A Novel Site, Mt, in the Human Desmin Enhancer Is Necessary for Maximal Expression in Skeletal Muscle*

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Previous investigations have shown that expression of the muscle-specific intermediate filament desmin gene in skeletal muscle is controlled in part by a 5′ muscle-specific enhancer. This enhancer activity can be divided into myoblast-specific and myotube-specific activation domains. The myotube-specific region contains a MyoD and MEF2 sites, whereas the myoblast-specific region contains Sp1, Krox, and Mb sites. In the present study, we designed mutations in the conserved portion of the myotube-specific region; transfection analysis of these mutations showed that a novel site located between the MyoD and MEF2 sites, named Mt (G GTATTT), is required for full transcriptional activity of the desmin enhancer in skeletal muscle. Although gel mobility shift assays demonstrate that myotube, myoblast, fibroblast, and HeLa nuclear extracts contain a nuclear factor that binds specifically to Mt, four copies of the Mt site function as the native enhancer only in myotubes. Functional synergy among the MyoD, MEF2, and Mt sites in myotubes has been demonstrated. These results show that the novel Mt site cooperates with MyoD and MEF2 to give maximal expression of the desmin gene.

Skeletal muscle commitment, differentiation, and maturation are largely controlled by the transcriptional regulation of a large battery of unlinked muscle-specific genes. A variety of different DNA sequence motifs that are required for muscle-specific gene transcription have been identified in many genes. These include the E-box (CANNNTG), MEF2 site (CTA/ATCTAG), M-CAT-box (CCATCCT), and CARbox (CC(A/G)TGG) that serve as binding sites for the myogenic basic helix-loop-helix MyoD1 family proteins (1–5), MEF2 protein (6–9), TEF1 (10–12), and serum response factor (13), respectively. Other sequence elements required for the transcription of genes expressed in skeletal muscle have been defined by mutational analysis of promoter-reporter constructions, such as Trelx (14), CCAC-box (15, 16), MEF3 (15, 17), Mb (18) etc., but their corresponding binding factors await characterization. The aim of the present study is to find new elements necessary for desmin gene expression in skeletal muscle.

Desmin, a muscle-specific member of the intermediate filaments protein multigene family, is encoded by a single gene (19, 20). Desmin is located at the level of the Z-disc in striated muscles. It has been proposed that desmin maintains the integrity of muscle tissues upon stress. Cardiovascular lesions and skeletal myopathies have been found in mice lacking desmin (21). Desmin is expressed at early stages of myogenesis, such as in replicating myoblasts and satellite cells and at high levels in differentiated myotubes (22–27). In a previous study, we demonstrated that the sequence including 228 bp upstream of the transcription initiation site is sufficient to confer low level muscle-specific expression. A negative region was located between −693 and −228 bp. High level expression of the gene depends on a 280-bp muscle-specific enhancer located between −693 and −973 bp (28). This enhancer can activate either the desmin promoter or heterologous promoters in myogenic cells and can function not only in myotubes but also in myoblasts via the action of two independent activating regions (18). In the myoblast-specific domain, four regions are protected by nuclear factors from myogenic cells; three of these contain a GC-rich sequence sharing homology with the Krox binding site (29). Deletion and site-directed mutation experiments demonstrated that at least two Krox-like sequences are required for enhancer activity in myoblasts. In addition, another GC-rich sequence, designated Mb, sharing some homology with an Ets binding site, is also required for full enhancer activity in myoblasts. In the myotube-specific region, one MyoD site and one MEF2 site are necessary for full enhancer activity in myotubes.

In the present study, we demonstrate that a novel site Mt (G GTATTT) is necessary for maximal expression of desmin in myotubes. We show that this novel Mt site cooperates with MyoD and MEF2 sites to give maximal expression of desmin. Mutation of Mt in the myotube-specific enhancer resulted in the decrease of the transactivation level by the MyoD family in nonmyogenic cells and can function not only in myotubes but also in myoblasts via the action of two independent activating regions (18). In the myoblast-specific domain, four regions are protected by nuclear factors from myogenic cells; three of these contain a GC-rich sequence sharing homology with the Krox binding site (29). Deletion and site-directed mutation experiments demonstrated that at least two Krox-like sequences are required for enhancer activity in myoblasts. In addition, another GC-rich sequence, designated Mb, sharing some homology with an Ets binding site, is also required for full enhancer activity in myoblasts. In the myotube-specific region, one MyoD site and one MEF2 site are necessary for full enhancer activity in myotubes. Finally, gel shift experiments show that the Mt site could specifically bind nuclear factors.

