The Mouse Dystrophin Enhancer Is Regulated by MyoD, E-box-binding Factors, and by the Serum Response Factor*

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In vivo studies in the mouse have revealed that the muscle promoter of the mouse dystrophin gene can target the right ventricle of the heart only, suggesting the need for other regulatory elements to target the skeletal muscle as well as other compartments of the heart. In this study we report the identification of the mouse dystrophin gene enhancer that is located ~8.5 kilobases downstream from the mouse dystrophin gene muscle promoter. The enhancer was tested in myogenic G8, H9-C2, and nonmyogenic 3T3 cell lines and is mostly active in G8 myotubes. Sequence analysis of the mouse dystrophin gene enhancer revealed the presence of four E-boxes numbered E1–E4, a putative mEF-2 binding site, and a serum response element. Site-directed mutagenesis studies have shown that E-boxes 1, 2, and 3 as well as the serum response element are required for enhancer activity. Gel shift analysis revealed two binding activities at binding sites E1 and E3 which were specific to myotubes, and supershift assays confirmed that myoD binds at both these sites. Our study also shows that serum response factor binds the serum response element but in myoblasts and fibroblasts only, suggesting that serum response factor may repress enhancer function.

The 2.5-megabase gene encoding the cytoskeletal protein, dystrophin (1, 2) is the largest gene yet identified and is complex with a minimum of seven promoters. Three promoters, muscle (3), brain (4), and Purkinje (5), express full-length dystrophin isoforms that localize in the skeletal/cardiac muscle, the cerebral cortex, and in cerebellar Purkinje cells, respectively. Four other promoters located in introns 29, 55, 59, and 68 encode shorter isoforms Dp 260 (6), Dp 140 (7), Dp 116 (8), and Dp 71 (9, 10), which are expressed in retina, the central nervous system, the peripheral nervous system, and non-muscle tissues, respectively. The exact role of dystrophin has yet to be determined, but many studies suggest that in muscle it maintains the cytoarchitecture of the cell by bridging intracellular F-actin filaments to the extracellular matrix via contacts with the dystroglycan complex (11, 12). What is known is that mutation in the gene resulting in loss of dystrophin is the major cause of Duchenne muscular dystrophy or Becker muscular dystrophy but is more common in a group of X-linked dilated cardiomyopathy patients (13), in which promoter deletions (14) or splice site mutations (15) abolish expression of the muscle isoform of dystrophin in skeletal and cardiac muscle. These individuals continue to express full-length dystrophin in their skeletal muscle from the brain and/or Purkinje promoters (15, 16), normally silent in this tissue. This suggests that different regulatory elements are involved in regulation of dystrophin gene expression in skeletal and cardiac muscle. This conclusion is supported in mouse by in vivo studies showing that the muscle core promoter of the mouse dystrophin gene is capable of expressing a reporter gene only in the right ventricle of the heart (17), suggesting that other cis-acting elements are required for expression in other compartments of the heart and in skeletal muscle.

The intron-1 enhancer described previously in intron-1 of the human gene is a good candidate for this regulatory function. Because detailed regulatory studies of the dystrophin gene cannot be done in human, we have turned to the mouse gene and have mapped an intron-1 enhancer element located 8.5 kilobases (kb) downstream from the mouse dystrophin muscle promoter which shows 65% homology with its human counterpart located 6.5 kb downstream (18) from the human dystrophin muscle promoter. We have characterized the mouse enhancer in G8 and H9-C2 myogenic and 3T3 non-myogenic cell lines and found that enhancer activity is specific to differentiating myoblasts. Deletion and site-directed mutagenesis studies have confined the enhancer activity to a minimum of four putative binding sites. These include three E-boxes, E1–E3, and a serum response element (SRE). E1 and E3 match the consensus 5′-AACAagt c/g TGC a/t while E2 and a sequence contained in the SRE resemble the consensus 5′-GGAa/cANGTGGGC/gNa/g. Factor binding studies by mobility shift analysis suggest that ubiquitous factors bind E2 and the SRE whereas myotube-specific factors complexed with myoD bind E1 and E3.

**EXPERIMENTAL PROCEDURES**

Isolation of the Mouse Dystrophin Muscle Promoter and the Mouse Dystrophin Enhancer (MDE)—A bacterial artificial chromosome (BAC) (20) insert that contains the mouse dystrophin muscle promoter was identified by screening a BAC genomic library (Genomic Systems Inc.) with a probe that extends from position −500 to −900 relative to the transcription initiation site.* This work was supported by the Canadian Genetic Diseases Network and the Heart and Stroke Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: kb, kilobase(s); SRE, serum response element; MDE, mouse dystrophin enhancer; BAC, bacterial artificial chromosome; bp, base pair(s); PCR, polymerase chain reaction; DTT, dithiothreitol; CDTA, 1,2-diaminocyclohexane-N,N,N′,N″-tetraacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SRF, serum response factor; contig, group of overlapping clones.
The Mouse Dystrophin Enhancer

C2, a 3.0-kb BamHI fragment that was isolated from mdmE and contains the MDE was inserted in either orientation at the BamHI site of the pGL3-P vector.

