Characterization of a Muscle-specific Enhancer in Human MuSK Promoter Reveals the Essential Role of Myogenin in Controlling Activity-dependent Gene Regulation*

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Neuromuscular synaptogenesis is initiated by the release of agrin from motor neurons and the activation of the receptor tyrosine kinase, MuSK, in the postsynaptic membrane. MuSK gene expression is regulated by nerve-derived agrin and muscle activity. Agrin stimulates synapse-specific MuSK gene expression by activating GABP<sub>α</sub> transcription factors in endplate-associated myonuclei. In contrast, the mechanism by which muscle activity regulates MuSK gene expression is not known. We report on a 60-bp MuSK enhancer that confers promoter regulation by muscle differentiation, changes in intracellular calcium, and muscle activity. Within this enhancer, we identified a single E-box that is essential for this regulation. This E-box binds myogenin, and we showed that myogenin is necessary for not only MuSK but also nAChR gene regulation by muscle activity. Surprisingly, the same E-box functions in vitro to mediate muscle-specific and differentiation-dependent gene induction in zebrafish, suggesting an evolutionary conserved mechanism of regulation of synaptic protein gene expression.

Muscle activity impacts many aspects of muscle biology including formation of the neuromuscular junction, slow or fast fiber type, and muscle mass. Many of these effects are a result of activity-dependent control of gene expression. At the developing neuromuscular junction, muscle activity controls the expression of genes, the products of which make up the postsynaptic apparatus. In general, the expression of these genes is suppressed by muscle activity (1, 2). Prior to muscle innervation, they are highly expressed throughout the multinucleated fiber; however, after innervation, their expression is localized to endplate-associated myonuclei (1, 2). Denervation of adult muscle results in a return to the embryonic pre-innervation pattern of gene expression, and this is correlated with the ability of adult denervated muscle to become ectopically innervated.

One of the key components in initiating neuromuscular junction formation is the muscle-specific receptor tyrosine kinase (MuSK) (1). This kinase is activated by nerve-derived agrin and is necessary for nicotinic acetylcholine receptor (nAChR) clustering and the initiation of postsynaptic differentiation. MuSK expression is tightly controlled by a combination of nerve activity that suppresses extrajunctional differentiation (3, 4) and nerve-derived factors such as agrin and neuregulin that promotes synapse-specific expression (5). Neuregulin mediates its action via Erb receptor tyrosine kinases (6) that stimulate a Ras/mitogen-activated protein (MAP) kinase signal transduction cascade (7, 8), resulting in activation of GABP<sub>nα</sub> transcription factors in endplate-associated myonuclei (9, 10). These transcription factors bind to N-box sequences within the MuSK promoter, resulting in its activation (5). Agrin participates in this process by stimulating clustering of Erb receptors in the postsynaptic membrane (5) and by activating a c-Jun NH<sub>2</sub>-terminal kinase (JNK)-dependent signaling cascade that regulates GABP<sub>nα</sub> activity (5). Wnt signaling has also been suggested to contribute to synapse-specific MuSK gene expression (11).

In contrast to these well described mechanisms by which agrin and neuregulin control synaptic expression of the MuSK gene, extrajunctional suppression by muscle activity remains uncharacterized. In our studies of muscle activity and nAChR gene expression, we identified a calcium-dependent signal transduction cascade that depends upon CaMII to suppress nAChR gene expression in cultured myotubes (12–14). This signal transduction cascade ultimately regulates the expression of myogenin, a bHLH muscle-specific transcription factor that binds specific E-box sequences within the nAChR subunit promoters (13, 15). In cell culture, myogenin is essential for both differentiation- and activity-dependent nAChR gene regulation in aneural myotubes (15, 16). It is not known whether a similar mechanism contributes to activity-dependent nAChR gene expression in adult muscle and whether MuSK is regulated in a similar fashion.

Interestingly, the MuSK promoter was recently reported to harbor a critical E-box that mediates promoter activation by the myogenic factor MyoD (11). This E-box was shown to be important for inducing MuSK expression when myoblasts differentiate into multinucleated myotubes. MyoD is a muscle-specific bHLH transcription factor that can transactivate this promoter in transient transfection assays and is induced/activated upon myoblast differentiation. Recently, CREB was shown to bind directly to MyoD, suggesting a novel mechanism of MuSK promoter inhibition that may underlie extrajunctional activity-dependent MuSK suppression following muscle innervation (17). To further this analysis of activity-dependent regulation, we carefully characterized the MuSK promoter elements contributing to activity-dependent gene regulation.

Using a combination of in vitro and in vivo expression assays, we showed that a critical E-box residing in a 60-bp enhancer is crucial for mediating MuSK promoter regulation during muscle differentiation and in response to muscle activity. This same E-box also mediated calcium-dependent regulation of the MuSK promoter. We showed that...
myogenin binds this E-box and transactivates MuSK promoter activity. RNAi-mediated myogenin knockdown indicated that myogenin is essential for differentiation-dependent gene induction during development and denervation-dependent gene induction in adults. Finally, the human MuSK 60-bp enhancer conferred muscle-specific expression in developing zebrafish, suggesting that the mechanism responsible for MuSK induction is conserved between fish and mammals.

