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A muscle-specific enhancer within intron 1 of the human dystrophin gene is functionally dependent on single MEF-1/E box and MEF-2/AT-rich sequence motifs

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ABSTRACT

In previous studies we have described a 5.0 kb *Hin*dIII fragment downstream of muscle exon 1 that exhibits properties consistent with a muscle-specific transcriptional enhancer. The goal of this study has been to identify the sequence elements responsible for muscle-specific enhancer activity. Functional studies indicated that this enhancer is active in pre- and post-differentiated H9C2(2-1) myoblasts but functions poorly in L6 and C2C12 myotubes. The core enhancer region was delimited to a 195 bp *Spe*I–*Acc*I fragment and sequence analysis identified three MEF-1/E box and two MEF-2/AT-rich motifs as potential muscle-specific regulatory domains. EMSA competition and DNase footprinting indicated that sequences within a 30 bp region containing single adjoining MEF-1/E box and MEF-2/AT-rich motifs are target binding sites for *trans*-acting factors expressed in H9C2(2-1) myotubes but not in L6 or C2C12 myotubes. Site-specific mutations within these motifs resulted in a significant reduction in enhancer activity in H9C2(2-1) myotubes. These results suggest that the mechanisms governing DMD gene expression in muscle are similar to those identified in other muscle-specific genes. However, the myogenic profile of enhancer activity and *trans*-acting factor binding suggests a more specialized role for this enhancer that is consistent with its potential involvement in dystrophin gene regulation in cardiac muscle.

INTRODUCTION

The 14 kb transcript encoding the muscle isoform of the human dystrophin (Duchenne muscular dystrophy, DMD) gene is spliced from 79 exons spread over 2500 kb or ~1.5% of the entire length of the X chromosome (1,2). The muscle-specific transcript is one of several isoforms that arise from tissue-specific promoters within introns throughout the dystrophin gene (3–10). For example, a brain-specific transcript originates from a novel first exon positioned 120–150 kb upstream of muscle exon 1 and the first exon of a cerebellar Purkinje cell-specific transcript is located 30–60 kb downstream of muscle exon 1 (4,11,12). The first exons of each of these transcripts are spliced to the 62 bp exon 2 positioned 170–200 kb downstream of muscle exon 1. The functional roles of these and other non-muscle isoforms in their respective tissues have not been firmly established.

The muscle isoform of the dystrophin gene is expressed primarily in skeletal and cardiac muscle and to a lesser extent in smooth muscle (13) and coincides with the pathological profile of tissue involvement in the disease. Mutations that disrupt the abundance and/or integrity of the 427 kDa protein product dystrophin lead to progressive degeneration of muscle tissues which may be severe (DMD) or relatively mild (Becker muscular dystrophy, BMD) (14). DMD reduces life expectancy to 20–25 years, at which time patients succumb to respiratory or cardiac insufficiency.

Typical of muscle-specific genes, dystrophin gene transcription has been shown to be up-regulated as myoblasts differentiate into multinucleated myotubes *in vitro* (15–18). Studies of transcript accumulation with myoblast differentiation have verified the predicted transcription time of 14–24 h based on published elongation rates for RNA polymerase II (18) and established a mean half-life of 15 h for dystrophin gene transcripts in immature myotubes (19).

In a previous study we have described the isolation and characterization of the muscle promoter region upstream of muscle exon 1 in the human dystrophin gene. A 918 bp *Hin*dIII–*Bgl*II fragment containing the transcriptional start site and 850 bp of upstream sequence was shown to confer muscle-specific regulation upon promoterless and enhancerless reporter gene expression plasmids when transiently transfected into primary human, primary mouse and H9C2(2-1) rat myoblast cultures (17). The muscle promoter was inactive in fibroblasts and functioned poorly in both the L6 and C2 myogenic cell lines, despite the fact that several muscle-specific regulatory domain

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homologies were identified within this region [e.g. MEF-1/E box (20,21), MEF-2 (22,23), CArG box (24–26) and MCAT (27)]. Regulatory domains responsible for transcriptional induction of reporter gene expression during myoblast differentiation were found to lie within 150 bp of the transcription start site (17). This 'minimal' promoter region contains single GC box, MEF-1/E box and CArG box motifs and in an independent study the CArG box motif was shown to be an essential functional regulatory element and to have *trans*-acting factor binding properties similar to the cardiac actin promoter CArG box motif (28). The MEF-1/E box motif, on the other hand, was found to be non-responsive to *trans*-activation by MyoD.

The observation of low levels of dystrophin promoter activity in mouse skeletal muscle *in vivo* provided indirect evidence that additional muscle-specific regulatory control elements may be present in sequences surrounding muscle exon 1 (12). Functional analysis of *Hin*dIII fragments from within a 36 kb region surrounding muscle exon 1 resulted in the identification of a 5.0 kb fragment that generated high levels of reporter gene expression in both immature H9C2(2-1) myotubes and mature mouse skeletal muscle. This 5 kb fragment was functionally independent of position and orientation and inactive in fibroblasts, properties consistent with the definition of a muscle-specific enhancer. The identification of X-linked dilated cardiomyopathy patients having deletions through the muscle promoter and into intron 1 of the dystrophin gene (29,30) and reports that the H9C2(2-1) cell line retains residual cardiac-specific transcriptional properties (31–34) provided indirect evidence that this enhancer may play a role in regulating dystrophin gene expression in cardiac muscle.