**EXPERIMENTAL PROCEDURES**

Mutagenesis of the Desmin Myotube-specific Enhancer and Preparation of Constructions—Site-directed mutagenesis of the human desmin enhancer was performed according to the method described by Kunkel (30). The oligonucleotide MuMyoD was used to change the MyoD site from CAGCTG to aATCTG, and oligonucleotide MuMt1 was used to mutate the Mt site from GGTATTT to GGgATcc. Previous experiments involving transfection of the deletion mutants in myotubes have demonstrated that 70 bp (−919 to −850) of the human desmin myo-
tube-specific enhancer gives an activity similar to that of the complete enhancer in myotubes (18). We synthesized this 70-bp fragment by polymerase chain reaction using oligonucleotide 1, with a HindIII linker (5'-AACGTCTCCTCTCTGAGAATAC-3') corresponding to –919 to –901 bp, and oligonucleotide 2, with XhoI linker (5'-TG TAGAGACTCTTCTCCTCTCTGAGAATAC-3'). The polymerase chain reaction-amplified fragment was inserted into the HindIII-XhoI sites of pBLCAT2, which contains the herpes simplex virus thymidine kinase promoter and the chloramphenicol acetyltransferase (CAT) gene (31). To obtain the mutation in the MEF2 and Mt sites, the following oligonucleotides were used, respectively, as primers in combination with oligonucleotide 2 in a polymerase chain reaction: MuMEF2, 5'-GAAAGCTTCTCCTCTCTGAGAATACCCG-3'; MuMEFMT*, 5'-GAAAGCTTCTCCTCTCTGCAGAATACCCG-3'; MuMy, 5'-GAAAGCTTCTCCTCTCTGAGAATACCCG-3'; MuFL, 5'-GAAAGCTTCTCCTCTCTGAGAATACCCG-3'; MuMy3, 5'-GAAAGCTTCTCCTCTCTGAGAATACCCG-3'; MuMt, 5'-GAAAGCTTCTCCTCTCTGAGAATACCCG-3'; MuMt2, 5'-GAAGCTCCTCCTCTCTGAGAATACCCG-3'; MuMt3, 5'-GAAGCTCCTCCTCTCTGAGAATACCCG-3'; MuMt4, 5'-GAAGCTCCTCCTCTCTGAGAATACCCG-3'; MuMt5, 5'-GAAGCTCCTCCTCTCTGAGAATACCCG-3'. Mutated nucleotides are presented in lowercase letters. Mt* indicates a sequence (AAATCC) complementary to the Mt site that overlaps with the MEF2 site. FL indicates the flanking sequence around the Mt site. The polymerase chain reaction products were inserted 5' to the tk-CAT in the pBLCAT2 plasmid (31), and the resulting constructs are listed in Fig. 1. To study the transcriptional activity of the Mt motif, the oligonucleotides Mt (sense, 5'-ATACCCGGCCAGAACCG-3') and the oligonucleotides M2T, containing two palindromic Mt sites (sense, 5'-CTAGAGAATACCCTGCTCTGCTATCTGGTT-3'; antisense, 5'-CTAGAGAATACCCTGCTCTGCTATCTGGTT-3') were annealed and inserted into both the plasmid pBLCAT2 (31) and pDes-237CAT containing the human desmin pro- moter and CAT gene (28). The constructs containing one, two, and four Mt motifs were obtained.

RESULTS

The Novel Mt Site Cooperates with MyoD and MEF2 Sites to Give Maximal Expression in Differentiated Skeletal Muscle Cells—Previous studies involving transfecting the deletion mutants in myotubes have demonstrated that 70 bp (–919 to –850) of the human desmin myotube-specific enhancer produces an activity similar to that of the full enhancer in myotubes (28). Nucleotide sequence comparisons of this enhancer region with hamster (34) and mouse enhancers (35) showed that this region has been conserved during mammalian evolution, suggesting that it may therefore be important for enhancer function. Fig. 1A shows an alignment of this sequence. Within these 70 bp, 90% are conserved across the three species. Interestingly, a previous footprinting experiment had demonstrated that 42 bp of this region were protected by nuclear extracts from muscle cells (18). This 42-bp region includes MEF2 and MyoD binding sites. Due to its high degree of evolutionary conservation and its capacity to bind nuclear factors, we decided to analyze this region in more detail for its transcriptional activity.