MATERIALS AND METHODS

Primary Muscle Culture and Promoter Activity Assay—Rat primary skeletal muscle cultures were prepared as described previously (13, 15). Muscle cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, 10% fetal bovine serum, 25 mM HEPES (pH 7.4), and antibiotics. At 80% confluence, DNAs were transfected into primary myoblasts for reporter gene assay with FuGENE 6 (Roche Applied Science). The confluent myoblasts were induced to differentiate in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum, 5 μM tetrodotoxin (TTX) to inhibit muscle contraction, and 2.8 μg/ml Ara-C to inhibit fibroblast proliferation. Five days later, the well differentiated myotubes were incubated with or without TTX for the indicated time. The myotubes were then collected for luciferase and chloramphenicol acetyltransferase (CAT) assays as described previously (13, 15).

MuSK Genomic Clones and Plasmids—Human genomic DNA was extracted from HEK293 cells, and Hi-Fr PCR (Invitrogen) was used to amplify a 2.2-kb MuSK 5′-flanking DNA sequence, spanning from –2116 to +130. The amplified DNA fragment was cloned into pEGFPN1 (between HindIII and Sall) and PXP2 (between HindIII and Xhol) vectors, respectively. All of the DNA fragments for the derivative mutants, both truncation and mutation, were amplified with Hi-Fr PCR and cloned into either pEGFPN1 or PXP2 vectors. MuSK intron sequence was amplified from the first intron, spanning +728 to +1847. Mutations were confirmed by DNA sequencing. pcS2, pXP2, CMV, and U6 promoter-driven myogenin-targeted shRNAs were described previously (13–15, 18, 22). pcGNSp1 and the Sp DNA binding domain (SpDBD) cloned into pcS2 vector were used in co-transfection assays.

In Vivo siRNA Electroporation and RT-PCR—Steady siRNAs were purchased from Invitrogen. Twenty-five μl of siRNA (20 μM) was injected into adult mouse tibialis anterior muscle and uptake by muscle fibers facilitated by electroporation using a BTX840 square wave electrophorator. The parameters are 140 V/cm, 6 pulses, 60-ms duration, and 100-ms interval. pcS2GFP was co-injected with the siRNAs and allowed us to visualize siRNA/GFP-containing fibers using fluorescence microscopy. GFP-positive fibers were isolated in cold phosphate-buffered saline using fine forceps. Total RNA from these fibers was extracted from HEK293 cells, and Hi-Fr PCR (Invitrogen) was used to amplify a template for the PCR reactions. Radioactive PCR was performed using [α-32P]dCTP to spike the reaction mix. The products were separated by 6% PAGE, and gels were subsequently dried and exposed to x-ray film. Gene-specific primers were used to assay the transcript levels of myogenin, nAChR subunits, MuSK, γ-actin, muscle creatine kinase, and Sp family members. Primers are as follows: nAChR α-subunit, forward, 5′-GGTCTGGTGGGCCAAGCT, and reverse, 5′-CCGCTCTCCATGGAAGTT; nAChR γ-subunit, forward, 5′-ACGGTTGATCTACTGGCTG, and reverse, 5′-GATCCACTCAATGGCTTGC; nAChR δ-subunit, forward, 5′-GGATCTGTGATCCTGACT, and reverse, 5′-GGTCTCGTGGGAGTGTC; muscle creatine kinase, forward, 5′-GGTGTGCGATGAGTGTCCTACA, and reverse, 5′-GCAGTGCCAGGGGCAGGTG; TAA; γ-actin, forward, 5′-ACCCACCGATTTGCTACAGATGTC, and reverse, 5′-CCATCTAGAAGCATTTGCTGGACG; SP1, forward, 5′-AGGAGAAGAATTGCTTGAT, and reverse, 5′-TGTTAGTCTCTGCATATATAC; SP3, forward, 5′-CACTGGTGACTCTACCTTG, and reverse, 5′-TGTTGCTCACTAGTGAGG; Sp4, forward, 5′-TGACACATCACAACTGTTG; and reverse, 5′-CACTCAAAGGCTGACAGG.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA was performed as follows. The indicated E-box sequence was radiolabeled with [γ-32P]ATP using T4 polynucleotide kinase and purified over a G5 spin column. The radiolabeled DNA probe was incubated with C2C12 nuclear extract on ice for 40 min in the reaction buffer (10 mM HEPES (pH 7.9), 75 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 μg poly(dIdC), 300 μg/ml bovine serum albumin). The DNA binding mixture was then resolved on a 5% polyacrylamide gel and subsequently dried and exposed to x-ray film. Supershift assays were performed with anti-myogenin F5D (Developmental Studies Hybridoma Bank, University of Iowa) mouse monoclonal antibody. Mouse anti-Myc was used as control antibody.