The goal of this study has been to identify sequence elements within this enhancer responsible for transcriptional activity in immature cultured myotubes. The core enhancer region was defined as a 195 bp *Spe*I–*Acc*I fragment containing three MEF-1/E box and two MEF-2/AT-rich-like motifs as potential muscle-specific regulatory domains. EMSA competition, DNase footprinting and mutational studies demonstrated that single adjoining MEF-1/E box and Mef2/AT-rich sequence motifs are involved in mediating the activity of this enhancer in H9C2(2-1) myotubes.

MATERIALS AND METHODS

Plasmid construction

Chloramphenicol acetyltransferase (CAT) expression constructs were prepared in the pBLCAT2 vector containing the enhancerless Herpes simplex virus thymidine kinase (HSVtk) promoter upstream of the *CAT* gene (35). Deletion constructs were prepared utilizing internal restriction enzyme sites. Some fragments were initially subcloned into the pBluescript vector (Stratagene Cloning Systems, La Jolla, CA) and subsequently transferred to the pBLCAT2 vector using common polylinker restriction sites. Exonuclease III/mung bean nuclease deletions were prepared from the *Hin*dIII site of a 3.5 kb *Xba*I–*Hin*dIII fragment isolated from DME.H5 and subcloned into the pBluescript vector (36).

Mutant constructs were prepared by synthesizing oligonucleotides containing specific base changes and using these to generate overlapping PCR products which were subsequently annealed, extended and PCR amplified using vector-based primers. Mutant PCR primers used in these experiments were E box mut (5′-TGACTCACGGTTTGCAAAATAACTAGGAAG) and MEF-2 mut (5′-TGACTCACATGTTGCGGTACCACTAGGAAG), along with primers within the T3 and T7 promoters framing the polylinker in the pBluescript vector. Double mutant constructs were prepared using the primer 5′-TGACTCACGGTTTGCGGTACCACTAGGAAG and appropriate forward and reverse primers. These were subsequently combined and re-amplified using T3 and T7 primers alone. PCR products were cloned using the TA cloning vector PCRII (Invitrogen Corp., San Diego, CA) and each construct was sequenced to verify that appropriate nucleotide changes had been incorporated.

Cell lines and transfections

H9C2(2-1) myoblasts were seeded at a density of 100 cells/mm^2 on plastic culture dishes in αMEM containing 16 mM glucose, 10% fetal bovine serum and 40 µg/ml gentamycin (growth medium). Cells were transfected using the modified calcium phosphate precipitation protocol (37) using 10 µg test plasmid and 10 µg reference plasmid pXGH5 (38) per 100 mm^2 dish. The pXGH5 reference plasmid contains the human growth hormone gene driven by the mMT-1 promoter and was included as a control for transfection efficiency. Transfected cells were exposed to fusion medium (αMEM containing 16 mM glucose, 5% horse serum and 100 U/ml penicillin, 0.1 mg/ml streptomycin) for 72 h prior to harvesting for CAT and growth hormone assays. C2C12 and L6 myoblasts were grown and transfected under the same conditions. In experiments requiring pre-differentiated H9C2(2-1) myoblasts, cells were seeded at 25 cells/mm^2 and maintained in growth medium. NIH 3T3 fibroblasts were maintained in growth medium at all times.

Reporter gene assays

CAT assays were performed according to the dual phase diffusion procedure (39). Briefly, cell extracts (25 µg protein) were added to reaction mixtures containing 100 mM Tris–HCl, pH 7.8, 1.0 mM chloramphenicol and 0.1 mM acetyl-CoA (2–5 µCi [^{3}H]acetyl-CoA; 198 mCi/mmol; Dupont Canada Inc., Mississauga, Ontario, Canada) in a final volume of 250 µl. Reactions were overlaid with 5 ml Econofluor (Dupont Canada Inc.) and assayed at 30–60 min intervals. CAT activities were calculated using values which fell within the linear range of the reaction and were converted from c.p.m. to units CAT using standard CAT activity values determined in each experiment. Human growth hormone levels in 20 µl aliquots of fusion medium were determined using a dual antibody radioimmunoassay (Nichols Institute Diagnostics, Los Angeles, CA). Values were converted to ng growth hormone/ml using a standard curve prepared with each experiment. Cell extract protein concentrations were determined using the procedure of Lowry *et al.* (40). CAT activities are expressed as units CAT/mg protein/ng growth hormone.

DNA sequencing

Sequences within the 195 bp *Spe*I–*Acc*I fragment were determined from a series of overlapping fragments (1.4 kb *Eco*RI–exonuclease III, 710 bp *Hin*cII, 800 bp *Eco*RI–*Acc*I) subcloned into the pBluescript vector. Each fragment was sequenced from T3 and T7 primer sites within the vector, as well as from internal primer sites using the dideoxy chain termination procedure (41) and Sequenase (US Biochemical Corp., Cleveland, OH) according to the manufacturer's protocol.

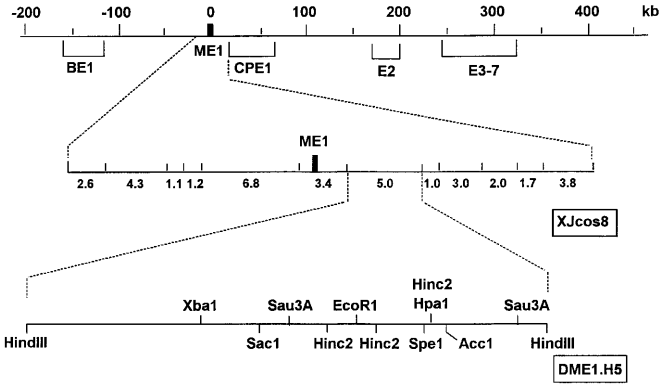

Figure 1. Localization and mapping of the intron 1 enhancer fragment relative to muscle exon 1 at the 5′-end of the human dystrophin gene. The relative positions of muscle exon 1 (ME1, 275 bp), exon 2 (E2, 62 bp) and exons 3–7 (E3–7, 556 bp total) representing the first 893 bp of the 14 kb mRNA expressed in skeletal, cardiac and smooth muscle are shown. Also shown are the relative positions of the first exons of the brain (BE1) and cerebellar Purkinje cell (CPE1) isoforms within a 600 kb region at the 5′-end of the human dystrophin gene. A *Hin*dIII restriction map of a 36 kb cosmid clone (XJcos8) containing muscle exon 1 illustrates the position of the 5 kb *Hin*dIII fragment which has been shown previously to display characteristics of a muscle-specific enhancer. Also shown is a detailed restriction map of this 5 kb *Hin*dIII fragment (designated DME1.H5).