To investigate the cooperation between the sites present in this region and the possibility that an additional regulatory site exists between the MEF2 and MyoD sites, we introduced different mutations in this 42-bp region. These constructs were tested by transient transfection assays in C2,7 myotubes and myoblasts, and the data are presented in Fig. 1C. When linked to the tk promoter (construct 1), this 70 bp can increase expression about 33-fold compared with the tk promoter alone in myotubes but not in myoblasts. Mutation of the MEF2 binding site resulted in a loss of 70–90% of the CAT activity in myo- tubes according to the mutated nucleotides (constructs 2 and 3); mutation of the MyoD site resulted in almost complete loss (95%) in myotubes (construct 10). These results show that the MEF2 and MyoD sites are important for the gene expression in myotubes. The mutations in constructs 6, 7, and 8 did not change gene expression in myoblasts but resulted in a 60–80% loss of CAT activity (from 33 to 10, 13, and 7, respectively) in myotubes. These three mutations were located in a GGTATT sequence. Mutations in 6, 7, and 8 convert wild-type sequence GGTATT into GAGATCT, GTGACC, and GGGATCC, respectively. These mutations abolished the binding activity to nuclear proteins (Fig. 5). We designate this sequence (GGTATT) as Mt (active in myotubes). We found this sequence present as a palindrome between the MEF2 and MyoD sites; one Mt sequence, AAATCC (complementary to the Mt site), overlaps with the MEF2 site. The mutation in construct 3 changes both the MEF2 and Mt site in the same time reduced CAT activity by 70% in myotubes. Double mutations in the MEF2 and Mt sites results in an almost complete loss of CAT activity (constructs 11 and 12) in myotubes. The mutation in construct 9 changes the flanking sequence of the Mt site does not alter CAT activity in myoblasts but resulted in a 60–80% loss of CAT activity (from 33 to 10, 13, and 7, respectively) in myotubes. The increase of CAT activity of mutant 4 in myotubes could be explained by the fact that this mutation converted the imperfect (nonconsensus) desmin MEF2 site CTATAAATAcCC to a perfect MEF2 site CTATAAATAGGA, which is 100% identical to the MEF2 consensus (6–9). It has been reported that the flanking sequences modulate the cell specificity of the M-CAT regulatory site (37). The increase in gene expression in myo- blasts and myotubes for mutants 4 and 5 could also be related in that the flanking sequences around the MEF2 and Mt sites could have some negative effect, or mutations could create new regulatory elements. These results demonstrated that the Mt sequence is necessary for the maximal expression in myotubes, and mutation of any one of the three sites (MEF2, Mt, and MyoD) results in a dramatic loss of activity, suggesting a synergistic effect between MEF2, Mt, and MyoD sites to give maximal expression in differentiated skeletal muscle cells.

Mutations of the Mt Site Diminish the Transactivation Effect of the MyoD Family on the Desmin Enhancer in Nonmyogenic Cells—The discovery of the MyoD family of proteins provided the first information about the molecular events that control myogenic commitment and muscle fiber differentiation. When expressed in many nonmyogenic cell lines, each member of the MyoD family is capable of converting the nonmyogenic cells into cells capable of myotube formation and muscle-specific gene expression (1, 38–41). The MyoD binding site alone is not sufficient to direct high transcription and should cooperate with other sites (42). To know if Mt site mutations influence MyoD transactivation on the desmin enhancer, the constructs containing the mutations either in the Mt site or MyoD site linked to the desmin promoter or tk promoter were carried out. These constructs were cotransfected with MyoD1 or myogenin expression vectors in nonmyogenic cells C3H101/2. We have
found that both factors could greatly increase CAT activity when wild-type desmin enhancer was present in C3H10T1/2 fibroblasts. However, when the MyoD binding site was mutated, the transactivation effect was diminished to the level obtained by the desmin promoter without enhancer (Fig. 2). When the Mt site was mutated, the transactivation effect was diminished about 50% compared with the wild-type enhancer. Similar results were obtained when wild-type or mutated enhancer was linked to the tk promoter (Fig. 2). These results suggest that the novel Mt site is also necessary to obtain the maximal transactivation effect by the MyoD family and that there is a potential cooperation between Mt and MyoD sites.

