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Common Core Sequences Are Found in Skeletal Muscle Slow- and Fast-Fiber-Type-Specific Regulatory Elements

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The molecular mechanisms generating muscle diversity during development are unknown. The phenotypic properties of slow- and fast-twitch myofibers are determined by the selective transcription of genes coding for contractile proteins and metabolic enzymes in these muscles, properties that fail to develop in cultured muscle. Using transgenic mice, we have identified regulatory elements in the evolutionarily related troponin slow (TnIs) and fast (TnIf) genes that confer specific transcription in either slow or fast muscles. Analysis of serial deletions of the rat TnIs upstream region revealed that sequences between kb −0.95 and −0.5 are necessary to confer slow-fiber-specific transcription; the −0.5-kb fragment containing the basal promoter was inactive in five transgenic mouse lines tested. We identified a 128-bp regulatory element residing at kb −0.8 that, when linked to the −0.5-kb TnIs promoter, specifically confers transcription to slow-twitch muscles. To identify sequences directing fast-fiber-specific transcription, we generated transgenic mice harboring a construct containing the TnIs kb −0.5 promoter fused to a 144-bp enhancer derived from the quail TnIf gene. Mice harboring the TnIf/TnIs chimera construct expressed the transgene in fast but not in slow muscles, indicating that these regulatory elements are sufficient to confer fiber-type-specific transcription. Alignment of rat TnIs and quail TnIf regulatory sequences indicates that there is a conserved spatial organization of core elements, namely, an E box, a CCAC box, a MEF-2-like sequence, and a previously uncharacterized motif. The core elements were shown to bind their cognate factors by electrophoretic mobility shift assays, and their mutation demonstrated that the TnIs CCAC and E boxes are necessary for transgene expression. Our results suggest that the interaction of closely related transcriptional protein-DNA complexes is utilized to specify fiber type diversity.

Skeletal muscle commitment, differentiation, and maturation are largely controlled by the transcriptional regulation of a large battery of muscle-specific genes (reviewed in references 10 and 49). Whereas our understanding of the factors regulating myoblast commitment and differentiation has advanced significantly in the past years, little is known about the mechanisms dictating diversity among fibers. The differences in the contractile and biochemical properties of different muscles originate from the selective expression of genes coding for contractile protein isoforms in distinct myofibers. The isoforms determine the rate of force generation with respect to depolarization, the relaxation rates, and the fatigability of the myofibers. Four myofiber phenotypes have been defined on the basis of the type of myosin heavy-chain isoform that they express. On the basis of this nomenclature, fast-twitch fibers (type II) are categorized into types IIA, IIB, and IIX, whereas only one type of slow fiber (type I) has been identified thus far (reviewed in reference 45). In avian muscle, there is evidence that fiber diversity may arise from the commitment of distinct myoblast lineages during embryogenesis that can differentially express myofibril proteins (54). However, the phenotypes of neonatal and adult muscles are plastic; epigenetic factors modulate their contractile and metabolic properties. The innervation of skeletal muscle by motoneurons, and more specifically the patterns of electrical stimulation used to depolarize muscle, regulates the expression of contractile protein isoforms and metabolic enzymes (reviewed in reference 45). Hormones can also modify the effects of innervation on skeletal muscle properties (35). Thus, one approach to understanding the molecular mechanisms controlling muscle diversification and plasticity is to identify the DNA regulatory sequences that confer fiber-type-specific expression of contractile protein genes.

The use of fetal myocyte cultures and skeletal muscle cell lines has been invaluable as a means for studying the transcriptional regulation of muscle-specific genes during commitment and differentiation (reviewed in references 22, 43, and 57). The identification and characterization of the family of muscle regulatory factors MyoD (15), myogenin (21, 58), myf-5 (9), and MRF-4 (8, 41, 48) have greatly contributed to our understanding of skeletal myogenesis. These factors, when heterodimerized to the related ubiquitous factors coded by the E2A (42) and HEB (31) genes, bind to a DNA motif (known as the E box) found in multiple muscle genes to activate transcription. In addition, other nuclear factors binding to DNA motifs present in numerous muscle genes, such as MEF-2/RSRF (23, 46, 59), SP1/CCAC (6, 52), SRF (37, 52), and M-CAT (40) sites, contribute to the transcriptional activation of genes during myogenesis. However, because muscle cultures do not manifest the specific properties of adult muscles, the identification of the *cis*-acting sequences conferring fiber specificity needs to be addressed by using an in vivo system, such as transgenic mice.

We have used the regulation of the troponin I (TnI) genes as a model to elucidate the mechanisms that generate fiber di-

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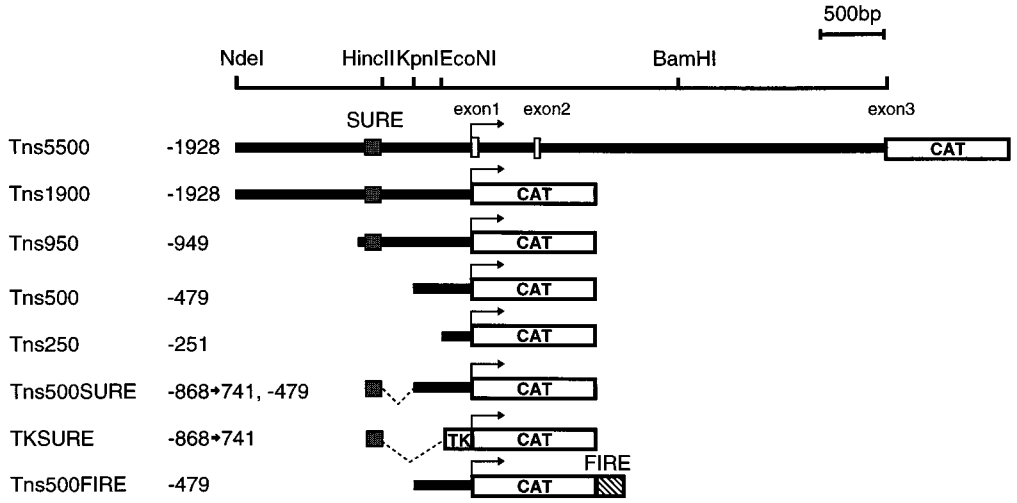