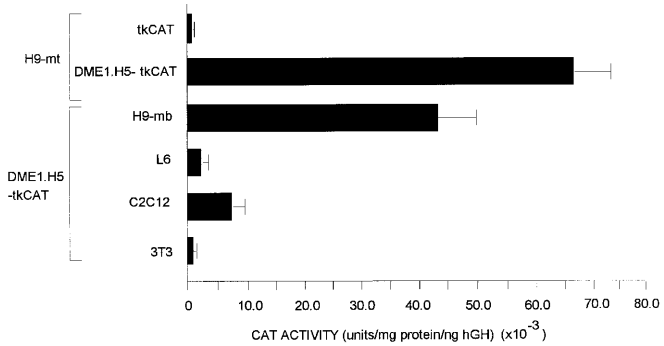

Figure 2. The dystrophin gene intron 1 enhancer displays myogenic cell line specificity. A DME1.H5–tkCAT construct was transiently transfected into near confluent H9C2(2-1), L6 and C2C12 myoblasts and CAT activities measured at 3 days post-transfection, by which time cultures had reached confluency and had begun to differentiate into multinucleated myotubes. For H9C2(2-1) myoblasts (H9-mb), cultures were transfected at low density such that cells had not reached confluence by 3 days post-transfection. Also shown are activities determined for the enhancerless tkCAT vector control in H9C2(2-1) myotubes and for dystrophin enhancer activity in 3T3 fibroblast cultures. Transfection efficiencies were normalized by inclusion of a mMTV–hGH control vector in all experiments. Values are expressed as units of CAT activity/mg cell extract protein/ng hGH measured in an aliquot of the culture medium at the time of harvest and represent the mean ± SEM of a minimum of five determinations.

Electrophoretic mobility shift assays (EMSA) and DNase footprinting

Protein extracts were prepared from H9C2(2-1) myoblasts and myotubes, L6 and C2C12 myotubes and 3T3 fibroblasts essentially as described by Lassar *et al.* (42) and stored at –80°C. Protein concentrations were determined according to the Bradford procedure using the reagent kit and manufacturer's protocol (BioRad Laboratories, Richmond, CA).

The 195 bp *Spe*I–*Acc*I fragment was end-labelled at a polylinker *Xba*I site by a Klenow fill-in reaction in the presence of [^{32}P]dCTP (3000 Ci/mmol), excised with *Hin*dIII and purified on a non-denaturing polyacrylamide gel. Sense and antisense unlabelled competitor oligonucleotides corresponding to *E-1* (5′-TGACTCACATGTTGCAAAATAACTAGGAAG), *E-2* (5′-AAAGAGCGTAATCAGATGCAAGAAA), *E-3* (5′-GGGAAGCTCAGACAGCTGGGGAATCCTGAT), *E-mut* (5′-TGACTCACGGTTTGCAAAATAACTAGGAAG) and *AT-mut* (5′-TGACTCACATGTTGCGGTACCACTAGGAAG) were annealed and gel purified prior to use. EMSA binding reactions in a total volume of 25 µl typically contained nuclear extracts (2 µg protein), labelled probe (~25 000 c.p.m./ng) and a 100-fold molar excess (3.25 µg) of poly(dI·dC) in 20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 0.6 mM DTT, 300 µg/ml BSA and 12% glycerol. When used, unlabelled competitor oligonucleotides were added at 10- to 500-fold molar excess over probe concentrations. Annealing of complementary competitor oligonucleotide strands was confirmed by native polyacrylamide gel electrophoresis. Binding reactions were incubated at room temperature for 30 min and electrophoresed on 4% non-denaturing polyacrylamide gels. Gels were dried and autoradiographed at –80°C with intensifying screens.

For DNase footprinting, 0.1 vol. of a solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added to the EMSA binding reaction followed by DNase for 1 min at room temperature, phenol/chloroform extraction and ethanol precipitation. DNA pellets were resuspended in 3 µl loading dye and the samples heated for 2 min at 80°C. At least 10 000 c.p.m. was loaded per lane of a pre-run (30 min, 40 W) 8% acrylamide–7M urea denaturing polyacrylamide gel. A Maxam–Gilbert G+A sequencing ladder prepared by 1 M piperidine cleavage of the end-labelled target was included as a marker. Gels were dried and processed for autoradiography at –80°C with intensifying screens.

RESULTS

Mapping the muscle-specific enhancer fragment within intron 1 of the human dystrophin gene

A schematic representation of the relative positions of muscle exon 1 (ME1), the first exons of the brain (BE1) and cerebellar Purkinje cell (CPE1)-specific transcripts and exons 2–7 within a 400 kb region at the 5′-end of the human dystrophin gene are shown in Figure 1. Below the long range map is a *Hin*dIII restriction map of a 36 kb cosmid clone (XJcos8) containing muscle exon 1. The 5 kb fragment downstream of muscle exon 1 (DME1.H5) displays characteristics typical of a muscle-specific enhancer and is the focus of this study. The DME1.H5 fragment is positioned immediately downstream of 3.4 kb *Hin*dIII fragment that contains muscle exon 1 and the core muscle promoter region. A restriction map of the DME1.H5 fragment is shown below XJcos8.

