO2	S(1012–1052)	GGCTTCTGAGGCGGCAGGATCCGCCAGGGGGCCAGATAACG
TCO9	S(1001–1036)	GGACTAATTTTGGCTTCGGATCCGGCACTAGCAGCC
TCO10	A(1188–1153)	ATAATAAAACCAGTGTGGAGGATCCCAAGCCACCCA
TCO17	A(1362–1326)	CGTGACCCAGCTGGATCCACTGCGACTGGGCTCAGGG
TCO18	A(1285–1244)	CCCTCAGTAACAATAGTCAGTGACCACGGATCCCCCTTTCGG
TCO19	S(1154–1192)	GGGTGGCTTGCCTGGATCCACACTGGTTTTATTATCCC
TCO23	S(933–972)	GGGAGTCCATGGAGCCGGGGGATCCACAGCCCTCCACCC
TCO30	A(1342–1303)	GCGACTGGGGGATCCGCCAGGGTGACAGGTGGGCACCCC
TCO31	A(1342–1303)	GCGACTGGGGGATCCGCCAGGGTGA*GAATTC*GGCACCCC[1]
TCO33	A(1232–1193)	CTCAAGCTGGGTGGGCCGGAAGGA*GAATTC*TCCCTAAGT[2]
TCO34	S(1223–1172)	CACACTGGTTTTATTATCCCAACTTAGGGA*GAATTC*TCCTTCGGCCCACCC[3]
TCO37	A(1153–1110)	CGGTAACCTGACCAGGGTGGCCACTGG*GAATTC*TTTAACGAGG[4]
TCO38	S(1095–1134)	GGTTTCACTTTGTTCCTCGTTAAA*GAATTC*CCAGTGGCC[5]
TCO52	S(1124–1165)	GCCCAGTGGCCACCCTGGTC*GAATTC*CCGTGGGTGGCTTGGC[6]
TCO53	A(1165–1124)	GCCAAGCCACCCACGG*GAATTC*GACCAGGGTGGCCACTGGGC[7]

*Sense strand: S; antisense strand: A. Numbers identify base locations of the sequences in the right-hand column.
†New *Bam*HI sites are underlined, new *Eco*RI sites are in italics.
[1]E-box destroyed by *Eco*RI site substitution.
[2]E-box destroyed by *Eco*RI site substitution.
[3]E-box destroyed by *Eco*RI site substitution.
[4]MEF2 site destroyed by *Eco*RI site substitution.
[5]MEF2 site destroyed by *Eco*RI site substitution.
[6]MEF3 site destroyed by *Eco*RI site substitution.
[7]MEF3 site destroyed by *Eco*RI site substitution.

supplemented with additional protease inhibitors, leupeptin (2 μg/ml) and aprotinin (2 μg/ml) (53). All C3H 10T1/2 cell lines were grown in DMEM with 10% fetal calf serum and 2 mM glutamine. For untransfected C3H 10T1/2-like and stably transfected C3H 10T1/2 myoblast-like cell extracts, cells were harvested at 85% confluency. To obtain C3H 10T1/2 myotube-like nuclear extracts, cells were permitted to grow to 100% confluency in DMEM with 10% fetal calf serum and 2 mM glutamine and then switched into differentiation medium, DMEM supplemented with 2% horse serum and 2 mM glutamine. They were harvested 48 h later at a time when the cells had fused into multinucleate cells resembling myotubes. The integrity of nuclear protein extracts for EMSA assays was verified by using a labeled Sp-1 consensus binding oligonucleotide.

Synthesis of Myogenin Fusion Proteins

The plasmid expressing the myogenin–GST fusion protein (9) was a kind gift of Eric Olson (University of Texas Southwestern Medical School). Protein synthesis and purification was carried out as previously described (26,58). Purified protein (150 ng) was used for the EMSA assays (see below).

Antibodies

The MEF-2 polyclonal antibody (68) was a kind gift from Dr. Bernardo Nadal-Ginard. The hybridoma

TABLE 2
TEMPLATE DNA AND PRIMERS USED FOR SYNTHESIS OF HcTnC II FRAGMENTS

Fragment	Template*	Sense Primer	Antisense Primer
1	HcTnCpB8B	TCO2	TCO17
7	HcTnCpB8B	TCO2	TCO18
8	HcTnCpB8B	TCO9	TCO10
9	HcTnCpB8B	TCO19	TCO1–4
10	HcTnCpB8B	TCO23	TCO1–4
11	HcTnCpB8B	TCO23	TCO10

*HcTnCpB8B [see (56)].

TABLE 3
TEMPLATE DNA AND PRIMERS USED FOR SYNTHESIS OF MUTAGENIZED HcTnC II FRAGMENTS

Fragment	PCR Reaction	Template	Sense Primer	Antisense Primer
2	2-1	HcTnCpB8B	TCO2	TCO37
	2-2	HcTnCpB8B	TCO38	TCO30
	2-3	Annealed products of 2-1 + 2-2	TCO2	TCO30
3	3-1	HcTnCpB8B	TCO2	TCO33
	3-2	HcTnCpB8B	TCO34	TCO31
	3-3	Annealed products of 3-1 + 3-2	TCO2	TCO31
4	4-1	HcTnC4500/G23	TCO38	TCO31
	4-2	HcTnC4500/G23	TCO2	TCO37
	4-3	Annealed products of 4-1 + 4-2	TCO2	TCO31
5	5-1	HcTnC4500/B3	TCO38	TCO31
	5-2	HcTnC4500/G23	TCO2	TCO37
	5-3	Annealed products of 5-1 + 5-2	TCO2	TCO31
6	6-1	HcTnCpB8B	TCO2	TCO53
	6-2	HcTnCpB8B	TCO52	TCO30
	6-3	Annealed products of 6-1 + 6-2	TCO2	TCO30