FIG. 1. Organization of the TnI CAT reporter constructs used to generate transgenic mice. A diagrammatic representation of the upstream region of the rat TnIs gene is shown at the top with restriction sites used to generate transgene constructs. Representations of the fragments used to generate transgenic mouse lines are shown below. The names of the transgenic mouse lines analyzed are on the far left, with the upstream-most position indicated to their right. The fragment Tns5500 was previously used to generate transgenic mice (4); this is the largest construct used, and it contains the first and second noncoding exons, represented by the open boxes, and the first two introns. The boxes labeled SURE and FIRE correspond to the TnI rat SURE and the quail FIRE, respectively. These regulatory sequences were linked to a CAT reporter gene plus the simian virus 40 large t-antigen intron and polyadenylation signal. In construct TKSURE, the SURE fragment was linked directly to the herpes simplex virus TK promoter.

versification. These loci are less complex than those encoding myosin heavy-chain isoforms, which consist of a large number of genes expressed at different developmental times, and myofibers (reviewed in reference 10). The three troponin genes encoding the isoforms that in the adult are specifically expressed in slow (TnIs), fast (TnIf), and cardiac muscles originated from a common ancestral gene (3). The different isoforms of TnI form part of a complex, in combination with troponins C and T, which is involved in the regulation of actomyosin calcium-mediated interactions during contraction (61). Transcription of TnI genes is activated during myoblast terminal differentiation (4, 12, 36). In adult muscle, expression of the TnIs and TnIf genes is confined to type I and II fibers, respectively (25). Previously, we demonstrated that transcription of the rat TnIs gene in cultured differentiated myotubes is regulated by sequences residing in the upstream and intronic regions of the gene. TnIs reporter constructs containing as little as 200 bp of upstream sequence, driving expression of the chloramphenicol acetyltransferase (CAT) reporter gene, were sufficient to confer muscle-specific expression in transiently transfected C2C12 myotubes. We also demonstrated that transgenic mice harboring a rat TnIs reporter construct that included sequences from bp −1900 upstream of the transcription initiation site and extended 3′ to the second noncoding exon were able to confer fiber-type-specific expression of the CAT reporter (4). Regulation of the quail TnIf isoform has also been studied in vitro and in transgenic mice. Characterization of the quail TnIf gene in cultured cells (60) demonstrated that there is an enhancer that confers muscle- and differentiation-specific expression located in the first intron. This enhancer was given the name IRE, for intronic regulatory element (60). Transgenic mice carrying a β-galactosidase reporter construct driven by the upstream 530 bp plus the first intron and noncoding exon of the quail TnIf gene (containing the IRE) were shown to specifically express the reporter in type II fibers (24).

To identify the sequences that direct the fiber-type-specific expression of the TnI genes, we have generated a series of transgenic mouse lines that harbor different CAT reporter constructs driven by sequences of the rat TnIs and quail TnIf genes. Deletion analysis of the TnIs gene in transgenic mice shows that sequences residing in the upstream region from bp −1900 and −500 are necessary for expression in slow muscle. Recent experiments performed in our laboratory using cultured Sol 8 cells demonstrated the presence of regulatory elements in this region (11), which upon sequencing revealed a span of about 128 bp that is highly homologous to the human TnIs gene (12). Addition of the 128-bp sequence to the inactive −500-bp promoter is shown to confer slow-fiber-specific expression, whereas addition of the quail TnIf IRE (144 bp) to the same promoter confers fast-fiber-specific transcription in transgenic mice. Interestingly, comparison of the slow and fast regulatory elements reveals they share multiple conserved DNA motifs. We demonstrate that these motifs are able to bind their cognate factors and furthermore are required for transcriptional activation, suggesting that the interaction of similar transcription factors is necessary to regulate expression of contractile protein genes in different fiber types.

MATERIALS AND METHODS

Sequencing of the 5′ flanking region of the rat TnIs gene. Plasmid Tn2700.CAT, previously described (4), is a CAT reporter construct built on the pCAT Basic vector (Promega) which contains TnIs sequences extending from 2.7 kb upstream of the transcription initiation site (position +1) to position +50 located in the first noncoding exon. Nested deletions in sequences residing in the upstream region were generated in both directions with exonuclease III and S1 nuclease. DNA sequencing was performed by standard dideoxynucleotide termination reactions. GeneWorks software (IntelliGenetics, Inc.) was used for the sequence and alignment analysis.

Isolation of TnIs fragments used for transgenic mouse analysis. A series of fragments was prepared from CAT reporter constructs containing different parts of the TnIs upstream region to generate transgenic mice (Fig. 1). All the fragments included the CAT reporter gene plus the simian virus 40 intron and polyadenylation site; all constructs except TKSURE were made in the pCAT Basic vector backbone. The fragment Tns1900 was obtained by digestion of construct Tn2700.CAT with *Nde*I (−1928) to isolate a fragment containing 1.9 kb of TnIs upstream sequence linked to the CAT reporter. One of the clones obtained by exonuclease III deletion of plasmid Tn2700.CAT (see above) was designated Tns950. Plasmid DNA from clone Tns950 was digested with *Hin*dIII and *Bam*HI to isolate a fragment containing 0.95 kb of TnIs upstream sequence linked to the CAT reporter gene. Fragments Tns500 and Tns250 were obtained by digestion of clones Tn500.CAT and Tn300.CAT (4) with *Bam*HI and either

*Hin*dIII or *Eco*NI, respectively. These constructs contain 479 and 251 bp of TnIs upstream sequence, respectively. Plasmid Tns500SURE was constructed by the insertion of a 128-bp PCR fragment (corresponding to nucleotides −868 to −741) into *Sph*I-*Sal*I-cleaved plasmid Tn500.CAT. Fragments for injection were obtained by digestion with *Hin*dIII and *Bam*HI. Plasmid TKSURE was constructed by insertion of the same PCR fragment into the *Sph*I-*Sal*I sites of vector pBLCAT2 (39). This reporter construct contains the herpes simplex virus thymidine kinase (TK) promoter at −105 to +51 directing transcription of the CAT gene. The TKSURE injection fragment was generated by *Hin*dIII and *Bam*HI digestion. Transgenic mice harboring a chimera of the rat TnIs Tns500 fragment with enhancer sequences from the quail TnIf gene were also generated. This chimeric construct, designated Tns500FIRE, was generated by cloning a 384-bp *Bam*HI-*Nde*I fragment from the quail TnICAT-23 construct (38) (kindly provided by S. Konieczny) into *Bam*HI-*Nde*I-cleaved plasmid Tn500.CAT to generate the construct Tns500FIRE. This construct contains the quail TnIf intronic regulatory element (IRE) in its natural orientation concerning the promoter. The construct was digested with *Sph*I and *Nde*I to generate the fragment used for injection. All the clones obtained with fragments generated by PCR were verified by sequencing. To prepare DNA for microinjection, constructs were digested to remove plasmid sequences, electrophoretically purified on agarose gels, electroeluted, and purified with ELUTIP-D columns (Schleicher & Schuell).