Dystrophin intron 1 enhancer activity displays myogenic cell line specificity

To more fully characterize the activity of this enhancer with respect to myoblast differentiation, enhancer activity was compared in H9C2(2-1) myoblasts and myotubes. CAT activities were normalized for transfection efficiency by co-transfection with a mMT1–hGH construct as described in Materials and Methods. In these experiments transfection efficiencies varied by <30%. As shown in Figure 2, mean enhancer activity in

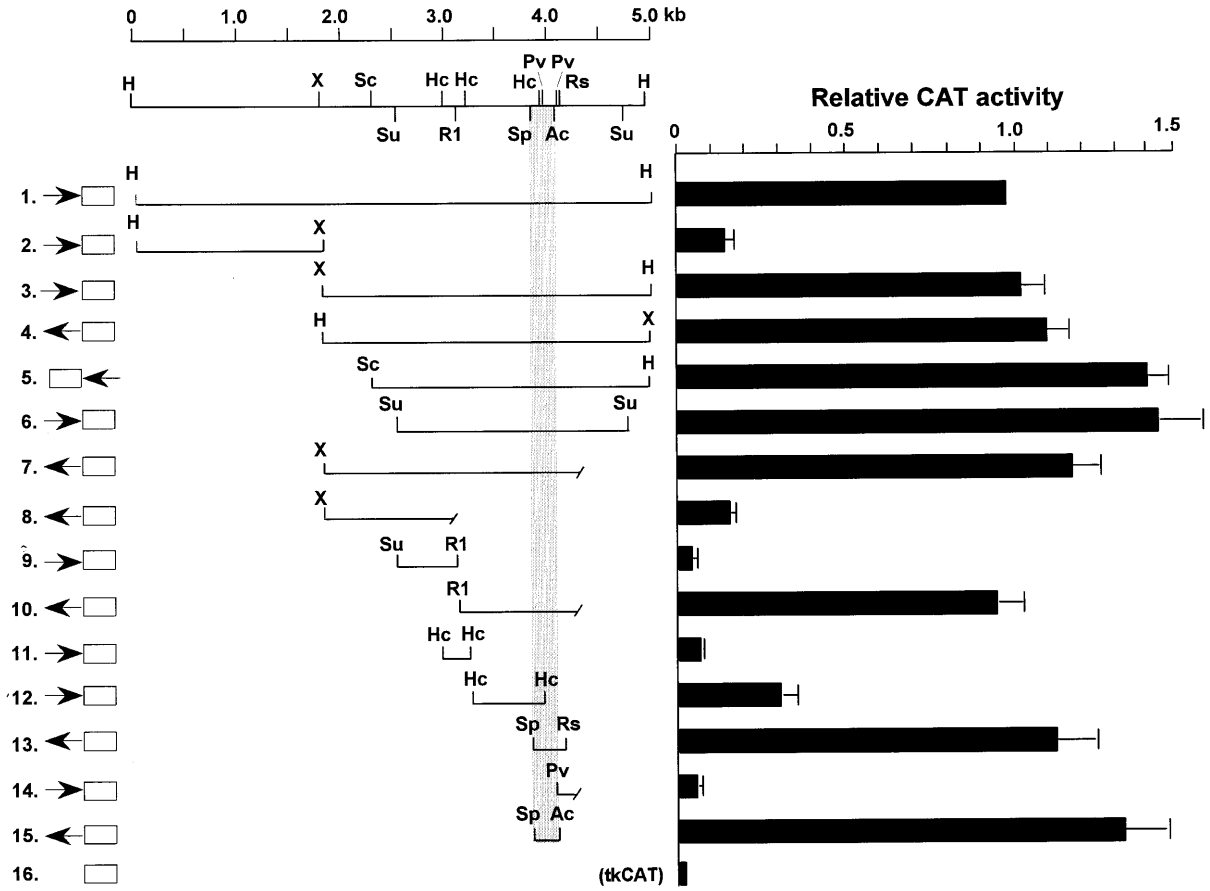

Figure 3. Localization of the core enhancer region to a 195 bp *Spe*I–*Acc*I fragment. Restriction enzyme and mung bean nuclease/exonuclease III-generated fragments isolated from the 5 kb DME.H5 *Hin*dIII fragment were subcloned in a variety of orientations relative to tkCAT in the pBL2CAT vector and tested for transient*CAT* gene expression in H9C2(2-1) myotubes. In these experiments CAT activities are expressed relative to that of DME.H5–tkCAT (construct 1). Constructs 1 and 16 (the enhancerless tkCAT vector alone) were included as controls in all experiments. The shaded area represents the position of the core enhancer region defined by a 195 bp *Spe*I–*Acc*I fragment which exhibits high levels of enhancer activity. Arrows represent enhancer orientation (5′→3′ relative to DMD gene transcription) relative to the tkCAT gene cassette (represented by the box). Restriction enzyme abbreviations: H,*Hin*dIII; X, *Xba*I; Sc, *Sac*I; Su, *Sau*3A; R1, *Eco*RI; Hc, *Hin*cII; Sp, *Spe*I; Rs, *Rsa*I; Pv, *Pvu*I; Ac, *Acc*I.