Western Blots—Cell lysates were prepared by harvesting cells in radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing a protease inhibitor mixture (P8340 Sigma) at a dilution of 1:500. Cells are sheared via passing through a 25-gauge needle and centrifuged briefly to remove debris. Protein concentration was determined using Bio-Rad DC protein assay. Proteins are resolved by SDS-PAGE (10%) and transferred electrophoretically to Immobilon-P membranes (Millipore). Membranes were blocked in 5% nonfat milk and incubated overnight at 4 °C with anti-GFP antibody (1:3000, Invitrogen). Immunodetection was accomplished using peroxidase-conjugated secondary antibody and subsequent chemiluminescent detection (ECL, Amer sham Biosciences).

Zebrafish Embryo Injection for Transient in Vivo Expression—Zebrafish embryo injection was performed as reported previously (31). For these experiments, zebrafish embryos, at the 1–2-cell stage, were injected with ~300 pl of a solution containing 25–50 ng/μl wild-type or mutant expression vector DNA using a glass micropipette and picopump. pcS2dsRed expression vector was included in all injections for identifying DNA injected fish. At 48 h after injection, fish were anesthetized and examined for GFP reporter gene expression using fluorescent microscopy. Fish exhibiting MuSK promoter-driven GFP expression were counted along with the total number of fish expressing dsRed from the pcS2 promoter.

RESULTS

A Conserved 200-bp 5′-flanking MuSK Promoter Sequence Is Sufficient for Muscle-specific Expression—To investigate MuSK gene regulation, we cloned a 2.2-kb genomic fragment of the human MuSK gene that spanned nucleotides –2116 to +130. Although there are no obvious TATA or CAAT promoter sequences immediately upstream of the transcription initiation site, we did identify a putative initiator sequence that is conserved with the initiator sequences of a number of other genes including the nAChR δ-subunit, terminal deoxynucleotidyltransferase, and adenovirus major late promoters (Fig. 1A) (18–20). Within this initiator resides the transcription start site of the MuSK promoter (5).

We confirmed that this putative MuSK promoter fragment (referred to as MuSK2.2) functioned in a muscle-specific manner by cloning the 2.2-kb promoter fragment into the pEGFP-1 vector (Clontech) and transfecting primary muscle cells growing in tissue culture. These cells
are a mixture of muscle and fibroblast-like cells. As expected, we observed robust reporter gene expression specifically in differentiated muscle (Fig. 1B). As a control, we compared this expression with that elicited by a vector harboring the simian CMV promoter (pCS2). Consistent with the promiscuous expression of the CMV promoter, we found that both muscle and non-muscle fibroblasts exhibit reporter gene expression (Fig. 1B).

Comparison of the MuSK promoter sequences across human, mouse, and rat identified a highly conserved DNA fragment that extended from the initiator sequence upstream to approximately nucleotide −175. Interestingly, this sequence harbors three E-boxes (CANNTG) at positions −43, −76, and −152. Based on our experience characterizing nAChR subunit promoters, we suspected that the E-boxes most proximal to the initiator sequence would be most important for mediating muscle-specific and activity-dependent MuSK gene expression. To test this idea, we deleted nucleotides −208 to −9 from the 2.2-kb promoter using convenient EcoRI restriction sites. Because this fragment harbors three E-boxes, we refer to the 2.2-kb MuSK promoter with this fragment deleted as MuSK2.2(del3E). Transfection of muscle cells with MuSK2.2(del3E) confirmed that this region harbors important regulatory elements that are necessary to drive promoter activity in differentiated muscle cells (Fig. 1C). In contrast, deletion of sequences −208 to −2116 (remaining promoter referred to as MuSK3E) did not significantly influence promoter activity in these quantitative assays (Fig. 1C). In addition, the three-E-box region of the MuSK promoter directs muscle-specific expression in both orientations, typical of an enhancer sequence (Fig. 1C, inset, MuSK3E with opposite orientation).

We used Western blotting to quantify MuSK promoter activity in 10T1/2 cells with and without myogenin co-transfection. Western blots were normalized to co-transfected CMV-CAT activity (Fig. 1D). This analysis showed that MuSK2.2, MuSK3E, and MuSK2.2(del3E) all had similar basal promoter activity; however, only MuSK2.2(del3E) was not induced upon myogenin co-transfection. Therefore, the three-E-box region of the MuSK promoter was sufficient to confer muscle-specific reporter gene expression and is indispensable for myogenin-dependent transactivation.

A Specific E-box in MuSK3E Is Essential for Promoter Activation—We found that the −208 to −9 region, harboring the three E-boxes, was able to confer muscle expression to the heterologous minimal enkephalin (MEK) promoter (Fig. 2A). To evaluate the significance of each E-box in mediating increased promoter activity, we used site-directed mutagenesis to convert each E-box sequence CANNTG to a non-E-box sequence GCNNTG. This analysis showed that only E-box 1, which is most proximal to the initiator sequence, is necessary for conferring high level promoter activity to MEK (Fig. 2A).