Mutagenesis of the TnIs CCAC- and E-box sequences. The PCR-based method of reference 33 was used to generate site-directed mutations in the TnIs regulatory element. The primers used to generate the mutations are as follows: for E box, OMEbox (CTTAAGTCGACCAGGGCCAACCCGTTTTCCTGG GT) and TNENH1C (ACTTAAGCATGCCCCACAAGATGACCGAC), and for CCAC box, OMCCAC1 (TCTGACTATATACCGGGTATTATG), OMC CAC2 (CATAATACCCGGTATATAGTCAGA), and TNENH2NC (CTTAAG TCGACCAGGGCCACACCTGTTT). Plasmids Tns500CCAC and Tns500E were constructed by the insertion of the mutated fragments into *Sph*I-*Sal*I-cleaved plasmid Tn500.CAT. The mutations were confirmed by sequencing. Fragments for generation of transgenic mice were obtained by digestion with *Hin*dIII and *Bam*HI.

Electrophoretic mobility shift assays (EMSAs). The MEF-2, myogenin, and E12 proteins for EMSAs were obtained by using the coupled in vitro transcription and translation reticulocyte lysate TnT system from Promega. The transcripts were synthesized from cDNAs encoding rat MEF-2A (1) in the pSK vector, mouse myogenin cDNA (21) in the pEMSVscribe II vector, and mouse E12 cDNA (42) in the pBSATg vector. Samples were electrophoresed in a Tris-glycine buffer system as described previously (2). EMSAs for the analysis of the TnIs CCAC site were performed with nuclear extracts generated from differentiated Sol 8 myocytes; extracts were prepared by the method of Dignam et al. (17). The double-stranded oligonucleotide probes were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The sequences of the sense strands (core sequences boldfaced) of the oligonucleotides used as probes and competitors in EMSAs were as follows: MEF-2/SURE, CAACAGTC**CTAAAAATA**CC CAGGAAA; MEF-2 Consensus, GATCGC**TCTAAAAATAA**CCCTGTCG; MEF-2 Mutant, GATCGC**TGTAAACATAA**CCCTGTCG; E box/SURE, CCCAGGAAA **CAGGTG**TGGCCCTG; E box Consensus, GATCCCCCCAA**CACCTG**CTGC CTGA; E box Mutant, GATCCCCCCAA**CACGGT**AACCCTGA; CCAC/SURE, TCTGACTATA**GGGTGGG**TATTATGT; and CCAC Mutant, TCTGACTATA**T ACCGGG**TATTATGT. Oligonucleotides MEF-2 Consensus, MEF-2 Mutant, E box Consensus, and E box Mutant were obtained from Santa Cruz Biotechnology, Inc.

Generation of transgenic mouse lines. Transgenic mice were prepared by the methods previously described (29). Lines containing fragments Tns1900, Tns500, Tns250, Tns500CCAC, and Tns500E were generated and maintained in an FVB/N background. All other lines were generated by injection into (C57BL/6 × SJL)F_1 embryos, and lines were propagated by mating to C57BL/6 mice. Putative founders and their generations were screened by slot blot analysis of tail DNA using a CAT probe. Adult transgenic mice were used to analyze tissue- and muscle-type-specific expression of CAT activity.

Preparation of tissue extracts and CAT analysis. Extracts were prepared essentially as described previously (4, 19). Briefly, the tissues were sonicated in 0.25 M Tris-HCl (pH 8.0) containing 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine, 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, and 1 μg of pepstatin A per ml for 10 s with a Branson Sonifier 450 (50% efficiency). The homogenates were centrifuged at 12,000 × *g* for 10 min, and the supernatants were collected for analysis. For each sample, protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) using bovine serum albumin as the standard. CAT activity was assayed as described previously (4). Initially, 10 or 100 μg of protein was incubated at 65°C for 10 min and centrifuged at 12,000 × *g* for 5 min to inactivate endogenous deacetylase activity. The CAT assays were performed with the supernatants at 37°C for 1 or 3 h. Thin-layer chromatography was used to separate the reaction products, and these were quantitated with a Molecular Dynamics PhosphorImager. Extracts that were beyond the linear range of the standard assay were diluted and reassayed.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with the accession number U49920.

RESULTS

TnIs upstream sequences and constructs used to generate transgenic mice. In a previous study we characterized TnIs transcription regulatory sequences that conferred tissue- and development-specific expression in the mouse muscle cell line C2C12 (4). Although primary muscle cultures and established muscle cell lines provide excellent models in which to study myogenic differentiation, mammalian cultured muscle cells fail to mature and manifest fiber-type-specific properties. For this reason, we used transgenic mice harboring a construct containing the upstream 1.9 kb plus the first three exons and introns of the TnIs gene to demonstrate that these sequences conferred fiber type specificity (4). A schematic representation of this construct, designated Tns5500, is shown in Fig. 1. The focus of these studies has been to delineate the TnIs sequences that direct slow-muscle expression, by generating transgenic mice harboring a series of deletions extending from the *Nde*I site (−1928) towards the transcription initiation site (Fig. 1). The complete nucleotide sequence covering this region of the TnIs gene and potential transcriptional regulatory elements are presented in Fig. 2. The restriction sites used to generate the constructs are also indicated.