H9C2(2-1) myotubes (H9-mt) was 50- to 60-fold greater than the enhancerless pBLCAT2 vector alone. High levels of enhancer activity were also observed in pre-differentiated H9C2(2-1) myoblasts, although on average this activity was only 56% of that observed in H9C2(2-1) myotubes. This indicated that this enhancer is not highly responsive to changes in *trans*-acting factor profiles associated with myoblast differentiation. However, this enhancer does function in a muscle-specific manner as evidenced by low activity in 3T3 fibroblasts. Furthermore, intron 1 enhancer activity was significantly higher in L6 and C2C12 myotubes as compared with 3T3 fibroblasts, although these activities were only 10–15% of those observed in H9C2(2-1) myotubes. While somewhat unexpected, this result paralleled our previous observation of relatively low dystrophin muscle promoter activities in these cell lines (17).

Enhancer activity is restricted to a 195 bp *Spe*I–*Acc*I fragment that contains MEF-1/E box and MEF-2/AT-rich sequence motifs

In order to more precisely localize transcriptional regulatory domains responsible for enhancer function several restriction endonuclease- and mung bean nuclease/exonuclease III-generated fragments were cloned into the pBLCAT2 vector and tested for *CAT* gene expression in H9C2(2-1) myotubes. The results, expressed relative to the activity of the DME.H5 fragment (construct 1), are summarized in Figure 3. A comparison of mean CAT activities generated by constructs 2–15 served to define the core enhancer as a 195 bp *Spe*I–*Acc*I fragment located near the 3′-end of DME.H5. This fragment (designated SA195) lies ~6.5 kb downstream of the transcriptional start site in muscle exon 1. The observation of reduced transcriptional activity (30% of DME.H5) from a 750 bp *Hin*cII fragment (construct 12) that contains 144 within SA195 indicated that the 51 bp region between the *Hin*cII and *Acc*I sites required for full enhancer function. As shown in Figure 4a, sequence analysis identified three MEF-1/E box and two MEF-2/AT-rich motifs within the SA195 fragment. These motifs have been defined as binding sites for myogenic determination gene products (MyoD1, myogenin, myf5 and MRF4; 43–45) and MADS box-type myogenic factors (e.g. MEF-2; 46). A comparison of MEF-1/E box and MEF-2/AT-rich motifs within the enhancer core with their respective consensus sequences is shown in Figure 4b. All three MEF-1 homologies contain an intact E box (CANNTG) consensus motif

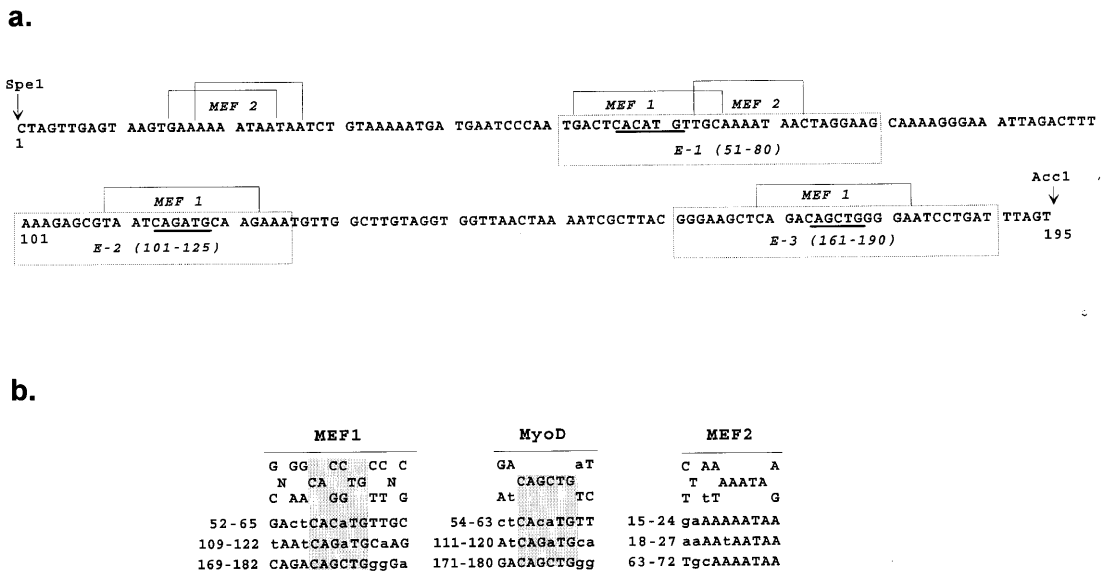

Figure 4. Sequence analysis of the 195 bp core enhancer region reveals multiple E box and AT-rich motifs. (**a**) A number of restriction fragments spanning (and including) the 195 bp *Spe*I–*Acc*I core enhancer fragment were subcloned into the pBluescript vector and sequenced. A search for sequence homologies to regulatory domains found in other muscle-specific genes identified three MEF-1/E box and two MEF-2/AT-rich motifs within the 195 bp core enhancer region. E box motifs within the MEF-1 homology regions are underlined. E-1, E-2 and E-3 represent sequences that were used to prepare double-standed oligonucleotides for EMSA competition experiments. (**b**) Alignment of the MEF-1/E box and MEF-2/AT-rich motifs identified within the enhancer core with MEF-1, MyoD and MEF-2 consensus sequences. The shaded region in the MEF-1 and MyoD alignments distinguishes the E box (CANNTG) motif from surrounding sequences. Nucleotide mismatches to the consensus are indicated in lower case. The MEF-1/E box motif at 169 bp displays the highest homology to both the MEF-1 and MyoD binding site consensus sequences. The two MEF-2/AT-rich motifs at 15 and 63 bp contain mismatches within the first 2–3 nt. A third potential MEF-2/AT-rich motif at 18 bp is also shown. In view of the considerable overlap of this motif with the MEF-2/AT-rich motif at 15 bp, these two regions have been considered a single domain for the purposes of this study.