Four Mt Motifs in Palindromic Form Can Function as the Native Enhancer in Myotubes—To test the capacity of the Mt sequence to direct gene expression, one, two, and four copies of the Mt site were linked to either the tk promoter or desmin promoter. Because the Mt site is present in a palindrome in the human desmin enhancer, two copies and four copies of the Mt site were linked either in tandem or in palindrome fashion as indicated in Fig. 3. These constructs were transfected transiently in HeLa cells, fibroblasts, myoblasts, and myotubes. The results show that one copy of the Mt site linked to both tk and desmin promoters cannot activate gene expression in these four cell types. Two copies of the Mt site arranged in tandem or as a palindrome can give rise to a slight increase in CAT activity (three times) only in myotubes. Four Mt copies linked in tandem can increase about 9-fold the expression in myotubes, but not in myoblasts, fibroblasts, and HeLa cells. Interestingly, four Mt copies linked in palindromic fashion function as native myotube-specific enhancers of desmin and can increase 30 times the CAT expression in myotubes. However, in contrast to the native desmin myotube-specific enhancer, these four copies of palindromic Mt sequence give rise to a slight increase in CAT activity (2–6 times) in myoblasts, fibroblasts, and HeLa cells. This suggests that sequences surrounding the Mt site in the myotube-specific enhancer of the human desmin gene could function as negative elements in undifferentiated muscle cells and nonmyogenic cells.
The Mt Site Exhibits Specific Nuclear Protein Binding—DNA-protein binding experiments were performed to characterize trans-acting factors that interact with the Mt site. A 26-bp oligonucleotide containing the Mt site was bound in gel mobility shift assays by nuclear proteins from HeLa cells, fibroblast 3T3, C2,7 myoblasts, and myotubes (Fig. 4A, lanes 1, 4, 7, and 10). The binding of nuclear proteins was sequence-specific as assessed by competition with excess unlabeled oligonucleotides bearing the wild-type Mt sequence (Fig. 4A, lanes 2, 5, 8, and 11) and the lack of competition by excess oligonucleotides bearing the mutated Mt sequence (Fig. 4A, lanes 3, 6, 9, and 12). Since the Mt binding complex migrated to approximately the same gel location irrespective of whether the nuclear extracts were derived from HeLa, fibroblasts, myoblasts, or myotubes, it seems likely that the same or similar Mt-binding protein, termed MtBF (Mt binding factor), is present in these diverse cell types.

The Effect of the Mutation within the Mt Site on the Binding Activity to Nuclear Proteins—To try to obtain a consensus sequence for Mt, we tested the binding capacity of a series of oligonucleotides containing different mutations in the Mt site and in the surrounding sequences. Gel mobility shift assays showed that the specific complex formed by Mt and nuclear proteins from the C2,7 myotubes was abolished by competition with unlabelled wild-type Mt sequence (Fig. 4C, lanes 2 and 3). A/T-rich sequence from myoglobin promoter (Fig. 4C, lanes 4 and 5) (43), MuFL1 (Fig. 4C, lanes 6 and 7), and MuFL2 (Fig. 4C, lanes 8 and 9). MuFL1 and MuFL2 change only the flanking sequence around the GTTATTT sequence. The A/T-rich sequence contains a GTTATTT sequence. This complex is also abolished by the unlabelled MEF2 oligonucleotide of the human desmin enhancer (Fig. 4C, lanes 18 and 19), which contains an overlapping Mt sequence, GTTATTT, but is not abolished by the MEF2 site of the MCK enhancer, which contains a GTTATTT (Fig. 4C, lanes 16 and 17). To show that MtBF is not related to the MEF2 family, gel supershift analysis was performed, and the results demonstrated that the antibody against MEF-2 did not change the Mt binding activity (Fig. 4B, lane 2), whereas this antibody can supershift the band formed by MEF-2 site from the MCK and desmin enhancer (Fig. 4B, lanes 10 and 15). In addition, the Mt site cannot compete with the MEF2 site of MCK and vice versa (Fig. 4B). The MEF2 site of the desmin enhancer containing the GTTATTT sequence can form both MEF-2 and Mt complexes (Fig. 4B, lane 9). These results demonstrated MtBF is different from MEF-2. The Mt-protein complex is not abolished by a MyoD site of the human desmin enhancer (Fig. 4C, lanes 20 and 21), but surprisingly mutation of the MyoD site GGCAGCTG to GGAATCTG abolished the complex (Fig. 4C, lanes 22 and 23). The mutations that transformed GTTATTT to GTGACcT, GGGATcc, ccTATT, and GAGAtt (Fig. 4C, lanes 9–12, 24, 25, 28, and 29) cannot compete with the Mt sequence. The mutation from GTTATTT to cGTATTT exhibits a slight competition with Mt (Fig. 4C, lanes 26 and 27). To show that the Mt binding activity is different from TATA-box binding activity, an oligonucleotide containing a potential TATA-box (GGTATAAAA) was used as competitor and shows no competition with Mt (Fig. 4C, lanes 30 and 31). MtBF is also different from the HMG-box proteins and Oct1–2 factors, because the oligonucleotides containing an HMG-box binding site (TTCAAG) and Oct binding site (ATGCAAT) (44) cannot compete with the Mt site (Fig. 4B). The Mt motif (GTATTT) differs in one nucleotide from the GT2-box (GGTATTT), which is present in the promoter of phytochrome polypeptide-encoding gene A of rice. The GT2 binding factor shares a trihelix motif (HLHLH, helix-loop-helix-loop-helix) and might be representative of a new class of DNA-binding proteins (45). But the competition assay showed that MtBF is not related to the GT2 factor (Fig. 4B, lane 8). The oligonucleotides used in the gel mobility shift assays were listed in the Fig. 5. A preliminary consensus sequence (G/G/C/T/A/GTTT/C/T) for the Mt site has been deduced. No homology with Mt has been found within the known DNA binding sequences (for a review, see Ref. 44). Using the preliminary consensus, we were able to identify several potential Mt sites in other muscle-specific control regions (Fig. 6). These sites are located within regions that have been shown to be functionally
important for transcription of the associated genes, most of them overlapped with a potential MEF2 site.