Sequences residing upstream of the transcription initiation site are sufficient to confer expression in slow muscle. Since construct Tns5500 contained both upstream and intronic sequences, we began by generating line Tns1900, which utilized exactly the same upstream boundary but lacked the sequences residing downstream of the first exon. Adult mice from the three independent transgenic mouse lines generated expressed the CAT reporter specifically in skeletal muscle (Fig. 3A). At this age reporter activity was not detectable in cardiac muscle. To determine if these TnIs sequences retained their capacity to direct transcription to slow-twitch muscles, we compared the levels of CAT activity in different hind limb crural muscles of adult transgenic mice (Fig. 3B). Reporter levels were compared in the soleus (SOL), extensor digitorum longus (EDL), superficial tibialis anterior (TBL), and superficial gastrocnemius (GAS). The last three muscles are predominantly composed of fast (type II) myofibers, with less than 5% of the fibers being of the slow type, whereas over 60% of the mouse SOL is composed of slow (type I) myofibers (18, 26). In all three Tns1900 transgenic lines the levels of reporter activity in the SOL were approximately 100-fold higher than those found in the three fast muscles, indicating that the sequences residing between −1928 and +50 are sufficient to direct fiber type transcription. Similar qualitative results were obtained with two independent transgenic mouse lines harboring a construct extending from positions −950 to +50 (Fig. 3B). The levels of CAT reporter activity were between 20- and 150-fold higher in the SOL than in the EDL, TBL, and GAS muscles.

Next, mice harboring constructs extending from 479 and 251 bp upstream of the start site were analyzed in transgenic mice. We previously had shown that these CAT constructs confer muscle-specific expression in transiently transfected C2C12 myotubes. As shown in Fig. 2, a MEF-2-like site surrounded by two E boxes is located directly upstream of the basal promoter; recent experiments showed that mutation of any of these sites eliminates expression in C2C12 myotubes (data not shown). However, it is surprising that mice harboring these constructs fail to express the CAT reporter in hind limb muscles, as well as any of the other tissues tested (Fig. 3B). Since there is a precedent for reporter constructs to be differentially expressed in caudal and rostral muscles, as has been shown for transgenic mice expressing the CAT reporter driven by the myosin light-chain promoter plus enhancer (19), we also tested muscles of

FIG. 2. Upstream sequence of the rat TnIs gene. The nucleotide sequence extending from the *Nde*I site (−1928) to the transcription initiation site is shown. The TnIs upstream regulatory element necessary for slow-type expression (SURE) is underlined. Transcription regulatory sequence motifs obtained from a computer search are boxed; these include E boxes (CANNTG), MEF-2-like sites, and a CCAC box. Restriction sites used to generate the fragments for analysis in transgenic mice are shown. The arrow denotes the 5′ end of the Tns950 sequence obtained by exonuclease digestion; this construct was used to generate the Tns950 transgenic mice (see Materials and Methods).

the forelimb, diaphragm, and head. These results, as well as those of experiments performed in 15.5 and 18.5 embryos, were negative (data not shown). In addition to the results shown in Fig. 3B, we obtained negative results with two additional lines harboring *lacZ* constructs driven by the same −479 sequence. Thus, elements residing between positions −949 and −479 are necessary in vivo to drive transcription from the TnIs gene in skeletal muscle.

An upstream 128-bp sequence of the rat TnIs gene is necessary to direct slow-muscle expression in transgenic mice. Using transiently transfected Sol 8 myocytes, we recently identified a regulatory region between −949 and −720 in the rat TnIs gene (11). Comparison of this regulatory region with the human TnIs gene (12) revealed a region of approximately 128 bp that is approximately 85% homologous. Addition of this rat slow upstream regulatory element (SURE) to the Tn500.CAT construct enhanced transcription of the CAT reporter in Sol 8 myotubes (data not shown). Transgenic mouse lines harboring the CAT reporter gene driven by the −479-bp fragment plus the 128-bp rat TnIs regulatory element (Tns500SURE) were generated to test if these sequences confer muscle-type-specific transcription. Analysis of multiple tissues from the Tns500 SURE transgenic mice demonstrated that the CAT reporter was specifically expressed in skeletal muscle and not other tissues (Fig. 4A). Moreover, the 128-bp SURE, linked to the inactive 479-bp proximal region, was sufficient to restore transcription of the reporter gene preferentially in the SOL muscle. The levels of activity in the SOL muscle were 14- to 33-fold higher than in the EDL; the difference was slightly higher in comparisons with the TBL and GAS muscles. The levels of reporter activity between muscles are qualitatively similar to those obtained in the Tns950 mice. These results indicate that the 128-bp sequence, which is highly homologous to the core of the human TnIs enhancer (12), functions to direct slow-muscle-specific expression.

To test if the TnI SURE is not only necessary but also sufficient to convey slow-muscle-specific expression, the fragment was cloned into a CAT vector containing the herpes simplex virus TK promoter (TKSURE). The three transgenic mouse lines harboring this construct failed to express the CAT reporter in a skeletal-muscle-specific fashion (Fig. 4B); activity was extremely low in all tissues tested (data not shown). Furthermore, no significant differences were observed among the different crural muscles, suggesting either that the 128-bp reg-

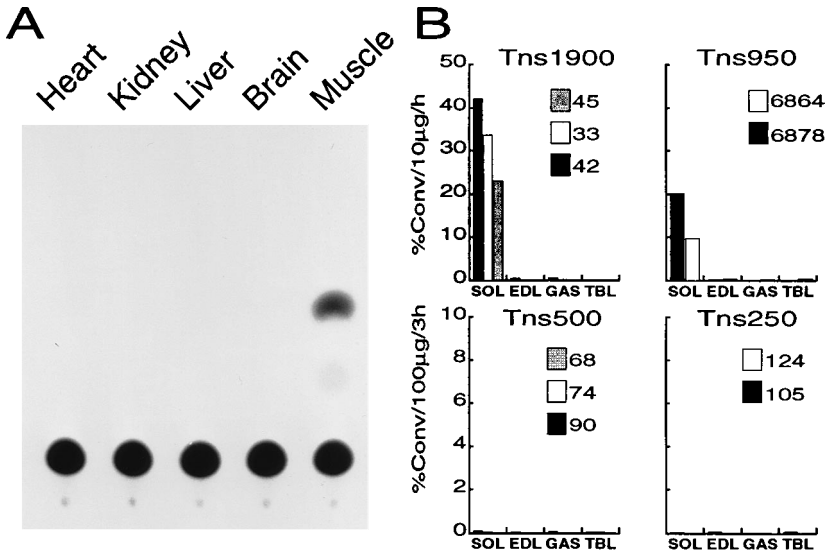

FIG. 3. The upstream region of the rat TnIs gene confers skeletal-muscle- and fiber-type-specific transcription in adult transgenic mice. (A) An autoradiogram of a representative CAT assay showing the distribution of reporter activity in several tissues derived from the Tns950 transgenic mouse line 6878. The assay was performed with 10 μg of protein for 1 h at 37°C. (B) CAT reporter activity was quantitated in the hind limb muscles of transgenic mice harboring CAT constructs driven by TnIs upstream fragments extending from positions −1928 (Tns1900), −949 (Tns950), −479 (Tns500), and −251 (Tns250). Multiple transgenic lines (line numbers are shown next to the boxes) were tested for each construct. Extracts were prepared from the SOL, EDL, GAS, and TBL muscles. CAT assays were performed with 10 or 100 μg of extract, and mixtures were incubated for 1 to 3 h at 37°C, as indicated on the ordinate. The levels are expressed as the rate of enzyme activity (percentage of acetylated chloramphenicol per amount of protein per time of assay) in each muscle type. Equal amounts of protein extract were assayed for muscles derived from mice within a construct group; however, protein levels and/or assay times were adjusted between different construct groups to perform the assay within its linear range.