(47) at their core and display 71–79% homology to the 14 bp MEF-1 binding site consensus described by Buskin and Hauschka (20). The MEF-1 motifs at 54 and 111 bp are only 60% homologous to the MyoD binding concensus established by Blackwell and Weintraub (47), whereas the MEF-1 motif at 171 bp shows 80% homology overall and 100% homology within the 6 bp E box core. The two MEF-2/AT-rich binding site homologies each display 80% identity to the 10 bp consensus described by Yu *et al.* (48) A third MEF-2/AT-rich motif at bp 18 has significant overlap with the motif at bp 15 and has an additional mismatch in the core AAATA region. Due to the extent of the overlap we have treated these two motifs as a single MEF-2 homology region. All three MEF-2/AT-rich motifs have mismatches in two of the first three positions of the MEF-2 consensus.

Sequences within a 30 bp region of the core enhancer are target binding sites for *trans*-acting factors expressed in H9C2(2-1) myotubes

To determine whether these MEF-1/E box and MEF-2/AT-rich motifs behave as functional *trans*-acting factor binding sites within the context of the SA195 fragment, synthetic double-stranded oligonucleotides spanning each of the three MEF-1/E box motifs within the SA195 region were prepared and used as unlabelled competitors in EMSA binding reactions. The positions of each of these competitor oligonucleotides (designated E-1, E-2 and E-3) are indicated in Figure 4a. The profile of protein–DNA complexes formed in binding reactions containing H9C2(2-1) myotube nuclear extracts is shown in Figure 5a, as well as in positive control lanes in Figure 5b and c. Four major protein–DNA complexes (bands B–E) were consistently observed in these experiments. Formation of a fifth protein–DNA complex (band A) was less consistent in terms of its intensity relative to bands B–E. Inclusion of a 100-fold molar excess of unlabelled SA195 DNA in the binding reaction completely abolished EMSA complex formation (Fig. 5b), demonstrating the specificity of nuclear factor binding to this probe. Competitor oligonucleotides were tested over a range of 10- to 500-fold molar excess over labelled SA195 probe. As shown in Figure 5b, inclusion of a 100-fold molar excess of unlabelled competitor E-1 (bp 51–80) in EMSA binding reactions resulted in the complete loss of three bands corresponding to complexes C–E and a reduction in the intensity of band B. Competitor oligonucleotides E-2 and E-3, on the other hand, had no effect on EMSA complex formation when present at either 100-fold (Fig. 5b) or 500-fold (not shown) molar excess over labelled probe. The introduction of mutations into either the MEF-1/E box (E-mut) or the MEF-2/AT-rich (AT-mut) motifs within the E-1 oligonucleotide abolished competition for EMSA complexes C–E (Fig. 5b). These results pointed to the specific involvement of these *cis*-acting sequence motifs in factor binding to the SA195 enhancer core.

To examine whether differences in enhancer function in myogenic cell lines are reflected in changes in *trans*-acting factor binding to the 195 bp enhancer core, EMSAs were performed using nuclear extracts prepared from L6 and C2C12 myotubes and 3T3 fibroblasts. As shown in Figure 5c, L6 myotube extracts support the formation of four EMSA complexes with similar mobilities to bands A, B, D and E formed with H9C2(2-1) myotube extracts. Protein–DNA complexes with mobilities resembling bands A and B, along with a complex with slightly greater mobility than band D, were observed in EMSAs using C2C12 myotube extracts. Nuclear extracts prepared from 3T3 fibroblasts supported the formation of complexes resembling

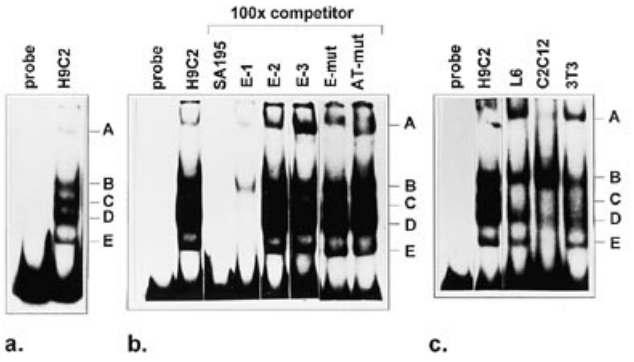

Figure 5. A 30 bp region containing single E box and AT-rich motifs mediates H9C2(2-1) myotube-specific *trans*-acting factor binding. (**a**) The 195 bp *Spe*I–*Acc*I (SA195) core enhancer region was found to mediate the formation of five protein–DNA complexes (designated bands A–E) in electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from H9C2(2-1) myotubes. Band A intensities varied considerably relative to bands B–E in different experiments. (**b**) EMSA competition experiments in which EMSA binding reactions contained either a 100-fold molar excess of unlabelled SA195 DNA (SA195) or double-stranded oligonucleotides (designated E-1, E-2 and E-3) spanning the three MEF-1/E box motifs within the SA195 enhancer core. No EMSA complex formation was observed in the presence of unlabelled SA195 DNA. Inclusion of the E-1 oligonucleotide consistently resulted in the absence of bands C–E in the EMSA profile. No competition for any of these five protein–DNA complexes was observed with oligonucleotides E-2 and E-3. (**c**) EMSA profiles generated by the SA195 probe and nuclear extracts prepared from H9C2(2-1), L6 and C2C12 myotubes and 3T3 fibroblasts. Band C was absent in EMSA profiles generated by L6 and C2C12 myotube and 3T3 fibroblast nuclear extracts.