In constructs of the A series, a fragment of 500 bp was tested for enhancer activity. In A1 and A2, the 500-bp fragment was amplified by polymerase chain reaction (PCR) using primers 5′-ATCCGATCCCTGAGCTGTAGCTGTAGCTGAAATG and 5′-ATCGTAAACGCCGATACCTTGATCCGGTGTGAAAATG, which contain the Miu restriction site (underlined). In A3 and A4, the 500-bp fragment was amplified using oligonucleotide primers 5′-ATCGTGGATCCCTGAGCTGTAGCTGTAGCTGAAATG and 5′-ATCGTGGATCCCTGAGCTGTAGCTGTAGCTGAAATG, which contain a BamHI restriction site. PCR products were gel purified using QIA-QUICK gel extraction cartridges (Qiagen Inc.) and subsequently cleaved with either Miu or BamHI. Miu fragments were inserted at the Miu site of pGL3-P upstream of the of the SV40 promoter to generate A1 and A2. BamHI fragments were inserted at the BamHI site of pGL3-P downstream from the luciferase gene to generate A3 and A4.

The constructs B1 and B2 were made by deleting 220 bp from the MDE contained in constructs A1 and A2. Thus oligonucleotide primers that define the deletion end points 5′-ATCGTGAATATTCCTTCCCAAG-GTAAAAGTCTGAACTGAG and 5′-ATCGTGAATATTCCTTCCCAAG-GTAAAAGTCTGAACTGAG were used to amplify a DNA fragment of 5.2 kb which is missing 220 bp of the 500 enhancer sequencer. Subsequently the fragment was cleaved with EcoRI, self-ligated, and transformed into Max Efficiency Escherichia coli DH5α (Life Technologies Inc.). Deletions of the enhancer sequences were confirmed by sequence analysis with an automatic Applied Biosystems 373 sequencer.

Site-directed Mutagenesis of Enhancer Protein Binding Sites—Binding determinants within the putative protein binding sites of the MDE were replaced with restriction sites by PCR. The oligonucleotide primers that were used to introduce base changes in the MDE binding sites E1–E4, mef-2, and SRE are listed in Table I. As a result, the bindings sites E1–E4 were replaced by a BamHI restriction site, whereas the mef-2 binding site and the SRE were replaced by Miu and PstI restriction sites, respectively. The template used was the B1 construct in which the BamHI site was destroyed by filling in the cohesive ends with Klenow DNA polymerase (New England Biolabs Inc.). The PCR products that encode mutations in E-boxes E1–E4 were cleaved with BamHI, and products encoding mutations of the putative mef-2 and SRE binding sites were cleaved with MiuI and PstI. Cleaved DNA products were gel purified using QIAQUICK cartridges, self-ligated using T4 DNA ligase (New England Biolabs Inc.), and transformed into Max Efficiency Escherichia coli DH5α. All mutations that were introduced in the MDE were confirmed by sequence analysis.

Transfections and Biochemical Assays—For liposome-mediated transfections, 5 μg of recombinant plasmid and 2 μg of pGK β-galactosidase were incubated with 0.014 μl DODAC:DOPE liposomes (Inex Pharmaceuticals Inc.) in 0.9% NaCl at room temperature for 10 min in a final volume of 50 μl. 750 μl of α-minimal Eagle’s medium and 25% fetal bovine serum was added, and the mix was applied to confluent cells on the surface of a well (six-well dish). Cells were incubated with the transfection mix for 3 h at 37 °C, 5% CO2. The mix was removed, and α-minimal Eagle’s medium containing 10% fetal bovine serum, 10% horse serum was added, and the cells were incubated at 37 °C, 5% CO2 for 20 h. Luciferase activity was measured in both myoblasts and myotubes. Myoblasts were harvested after 24 h with a cell scraper in luciferase lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100). Alternatively, cells were induced to differentiate into mature myotubes in α-minimal Eagle’s medium containing 1% horse serum over 4 days and were harvested as above. Fibroblasts were harvested 1 day after transfection and harvested as above. Cellular extracts were prepared as described by the manufacturer (Roche).