ulatory element is not sufficient to confer muscle specificity or that these sequences fail to properly interact with the TK promoter. Precedents for regulatory sequences from other genes that are active with their innate promoters but fail to properly interact with the TK promoter have been observed in cultured cells (56) and in transgenic mice (47).

We observed a weak copy number dependence among different lines of mice generated with the same construct; however, the SOL/EDL CAT activity ratio remained fairly constant, indicating that reporter expression in the mice was position independent. In addition, we used multiple lines of transgenic mice harboring the same construct to circumvent

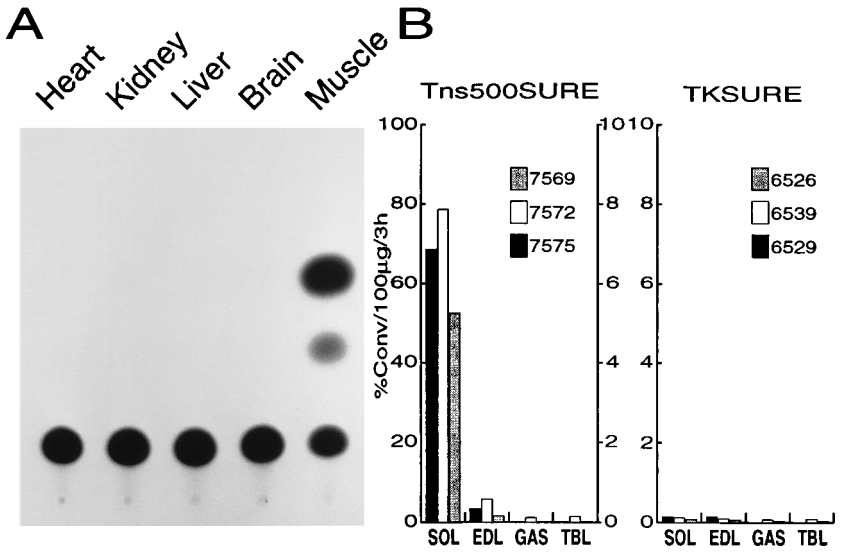

FIG. 4. Addition of the TnIs upstream regulatory element to the inactive Tns500 construct restores slow-muscle-specific activity. (A) CAT analysis was performed using different tissue-extracts derived from mice harboring SURE linked to the Tns500 fragment (Tns500SURE). The assay shown was performed with muscles from line 7575 using 100 μg of protein for 3 h at 37°C. (B) Quantitation of CAT reporter levels in different hind limb muscles obtained from multiple transgenic lines harboring either the Tns500SURE or TKSURE construct. The muscle utilized and the assay conditions used are as specified in the legend to Fig. 3. The results obtained with the three independent Tns500SURE lines are shown with two different scales since mice from line 7575 had CAT activity levels that were approximately 10-fold higher than those found in lines 7572 and 7569. The activity in the three lines was restricted to slow muscle.

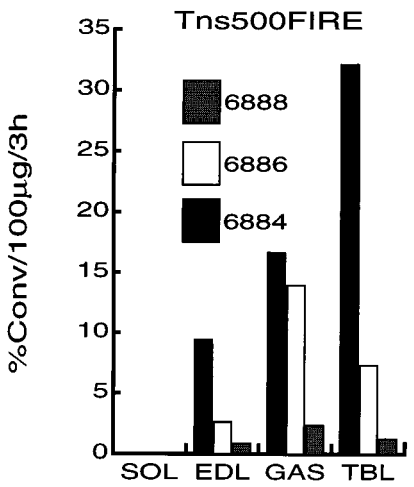

FIG. 5. Transcription of the CAT reporter is restricted to fast muscles when the quail TnIf intronic enhancer is linked to the Tns500 construct. CAT reporter activity was measured in extracts made from different muscles of Tns500FIRE transgenic mouse lines. The assays were performed using 100 μg of protein for 3 h at 37°C.

the issue of integration-dependent effects on the levels of reporter expression.

The quail TnIf intronic enhancer, linked to the TnIs promoter, confers fast-muscle specificity in transgenic mice. From the previous results, it is unclear if sequences conferring fiber specificity reside exclusively within the SURE or if the specificity results from the interactions between the SURE and the −479 region, with this region acting to restrict activity only to slow muscle. Experiments to test these questions were designed by using the evolutionarily related quail TnIf regulatory sequences. Hallauer et al. (24) recently showed that transgenic mice harboring a quail TnIf construct extending from 530 bp upstream of the transcription site to the second noncoding exon (a total of ~2.3 kb) direct expression of a β-galactosidase reporter specifically to fast myofibers. Characterization of the same gene in cultured cells (60) demonstrated that there is a muscle-specific enhancer in the first intron; this region was called the IRE (intronic regulatory element). To determine if the −479 region of TnIs directs fiber specificity or if the specificity resides within the SURE, a construct containing this promoter-proximal region of TnIs (Tns500) and the TnIf IRE were cloned into the CAT reporter vector (Fig. 1; Tns500FIRE). Three independent Tns500FIRE transgenic lines were generated and analyzed. As shown in Fig. 5, transgenic mice harboring this construct expressed practically undetectable levels in the SOL, and the CAT reporter was specifically expressed in the fast-twitch EDL, GAS, and TBL muscles. Thus, these results lead to two important findings: sequences sufficient to direct fiber-type-specific expression reside within the 128- and 144-bp TnI regulatory elements, and the promoter proximal sequence of TnIs (−479) does not contain sequences that restrict expression to slow-twitch muscles.