Inspection of the three different E-boxes indicates that E-box 1 harbors a sequence (CAGCTG) that is very similar to those found in nAChR promoters that are transactivated by myogenin and mediate muscle-specific and activity-dependent gene expression (21–25). In contrast, E-box 2 (CACTTG) and E-box 3 (CATCTG) are less similar. Interestingly, E-box 1 bound significantly more nuclear protein than the other E-boxes, and supershift assays confirmed that myogenin is a major component of the proteins binding to E-box 1 (Fig. 2B). We do not see a very small amount of myogenin binding to E-box 3.

FIGURE 1. A conserved 200-bp 5′-flanking sequence of the MuSK promoter is sufficient for muscle-specific expression. A, MuSK harbors an initiator-like sequence that is similar to the initiator sequences identified in TdT, ADML, and nAChR β-subunit genes. Sequences are aligned, and the transcription start site is highlighted. Transcription start sites were derived from the literature: terminal deoxynucleotidyltransferase (TdT) (19), adenovirus major late (ADML) (20), and nAChR β-subunit (18). B, comparison of simian CMV (CS2) promoter with MuSK promoter in directing gene expression to muscle in primary muscle cultures that contain both fibroblast and muscle cells. C, promoter deletions identify a 200-bp 5′-flanking MuSK promoter region that mediates muscle-specific expression. The inset for MuSK3E shows muscle-specific expression after flipping the orientation of the three-E-box sequence. D, quantification of MuSK promoter activity in the presence and absence of myogenin (Mgn) co-expression. Promoter activity is reported as reporter GFP protein levels derived from Western blots normalized to CAT activity from a co-transfected CMV-CAT vector. Con, control.
In addition to its sequence, the location of the E-box relative to the basal promoter may be important for its function. Because E-box 3 appeared to bind myogenin at a low level, we asked whether E-box 3 could compensate for E-box 1 when placed in the E-box 1 position. In these experiments, we only replaced the E-box sequence, not the flanking nucleotides. Surprisingly, E-box 3 was able to restore MEK promoter activity in muscle when placed in the E-box 1 position (Fig. 2A). We next asked whether E-box 1 could function if placed in the E-box 3 position and found that it could not (Fig. 2A). Consistent with these latter data, we also showed that E-box 1 sequences placed in both E-box 1 and E-box 3 positions were not able to confer increased promoter activity beyond that of E-box 1 in the E-box 1 position (Fig. 2A). These data suggest that although a particular E-box sequence may be important, its location/flanking nucleotides is also critical for defining function.

Although myogenin can mediate increased MuSK promoter activity via binding to E-box 1, we were interested in determining whether other muscle-specific bHLH proteins activate MuSK promoter activity via E-box 1 or whether they might utilize E-box 2 or 3. To address this question, we compared MuSK3E promoter activity with MuSK(E1m), which harbors a mutation in E-box 1. HEK293 cells were transfected with these expression vectors with and without bHLH proteins myogenin, MyoD, MRF4, or myf5. All bHLH proteins were able to transactivate the MuSK3E promoter (Fig. 2C); however, they were unable to activate the MuSK3E promoter harboring a mutant E-box 1. Although all the muscle-specific bHLH proteins tested could transactivate this promoter when overexpressed, our gel shift assays suggested that myogenin preferentially binds this particular E-box under more physiological protein concentrations (Fig. 2B).

**Identification of a 60-bp Enhancer from the MuSK Promoter**—We used a promoter deletion strategy to identify a minimal sequence flanking the first MuSK E-box to further define the sequences nec-
cessary for high level MuSK promoter activity in muscle. For this analysis, we started with the −208 to −9 fragment of the MuSK promoter that harbors all three proximal E-boxes (Fig. 3A, MuSK3E). Deletion of 120 bp from the 5′ end of this sequence, along with mutation of E-box 2, had little effect on promoter activity (Fig. 3A, (E2m)E1MEKPXP2). The remaining 80 nucleotides of this promoter were sufficient to confer high level reporter gene expression in both orientations, suggesting that this region of DNA is functioning as a classical enhancer. In contrast, upstream sequences that harbor the 120-bp DNA sequence that was deleted in the construction of MuSK3E did not confer increased activity onto the heterologous MEK promoter (Fig. 3A, E3(E2m)(delE1)MEKPXP2).

Although the above data suggest that E-box 1 of the MuSK promoter is essential for maximal promoter activity, we were concerned that additional regulatory elements may reside in the first intron of the MuSK gene. Previous studies have suggested that the first intron harbors the N-box sequence that is responsible for synapse-specific expression of the MuSK gene in innervated muscle (5). We also identified several E-box sequences within the first intron. To evaluate whether these intronic E-boxes can influence promoter activity, we cloned a 1-kb intronic fragment that harbored the N-box and E-box sequences. These sequences were cloned upstream of the minimal MEK promoter or the 3E MEK promoter. Transfection of primary muscle cells showed that regardless of which promoter was employed, E-boxes residing in the first intron did not result in promoter activation (Fig. 3B). In fact, these sequences may hinder promoter activity when juxtaposed to the 3E sequence (Fig. 3B).