Luciferase activity was measured in a 5-μl aliquot of cellular extract mixed with 100 μl of luciferin reagent (20 mM Tricine, 1 mM (MgO3)4Mg(OH)2, 5H2O, 2.7 mM MgSO4, 7H2O, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzymes A, 470 μM luciferin, 530 μM ATP) using a ECG Berthold Lumat 9700 luminometer. The protein concentration of the cellular extracts was determined with the Bio-Rad protein assay kit. β-Galactosidase activity was determined by diluting 10–30 μl of the extract in luciferase lysis buffer in a final volume of 150 μl. The diluted extract was then mixed with 150 μl of 2 × β-galactosidase buffer (200 mM sodium phosphate, pH 7.3, 2 mM MgCl2, 100 mM β-mercaptoethanol, 1.33 mM/ml o-nitrophenyl-β-D-galactopyranoside). The luciferase activity of each extract was normalized for protein concentration and β-galactosidase. The luciferase activity obtained from constructs that contained transcriptional start site of the mouse dystrophin muscle promoter. To map the promoter within the BAC clone, the BAC insert was digested with EcoRI, BamHI, or HindIII restriction endonucleases, and fragments were separated by agarose gel electrophoresis, transferred to a GeneScreen Plus membrane (NEN Life Science Products). This was hybridized to the same probe in the presence of 6 × sodium citrate, 5 × Denhardt’s, 0.5% SDS, 10% dextran sulfate, and 100 μg/ml herring sperm at 65 °C for 20 h. The membranes were washed in 1 × sodium citrate, 0.1% SDS at 65 °C for 20 min following a wash at room temperature. A positive EcoRI fragment of 7.0 kb was subcloned into the pBluescript SK+ (bold line) and mapped by partial cleavage using restriction endonucleases, and a 3.0-kb fragment that includes the human dystrophin intron-1 base pair (bp) fragment that contains the human dystrophin intron-1 enhancer in the presence of 6 × sodium citrate, 5 × Denhardt’s, 0.5% SDS, 10% dextran sulfate, and 100 μg/ml herring sperm at 65 °C for 20 h. Subsequently the membranes were washed in 6 × sodium citrate, 0.1% SDS at 50 °C for 20 min following a wash at room temperature. Positive fragments include a HindIII fragment of 7.0 kb and a BamHI fragment of 11 kb which were subcloned into pBluescript SK+ to generate pmdeH and pmdeB, respectively (Fig. IA).

To identify restriction fragments that include the mouse counterpart to the human enhancer, the BAC clone was incubated with restriction endonucleases EcoRI, BamHI, and HindIII. Restriction fragments were separated by agarose gel electrophoresis and transferred to a GeneScreen Plus membrane. The latter was hybridized to a SpeI-SacI 195-base pair (bp) fragment that includes the human dystrophin intron-1 enhancer in the presence of 6 × sodium citrate, 5 × Denhardt’s, 0.5% SDS, 10% dextran sulfate, and 100 μg/ml herring sperm at 50 °C for 20 h. Subsequently the membranes were washed in 6 × sodium citrate, 0.1% SDS at 50 °C for 20 min following a wash at room temperature. Positive fragments include a HindIII fragment of 7.0 kb and a BamHI fragment of 11 kb which were subcloned into pBluescript SK+ to generate pmdeH and pmdeB, respectively (Fig IA). Both fragments were mapped by partial cleavage using EcoRI, BamHI, and HindIII restriction endonucleases, and a 3.0-kb EcoRI that hybridized to the human dystrophin enhancer was subcloned from pmdeB into the EcoRI site of pBluescript SK+ to generate pmdeE (Fig IB).

Luciferase Constructs—Recombinant plasmids that were used to characterize the dystrophin muscle enhancer activity are derived from the pGL3 vector series containing the firefly luciferase gene (Promega). The C3 construct (Fig. 2) was made by inserting a 3.0-kb SacI-XhoI fragment that was isolated from pmdeE between the SacI and XhoI sites of the pGL3-P vector (Promega) upstream of the SV40 early promoter and the luciferase gene. To generate the constructs C1 and
tained the SV40 promoter/dystrophin enhancer was expressed relative to the activity obtained from constructs that express the luciferase gene from the early SV40 promoter.

**Nuclear Extracts and Gel Mobility Shift Assays—**

Nuclear extracts from all cell lines were prepared as follows. Cells from a 10-cm dish were washed twice with 3 ml of phosphate-buffered saline and harvested by scraping in 500 μl of cold phosphate-buffered saline. Cells were centrifuged at 3,000 rpm at 4 °C for 3 min, resuspended in 400 μl of buffer A (10 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 5 mM EDTA, 1 mM DTT), and binding reactions were carried out as described earlier. Reaction products were loaded on a 6% polyacrylamide 0.5 x TBE gel and run at 150 V at 4 °C.