Sequences of the TnIs and TnIf regulatory elements were aligned to determine if they share common motifs previously determined to interact with transcription factors (Fig. 6). Interestingly, four sequences that resemble previously described *cis*-acting motifs were identified; these include a MEF-2-like AT-rich sequence (CTAAAAATA), a CCAC box (GGGT GGG; opposite strand), and an E box (CAGNTG). In addition, there was a conserved GCAGGCA sequence that had not been previously identified. Thus, the regulatory elements directing slow- and fast-fiber-type-specific expression of both troponin genes share common core sequences.

Specific binding to the conserved core elements. Competition EMSAs were used to analyze if the conserved MEF-2-like sequence, E box, and CCAC site in the TnI SURE have the capacity to bind specifically to their cognate factors. As shown in Fig. 7A, in vitro-translated MEF-2 binds to the MEF-2-like SURE sequence. This binding is specifically competed for by a 100-fold molar excess of the same unlabeled oligonucleotide (not shown) and the consensus MEF-2 site from the muscle creatine kinase (MCK) enhancer originally used to identify MEF-2 (23); a mutant MEF-2 oligonucleotide fails to compete for binding (Fig. 7A). Binding to the TnI SURE E-box-labeled probe was observed when incubated with in vitro-translated myogenin/E12; binding was again specifically competed for by

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                                                          CAGG conserved sequence
SURE      -868 CCCACAAGATGACCGACTATAATAGCTACCGGATTAACATAGCAGGCAT-
FIRE      +776           TCCCTGAGGAAACCTTATCCTGGA-AAATGT-GCAGGCACA
                         *  * **  * *     * * ***  **  * *******


                                        CCAC box
SURE           -TGTCTTTCTCTGA----CTATAGGGTGGGTATTATGTGTTCATCAACAG
FIRE           ACACATTGCTGGGAAGAGCAAGGGGGTGGG------GGGGGAA--AGTGC
                    ** **  **    * *  *******      * *   *  *
                           [          Site II         ]

                  MEF-2-like                              E box
SURE           TCCTAAAAATACCCAGGAA--------------ACAGGTGTGGCCCTG-
FIRE           TTCTAAAAATGGCTGGGGAGCTGCACAAGGAGCTGCAGCTGTCTCCTCAG
               * ********  *  ** *                 *** ***  **
                    [    Site I        ]

SURE           ------------  -741
FIRE           ACGCAGCCAGGC  +633
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FIG. 6. Core elements are conserved between the rat TnIs upstream regulatory region and the quail TnIf intronic regulatory region. Sequences from the sense strand of the rat TnI SURE and the nonsense strand of the quail FIRE have been aligned; the location of these regions is numbered with respect to the transcription start site. The common core elements between both sequences are marked in boldface characters. These include consensus binding sites known as the CCAC box, MEF2 site, and E box in the rat SURE and the quail IRE sites I and II, described by Yutzey et al. (60), which are demarcated by brackets.

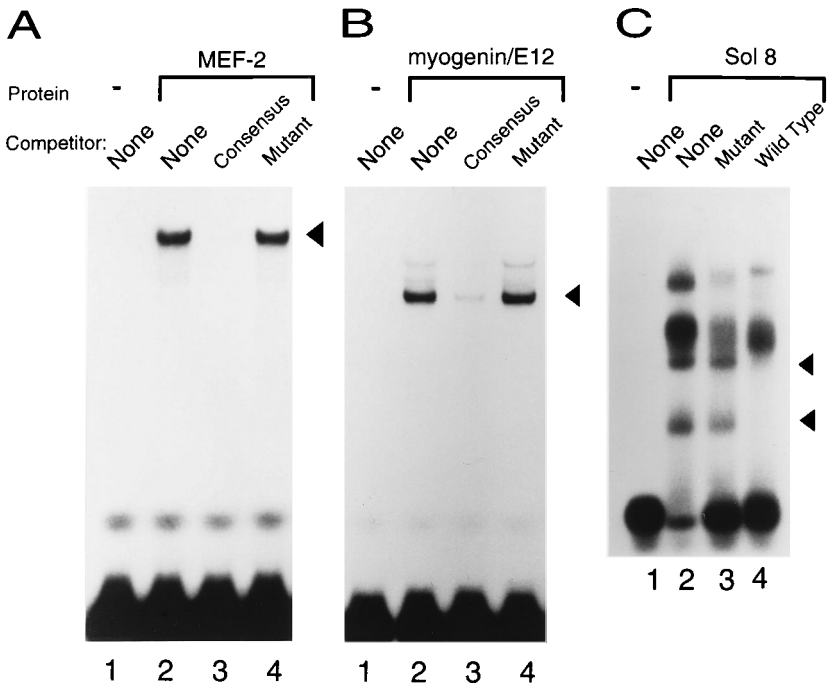

FIG. 7. EMSAs using the MEF-2-like, E-box, and CCAC sequences from the TnIs regulatory element. EMSAs were performed with labeled oligonucleotides corresponding to the MEF-2-like (A), E-box (B), and CCAC-box (C) sequences from the TnI SURE, using in vitro-transcribed-translated products from cDNAs encoding MEF-2A (panel A, lanes 2 to 4) and myogenin/E12 (panel B, lanes 2 to 4) or Sol 8 myotube nuclear extracts (panel C, lanes 2 to 4). The specific bands are indicated by arrowheads. A 100-fold molar excess of the wild-type or mutated oligonucleotides originally reported in the MCK gene was used for competition assays in panels A and B. A 500-fold molar excess of the wild-type or mutated oligonucleotide competitor was used as indicated in panel C. The competitors used are the MCK MEF-2 consensus sequence and mutated MEF-2 site (A), an MCK E-box consensus and E-box mutant (B), and the wild-type and mutated TnI SURE CCAC sequences (C). (C) EMSA using the TnI SURE CCAC sequence and nuclear extracts derived from Sol 8 myotubes (lanes 2 to 4): two specific bands were observed (lane 2). Binding to the labeled probe was not competed for by the mutated CCAC sequence (lane 3) but was competed for by the wild-type sequence (lane 4; arrowheads).

a consensus MCK E-box sequence but not by a mutated unlabeled probe (Fig. 7B). Nuclear extracts made from Sol 8 myotubes, which have been shown to express factors that bind the myoglobin enhancer CCAC box (6), were used to test the TnI SURE CCAC box. As expected, a more complex pattern of shifted bands was observed (Fig. 7C). However, two bands were specifically competed for by the wild-type sequence but not by the competitor containing the mutated CCAC site, indicating that the three conserved motifs function as bona fide binding sites.