The above data led us to focus on the 80-bp MuSK promoter regulatory sequence that harbors two E-boxes. Prediction of transcription factor binding sites within this sequence using the TRANSFAC data base suggests that in addition to the two E-boxes, this sequence harbors putative regulatory elements that bind Sp1, CCAAT enhancer-binding protein α, and a half-site for NF1 binding (Fig. 4A). Interestingly, the CCAAT enhancer-binding protein α site overlaps E-box 2 and may explain why this particular E-box is not functional. To begin to evaluate the significance of these elements in MuSK promoter regulation, we deleted them and assayed promoter activity (Fig. 4A and B). Because E-box 1 is necessary for muscle-specific expression (Fig. 2A), we kept this sequence intact in all deletions. We assayed the activity of these constructs in undifferentiated myoblasts and differentiated myotubes (Fig. 4B). All mutant promoters maintained differentiation-dependent activation, suggesting that E-box 1 mediates this activation. However, deletion of the most 5′ end of this 80-bp enhancer that harbors a putative Sp1 binding site reduced promoter activity by about 50%, whereas further deletions up to E-box 1 had no additional effect. Finally, deletion of the most 3′ end of this enhancer, harboring the putative NF1 half-site, resulted in promoter activation, suggesting that this site may be inhibitory in this context. To determine whether these regulatory sequences

**FIGURE 3.** An 80-bp MuSK promoter fragment mediates muscle-specific gene expression. A, various deletions and mutations identify an 80-bp sequence of the MuSK promoter that is capable of activating a heterologous promoter in both orientations. Primary muscle cells were co-transfected with the indicated promoter constructs along with CMV CAT for normalization. B, the first intron of the MuSK gene harbors four E-box consensus sequences and the functional N-box responsible for synapse specific expression. Primary muscle cells were transfected with the indicated constructs and CMV CAT for normalization. Promoter activity is reported as normalized luciferase activity.
collaborated with myogenin-dependent regulation, we assayed the effect myogenin co-expression had on promoters harboring these various deletions after transfection into 10T1/2 cells (Fig. 4C). Interestingly, basal promoter activity was not dramatically affected by the various deletions; however, myogenin-dependent induction was affected. For example, the wild-type enhancer allowed for a 6-fold increase in promoter activity, whereas the Sp-deleted enhancer only allowed for a 2-fold increase in promoter activity by myogenin (Fig. 4C). These data indicate that protein binding to the Sp-like sequence may collaborate with myogenin to allow the highest possible level of promoter activity. In addition, our data suggest that the 3’ NF1 half-site is dispensable and that the remaining 60-bp enhancer is sufficient for mediating muscle-specific and myogenin-dependent gene induction.

Sp Proteins Contribute to the MuSK Enhancer Activity—To determine whether Sp proteins participate in MuSK promoter regulation, we used gel electrophoretic mobility shift and transfection assays to evalu-
ate Sp-dependent promoter regulation. Gel electrophoretic mobility shift assays, using myotube nuclear extracts and the GC-rich Sp-like sequence of the 60-bp MuSK enhancer, indicated that two complexes were formed (Fig. 5A) that could be competed with either cold probe (Self) or the 60-bp MuSK enhancer (60 bp). Interestingly, only the upper band (Fig. 5A, arrow) was competed with a consensus Sp1 binding site competitor, suggesting that proteins other than Sp family members are responsible for the faster migrating band (Fig. 5A, arrowhead). We also assayed Sp-dependent enhancer activity in transfected muscle cells overexpressing Sp1 or a dominant/negative Sp protein harboring only the SpDBD (Fig. 5B). Consistent with the idea that Sp1 contributes to MuSK enhancer activity, we found a 70% increase in enhancer activity when Sp1 was overexpressed and a dramatic suppression of enhancer activity when the SpDBD was overexpressed (Fig. 5B). SpDBD had no effect on muscle differentiation (Fig. 5B, inset).

The more dramatic suppression observed with the SpDBD versus deletion of the Sp-like sequence (compare Fig. 4B with Fig. 5B) may be explained by the finding that the SpDBD can interact with myogenin (28) and therefore may compromise its transactivating function. In addition, the relatively modest increase in enhancer activity upon overexpression of Sp1 suggests that Sp proteins are normally expressed in myotubes. We tested this using RT-PCR and confirm that Sp1, -3, and -4 are all induced following muscle differentiation (Fig. 5C).