Fragments 2, 3, 4, 5, and 6 are altered from the WT sequence by site-directed mutagenesis. They were synthesized in a multistep procedure: for each fragment, the template of the final (third) PCR reaction was the annealed product of individual, purified PCR fragments synthesized in two previous steps. In synthesis of fragments containing site-directed mutations, the amount of template, primers, and other reagents in each 100 µl PCR reaction was the same as for WT fragment synthesis (see Materials and Methods). Fragments were prepared for subcloning by purification and *Bam*HI treatment.

cells synthesizing the monoclonal anti-myogenin antibody FD5 (65) was a kind gift of W. Wright (University of Texas Southwestern Medical School). This monoclonal antibody was purified by a procedure previously described (2). The rabbit polyclonal IgG Sp-1 antibody was from Santa Cruz Biotechnology, Inc.

Gel Electrophoresis Mobility Shift Assays (EMSA)

The top strand of a synthetic oligonucleotide (Table 4) was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and annealed with a 4 molar excess of the corresponding bottom strand unlabeled oligonucleotide. For EMSA assays in which crude nuclear extract was used, a reaction mixture included 1 ng of annealed oligonucleotide, 8 µg of CV1 or C2 myotube nuclear extract, 3 µg of poly(dI/dC) as nonspecific competitor, and varying amounts of specific synthetic double-stranded oligonucleotides or unlabeled DNA fragments as competitor DNAs adjusted to a final volume of 30 µl with buffer C (16). Reactions involving the purified GST–myogenin protein contained 1 ng of labeled oligonucleotide without addition of poly(dI/dC) and 150 ng of purified GST–myogenin protein. Reaction mixtures were incubated at room temperature for 20 min. In some experiments antibodies were then added and the reactions allowed to continue for an additional 20 min. Samples were subjected to polyacrylamide gel electrophoresis. Gels were stained in 10% acetic acid and 10% methanol, dried, and autoradiographed.

RESULTS

Requirement for the MEF-2, MEF-3, and E-box Sites to Sustain Maximal HcTnC II Enhancer Activity

We placed the full 3′IIe 495 bp enhancer fragment (designated WT in Fig. 1) downstream of a CAT reporter gene linked to the 4500 bp upstream elements to create a full-strength expression vector. The expression of all other HcTnC fragments (constructs 1–11 in Fig. 1) was scored relative to WT. In transient transfection assays of C2 myogenic cells, and in accordance with our previous findings (13), the pHcTnC4500CAT construct without an intron expresses at 14% relative to WT and significantly higher than the 3% background level. The enhancer fragment 3′IIe appears to be a composite element because it contains at least seven highly conserved sequence elements that correspond to known transcription factor binding sites (13).

We created a series of reporter constructs similar to WT but each containing various mutations and deletions of the conserved sequence elements. In addition, we tested the enhancer capability of subsegments of 3′IIe. The results of transfection studies of

TABLE 4
OLIGONUCLEOTIDES USED FOR EMSA ASSAYS

Name	Description	Reference or Location	Nucleotide Sequence (5′ to 3′)
Sp1		(7)	ATTCGATCGGGGCGGGGCGAGC
AP2		(63)	GATCGAACTGACCGCCCGCGGCCCGT
TCO13	HcTnC consensus MEF-2 binding sequence	(1109–1131)	TCCTCGTTAAAAATAGCCCAGTG
TCO15	HcTnC MEF3 site	(1133–1160)	CCACCCTGGTCAGGTTACCGTGGGTGGC
TCO44	Destruction of HcTnC consensus MEF-2 binding site with new *Eco*RI site (in italics)	(1107–1132)	GTTCCTCGTTAAA*GAATTC*CCAGTGG
TCO46	Rat muscle creatine kinase consensus MEF-2 binding sequence	(1)	GACAGGGTTATTTTTAGAGCGAGC
TCO40	HcTnC consensus MyoD binding sequence	(1196–1213)	TAGGGACAGCTGTCCTTC
TCO42	Destruction of HcTnC consensus MyoD binding sequence binding with new *Eco*RI site (in italics)	(1194–1214)	ATTAGGGA*GAATTC*TCCTTCG
TCO49	Rat muscle creatine kinase consensus MyoD binding site	(59)	TCAGGCAGCAGGTGTTGGGGGGATC
TCO54	Destruction of HcTnC consensus MEF-3 binding sequence with new *Eco*RI site (in italics)	(1136–1156)	CCCTGGTC*GAATTC*CCGTGGG

constructs 1–8 are shown in Fig. 2. These results can be summarized as follows.

1. At least two of four E-boxes must be intact for full WT expression. Deletion of two E-boxes has no effect (construct 1) but deletion of three E-boxes (construct 7) or destruction of all four E-boxes either by deletion (construct 8) or by a combination of deletion and mutation (construct 3) progressively greatly reduces expression.
2. A MEF-2 site must be intact for full WT expression. Mutation of the MEF-2 site alone (construct 2) reduces expression to about 30% of WT.
3. A MEF-3 site (4,22) must also be intact for full WT expression. Mutagenesis of this site (construct 6) appears to inactivate the 3′Ile enhancer activity completely.