The CCAC box and E box in the TnI SURE are required for transgene expression. Transgenic mice harboring Tns500SURE CAT constructs with point mutations in either the CCAC box (Tns500CCAC) or the E box (Tns500E) were generated to determine the role of these putative *cis* elements in TnIs SURE function (Fig. 8A). As shown in Fig. 8B, in two independent Tns500CCAC transgenic lines the levels of CAT activity in extracts derived from SOL muscle were approximately 100-fold lower than those observed in the Tns500SURE transgenic mice; CAT activity was undetectable in extracts derived from the EDL, GAS, and TBL muscles. Despite the low CAT levels, transgene expression continued to be confined preferentially to the slow-twitch muscle. Mutations of the E box had even more dramatic effects on the SURE function (Fig. 8B). In two Tns500E transgenic mouse lines (2064 and 2041) no CAT activity was detected in any of the muscles tested. In extracts made from line 2060, reporter levels were slightly higher than those obtained in CAT assays containing no extract or muscle extracts from wild-type mice. Thus, these results demonstrate that the conserved CCAC- and E-box motifs found in the TnI SURE are essential for its transcriptional activity.

DISCUSSION

Although there has been enormous progress in understanding the molecular mechanisms that regulate myogenic determination and commitment, little is known of how fiber type diversity is generated. In mammalian muscle, fiber-type-specific properties are manifested around birth, after skeletal muscles have received motoneuron innervation. This may account for the fact that established cell lines and mammalian primary muscle cultures fail to exhibit fiber-type-specific properties, which has made studies of fiber diversification in vitro increasingly difficult. Thus, most studies directed at identifying *cis*-acting sequences that confer muscle-type-specific expression have been performed in transgenic mice (4, 19, 25, 34, 50, 51). As a model to understand the mechanisms that selectively direct expression of contractile protein isoforms to either slow or fast muscles, we have focused our analysis on the TnI family. To date, this report describes the smallest *cis*-acting sequences that are sufficient to confer either slow- or fast-muscle-specific transcription and is the first to directly compare the regulatory elements conferring slow- and fast-muscle-specific transcription.

We previously reported that 5′ flanking and intronic sequences of the rat TnIs gene conferred expression specifically in slow-twitch muscles (4). In the present studies, we initially demonstrate that TnIs 5′ flanking sequences are sufficient to direct slow-twitch-specific expression. Although the levels of CAT activity decreased as deletions of the upstream sequences were tested, in all cases there were levels of reporter activity greater than 20-fold higher in the SOL than in the fast-twitch muscles (EDL, GAS, and TBL). Delineation of the 5′ flanking

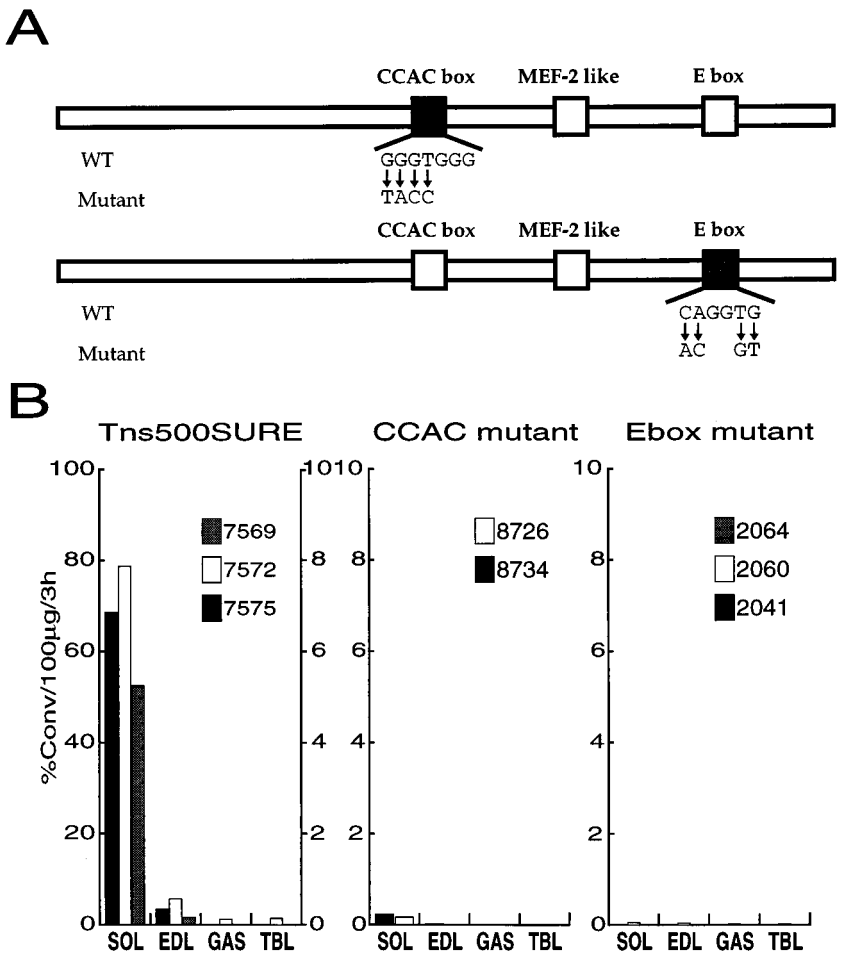

FIG. 8. The CCAC box and E box in the TnI SURE are functionally required for the function of the element in transgenic mice. (A) Schematic diagram of mutations of core elements within SURE in the rat TnI slow gene. The positions of the CCAC box, MEF-2-like site, and E box are indicated. Nucleotide substitutions used to produce the mutant CCAC and E boxes are noted below each sequence. (B) CAT reporter activity was measured in extracts made from different muscles of Tns500CCAC and Tns500E transgenic mouse lines. The assays were performed using 100 μg of protein for 3 h at 37°C.