MuSK 60-bp Enhancer Mediates Activity- and Calcium-dependent Promoter Regulation—Like many postsynaptic components, the MuSK gene is regulated by muscle innervation/denervation (Fig. 6D). To determine whether the 60-bp enhancer is sufficient to confer activity-dependent gene regulation onto the minimal MuSK promoter, we co-transfected the 2E(delNF1)MuSK promoter-luciferase (only contains the 60-bp enhancer and initiator sequences) and CMV-CAT (for normalization) expression vectors into the tibialis anterior muscle of adult mice. Muscles either remained innervated or were denervated for 10 days prior to harvesting for luciferase and CAT assays. Consistent with the 60-bp enhancer mediating activity-dependent gene expression, we found that this construct is expressed at a higher level in denervated muscle than in innervated muscle (Fig. 6A).

We confirmed this regulation in vitro using muscle cultures that were allowed to spontaneously contract or were kept inactive by TTX administration (Fig. 6B). These studies showed that the 2.2MuSK and MuSK3E promoters were regulated by muscle activity, whereas the 3E(E1m)MuSK promoter was not (Fig. 6A). Interestingly, myogenin induction correlates with increased MuSK promoter activity in denervated muscle, whereas Sp genes are constitutively expressed in innervated and denervated muscle (Fig. 6D).

We had previously shown that nAChR gene promoters are regulated by muscle activity via a calcium-dependent signal transduction cascade (12–14). Therefore, we assayed the effects of increasing calcium on MuSK promoter activity. Primary muscle cells were treated with various concentrations of A23187 to increase intracellular calcium. As expected, all promoters were suppressed by increased intracellular calcium, except those that had a mutant E-box sequence (Fig. 6C). As we observed for activity-dependent gene expression, increasing intracellular calcium predominantly regulated myogenin but not Sp transcription factors (Fig. 6D).

Myogenin Is Essential for Differentiation- and Activity-dependent Gene Regulation in Vitro and in Vivo—Previous studies of nAChR promoter regulation identified critical E-boxes, which are most proximal to the transcription initiation sites, as important mediators of bHLH binding and gene activation (21–25). Chromatin immunoprecipitation assays have suggested that all four bHLH MyoD family members can bind and activate nAChR promoters, suggesting a redundant function for these proteins in regulating nAChR gene expression (26). Consistent with this observation is our finding that myogenin, MyoD, MRF4, and myf5 all transactivate the MuSK promoter via E-box 1 (Fig. 2C). Comparing the sequence of these critical E-boxes with the MuSK E-box 1 sequence shows a preference for GG and GC as the variable NN nucleotides in the E-box sequence (CANNTG) (Fig. 7A). We suspect that these E-boxes preferentially bind myogenin to mediate gene induction in neuronal myotubes and following muscle denervation. Indeed, we showed in Fig. 2B that myogenin prefers to bind this type of E-box. If myogenin mediates MuSK gene induction during muscle differentiation and in adult denervated muscle, we should be able to prevent MuSK gene induction by blocking myogenin protein expression.

Primary myotubes were co-transfected with expression vectors harboring the MuSK2.2 promoter driving luciferase expression and a CMV promoter driving CAT expression (for normalization), along with an expression vector harboring the U6 promoter driving either an shRNAi targeting myogenin or a control shRNAi. We previously validated the specificity of the myogenin-targeted shRNAi (15). These experiments showed that myogenin suppression by RNAi reduced MuSK promoter activity in differentiated myotubes (Fig. 7B). We confirmed that E-box 1 mediated this effect by using various promoter constructs harboring specific E-box mutations (Fig. 7C).

Might myogenin also mediate MuSK activity-dependent gene regulation in adult muscle? This is a very important question that has not yet been addressed experimentally. Myogenin knock-out animals (16) illustrate the importance of myogenin gene induction in muscle differentiation and regulation of nAChR gene expression early in development;
Activity-dependent Gene Regulation

however, myogenin knock-out mice die at birth, precluding their use to study gene regulation in adult muscle. To surmount this problem, we took advantage of myogenin-targeted RNAi to suppress myogenin expression in adult muscle following denervation. Innervated and denervated adult skeletal tibialis anterior muscles were electropropated with two different siRNAs that targeted myogenin and were initially validated using muscle cells in tissue culture. Eight days later, muscles were harvested, and gene expression was assayed by RT-PCR. Consistent with myogenin playing an integral role in mediating activity-dependent gene induction is the observation that denervation-induced MuSK and nAChR gene induction is blocked by myogenin knockdown (Fig. 7D). In contrast, myogenin knockdown had little effect on muscle genes such as actin and muscle creatine kinase that are not regulated by muscle activity.

The Human 60-bp Enhancer Is Sufficient to Drive Muscle-specific and Differentiation-dependent Gene Expression in Zebrafish—We were intrigued by the observation that the functional myogenin-binding E-boxes of the nAChR subunits and MuSK promoters were restricted to G or C residues for the internal and variable dinucleotides of the E-box (CANNTG). We were interested in determining whether this sequence served a similar function among vertebrates. In particular, we were interested in determining whether E-box 1 was necessary for appropriate developmental expression of the MuSK promoter in a distant relative of mammals such as zebrafish.