Four E-boxes Contribute to HcTnC Promoter Expression But a CArG Box Does Not

We tested whether a DNA fragment containing the four E-boxes alone could activate transcription directed by the 4500 bp HcTnC 5′ promoter. This DNA segment (Fig. 1, fragment 9) boosts expression of the intronless pHcTnC4500CAT construct more than

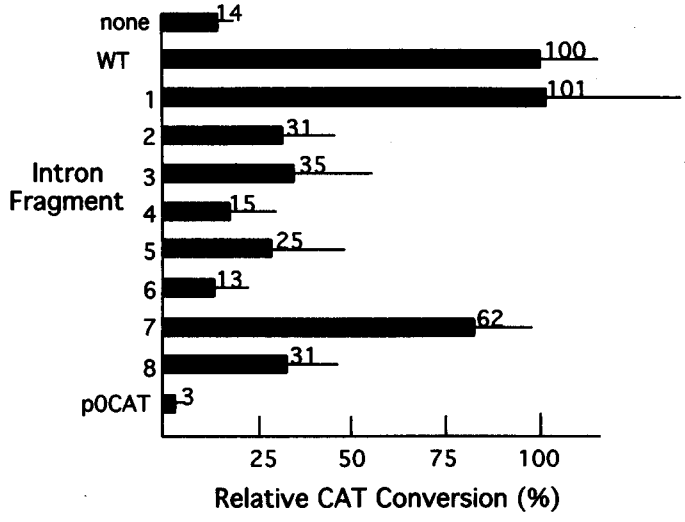

FIG. 2. Relative enhancer activities of fragments of HcTnC intron 1. pHcTnC4500CAT linked to various intron fragments (see Fig. 1) were transfected into C2 myogenic cells and transient CAT activity measured as described in Materials and Methods. All intron constructs carry the HcTnC4500 full promoter cloned into pCAT Basic (Promega) (12). Relative CAT expression was compared to the wild-type reference construct pHcTnC4500CAT/I1-500-S defined at 100% expression. The figure displays the mean values and the SD based on observations for four or more independent experiments. The intronless-promoterless pCAT Basic (p0CAT) served as a negative control.

fivefold (5.5 ± 3; $n = 6$; data not shown). Thus, E-boxes independent of the MEF-2 and MEF-3 sites can positively interact with the promoter elements and enhance transcription.

A CArG box is located just upstream of the MEF-2 site in 3′I1e and is conserved in mouse and human sequences. This site is known to interact with serum response factor and similar elements are critical components of several muscle-specific promoters (33,36). To test whether this element contributed to 3′ activity we included it in the WT fragment to create construct 10 and added it to the E-box-deleted fragment (construct 8) to create construct 11 (see Fig. 1). Each of these constructs was tested with the 4500 bp promoter element. The ratio of CAT activity of construct 10 to WT was 0.9 ± 0.3. The ratio of CAT activity of construct 11 to construct 8 was 1.3 ± 0.3. We conclude that the CArG box does not measurably contribute to enhancer activity in this cellular assay.

Muscle Cell Nuclear Protein Interaction With the MEF-2 Site

Based on our functional CAT activity data, the putative MEF-2 binding site plays an important role in the enhancer activity of intron 1. MEF-2 (14,22,69) is a transcription factor that activates a number of muscle-specific genes, and cotransfection of an expression vector encoding MEF-2 transactivates muscle-specific genes in nonmuscle cells (31,68). To determine whether the putative MEF-2 site in the HcTnC 3′I1e enhancer does bind MEF-2 we examined the binding of proteins in nuclear extracts of C2 myogenic cells to the HcTnC MEF-2-like site (Fig. 3). Three shifted bands (x, y, and z) were observed. These appear to be specific complexes because all bands were competed by unlabeled oligonucleotide but not by binding sites for transcription factors AP-2 (35,63,64) or Sp1 (7,26). To further identify the binding factor involved, we successfully competed the binding activity with the classic MEF-2 site from the muscle creatine kinase enhancer (14). Finally, we synthesized an oligonucleotide carrying the same mutation that had crippled expression in constructs 2, 4, and 5. This oligonucleotide failed to compete for nuclear factor binding. Thus, proteins that specifically bind the HcTnC enhancer are capable of binding to known MEF-2 sites.

As an additional test of the identity of the proteins binding to this site in the HcTnC enhancer we used an anti-MEF-2 antibody in the DNA protein binding assay. Following preincubation of the labeled oligonucleotide with C2 nuclear extract, we added an anti-MEF-2 polyclonal antibody (68), which has been demonstrated to bind to MEF-2 proteins. As seen in Fig. 3B, the anti-MEF-2 antiserum created a supershifted band (W) and loss of band y. An anti-Sp1 antibody had no effect on the binding pattern. We conclude that MEF-2 is a likely activator of the 3′I1e enhancer.