**DISCUSSION**

A Novel Regulatory Element, Mt, Cooperates with MEF2 and MyoD Sites—This study has identified a novel cis-acting regulatory element Mt, between the MEF2 and MyoD sites within the myotube-specific enhancer. Mutation analysis reveals that a sequence, GGTATTT, located between the MyoD and MEF2 sites is required for full transcriptional activity of the desmin myotube-specific enhancer in myotubes; the relative loss of transcriptional activity in myotubes due to Mt mutations that abolished the Mt-protein complex formation is greater than 60% compared with the 70-bp wild-type enhancer (Fig. 1C). The same Mt mutation neither decreases nor increases the low level of reporter gene expression in proliferating myoblasts or NIH 3T3 fibroblasts and HeLa cells. Mutation analysis also showed that the MyoD site is important for enhancer activity in myotubes. Mutation of this site resulted in the loss of almost all of the enhancer activity despite the presence of MEF2 and Mt sites. Mutation of the MEF2 site resulted in a 90% loss of CAT activity, suggesting that this site is also necessary for enhancer activity in myotubes. The fact that mutation of one of these three sites resulted in dramatic loss of reporter gene activity suggests the existence of a synergy among the MyoD, MEF2, and Mt sites.

**Fig. 3.** Four Mt motifs in palindromic form can function as a native enhancer in the myotubes. A, expression vector bearing the Mt site. The monomer, dimers, and tetramers of the Mt site were linked to either the tk promoter or the desmin promoter. Because the Mt sites are present in a palindrome in the human desmin enhancer, dimers and tetramers of the Mt site were arranged either in tandem or in palindromic fashion. These constructs were transfected transiently in HeLa, fibroblasts, myoblasts, and myotubes. *Hatched box,* myotube-specific enhancer region; *waved box,* myoblast-specific enhancer region. B, relative CAT activity in four cell types. Note that four Mt copies linked in tandem can increase gene expression about 9-fold in myotubes. Four Mt copies linked in palindromic fashion, which function as a native myotube-specific enhancer of desmin, can increase CAT expression 30-fold in myotubes. The efficiency of transfection was controlled by cotransfection with pRSVLacZ vector. Data are expressed as the mean ± S.D of five experiments.
It is interesting to note that the 70-bp fragment containing these three sites acts as a powerful myotube-specific enhancer.

Mt Binding Factor Is Present in Cultured Skeletal Muscle and in Nonmuscle Cells—

One Mt-binding complex of similar mobility was observed in myotubes, myoblasts, fibroblasts, and HeLa cells in a gel shift mobility assay. Although one- and two-Mt motifs do not appear to be active on their own, four-Mt sites arranged in tandem are active only in myotubes. Interestingly, four Mt sites linked in a palindrome can function as a wild-type desmin enhancer in myotubes. The reason why a four-Mt site in a palindrome is more active than that in tandem awaits determination and could be related to fact that the Mt motif in a palindrome favors the spatial interaction of the transactivators in the transcriptional machinery. It seems that this could not be related to novel factors binding to the oligonucleotides containing two or four Mt motifs in a palindrome, because they give a DNA-protein complex similar to the oligonucleotide containing one Mt site in gel shift assays (data not shown). MtBF is present in various cell extracts; why then are four copies of Mt very active only in myotubes? It is possible that MtBF’s activity is regulated in these cell types through several mechanisms, for example by post-translational modification (e.g. phosphorylation) or by interaction with ubiquitous or cell type-specific coactivators. Considering the fact that Mt tetramers are very active in myotubes but not in myoblasts, mitogen-regulated phosphorylation could be involved in MtBF activity.

In the case of the MyoD family, cAMP-dependent kinase can inhibit the activity of myogenic helix-loop-helix proteins (64).