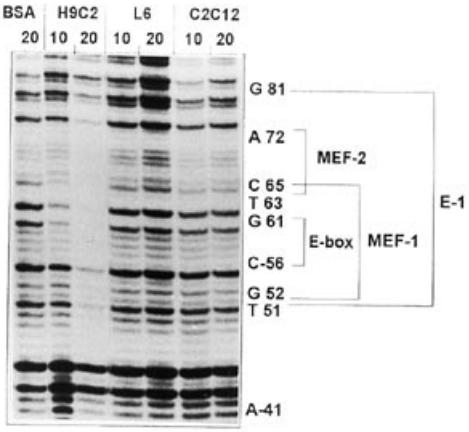

Figure 6. DNase footprinting verifies that the E-1 region contains sequences that bind factors present exclusively in H9C2(2-1) myotube nuclear extracts. DNase footprinting experiments utilizing H9C2(2-1) myotube nuclear extracts and the 195 bp *Spe*I–*Acc*I fragment end-labelled at the *Spe*I site as probe resulted in protection of sequences from 46 to 73 bp within the SA195 sequence. No discernable protection of sequences in this region was observed in experiments containing L6 and C2C12 myotube nuclear extracts. Shown are the results of DNase footprinting reactions containing either 20 µg BSA or 10 and 20 µg H9C2(2-1), L6 and C2C12 myotube nuclear extracts. Nucleotide positions were determined by inclusion of an A+G Maxam–Gilbert sequencing ladder generated from the SA195 probe (not shown). Nucleotides are numbered according to the sequence presented in Figure 4. Positions of MEF-1/E box and MEF-2 motifs within the E-1 region are indicated.

bands A, B and E as well as a complex with slightly greater mobility than band D, similar to that seen with C2C12 myotube extracts. The absence of band C in L6, C2C12 and 3T3 EMSAs suggested that formation of this complex is necessary for full enhancer function. The observation that band C is one of the three protein–DNA complexes competed by oligonucleotide E-1 provided further support for the notion that sequences within this 30 bp region play an important role in establishing the transcriptional activity of this enhancer in H9C2(2-1) myotubes.

The involvement of this 30 bp region (bp 51–80) in *trans*-acting factor binding was explored further in DNase fooprinting experiments utilizing H9C2(2-1), L6 and C2C12 myotube nuclear extracts. As shown in Figure 6, DNase protection of a region corresponding to bp 45–73 was seen with H9C2(2-1) myotube extracts but not with equivalent concentrations of L6 or C2C12 myotube extracts. The positions of MEF-1/E box (bp 52–65) and MEF-2/AT-rich (bp 63–72) motifs within the footprinted region are indicated. These results confirmed that sequences within the E-1 region specifically bind *trans*-acting factors expressed in H9C2(2-1) myotubes and further implicated these MEF-1/E box and MEF-2/AT-rich motifs in enhancer function. The absence of DNase protection through this region with L6 or C2C12 extracts indicated that formation of EMSA complexes resembling bands D and E in these cell lines involves *cis*-acting sequences positioned elsewhere within the enhancer core.

Dystrophin intron 1 enhancer activity is functionally dependent upon single MEF-1/E box and MEF-2/AT-rich factor binding motifs

To more specifically address the involvement of MEF-1/E box and MEF-2/AT-rich motifs in enhancer function, SA195 fragments containing site-specific mutations within each motif were prepared and tested in H9C2(2-1) myotubes. Sequence changes introduced into each motif were the same as those introduced into mutant E-1 oligonucleotides in EMSA competition studies and are based on mutations that abolish *trans*-acting factor binding the muscle creatine kinase gene upstream enhancer (48–51). As shown in Figure 7, mutation of the MEF-1/E box motif (E-mut) resulted in a 6-fold decrease in mean CAT activity, while mutation of the MEF-2/AT-rich motif (AT-mut) resulted in a 9-fold decrease in CAT activity relative to the wild-type SA195 fragment. CAT activities generated by a construct mutated for both motifs (E-mut + AT-mut) were reduced even further to levels that were 17-fold lower than the wild-type enhancer. These results provided direct evidence for the involvement of these regulatory motifs in enhancer function in H9C2(2-1) myotubes.

DISCUSSION

In a recent report we presented functional evidence for the existence of a muscle-specific transcriptional enhancer within intron 1 of the human dystrophin gene (12). This enhancer element was found to be active in both immature H9C2(2-1) myotubes *in vitro* and mature mouse skeletal muscle *in vivo* and to increase transcription from the core muscle promoter of the human dystrophin gene as well as from heterologous viral promoters. The description of X-linked dilated cardiomyopathy (XLCD) arising from a deletion that extends through the muscle promoter and into muscle intron 1 raised the possibility that this enhancer plays a role in regulating dystrophin gene transcription in cardiac muscle (17). In this study we have delimited enhancer activity to within a 195 bp *Spe*I–*Acc*I fragment and provide evidence that a 30 bp region within the core enhancer contains