MyoD or the Cognate Myogenic HLH Protein, Myogenin, Binds to the 5′-Most E-box of the HcTnC I1

We carried out a similar series of experiments to demonstrate that the HcTnC E-boxes bind bona fide members of the myogenic determination family. MyoD1 and its congeners are capable of initiating the myogenic program (4,5,17,32,51,66), and can transactivate a number of muscle-specific genes in nonmuscle cells (4,6,7,9,10,18,28,29,52,54,59,61). These factors bind to the E-box that has the consensus sequence, CANNTG. EMSA assay was carried out with a labeled oligonucleotide corresponding to the sequence of the 5′-most E-box sequence and a C2 cell nuclear extract. The results (Fig. 4A) show several retarded DNA–protein complexes (q, r, and s) that were specifically competed with an excess of unlabeled identical oligonucleotide or the rat MCK MyoD binding site, but not by oligonucleotides containing a mutant E-box, an AP-2 site, or an Sp-1 site. Further evidence that the HcTnC E-box can bind a legitimate myogenic determination factor was provided by a EMSA assay (Fig. 4B) using a purified glutathione–myogenin fusion protein in the presence and absence of an anti-myogenin monoclonal antibody. The antibody specifically prevented the formation of protein–DNA complexes that formed with the GST fusion protein. Because the only protein in the reaction was the GST–myogenin, the bound complex is presumably a homodimer.

The HcTnC MEF-3 Site Binding Factors Are Found in Both Muscle and Nonmuscle Cells

Hidaka et al. (23) were the first to demonstrate that the sequence TCAGGTTT(T/A)CA, referred to as a MEF-3 site, was the functional binding site for a transcription activator for the muscle-specific expression of the rat aldolase A gene and the mouse myogenin gene. This sequence is conserved in the cTnC enhancer (13) and was suggested as a required element in enhancer function (44). In transient transfections of C2 myogenic cells, the MEF-3 site is required for HcTnC 3′I1e enhancer function (Fig. 2). C2 cell nuclear extracts form specific DNA–protein complexes with the MEF-3 sequences (designated t and u; Fig. 5A) that are competed from binding by self but not by an oligonucleotide carrying the same