**RESULTS**

**Isolating the Mouse Enhancer**

Because of the high degree of conservation between mouse and human (19), we hypothesized that a mouse counterpart to the human dystrophin intron-1 enhancer would lie at a similar distance downstream from the mouse promoter. To identify a large mouse clone that might contain the mouse promoter plus exon-1 and intron-1, we screened a mouse BAC genomic library (20) using as probe a DNA fragment containing sequences that extend from −500 to −900 of the mouse dystrophin muscle promoter. A positive clone of 120 kb was cleaved with EcoRI, HindIII, and BamHI and hybridized to the mouse muscle promoter or the human enhancer (see “Experimental Procedures”). A 7-κb EcoRI fragment that hybridizes with the promoter sequence was subcloned into Bluescript SK+ (Fig. 1A). Two fragments hybridized with the human dystrophin enhancer and therefore contain the putative MDE. A 7-κb HindIII fragment and an 11-κb BamHI fragment that hybridize with the human enhancer were subcloned into pBluescript SK+ to yield pmdeH and pmdeB, respectively (Fig. 1A). All three clones were restriction mapped by partial digestion with EcoRI, BamHI, and HindIII, and the alignment of restriction sites from the three fragments allowed the fine mapping of a contig of −20 kb (Fig.

**Table I**

**Oligonucleotide primers used to mutagenize putative binding sites of mouse dystrophin enhancer**

| Table I | Oligonucleotide primers used to mutagenize putative binding sites of mouse dystrophin enhancer |
|---------|-----------------------------------------------------------------------------------------------|
| E1 mutant forward | 5' - ATGTAACCGGCTAGAGCAACAAATAGTATACGTTTGTAGG |
| E1 mutant reverse | 5' - ATGTAACCGGCTACAGGTGTAAAGGTGTAAGG |
| E2 mutant forward | 5' - ATGTAAGAAGCTTTTCCTCTCTG |
| E2 mutant reverse | 5' - ATGTAAGAAGCTTTTCCTCTCTG |
| SRE mutant forward | 5' - ATGTAAGAGCCGTTCCCTGTCAGCTCACCTT |
| SRE mutant reverse | 5' - ATGTAAGAGCCGTTCCCTGTCAGCTCACCTT |

**Fold Activation**

| Construct | G8 mt | G8 mb | H9 mt | H9 mb | ST3 |
|-----------|-------|-------|-------|-------|-----|
| A1        | 7.03 ± 3.76 | 3.11 ± 01 | 1.28 ± 0.18 | 1.19 ± 0.10 |
| A2        | 4.79 ± 2.03 | 2.76 ± 0.51 | ND | 0.38 ± 0.04 |
| A3        | 0.89 ± 1.87 | 3.75 ± 0.38 | ND | ND |
| A4        | 5.90 ± 1.80 | 3.98 ± 0.10 | ND | ND |
| B1        | 0.79 ± 0.91 | 2.10 ± 0.06 | 0.69 ± 0.06 | 0.69 ± 0.06 |
| B2        | 9.35 ± 1.54 | 2.52 ± 0.82 | 0.64 ± 0.06 | 0.67 ± 0.07 |
| C1        | 30.04 ± 3.66 | ND | ND | ND |
| C2        | 12.00 ± 1.85 | ND | ND | ND |
| C3        | 11.04 ± 2.75 | 1.76 ± 0.72 | 0.79 ± 0.29 | 0.88 ± 0.29 |

N.D. = not determined

Using the Bio-Rad protein assay kit, and the supernatant was stored into aliquots at −80 °C.

To generate a binding substrate, 10 pmol of a single stranded oligonucleotide was end labeled at the 5'-end using T4 polynucleotide kinase (New England Biolabs Inc.) and γ-ATP (Amersham Pharmacica Biotech) in kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT) in a final volume of 10 μl. 10 pmol of the complement strand was prepared in 10 μl of 1× annealing buffer (10 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA) and added to the labeled oligonucleotide. Both strands were heat denatured by boiling for 5 min and allowed to cool slowly at room temperature. Competitor duplexes were generated by heat boiling 500 pmol of complementary strands for 5 min and allowing the strands to cool slowly to room temperature at a final concentration of 1 pmol/μl in 1× annealing buffer. Binding reactions were carried out on ice for 50 min by incubating 0.05 pmol of labeled oligonucleotide with 1 pmol or 20-fold molar excess of competitor DNA and 3 μg of extract in binding buffer (10 mM Hepes, pH 7.8, 50 mM potassium chloride, 5 mM MgCl₂, 1 mM EDTA, 5% glycerol). Supershift reactions were carried out by preincubating myoD, E12, or serum response factor (SRF) antibodies (Santa Cruz Biotechnologies Inc.) with the extract for 60 min at 4 °C. The remainder of the binding reaction mix was subsequently added, and binding reactions were carried out as described earlier. Reaction products were loaded on a 6% polyacrylamide 0.5 x TBE gel and run at 150 V at 4 °C.
activity in G8 myotubes as described in Fig. 2.

fragment using the B1 construct as template and tested for enhancer enhancer.