sequences reveals that the regulatory region is located between −949 and −479. Interestingly, transient transfection assays in C2C12 myotubes with CAT constructs containing the −479 domain had shown that it conferred specific activity in myotubes (4). However, all attempts using muscles of E11.5 and E18.5 embryos or rostral, caudal, or myotomal adult muscles of transgenic mice harboring the −479-bp sequence driving CAT (three tail-positive lines) or β-galactosidase (two tail-positive lines) failed to show any reporter activity or RNA. Comparison of the rat sequences between −949 and −479 with the human TnIs gene (12) revealed a highly homologous region of 128 bp. We find that addition of the 128-bp SURE to the inactive −479 rat TnI promoter is sufficient to reestablish transcription specifically in slow-twitch muscle. Interestingly, when the SURE is linked to a CAT construct containing the TK basal promoter, the reporter fails to be expressed in any of the tissues tested; there are precedents for other enhancers that fail to interact with the TK promoter but function in the context of their natural promoters (56). In contrast to these results, Corin et al. (13) recently reported that a luciferase reporter construct, driven by the TK minimal promoter and a 157-bp fragment from the human TnIs gene (containing the SURE), was preferentially expressed in slow muscles when introduced into adult muscles by direct DNA injection. The two experimental approaches differ markedly in that the somatically injected DNA remains episomal. Thus, we cannot exclude the possibility that the rat TnI SURE on its own is sufficient to direct slow-muscle-specific transcription.

Transcription regulatory sequences in the quail TnIf gene have been previously studied in cultured cells (36) and in transgenic mice (24, 25). From studies in transiently transfected cultured cells, Lin and colleagues demonstrated the presence of a 144-bp enhancer, located in the first intron of the quail gene, that confers transcription specifically to differentiated muscle cells (38). Using transgenic mice, Hallauer et al. (24) recently showed that the TnIf 530 bp of 5′ flanking sequence plus the first exon and intron (a total of approximately 2.3 kb) containing the aforementioned enhancer confers expression specifically in type II myofibers. In the present study we demonstrate that just the 144-bp enhancer linked to the rat TnIs −479 promoter is sufficient to confer fast-twitch muscle expression. These results, taken together with our results with the rat TnIs regulatory elements, strongly suggest that both elements are necessary and sufficient to direct muscle-type-specific expression.

Presently, the 128-bp SURE and the 144-bp fast IRE (FIRE) constitute the shortest sequences known to specifically confer transcription in either slow- or fast-twitch muscles. In

addition, these results permit the comparison for the first time of regulatory sequences present in evolutionarily related contractile genes which may have diverted prior to the generation of different muscle types. As shown in Fig. 6, comparison of the two regulatory elements (in opposite orientations relative to each other) reveals that they share an E box, a MEF-2-like site (14), a CCAC box motif, and an additional GCAGGCA sequence that is not similar to previously described elements; the middle CAGG portion of this motif weakly resembles a site previously described (28, 44). Using EMSAs, we have shown that the MEF-2-like site, E box, and CCAC site in the TnI SURE bind their cognate factors (Fig. 7) and that the last two motifs are necessary for the function of the TnIs regulatory element (Fig. 8). Most of these core motifs present in the TnI genes have been found in the regulatory sequences of muscle genes studied in cultured myotubes, many of which are not expressed specifically in either fiber type. The E boxes present in multiple muscle genes are necessary for expression in skeletal muscle; however, myoglobin (5, 6, 16) and the δ subunit of the nicotinic acetylcholine receptor do not require this *cis* element for muscle expression (27, 55). Although the distribution of MyoD and myogenin mRNAs varies among muscles composed of different fiber types (32, 48), there is no evidence that these factors are involved in conferring muscle type specificity. Because mutation of the E box in the TnI SURE abolishes transgene expression, it is not possible to determine its role in the fiber specificity of the TnIs gene. MEF-2-like (or AT-rich) sequences are also necessary for the expression of other skeletal-muscle-specific genes (23, 59). In the case of the creatine kinase gene, an AT-rich site on the upstream enhancer was found to interact with multiple transcription factors, including MEF-2, M-Hox, and Oct-1, but to be specifically activated by MEF-2 (14). Studies of the human aldolase A pM promoter, where a 280-bp region is sufficient to direct expression of a reporter to fast muscles of transgenic mice, have shown that a MEF-2 site in the regulatory region is unnecessary for muscle type specificity (51). The role of the MEF-2-like sequences in the rat TnI SURE will need to be evaluated. The CCAC motif is present in the enhancers for myoglobin, cardiac troponin C (44, 53), myogenin (20), and MCK (30) but absent in a pM aldolase A promoter construct which is expressed specifically in fast muscles (51). A factor that binds to the CCAC motif present in the myoglobin enhancer has recently been isolated (7). Interestingly, antisera generated against the cloned factor recognize a higher-molecular-weight protein that accumulates in the tibialis when this muscle is depolarized with electrical patterns that mimic those used by slow motoneurons, a condition that causes the muscle to adopt slow-muscle-like properties. The role of this factor in conferring slow properties to muscle has yet to be demonstrated. However, the fact that the TnIsCCAC transgenic mice, which harbor a mutation in the CCAC site of the TnIs SURE, continue to show preferential reporter expression in the SOL versus the fast-type muscles (Fig. 8) suggests that the CCAC motif is not essential to direct fiber type specificity. An alternate mechanism that needs to be considered for the role of the SURE and FIRE is that these sequences function as locus control regions and not as classical enhancers. These two regulatory elements may control the chromatin structure of TnI genes differentially in distinct populations of myocytes which give rise to diverse types of myofibers.

Interestingly, the relative order of the motifs to each other in the TnI regulatory elements is conserved (Fig. 6) and suggests that spatial interactions between multiple factors on these enhancers are required to confer skeletal-muscle specificity, an idea that is supported by the fact that mutations of either the E box or the CCAC site in the TnI SURE result in the complete or dramatic reduction of transgene expression. How fiber type specificity is achieved is another issue. At present it is impossible to know if fiber type specificity results from the binding of unrelated factors to distinct *cis*-acting elements in the TnI SURE and FIRE. Another possibility is that the differential binding and interaction of heteromeric or posttranslationally modified factors with the related *cis*-acting sites determine fiber specificity. The results obtained with the aldolase A promoter (51) and our work on the TnI SURE suggest that mutation of single motifs is not a feasible approach to map specific elements conferring fiber specificity, since transgene expression is eliminated (possibly because of the disruption of higher-order complexes). Because the linear arrangement of motifs in both TnI regulatory elements conferring fiber specificity is conserved, the generation of chimeras between the SURE and the FIRE may be a promising experimental approach for identifying sequences directing fiber type specificity.

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