Direct injection of expression vector DNA into single cell zebrafish embryos allows one to evaluate promoter activity during development, which is very rapid in the zebrafish. We found that the MuSK2.2 promoter is functional in zebrafish and directs reporter gene (GFP) expression to developing myotubes (Fig. 8A). Simian CMV promoter driving dsRed expression was co-injected with our MuSK vectors and results in both muscle and non-muscle expression (Fig. 8A, pCS2dsRED). Interestingly, muscle-specific expression was retained with the smaller MuSK3E promoter (Fig. 8A). However, deletion of the three-E-box region from MuSK2.2 reduced transgene expression to undetectable levels (Fig. 8A). These data suggest that the MuSK promoter E-box region is necessary for muscle differentiation-dependent gene induction in both mammals and fish.

Within the 3E region of the MuSK promoter is a 60-bp enhancer that is sufficient to drive muscle-specific and differentiation-dependent gene expression in mammals. We tested whether this 60-bp sequence was able to rescue MuSKInitiator (harbors just the initiator of the MuSK promoter) and MuSK2.2(del 3E) expression in developing zebrafish muscle. Consistent with this enhancer directing high level gene expression to muscle, we find that it rescued the muscle expression pattern of MuSKInitiator and MuSK2.2(del3E) expression in developing zebrafish muscle. In addition, mutation of E-box 1 in this enhancer abrogates its ability to rescue muscle expression (Fig. 8, B and C). Thus E-box 1 appears to be a critical E-box that functions in vertebrates to direct promoter activity to differentiating myotubes.

DISCUSSION

The work described here allowed us to draw the following conclusions. 1) A 60-bp DNA enhancer in 5’ MuSK promoter can mediate differentiation-, calcium-, and muscle activity-dependent gene regulation; 2) an evolutionary conserved E-box within this 60-bp enhancer is essential for enhancer activity, whereas an Sp1-like binding site modulates enhancer activity; and 3) myogenin, via binding to its E-box target, controls denervation-induced expression of MuSK and nAChR genes.

A few relatively recent studies have begun to examine the MuSK
promoter and its regulation (5, 11, 17). These studies have identified an N-box sequence in the first enhancer of the \textbf{MuSK} gene that mediates synapse-specific expression and a promoter proximal E-box (E-box 1 in this report) that is necessary for muscle-specific and differentiation-dependent induction in muscle cells grown in tissue culture. In addition, it was reported that a Wnt and CREB-dependent signaling cascade regulates \textbf{MuSK} promoter activity. Although the significance of the Wnt signaling is not clear, it was proposed that CREB-mediated \textbf{MuSK} suppression was a mechanism for suppressing \textbf{MuSK} gene expression in innervated muscle.

The results presented here have extended these observations in a couple of important ways. First, we confirmed that E-box 1 of the \textbf{MuSK} promoter is similar to other promoter E-boxes that mediate activity-dependent gene expression in muscle (H, human; R, rat; M, mouse; C, chicken). 8, primary muscle cells were co-transfected with \textbf{MuSK} and \textbf{CMVCAT} expression vectors along with various amounts of an shRNAi expression vector targeting myogenin or a control shRNAi expression vector. Two days after transfection, cells were harvested for luciferase and CAT assays. C, E-box 1 mediates the effects of myogenin on \textbf{MuSK} promoter activity. Promoter constructs containing wild-type and various E-box mutants of \textbf{MuSK} upstream of the \textbf{MEX} promoter were co-transfected into primary muscle cells with \textbf{CMVCAT}. 9, myogenin targeted RNAi (MgnRNAi). Two days later, cells were harvested and assayed for luciferase and CAT activity. Promoter activity is reported as normalized luciferase activity. D, myogenin knockdown in adult skeletal muscle blocks \textbf{nAChR} and \textbf{MuSK} gene induction after muscle denervation. Adult tibialis anterior muscle was electroporated with two different siRNAs (246 and 654) that targeted myogenin specifically or a control siRNA along with pCS2GFP. Control siRNA contains a similar GC content as the experimental siRNAs but does not target any known genes; pCS2GFP was used to identify electroporated fibers using fluorescent microscopy. Eight days after electroporation, muscle was harvested, and the GFP-positive portions of the muscle were enriched by further dissection using fluorescent microscopy. RNA was prepared from the GFP-positive muscle and gene expression assayed by RT-PCR.