Panel B

tively. The boxed Mlu disrupted by the insertion of

Asterisks (* show homology between the two sequences. Putative bind-

FIG. 3. The nucleotide sequence of the MDE. Panel A, the MDE (MOUSE) was aligned with the human dystrophin enhancer (HUMAN). Asterisks (*) show homology between the two sequences. Putative binding sites (boxed) that interact with mef-2 and E-boxes 1–4 (E1–4) were disrupted by the insertion of MluI and BamHI restriction sites, respectively. The boxed sequence that contains the SRE was deleted from the enhancer. Panel B, all enhancer mutations were introduced in a 280-bp fragment using the B1 construct as template and tested for enhancer activity in G8 myotubes as described in Fig. 2.

1A). According to this restriction map, the putative MDE resides in the region of overlap of clones pmdeH and pmdeB. Digestion of pmdeB with EcoRI resulted in the subclone pmdeC containing this region (Fig. 1B).

Characterization of the Mouse Dystrophin Intron-1 Enhancer

To determine whether the mouse counterpart to the human enhancer could enhance transcription, a 3.0-kb EcoRI fragment (Fig. 1B) that was isolated from pmdeE and contains the putative mouse enhancer was inserted in reverse orientation upstream of the SV40 early promoter and in both orientations downstream from the firefly luciferase reporter gene of the pGL3-P vector series (Promega) to generate recombinant constructs C1, C2, and C3 (Fig. 2). The latter were transfected into mouse skeletal muscle-derived G8 myoblasts (21) as well as into rat heart-derived H9-C2 myoblasts (22) and mouse NIH 3T3 (23) embryonic fibroblasts using DODAC:DOPE liposomes. Myoblasts were harvested after 24 h or induced to differentiate into mature myotubes over 4 days. Fibroblasts were harvested 24 h after transfection. Our results show that in construct C2 and C3 transcription increases by ~11 fold in differentiated G8 myotubes but not in myoblasts (Fig. 2). Interestingly in the C1 construct, transcription from the SV40 promoter increases more than 25-fold. In lines H9-C2 and NIH 3T3 cells, the enhancer has no effect on transcription of the luciferase gene (Fig. 2).

Small fragments of 500 and 280 bp (Fig. 1B) which contain the putative MDE were used to generate the constructs of the A and B series, respectively. These were all tested for enhancer activity. Our results show that both the 500- and 280-bp fragment increase transcription by a factor of 5–10-fold in differentiated G8 myotubes and 2–3-fold in differentiated H9-C2 myotubes (Fig. 2). No enhancer activity was observed in G8 or H9-C2 myoblasts or 3T3 fibroblasts (Fig. 2). Our results suggest that sequence elements contained in the 3-kb fragment are required for full enhancer activity and restrict enhancer activity to G8 myotubes. Our results also show quite clearly that sequence elements contained in a shorter fragment of 280 bp contain the minimal elements for enhancer activity.

Identification of Sequences Responsible for Enhancer Activity

Sequence analysis of the MDE revealed a putative mef-2 binding site (24), four E-boxes (25–30) numbered E1–E4, a SRE (31, 32) (Fig. 3A) (Table II). E-boxes 1 and 3 match the consensus 5'-AACAc/gc/gTGCa/t, whereas E-box 2 matches the consensus 5'-GGGa/cCANGTGGGc/gNa/g (33). E4 does not appear to match either of these two consensus sequences. Comparison of mouse and human enhancer sequences reveals a 65% homology. The analysis also reveals that the mef-1/mef-2 box (21) of the human enhancer is replaced by a single mef-2 site in the mouse enhancer.

To determine which of the putative binding sites is required for enhancer activity we replaced the binding sites E1–E4 by a BamHI restriction site and replaced the A-T-rich sequences of the mef-2 site with a MluI restriction site by performing site-directed mutagenesis on the 280-bp enhancer (34). A 27-bp region that contains the SRE was deleted and replaced with a PsI site. The resulting constructs were transfected into G8 myoblasts, and enhancer activity was monitored in differentiated myotubes. Our results show that disruption of E1, E2, E3, and SRE abolishes enhancer activity, whereas disruption of the mef-2 or E4 binding site had no effect on transcription (Fig. 3B). Thus, the MDE requires a minimum of four binding sites to activate transcription in G8 myotubes.