**FIG. 4.** Panel A, specific binding of nuclear factors to the Mt sequence. The end-labeled Mt oligonucleotide was incubated with 4 μg of nuclear extract from HeLa cells (lanes 1–3), NIH3T3 fibroblasts (lanes 4–6), C2,7 myoblasts (lanes 7–9), and C2,7 myotubes (lanes 10–12) and analyzed on a 5% polyacrylamide gel. One DNA-protein complex was observed in four cells. The Mt binding complex, indicated by an arrow, is abolished by the addition of a 50-fold molar excess of unlabeled Mt probe (lanes 2, 5, 8, and 11) but not by the addition of an equivalent amount of mutated muMt1 (lanes 3, 6, 9, and 12). C, DNA-protein complex; F, free probe. Panel B, gel supershift assay shows that Mt-binding protein is different from MEF-2 factor. Labeled Mt (lanes 1–8), MEF2 (Des) (lanes 9–13), and MEF2 (MCK) (lanes 14–18) sequences, respectively, were incubated with 4 μg of nuclear extract from C2,7 myotubes. Antibody against MEF2 supershifted the MEF2-protein complex indicated by the asterisk (lanes 10 and 15) but did not change the Mt-protein complex (lane 2). The sequences containing MEF2 (MCK), Oct1, HMG, and GT-2 sites did not compete with Mt (lanes 5–8 and 13). The Mt site cannot compete with MEF2 site (lanes 11 and 16). The MEF2 (Des) site containing an overlapping Mt site can form Mt- and MEF2-protein complexes (lane 9). The Mt site is underlined, and the MEF2 site is boxed; nucleotides different from the consensus sequence are indicated by lowercase letters. Panel C, effect on the binding activity to nuclear protein of the mutation in the Mt site and surrounding sequence. The different oligonucleotides were used as unlabeled competitors with a 10- and 50-fold molar excess. Lane 1 indicates the complex formed with the nuclear extract of C2,7 myotubes without competitors. The following unlabeled oligonucleotides can abolish the complex: Mt; A/T-rich sequence, MuFL1; MuFL2; MuMt4; MEF2 (Des); and MuMyoD(Des). MuMt5 gives a slight competition. The following oligonucleotides can not abolish the complex formation: MuMt; MuMt2; MEF2 (MCK); MyoD1 (Des); MuMt3; MuMt6; and TATA.
Mt ATACCCGCTCTGTTAATTGGTG ***
A/T tcGATGgggATATCAATGGG ***
MfU1 ATACCCGCTCTGTTAATTGGTG ++
MfU2 ATACCCGCTCTGTTAATTGGTG ***
MfU3 ATACCCGCTCTGTTAATTGGTG ++
MfU4 ATACCCGCTCTGTTAATTGGTG ***
MEF2 (MCK) ccaAGGGgcAGTTTGCATGC C
MEF2 (Des) ccaAGGGgcAGTTTGCATGC C
MfU Old (Des) cgcGATGgggATATCAATGGG -
MfU New (Des) cgcGATGgggATATCAATGGG -
Oct1 gcAGTGGgGcAGTTTGCATGC C
HMG tTcGATGgggATATCAATGGG -

Preliminary consensus GTTATT

CA C

Mo MCK (intron) 1001 TGGGATTTATGAT 987
rat MLC1/3 -146 GGGGATTTATGAT 160
Mo MLC1/3 -147 GGGGATTTATGAT 161
Hu MLC1/3 -148 GGGGATTTATGAT 162
Ch MLC1/3 -159 TGGGATTTATGAT 173
Mo sTnC 662 AAGCGTTTTATGAT 648
Hu sTnC 662 AAGCGTTTTATGAT 648
Mo GLUT4 -425 TGGGATTTATGAT 439
Hu desmin -989 CGGGATTTATGAT 914
Mo desmin -897 TGGGATTTATGAT 883
HMV TGGGATTTATGAT 886
Mo desmin -849 TGGGATTTATGAT 834
Hu desmin -842 TGGGATTTATGAT 856
Hu sTnC -838 TGGGATTTATGAT 824
Hu sTnC -838 TGGGATTTATGAT 824
Mo sTnC -788 TGGGATTTATGAT 782
Hu sTnC -787 TGGGATTTATGAT 781
Hu myoglobin -858 TGGGATTTATGAT 872
Mo myoglobin -157 AAGCGTTTTATGAT 171
Hu myoglobin -142 AAGCGTTTTATGAT 156
Hu sTnC -151 AAGCGTTTTATGAT 165
CH MLC1/3f 2790 TGGGATTTATGAT 2687
CH cardiac MLC1c/ls -2593 TGGGATTTATGAT 2555
Hu Mo and rat dystrophin -149 TGGGATTTATGAT 163
MO cTnC 48 TGGGATTTATGAT 54
Hu cTnC 113 TGGGATTTATGAT 99
Mo cardiac α-actin -319 TGGGATTTATGAT 17
Hu sTnC -852 TGGGATTTATGAT 986
Hu and Mo β-endolase 589 AAGCGTTTTATGAT 683
α-actin 546 AAGCGTTTTATGAT 568