**FIGURE 7.** Myogenin is essential for activity-dependent regulation of \textbf{MuSK} and \textbf{nAChR} gene expression in vitro and in vivo. A, E-box 1 of the \textbf{MuSK} promoter is similar to other promoter E-boxes that mediate activity-dependent gene expression in muscle (H, human; R, rat; M, mouse; C, chicken). 8, primary muscle cells were co-transfected with \textbf{MuSK} and \textbf{CMVCAT} expression vectors along with various amounts of an shRNAi expression vector targeting myogenin or a control shRNAi expression vector. Two days after transfection, cells were harvested for luciferase and CAT assays. C, E-box 1 mediates the effects of myogenin on \textbf{MuSK} promoter activity. Promoter constructs containing wild-type and various E-box mutants of \textbf{MuSK} upstream of the \textbf{MEX} promoter were co-transfected into primary muscle cells with \textbf{CMVCAT}, 9, myogenin targeted RNAi (MgnRNAi). Two days later, cells were harvested and assayed for luciferase and CAT activity. Promoter activity is reported as normalized luciferase activity. D, myogenin knockdown in adult skeletal muscle blocks \textbf{nAChR} and \textbf{MuSK} gene induction after muscle denervation. Adult tibialis anterior muscle was electroporated with two different siRNAs (246 and 654) that targeted myogenin specifically or a control siRNA along with pCS2GFP. Control siRNA contains a similar GC content as the experimental siRNAs but does not target any known genes; pCS2GFP was used to identify electroporated fibers using fluorescent microscopy. Eight days after electroporation, muscle was harvested, and the GFP-positive portions of the muscle were enriched by further dissection using fluorescent microscopy. RNA was prepared from the GFP-positive muscle and gene expression assayed by RT-PCR. inn, innervation; Den, denervation. MCK, muscle creatine kinase.
E-boxes across different nAChR subunit and MuSK promoters, we found very little conservation of the flanking sequence. Perhaps the proximity of this E-box to the basal promoter and/or other regulatory elements dictates its function.

We were able to identify one additional regulatory element within the 60-bp MuSK enhancer that appears to collaborate with the E-box in mediating high level MuSK expression. This element is a GC-rich sequence that may bind Sp1 family transcription factors. Interestingly, both the nAChR α-subunit and the nAChR β-subunit promoters harbor a sequence that is a potential Sp1 family member binding site (21, 27), and deletion of this element can result in reduced nAChR promoter activity (27) similar to that seen with the MuSK promoter. In addition, Sp family members have been reported to interact with bHLH proteins and can influence their activity (28). Our finding that Sp family member genes are induced during muscle differentiation (Fig. 5C) is consistent with their proposed role in regulating MuSK and nAChR gene expression.

It is interesting that we were able to use a zebrafish expression system for the human MuSK promoter and demonstrate that the same E-box sequence that is necessary for mammalian muscle-specific and differentiation-dependent expression also functions in vivo in developing zebrafish. These data suggest that the core mechanism for directing gene expression to muscle is conserved between fish and mammals. Interestingly, inspection of the zebrafish MuSK gene sequence identified a putative promoter-proximal E-box that harbors the sequence CAGCTG. However, sequences flanking this E-box showed little similarity to the mammalian MuSK sequence and suggested that the E-box is a critical element in both mammals and fish that dictates this expression pattern.

We previously showed for nAChR subunit promoters, and report here for the MuSK promoter, that the same E-box that mediates developmental promoter regulation also mediates promoter regulation in adult muscle in response to muscle denervation. This naturally begs the question: are the same trans-acting factors that mediate gene induction during development also controlling gene induction in adult muscle following denervation? Clearly, an E-box-binding protein must be involved in regulating promoter activity. Because myogenin-null animals die at birth, they cannot be used to study adult gene expression (16). Therefore, we used an RNAi-mediated gene knockdown approach to show that myogenin expression is crucial for MuSK and nAChR α-
δ-, and γ-subunit promoter induction following muscle denervation in the adult. We previously showed that myogenin suppression in myotubes suppressed nAChR gene promoter activity (15). In addition to myogenin, aneural myotubes and adult denervated muscle induce MyoD, Mrf4, and myf5, yet they do not compensate for the loss of myogenin in mediating nAChR and MuSK gene induction in vitro or in vivo.

Because of reports that all or many of these myogenic factors can bind (26) and transactivate nAChR (21–25) and MuSK (11, 17) promoters, one may propose that they do not compensate for myogenin but may act independently to regulate nAChR and MuSK gene expression. We do not think this is the case based on the following knock-out studies. First, MyoD knock-out mice are viable and express nAChR and MuSK genes throughout the muscle prior to innervation and suppress extrajunctional expression after innervation (29). In addition, these studies found normal regulation of MuSK gene expression following muscle denervation in adults (29). These data suggest that MyoD is not essential for developmental or activity-dependent gene regulation. Second, MRF4 and myf5 knock-out mice continue to express nAChR and MuSK genes during development, suggesting that they too are not necessary for nAChR and MuSK expression (30). Therefore, myogenin emerges as the critical myogenic factor controlling nAChR and MuSK gene expression.

Since the expression of many synaptic proteins is under the control of myogenin during development and MuSK is a key initiator of synapse formation, myogenin emerges as a master regulator of synapse formation. Consistent with this idea is our recent finding that myogenin controls nAChR clustering in developing myotubes.3 Thus to further our understanding of the mechanisms by which neuromuscular synapses are formed and regulated by muscle activity, we will need to understand the activity-dependent mechanisms controlling myogenin expression.

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