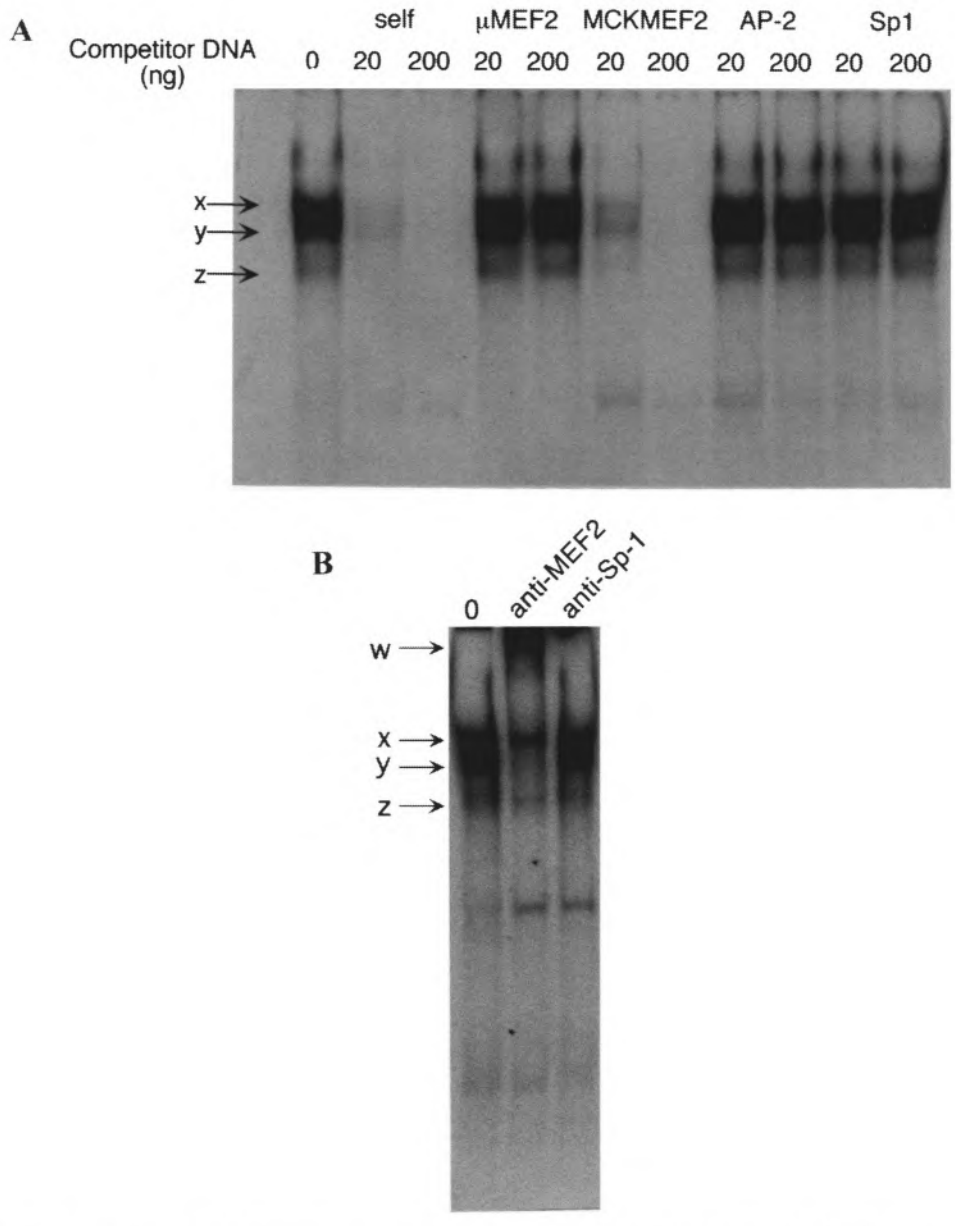

FIG. 3. Gel electrophoresis mobility shift (EMSA) assays of the putative HcTnC MEF-2 binding site. The EMSA assays were performed with C2 cell nuclear extracts as described in Materials and Methods. (A) All lanes contain 1 ng of [γ-^{32}P]ATP-labeled oligonucleotide TCO13 as described in Table 4 (HcTnC intron 1, bp 1109–1131) and 3 μg of poly(dI/dC) nonspecific competitor DNA in a final volume of 30 μl with buffer C. Each reaction contained 8 μg of C2 cell nuclear extract. Different types and amounts of competitor DNAs were added to some reactions as indicated, including a mutated MEF-2 site (TCO44) and the rat MCK MEF-2 site (TCO46). (B) Supershift of MEF-2 binding: following preincubation, 1 μg of polyclonal anti-MEF-2 or Sp-1 antibody was added to the binding reaction. The bands designated x, y, z, and w are described in the text.

mutation in the MEF-3 site (Fig. 1, construct 6) that strongly mutes WT function.

To determine whether the MEF-3 site binds only a muscle-specific factor, we compared the ability of the MEF-3 oligonucleotide to bind proteins from myogenic and nonmyogenic cells. We used nuclear extracts from C3H 10T1/2 cells (which do not support expression of HcTnC reporter genes; data not shown) as well as extracts from clones of the same 10T1/2 cells that had been stably transfected with a MyoD expression vector. These stable cells differentiate into cells that resemble multinucleate myotubes and support the expression of HcTnC promoter constructs (27). We isolated nuclear extracts from prolif-

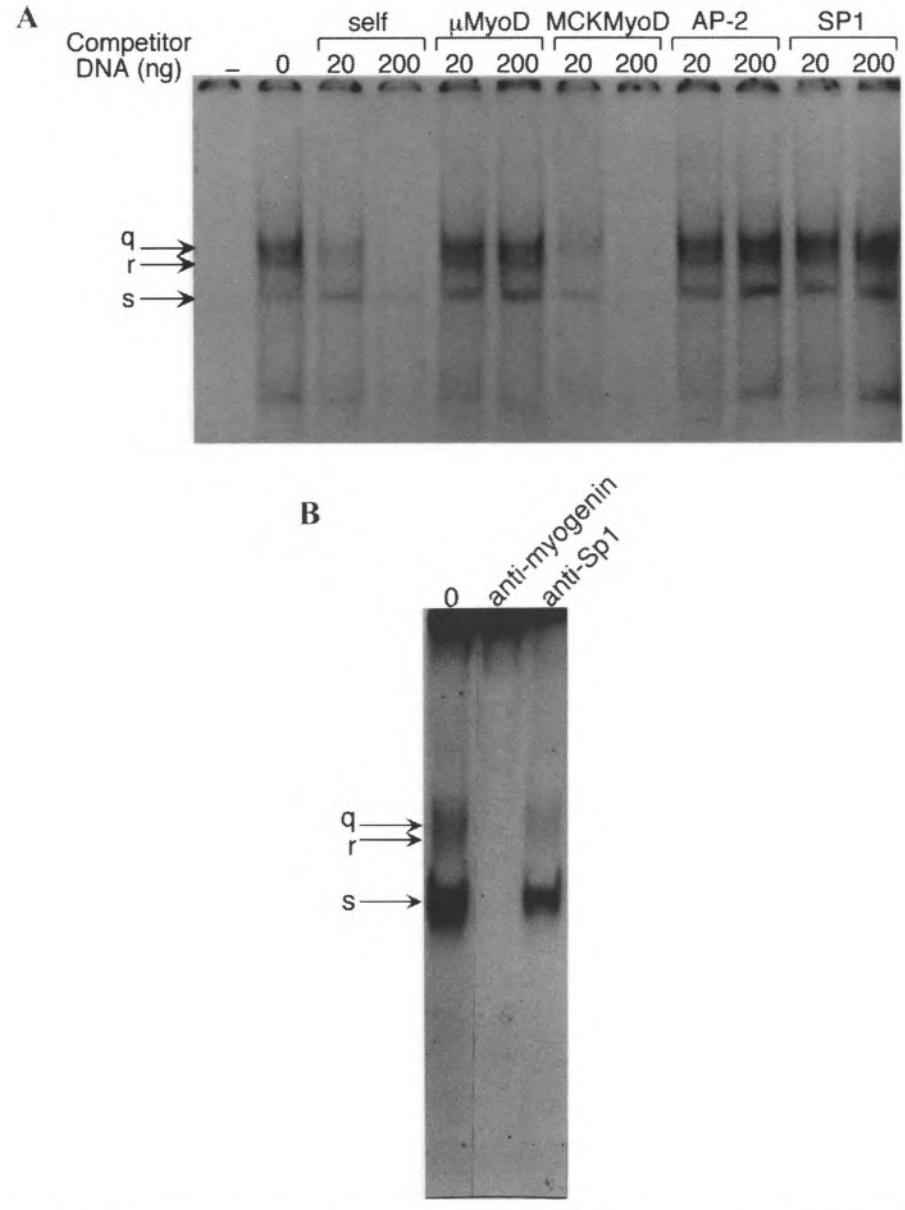

FIG. 4. EMSA assays of the 5′-most E box. The EMSA assays were performed with C2 cell nuclear extracts as described in Materials and Methods. (A) All details are as described in the legend to Fig. 3A except that the labeled HcTnC intron 1 oligonucleotide was TCO40 as described in Table 4 (bp 1196–1213). Mutant E-box oligonucleotide (TCO42) and the MCK E-box (TCO49) were used as competitors in some reactions as indicated. (B) Antimyogenin affects the ability of GST–myogenin to bind to the TC040 E-box. EMSA assay was carried out after incubation with a monoclonal anti-myogenin antibody. All reactions contained 1 ng of [γ-^{32}P]ATP-labeled HcTnC intron 1 oligonucleotide TCO40 and 150 ng of purified GST–myogenin protein; no nonspecific DNA was added. The final reaction volume was adjusted to 30 μl. Following a 20-min preincubation period aliquots were mixed with 1 μg of either a monoclonal antimyogenin antibody or a polyclonal anti-Sp-1 antibody. The bands labeled q, r, s are described in the text.