Characterization of the Binding Activities That Define MDE Function

The binding activities at the four putative binding sites were investigated further by gel shift assays (35) using three labeled oligonucleotides that contain E-box 1, E-boxes 2 and 3, or the SRE (Table II). The oligonucleotides that contain each of these sequences were incubated in the presence of nuclear extracts (36) that were prepared from non-myogenic NIH 3T3 fibroblasts, undifferentiated myoblasts, or differentiated myotubes of the G8 myogenic line. The specificity of binding was determined by performing binding reactions in the presence of a series of competitors that were added in 20-fold molar excess relative to the substrate.
Binding at E1 of the MDE—Binding reactions were carried out by incubating a labeled oligonucleotide that contains the E1 site in the presence of G8 myotube, G8 myoblast, or fibroblast extracts. In the presence of myotube extracts three protein-DNA complexes A1, A2, and A3 are apparent (Fig. 4A), whereas in the presence of myoblast and fibroblast extracts only A1 and A3 can be detected (Fig. 4B). A protein-DNA complex that migrates slightly faster than A3 is detected in all of the extracts in the presence every competitor. This suggests that this particular complex is not specific for any of the competitor duplexes used. The lower amounts of A1 in myotubes may come from myoblasts that have not differentiated. Thus, factors in the A1 and A3 complexes are likely to be ubiquitous, whereas certain factors in the A2 protein-DNA complex are likely to be specific to myogenic cells. Because the E1 binding site features an E-box of the mef-1 type, the factors that bind at this site are likely to be part of a mef-1 complex (37, 38). To confirm further that the A2 protein-DNA complex included factors that were specific to an E-box of the mef-1 type, we performed binding reactions in the presence of oligonucleotide competitors that feature E2, E3, mef-2, and the SRE. We notice that A2 is competed out by excess amounts of E3 and E1 competitors. Because both E1 and E3 feature an E-box of the mef-1 type, our results suggest that a mef-1 complex binds to E1.

Because mef-1 complexes are known to include myogenic differentiation factors of the myoD family, we repeated the binding reactions by using E2 competitor in the presence G8 myotube extracts that were preincubated with antibodies to myoD (39) or to E-box-binding factor, E12, which forms heterodimers with myoD (40, 41). The results show that antibodies to myoD block the formation of the protein-DNA complex A2 (Fig. 4C), whereas antibodies to E12 have little or no effect. Because myoD binds to E-boxes by forming heterodimers with E-box-binding factors, our results suggest that myoD heterodimerizes with an E-box-binding factor other than E12.

Binding to the Paired E-boxes, E2 and E3, in the MDE—Binding reactions aimed at characterizing protein-DNA complexes that occur at E2 and E3 were carried out by incubating a labeled oligonucleotide that contains the two putative binding sites in the presence of G8 myotube, G8 myoblast, and fibroblast extracts. In these experiments four protein-DNA complexes were detected which we have labeled B1, 2, 3, and 4. B1 and B4 appear with all three extracts and are therefore ubiquitous. B3 is specific to myoblasts and 3T3 and may therefore represent a complex that dissociates upon differentiation into myotubes. B2 is specific to myotubes and therefore represents a complex that forms upon differentiation into myotubes.

To determine which of the two E-boxes generated these complexes we carried out binding reactions in the presence of competitors that feature either the E2 or E3 boxes (Table III). Binding reactions were also carried out in the presence of competitors that contain the mef-2 binding site and the SRE of the MDE. The mef-2 binding site differs significantly from the E-box consensus and therefore should not compete for the binding of factors that recognize E2 or E3. The SRE, however, features a sequence that resembles the consensus 5'-GGa/ cCAGCTGGe/gNa/g shared by the E2 binding site and is therefore expected to compete for the binding of factors that recognize E2.

Protein-DNA complexes B3 and B4 are competed out by oligonucleotide competitors that contain either E2 or E3 (Fig. 5, B and C), whereas B1 complexes are competed out by oligonucleotide competitors that contain E2 and are partially competed by an oligonucleotide containing the SRE (Fig. 5, A–C). Thus, our results suggest that factors in the B1 protein-DNA complex are likely to bind E2 and that factors in the B3 and B4 complexes can bind both E2 and E3. The B2 protein-DNA complex is competed out by the competitors that feature E3, suggesting that factors that compose the B2 complex are likely to bind to E3 (Fig. 5A).