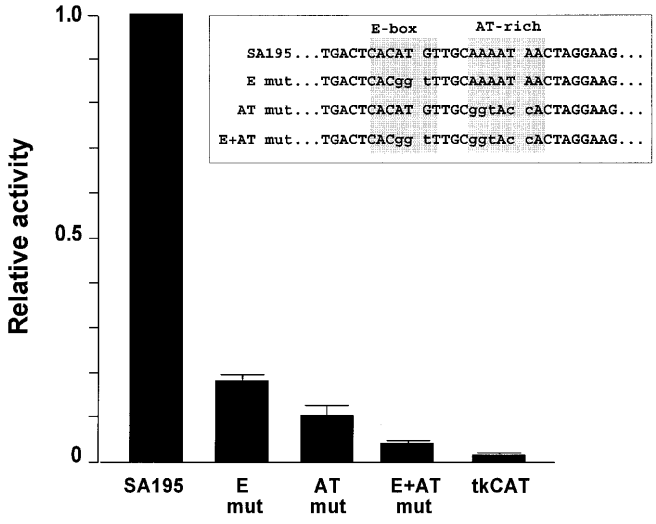

Figure 7. Mutation of MEF-1/E box and MEF-2/AT-rich motifs within the E1 region reduces enhancer activity in H9C2(2-1) myotubes. MEF-1/E box and MEF-2/AT-rich motif mutations were introduced into the SA195 enhancer core fragment and resulting constructs were tested for transient *CAT* gene expression in H9C2(2-1) myotubes. CAT activities were determined as described in Materials and Methods and are expressed relative to the wild-type construct (SA195). Values represent the mean ± SD of a minimum of three independent experiments. Mutation of the E box motif alone (E-mut), the MEF-2/AT-rich motif alone (AT-mut) or the two motifs together (E-mut + AT-mut) resulted in 6-, 9- and 17-fold reductions in CAT activities respectively, relative to wild-type SA195 construct. Relative tkCAT control vector activity is also shown.

binding sites for *trans*-acting factors expressed in H9C2(2-1) myotubes but not in L6 or C2C12 myotubes. This 30 bp region contains single adjoining MEF-1/E box and MEF-2/AT-rich motifs and mutation of one or both of these motifs resulted in a significant loss of enhancer activity in H9C2(2-1) myotubes. These results provide the first evidence for a role for the MyoD family of transcription factors (MyoD1, myf5, myogenin and MRF4; 52) in the regulation of dystrophin gene expression.

The MEF-2/AT-rich motif within the E-1 region is the second functional MEF-2 binding site identified within the dystrophin gene. The first is located within the core muscle promoter (28) in a region that also contains several E box consensus motifs. Like the intron 1 enhancer, sequences within this region of the muscle promoter are also functional in pre-differentiated H9C2(2-1) myoblasts when positioned upstream of a heterologous promoter (53). One of the questions raised in this study is the mechanism by which only one of three consensus E box motifs within the SA195 fragment binds *trans*-acting factors in H9C2(2-1) myotubes. Studies of the MCK and MLC1/3 gene enhancers have demonstrated cooperativity in MEF-2 and MyoD binding at their respective sites (22,46,54–57). Whether a similar mechanism is operating within the dystrophin gene and contributes to selective utilization of adjoining E box and MEF-2 motifs remains to be determined.

The profile of enhancer activity in H9C2(2-1), L6 and C2C12 myotubes also raises interesting questions regarding the nature of the *trans*-acting factors that bind to these E box and MEF-2 motifs. This profile is remarkably similar to that observed in studies of the muscle promoter (17). Electrophoretic mobility shift assays and DNase footprinting experiments indicated that H9C2(2-1) myotubes express one or more *trans*-acting factors that bind to sequences within the 30 bp E-1 region and, based on the absence of this binding activity in L6 and C2C12 myotubes, appear to be required for optimal enhancer activity. That no factor binding was observed through the E-1 region in DNase footprinting experiments involving L6 and C2C12 myotubes is also interesting in that both of these cell lines express *trans*-acting factors that bind to MyoD/E box and MEF-2 motifs within other muscle-specific genes (22,58). While it remains unclear how motifs within the E-1 region differ from similar motifs in other muscle-specific genes, it may be relevant that this MEF-1/E box motif displays relatively poor homology (60%) to the MyoD binding site consensus as compared, for example, with the MEF-1/E box motif within the E-3 region (80% homology). Furthermore, the H9C2(2-1) cell line was originally derived from an embryonic rat heart culture and retains residual cardiac-specific transcriptional properties (31–34). These observations raise the possibility that sequences within the E-1 region are target binding sites for cardiac-specific *trans*-acting factors, a function that would be consistent with a role for this enhancer in regulating dystrophin gene expression in cardiac muscle.

Finally, it should be noted that sequences within the E-1 region are not solely responsible for enhancer function. For example, a significant decrease in activity was observed upon deletion of 51 bp at the 3′-end of the SA195 fragment (construct 12, Fig. 3). In addition, EMSA competition with the E-1 oligonucleotide did not abolish complex B formation, suggesting that sequences involved in formation of this complex lie outside this region. Also, enhancer activity in L6 and C2C12 myotubes, while reduced relative to H9C2(2-1) myotubes, were significantly higher than in 3T3 fibroblasts. Studies aimed at determining the precise nature of *trans*-acting factors targeted to E-1 and other regions of the core enhancer in H9C2(2-1), L6 and C2C12 myotubes could provide important insights into the regulation of gene expression in cardiac and skeletal muscle.

In summary, the remarkably large size of the dystrophin gene presents special problems for both maintenance of transcript levels in muscle tissues and for identification of transcriptional control regions. The presence of a muscle-specific enhancer containing functional MEF-1/E box and MEF-2/AT-rich motifs suggests that the mechanisms regulating this gene are similar to those identified in other muscle-specific genes studied to date. On the other hand, the unusual profile of enhancer activity and of *trans*-acting factor binding to these regulatory motifs in different myogenic cell lines suggests a more specialized role for this enhancer that is consistent with its potential involvement in regulating dystrophin gene expression in cardiac muscle.

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