erating undifferentiated myogenic 10T1/2 cells as well as from the fully differentiated myotube-like cells.

We reasoned that a muscle-specific MEF-3 binding factor induced during myogenic differentiation would display differences in EMSA patterns among the three nuclear extracts. However, as seen in the data presented in Fig. 5B, all three nuclear extracts form indistinguishable and specific protein–DNA complexes with the MEF-3 oligonucleotide (arrows). These complexes are all competed with an excess of unlabeled oligonucleotide, but not with an Sp-1 binding sequence. Similarly, when we directly compared the nuclear extracts from myogenic C2 cells and the

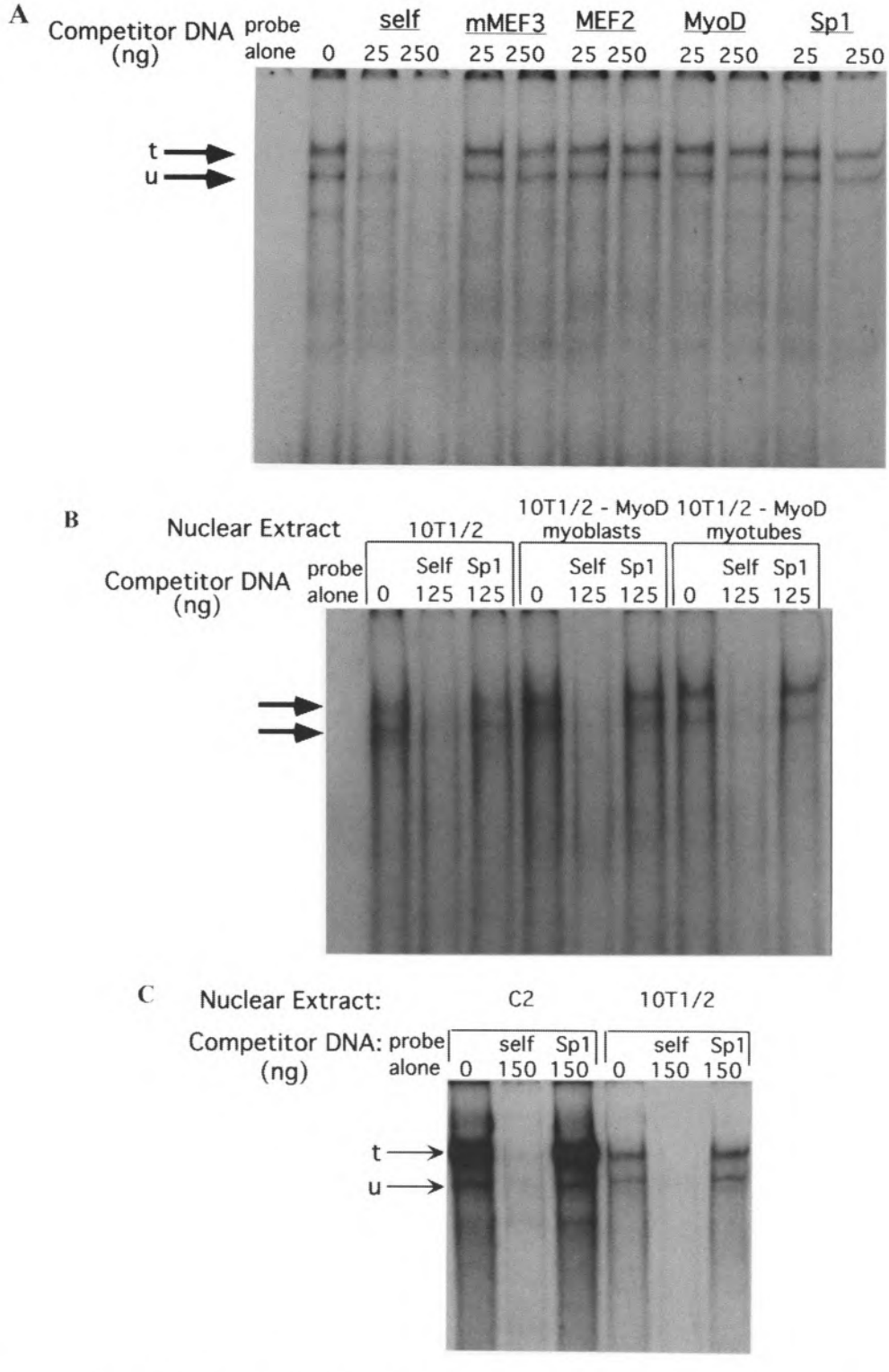

FIG. 5. EMSA assays of the HcTnC MEF-3 site. The EMSA assays were performed with 8 μg of nuclear extracts as described in Materials and Methods. All lanes contain 1 ng of [γ-^{32}P]ATP-labeled HcTnC wild-type oligonucleotide containing the MEF-3 site (TCO15 from bp 1133–1160; see Table 4) plus 3 μg of poly(dI/dC) in a final volume of 30 μl with buffer C. Various competitor oligonucleotides were added to some reactions as indicated. (A) C2 cell nuclear extract. The mutated MEF-3 site oligonucleotide (TCO54) is indicated as μMEF3. Two specific complexes (t and u) are designated by arrows. (B) Nuclear extracts were derived from 10T1/2 cells or 10T1/2 cells stably transfected with a MyoD expression vector and harvested as either myoblasts or myotubes. Two specific complexes are designated by arrows. (C) Direct comparison of binding activity in C2 and 10T1/2 cell nuclear extracts. Two specific complexes with identical mobilities are formed by both extracts (t and u, designated by arrows).

nontransfected 10T1/2 cells, we also observed binding complexes with identical mobilities (Fig. 5C). However, C2 nuclear extracts appear to have a higher binding activity than the 10T1/2 cell extracts, raising the possibility that there may be differences in the quantity or binding activity of MEF-3 in the myogenic cells. We conclude that the MEF-3 binding factor is not muscle specific and exists in fibroblastic 10T1/2 cells. Accordingly, the cTnC skeletal muscle enhancer is regulated by the combined effects of muscle-specific (MEF-2 and myogenic bHLH) and nonmuscle-specific (MEF-3) transcriptional activators.

DISCUSSION

In this study, we have extended our earlier studies and defined the specific control elements of a first intron enhancer of the HcTnC gene that specifically regulates the gene during skeletal myogenesis. Previously, we reported that the 1523 bp first intron of the HcTnC gene contains two enhancers. One is located in the 5′ end of intron 1 (between +58 and +1028) and augments transcription in both cardiac and skeletal muscle cells. A second enhancer is located at the 3′ end of I1 [between +1029 and +1523; (13)] and is the topic of this report. This second enhancer, a 495 bp DNA element (3′I1e), contains evolutionarily conserved sequences known to bind two types of well-characterized muscle-specific transcription factors: MEF-2 (14,22,24,25) and any of the family of the myogenic bHLH proteins (41). It also contains a sequence between bp +1142 and +1151, TCAGGTTACC, which is highly conserved between cTnC genes (45), the myogenin gene, and the rat aldolase A gene (23). This sequence has been previously suggested as important for expression of the mouse cTnC gene (45) and was designated as a MEF-3 site. Our experimental findings can be summarized as follows.