Because B2 complexes can be detected as B1 complexes are competed out by oligonucleotide competitors that include the SRE or E2 binding sites (Fig. 5A), we examined whether the dissociation of B1 complexes is required for the formation of B2 complexes. To this end, labeled oligonucleotides that contain both E2 and E3 were incubated with myotube extracts and increasing amounts of oligonucleotide competitor that contains the E2 site only. The results obtained clearly show that as the amounts of B1 complexes decrease, the amounts of B2 complexes increase (Fig. 6). Therefore, as factors bind E2, E3 is unavailable for binding by other factors. As factors that bind E2 are competed out, however, E3 can interact with factors that yield the B2 protein-DNA complex, indicating that the
FIG. 5. Binding activities at the binding sites E2 and E3. Gel mobility shifts were carried out by incubating a labeled oligonucleotide that contains the E2 and E3 (2+3) binding sites with nuclear extracts that were prepared from G8 myotubes (G8 mt; panel A), G8 myoblasts (G8 mb; panel B), and 3T3 (panel C). The competitors (Table II) that were used in binding reactions are indicated at the top of the gel, and the protein-DNA complexes are indicated on the left. NS is a nonspecific competitor that contains sequences unrelated to the E2 or E3 binding sites. In panel D, nuclear extracts from G8 myotubes were preincubated with myoD or E12 antibodies before being incubated with the labeled oligonucleotide that contains the E2 and E3 binding sites. The competitors (Table III) that were used in the latter reactions are indicated at the top of the gel.

FIG. 6. Competition between E2 and E3 binding activities. Gel mobility shifts were carried out by incubating a labeled oligonucleotide that contains the E2 and E3 (2+3) binding sites with nuclear extracts that were prepared from G8 myotubes (G8 mt). Binding reactions were performed in the presence of a nonspecific competitor (NS) or in the presence of increasing amounts (0.01, 0.05, and 1 pmol) of a competitor that contains the E2 site as indicated at the top.

binding of protein factors to E2 may prevent the binding of protein factors to the E3 binding site.

According to sequence comparisons between the muscle creatine kinase (41) and myosin light chain 1/5 (42) muscle-specific enhancers, the E3 binding site of the MDE matches a consensus 5'-AACAc/gc/gTGCa/t that is recognized by myogenic differentiation factors of the myoD family. Myogenic differentiation factors, however, do not recognize the consensus 5'-Gga/cCANGTGGo/gNa/g shared by the E2 binding site of the MDE. Because B1 and B2 protein-DNA complexes are proposed to occur at binding sites E2 and E3, respectively, the presence of myoD in these complexes was investigated further by supershift analysis. Thus, we carried out two distinct sets of binding reactions in which a labeled oligonucleotide that contains the E2 and E3 binding sites was incubated with G8 myotube extracts in the presence of competitors NS and E2 that yield protein-DNA complexes B1 and B2, respectively. The extracts used were preincubated with antibodies to myoD or to E-box-binding factor, E12, which forms heterodimers with myoD. Our results indicate that B2 but not B1 complexes are blocked by the presence of antibodies to myoD but unaffected by presence of antibodies to E12, suggesting that myoD recognizes the E3 binding site (Fig. 5D). Thus, our results suggest that myoD interacts with the E3 binding site by forming heterodimers with E-box-binding factors other than E12.

Binding to the SRE of the MDE—Binding reactions that were carried out in the presence of myotube extracts yield a protein-DNA complex C1 (Fig. 7A). Binding reactions that were carried out in the presence of myoblasts and fibroblasts extracts yield complexes C2, C3, and C4 (Fig. 7, B and C) and C5, and an additional complex that migrates below C1 is detected in the presence of fibroblasts extracts only. The C1 complex is competed out by the E2 binding site as well as by the SRE. This may be explained by the fact that both the SRE and the E2 binding site resemble the consensus 5'-GGa/cCANGTGGo/gNa/g. Thus, factors that bind the E2 site may also bind the SRE. To determine whether SRF was present in complexes C1–C5, we performed binding reactions by incubating a labeled oligonucleotide that contains the SRE with extracts prepared from myotubes and myoblasts in the presence of SRF antibodies. The results show that SRF antibodies block C5 complexes but do not affect C1–C4 complexes, suggesting that C5 complexes result from SRF interacting with the SRE (Fig. 7D). Protein factors involved in the C1 complex, however, interact with the E2 binding site but not with the antibodies to SRF, indicating that protein factors in the C1 complex may differ from those in the C5 complex. Thus, SRE may interact with E-box-binding factors as well as with SRF. The protein-DNA complexes C2, C3, C4 were not competed out by any of the competitors.