FIG. 5. The oligonucleotides and their capacity to bind nuclear proteins. The oligonucleotides used in the gel mobility shift assays (Fig. 4) are listed. Only one strand of the oligonucleotide is indicated, and the antisense strand of MEF2 (MCK), MEF2 (DES), and A/T-rich sequence are shown HT. The oligonucleotides and their capacity to bind nuclear proteins are shown in Fig. 4. The oligonucleotides used in the gel mobility shift assays are indicated by a plus symbol (competition) and minus symbol (no competition). A preliminary consensus sequence (G/C/G/T/A)AT(T/C)T for the Mt site has been deduced. Oligonucleotides MyoD (Des) and MyoDmut (Des) correspond to a wild-type and a mutated MyoD binding site, respectively, of the desmin enhancer. Oligonucleotides MEF2 (DES) and MEF2 (MCK) represent the MEF2 binding sites from the enhancer of the desmin and the MCK gene (46), respectively. A/T-rich sequence is from the myoglobin upstream region (43). The nucleotides different from the Mt oligonucleotide are indicated by lowercase letters. The Mt site and the mutated sequences within this Mt site are underlined.

The alternative mechanism, protein-protein interaction, could also be involved in MtfB activity. In fact, it has been reported that MyoD and MEF2 proteins need the coactivator p300 to activate gene expression (65). In addition, protein-protein interaction mechanisms have been proposed for regulation of the cardiac troponin T gene. In this gene, the ubiquitous TEF-1 factor binds to an M-CAT control element (10), but its full transcriptional activity requires intermediary factors, termed TIFs (66). Coactivators of MtbF are perhaps present only in myotubes. Post-translational modification and protein-protein interaction could both be involved in the regulation of the MtbF. Current efforts in our laboratory are directed at cloning this apparently unknown factor. Understanding the mechanisms involved in regulating MtbF function awaits its cloning and characterization.

Mt Site Is Present in Numerous Muscle-specific Regulatory Control Regions—We have established a preliminary consensus sequence (G/C/G/T/A)AT(T/C)T for the Mt site from the competition analysis of gel retardation assays. Using the preliminary consensus sequence, we were able to identify several potential Mt sites in other muscle-specific control regions (Fig. 6). All of these potential Mt sites are located within regions that have been shown to be functionally important for transcription of the associated genes; most of them overlapped with an A/T-rich sequence or a potential MEF2 site. The consensus sequence of MEF2 is (C/T/T/A/T/AATAAGG/G) (67). The following sequence CcTAAAATAGC bearing an imperfect MEF2 site can abolish the Mt protein complex (18, 35), rat sTnC (54), chicken MLC1f/3f 5′-flanking sequence (56), and β-endolase first intron enhancer (63) containing an imperfect MEF2 site (C/T/T/A/T/AATAAC) can compete with the Mt site (Fig. 4B, lanes 18 and 19). The sequence complementary to the Mt site is underlined. However, the sequence (C/T/C/T/A/T/AATAAAC) bearing a MEF2 site found in the MCK enhancer, cannot abolish the Mt-protein complex (Fig. 4B, lanes 18 and 17). These results indicate that there could be some subtle differences between the different potential MEF2 sites present in the control region of muscle-specific genes. The MEF2 site of the desmin enhancer is capable of forming two complexes with the myotube nuclear extracts; one band is abolished by the unlabeled MCK MEF2 site and the other by unlabeled Mt site, whereas the complex formed by the MCK MEF2 site cannot be abolished by the addition of excess unlabeled Mt sequence (Fig. 4B). The significance of this difference between the MEF2 sites in the control of muscle-specific gene expression awaits study. It is interesting to note that the common core regulatory sequences are found in skeletal slow and fast fiber-specific regulatory elements (54). This common core sequence contains some conserved regulatory elements such as an E-box, CCAC-box, and MEF2-like site. Transgenic mice bearing this common core sequence of quail fast fiber-specific troponin I express preferentially the transgene in fast fibers (68), whereas transgenic mice containing the core element of rat sTnC are preferentially in slow fibers. These transgenes contain different MEF2 sites. The MEF2 site in the quail fast fiber-specific troponin I control
region is perfectly homologous to the MEF2 consensus sequence and does not contain an Mt sequence, whereas the MEF2 site in rat sTnI enhancer is an imperfect MEF2 site (one nucleotide difference) and contains an Mt sequence. In addition, three sequences sharing the homology (six bp out of seven; Fig. 6) with the Mt sequence are found in the rat sTnI control region but not in quail fast fiber-specific tropinin I.