At least three of four E-boxes contribute to enhancer function. The presence of four E-boxes in the enhancer element suggested that they might contribute to enhancer activity and might be redundant. Deletion of the DNA segment containing E3 and E4, the two most distal E-boxes, had no effect on enhancer strength. However, additional deletion of E2 and E1 progressively reduced enhancer activity. The importance of the E-boxes rather than their flanking sequences was directly verified by site-directed mutagenesis. Such double E-box-mutagenized segments had the same reduced activity as the E-box-deleted segments (Fig. 2). Furthermore, E1 was shown to bind to myogenin in a mobility shift assay (Fig. 4B). It is useful to point out, however, that E-boxes E3 and E4 are not entirely nonfunctional because, when other elements are eliminated (MEF-2 and MEF-3), E1 and E2 alone cannot support enhancer activity without E3 and E4 (Fig. 2; compare the activities of fragment 6 and fragment 9). Because we did not compare the function of an element containing three rather than four E-boxes, the possibility remains that one of four E-boxes may not contribute to enhancer activity.

A MEF-2 binding site contributes to enhancer function. The complex formed between the nuclear factors bound by the WT MEF-2 sequence element was specifically supershifted in EMSA assays by a polyclonal anti-MEF-2-antibody. In addition, when we subjected the MEF-2 binding site to site-directed mutagenesis, MEF-2 binding was also eliminated. Reporter constructs carrying just this mutation (Fig. 2, fragment 2) had low levels of transcriptional activity compared to a WT promoter.

A MEF-3 binding site also contributes to enhancer function. Site-specific mutagenesis of the MEF-3 site eliminates enhancer activity, suggesting the importance of a nontissue-specific binding factor to the expression of the cTnC gene in muscle cells. Although the MEF-3 element is essential for enhancer activity in the context of fragment 6, it does not appear to be absolutely required because even in the absence of this site the element containing four E-boxes (Fig. 1, fragment 9) can support significant transcriptional activity.

An upstream CArG box has no impact on enhancer function. A CArG box located just upstream of 3′I1e did not add to the enhancer activity and could not support transcription in the absence of E-boxes.

The 3′I1e Activity Involves Nontissue-Specific, as Well as Muscle-Specific, Factors

In EMSA assays using nuclear extracts from myogenic cells, we found specific binding of at least two nuclear protein complexes to the MEF-3 site (Fig. 5A, bands marked t and u). However, there was no apparent change in binding activity of either band t or band u when we compared extracts obtained from proliferating myoblasts versus differentiated myotubes (Fig. 5B). Specific MEF-3 binding activities with identical mobilities in the EMSA assay were found in nuclear extracts from fibroblasts. Thus, the factor(s) binding to MEF-3 appear to be ubiquitously expressed. However, when we compared the binding activities in extracts from fibroblasts with those from C2 cells, it was clear that the more slowly migrating band (t) in C2 extracts (Fig. 5C) has a relatively higher avidity or concentration in the myogenic lineage. Identical mobilities in EMSA do not prove protein identity. However, whether or not the MEF-3 activities are identical in myogenic and nonmyogenic cells, the tissue specificity of cTnC expression can still clearly be explained by its dependence on one or more of the muscle-specific transcription factors.