DISCUSSION

Recent studies have shown that the mouse dystrophin muscle promoter targets expression in the right ventricle of the heart only, suggesting that other sequences are needed to target the skeletal muscle and/or other compartments of the heart. Our earlier studies in human have identified an enhancer element downstream from the muscle promoter which was specific to cell lines derived from rat heart. We now report the identification of a MDE that maps in the first intron ~8.5 kb downstream from the muscle promoter of the mouse dystrophin.
gene. The MDE shows 65% homology with its human counterpart, but the sequence elements that confer enhancer function in both species differ. In human the enhancer is defined by overlapping mef-1/mef-2 binding sites, whereas in the mouse the enhancer is defined by a minimum of four binding sites. These include three E-boxes, two (E1 and E3) of which are of the mef-1 type, and a SRE. A sequence comparison among the mouse dystrophin, the muscle creatine kinase, and myosin light chain-1/3 muscle-specific enhancers reveals that all three feature an E-box that resembles the consensus 5'-AACAc/gc/gTGCa/t paired to an E-box that resembles the consensus 5'-GGa/cCANGTGGc/gNa/g which is not recognized by any of the known myogenic differentiation factors, it remains to be determined which E-box-binding factors actually bind the SRE. To this end, previous studies on the muscle creatine kinase enhancer have demonstrated that the methylation protection patterns that occur at this particular sequence are similar but not identical to myoD, confirming that factors do bind at this E-box (33). We are currently investigating the Duchenne muscular dystrophy protection patterns that occur in vivo in order to identify the factors that are most likely to interact with the SRE in both myotubes and myoblasts. (46)

A third factor that may be involved in enhancer function is mef-2. Mef-2 belongs to the family of transcription factors with a MADS box and plays a key role in the regulation of many muscle-specific genes (24, 47). Interestingly, mutations of the mef-2 site in the MDE had no effect on enhancer activity. More surprising is the fact that mef-2 appears to interact with DNA do not necessarily prevent mef-2 from activating transcription. Because mef-2 is frequently associated with myogenic basic helix-loop-helix factors to activate transcription, studies have shown that mef-2 does not require direct binding to DNA (49). Thus, mutations that abolish mef-2 binding to DNA do not necessarily prevent mef-2 from activating transcription. Because mef-2 is frequently associated with functional E-boxes bound by myogenin, we suspect mef-2 may act in combination with myogenin differentiation factors can bind at both these sites. The protein-DNA complex that was detected by the E3 binding site, however, occurs only when factors that bind to the adjacent site, E2, are competed away, suggesting that factors that bind E2 may regulate the binding of factors to E3. Factors that bind E2, however, are not yet known because they bind a consensus 5'-GGA/cCANGTGGc/gNa/g that is not recognized by the known myogenic differentiation factors. Although our study points out the importance of mef-1 type E-boxes for enhancer function, in vivo studies must be carried out to verify this hypothesis.

The SRE may also play a role in regulating enhancer function. In G8 myotubes, the SRE is recognized mostly by E-box-binding factors. In G8 myoblasts, however, the SRE is also recognized by the SRF. The latter is a transcription factor of the MCM1, gapajous, deficiens, serum response factor (MADS) box family that regulates gene expression of several muscle-specific genes (45). Because the activity of the MDE is specific to myotubes, E-box-binding factors may act as positive regulators, whereas SRF may act as a negative regulator by preventing the binding of E-box-binding factors to the SRE. Because the latter includes an E-box consensus 5'-GGa/cCANGTGGc/gNa/g which is not recognized by any of the known myogenic differentiation factors, it remains to be determined which E-box-binding factors actually bind the SRE. To this end, previous studies on the muscle creatine kinase enhancer have demonstrated that the methylation protection patterns that occur at this particular sequence are similar but not identical to myoD, confirming that factors do bind at this E-box (33). We are currently investigating the Duchenne muscular dystrophy protection patterns that occur in vivo in order to identify the factors that are most likely to interact with the SRE in both myotubes and myoblasts. (46)
conformation in transcription regulation of muscle-specific genes was demonstrated in studies that showed that the binding of the bHLH factors myoD, Twist, and E2A to their respective sites is mediated by the topology of these sites (55). Another study showed that the proper conformation of a TATA box is required by activator proteins to activate transcription of the myosin heavy chain gene (56). Finally, a third study has reported that transcription activation of the human dystrophin gene depends upon DNA bending of its promoter to activate gene expression (57, 58).

The understanding of how transcription factors control the MDE is likely to shed more light on the mechanisms that regulate transcription of the dystrophin gene in skeletal and cardiac muscle. Such findings may help elucidate complex phenotypes such as those observed in X-linked dilated cardiomyopathy patients who fail to produce dystrophin in the heart but up-regulate the expression of the dystrophin gene in skeletal muscle from Purkinje and brain promoters that are normally silent in skeletal and/or cardiac muscle. The characterization of the MDE is also likely to assist the engineering of therapeutic vectors aimed expressing genes in skeletal and cardiac muscle.

Acknowledgments—We are very grateful to Dr. H. J. Klamut for providing the plasmid SA195 and to Inex Pharmaceuticals Inc. for providing liposomes throughout this study. We thank Drs. David Picketts and Karen Copeland for helpful advice on gel mobility shifts.

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