The MtBF is different from that of Mhox, a mesodermally restricted homeodomain protein that binds an A + T-rich element (TAT/ATAAT/ATAAT) in the MCK enhancer. In experiments reported by Cseresj et al. (69), oligonucleotides containing an Mt motif (mutant 10 and 12 GCTATTT) cannot abolish the Mhox-protein complex (69). The MtBF is also different from the TATA-box binding factor, HMG-box-binding proteins, and Oct1–2 factors, because a standard TATA-box sequence (T(A/T)ATAAT(A/T)A) in the MCK enhancer. The MtBF and ATF35 could be the same or similar protein. Nevertheless, there are some differences. ATF35 was reported to be a muscle-specific protein, whereas our experiments show that the Mt binding factor is present in nonmuscle cells like fibroblasts and HeLa cells.

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REFERENCES
1. Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Cell 51, 987–1000.
2. Blackwell, T. K., and Weintraub, H. (1990) Science 250, 1104–1110.
3. Funk, W. D., and Wright, W. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9484–9488.
4. Buckingham, M. E. (1994) Curr. Opin. Genet. Dev. 4, 745–751.
5. Molkentin, J. D., and Olson, E. N. (1996) Curr. Opin. Genet. Dev. 6, 445–453.
6. Pollack, R., and Treisman, R. (1991) Genes Dev. 5, 2327–2341.
7. Yu, Y. T., Breithart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Genes Dev. 6, 1783–1798.
8. Martin, J. F., Schwarz, J. J., and Olson, E. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5282–5286.
9. Andrés, V., Cervera, M., and Mahdavi, V. (1995) J. Biol. Chem. 270, 23246–23249.
10. Farrance, I. K. G., and Ordahl, C. P. (1992) J. Biol. Chem. 267, 17234–17240.
11. Stewart, A. F. R., Larkin, S. B., Farrance, I. K. G., Mar, J. H., Hall, D. E., and Ordahl, C. P. (1994) J. Biol. Chem. 269, 3147–3150.
12. Yockey, C. E., Smith, G., Izumo, S., and Shimizu, N. (1996) EMBO J. 15, 23247–23253.
13. Parmacek, M. S., Ip, H. S., Jung, F., Shen, T., Martin, J. F., Vora, A. J., Olson, E. N., and Leiden, J. M. (1994) J. Biol. Chem. 269, 10651–10659.
14. Bassel-Duby, R., Hernandez, M. D., Gonzalez, M. A., Krueger, J. K., and Kedes, L. (1993) Cell 78, 1625–1650.
15. Kastrinou, K. H., Christy, R. J., and Lane, D. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 251–255.
16. Ianello, R. C., Mar, J. H., and Ordahl, C. P. (1991) J. Biol. Chem. 266, 3309–3316.
17. Tsujino, S., Sakoda, S., Mizuno, K., Kobayashi, T., Suzuki, S., Kishimoto, S., Shanske, S., DiMauro, S., and Schwenke, C. (1991) J. Biol. Chem. 266, 251–256.
18. Martin, J. F., Schwarz, J. J., and Olson, E. N. (1993) Mol. Cell. Biol. 13, 5616–5622.
19. Lucknow, B., and Schuetz, G. (1987) Cell 55, 563–568.
20. Nakayama, M., Stauffer, J., Cheng, J., and Chambon, P. (1991) EMBO J. 10, 4478–4485.
21. Devlin, B. H., Wefald, F. C., Kraus, W. E., Bernard, T. S., and Williams, R. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7816–7820.
22. Gazzolo, S., Coˆ niga, J. L., Babinet, C., and Paulin, D. (1993) J. Biol. Chem. 268, 2698–2703.
23. Li, Z., Coluci-Guyon, E., Pincon-Raymond, M., Mericaksay, M., Pourrin, S., Paulin, D., and Babinet, C. (1996) Dev. Biol. 175, 365–366.
24. Kaufman, S. J., and Foster, R. F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9606–9610.
25. Babai, F., Musevi-Aghdam, J., Schurch, W., Royal, A., and Gabbiani, G. (1990) J. Cell. Physiol. 132–142.
26. Allen, R. E., Rankin, L. L., Greene, E. A., Boxhorn, L. K., Johnson, S. E., Taylor, R. G., and Pierce, P. R. (1991) J. Cell. Physiol. 149, 525–535.
27. Li, Z., Marchand, P., Humbert, J., Babinet, C., and Paulin, D. (1993) Development 117, 947–959.
28. Li, Z., and Paulin, D. (1991) J. Biol. Chem. 266, 5652–5670.
29. Lemaire, P., Deschamps, S., and Stournaras, C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 132–142.
30. Varshavsky, A., and Bloom, H. (1996) Mol. Cell. Biol. 16, 3476–3480.