MEF-2 belongs to the MADS box family of transcription factors [(68); also reviewed by (48,57)] and enhances transcription of several muscle-specific

genes (22). Only following differentiation do muscle cells rapidly accumulate detectable MEF-2 binding activity (14,22). MEF-2 is also present in cardiomyocytes. The activation of muscle-specific genes conferred by MEF-2 is relatively weak (22,68), and several pieces of evidence suggest that other muscle or nonmuscle transcription factors are a prerequisite for MEF-2 activity at enhancer sites (39,40,49,68). Although MEF-2 activity is highly muscle restricted, MEF-2 transcripts are widely expressed and it is not completely understood in which way MEF-2 confers tissue-specific expression (30). Because there are four MEF-2 genes capable of generating a variety of alternatively spliced transcripts, the mechanisms of tissue-specific control have not been easily understood. The expression of MyoD and myogenin, on the other hand, which are both members of the bHLH family of proteins, is restricted to the myogenic lineage and is not found in cardiac cells [reviewed in (8,41,60)]. These proteins bind to the CANNTG motif, or E-box, and activate the muscle-specific transcriptional program in numerous muscle promoters and enhancers [see (13) and references therein].

Several reports suggest that, in the absence of E-boxes, MEF-2 can contribute to the activity of muscle-specific enhancers but only in the presence of other ubiquitously expressed nuclear factors. Previous work from this laboratory found that expression of the skeletal α-actin gene depends on both a MEF-2 site and a ubiquitous factor binding to the DRF-1 enhancer element (39). Similarly, cardiac-specific expression of the MLC-2 gene depends not only on MEF-2, or a factor closely related to MEF-2, but must be supported also by a ubiquitously expressed factor (HF-1a) binding to a site in close proximity to the MEF-2 site (40). Hidaka et al. (23) discovered that the distal-most of two alternative promoters of the rat aldolase A gene is highly active only in skeletal muscle and contains a muscle-specific enhancer with a MEF-2 site and no E-boxes. However, MEF-2 cannot sustain maximal aldolase A transcription without the presence of a nearby MEF-3 binding site. These observations support the general paradigm that tissue-specific gene expression of the kind exhibited in skeletal muscle and myocardium depends on the use of tissue-specific activator proteins to attach the RNA polymerase holoenzyme to the correct promoter. In the cited instances the genes have evolved to require both tissue-specific and nontissue-specific activators to accomplish this task. A derivative of this notion is that a survey of the enhancer sites on a particular tissue-specific gene might well predict the timing of onset of its expression in development: the onset of expression or activation of a tissue-specific activator would predict the onset of expression of the gene.

The Role of E-boxes in Supporting HcTnC Muscle-Specific Enhancer Activity

We suspected that E-boxes played a supporting role in activating the 3′Ile enhancer because, as we have pointed out previously (13), the nucleotide sequence of the HcTnC gene closely resembles the mouse cTnC gene (McTnC) and, in particular, the 3′ region of intron 1 is highly conserved. This conserved region contains a MEF-2 and a MEF-3 site and three (McTnC) or four (HcTnC) E-boxes. The mouse homolog of the human cardiac troponin C gene has been intensely studied by Parmacek and Leiden and colleagues. They recently defined a 145-bp intron 1 enhancer element of the mouse gene (43) that corresponds to bp 1015–1159 of the human gene (corresponding closely to fragment 8 of Fig. 1). Previously they had reported also that the intron sequences downstream of this location, containing three E-boxes, do not contribute to enhancer activity (44,47). The 145-bp enhancer they defined in the mouse gene corresponds to only the upstream portion of the human 3′Ile enhancer described in our present study, and the nucleotide sequences and function of additional sites conserved between the two species were not considered by these workers. As described in this report, absent any E-boxes, the human homolog of the shorter mouse segment (fragment 8) only retains approximately 30% of the enhancer activity of the full-length human I1 enhancer. Parmacek et al. (43) concluded that, because the short mouse enhancer element contains no E-box sites and hence is not a direct target for myogenic bHLH interaction, "a novel myogenic regulatory circuit controls cardiac troponin C gene transcription in skeletal muscle." In contrast, our data support the critical role of E-box sites in conjunction with the adjacent elements. Hence, we reassign CTnC gene regulation to the "ordinary" rather than to the "novel" category of transcriptional regulation during skeletal myogenesis.

We conclude from these observations that the cTnC 3′Ile element is a composite enhancer that functions through the combined interactions of five or more regulatory elements and their cognate binding factors: three or four E-boxes, a MEF-2 site, a MEF-3 site, and, untested in this model, a conserved CACCC site tested by Parmacek et al. (43). Our data support the notion that all of these sites contribute to enhancer function in an additive way but that none are absolutely required for enhancer activity.

Whether the implicit redundancy of regulatory elements is ever operational during myogenesis in the organism remains to be determined. The data imply that the levels of transcription of cTnC in myogenic tissues in which the activities of one of the transcriptional factors is lacking would be noticeably suppressed. Thus, cTnC expression in vivo would depend on the summed level of activity of several classes of transcription factors. Such a model also predicts that TnC gene expression in myogenic cells is directly related to the time of onset of expression of one or more of the tissue-specific activators.

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