Regulation of MuSK Expression by a Novel Signaling Pathway*

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MuSK is a receptor tyrosine kinase essential for neuromuscular junction formation. Expression of the MuSK gene is tightly regulated during development and at the neuromuscular junction. However, little is known about molecular mechanisms regulating its gene expression. Here we report a characterization of the promoter of the mouse MuSK gene. The transcription of MuSK starts at multiple sites with a major site 31 nt upstream of the translation start site. We have identified an E-box-like cis-element that is both required and sufficient for differentiation-dependent transcription. Interestingly, the promoter activity of the MuSK gene did not respond to neuregulin, a factor believed to mediate the synapse-specific transcription of acetylcholine receptor subunit genes. Rather, MuSK expression is increased in muscle cells stimulated with Wnt or at conditions when the Wnt signaling was activated. These results suggest a novel mechanism for the MuSK synapse-specific expression.

MuSK is a receptor tyrosine kinase discovered because of its abundance in the synapse-rich Torpedo electric organ, of which principal cells are modified muscle cells that are innervated by cholinergic synapses (1). Accumulated evidence indicates that MuSK plays an essential role in the formation of the neuromuscular junction (NMJ)3 (2–5). It becomes tyrosine-phosphorylated in muscle cells in response to Agrin, a protein utilized by motoneurons to mediate the NMJ formation. The kinase activity is required for Agrin-induced AChR clustering (6).

Moreover, the induction of AChR clusters can be inhibited by motoneurons to mediate the NMJ formation. The kinase activity is required for Agrin-induced AChR clustering (6). Also like the AChR, MuSK expression at both mRNA and protein levels increases after denervation or blockade of electrical activity (15, 16).

Although MuSK is essential in the formation of the NMJ, little is known regarding mechanisms regulating its gene expression. To address this question, we have isolated and characterized the MuSK promoter. We identified an E-box-like cis-element that is essential for differentiation-dependent expression. Interestingly, we found that unlike AChR genes, the promoter activity of the MuSK gene did not respond to neuregulin, a factor believed to mediate the synapse-specific transcription of AChR subunit genes (18, 19). Our results suggest that MuSK expression is regulated by a novel mechanism.

EXPERIMENTAL PROCEDURES

Cloning of the MuSK 5′-Flanking Region—A search of the Celera genomic sequence bank using MuSK N-terminal cDNA sequences identified two bacterial artificial chromosome clones containing the entire MuSK gene: RPCI-23:334K8 and RPCI-24:155M23. They were purchased from BACPAC (bacterial artificial chromosome P1-derived artificial chromosome) Resource Center at Children’s Hospital Oakland Research Institute (Oakland, CA) and used as templates for amplification of the MuSK 5′-flanking region and the intron between exons 1 and 2. Primers for the 4.8-kb 5′-flanking region were 5′-GGGT ACCGT CAAGC CATT A AAATA CCAAC TAT-3′ (sense, containing the KpnI site) and 5′-CAAGC CTTTG GAAGA AAGGT TGCTT CTGGA C-3′ (antisense, containing the HindIII site). The PCR fragment was subcloned into the KpnI and HindIII sites of pGL2-Basic (Promega) to generate −4830 to +36 MuSK-firefly luciferase (M4830-Luc). Reporter constructs with deletions in the MuSK 5′-flanking region were generated by PCR using the same antisense primer from the 3′-end (+36) and different sense primers containing the KpnI site with RPCI-23:334K8 as a template. M4830-Luc(N) was generated by inserting a 999-nt (−133 to +1132) DNA fragment containing the purative N-box sequence between BamHI and SalI sites behind the SV40 poly(A) signal in M4830-Luc. The 999-nt DNA fragment was amplified by PCR with sense primer (5′-CCGGG TCTCT GCCTG GTACC TCGGA CTTGG C-3′, containing the BamHI site) and antisense primer (5′-CCGG GACGC TAAAT TTTCTG AAGCT AAGAT TTTTT A-3′, containing the SalI site). Site-directed mutants in putative E-boxes were generated using the QuickChange Site Directed Mutagenesis Kit (Stratagene).

Cell Culture, Transfection, and Luciferase Assay—C2C12 cells were
maintained as described previously (17, 20). Myoblasts (50% confluency) were transiently co-transfected with a MuSK promoter luciferase reporter transgene and pRL-CMV (as a control for transfection efficiency and sample handling) in a ratio of 20:1 using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. In some experiments, co-transfection included mutant Ras or MyoD (in a ratio of 100:20:1, mutant Ras or MyoD:MuSK transgene:pRL-CMV). Twenty-four h after transfection, myoblasts were switched to the differentiation medium (Dulbecco’s modified Eagle’s medium with 2% horse serum) and luciferase assays were performed after myotubes were fully developed using the dual luciferase kit (Promega) following the manufacturer’s instructions. The firefly luciferase activity was normalized by Rontilda luciferase activity.

RNAse Protection and Primer Extension Assays—The probe of RNAse protection assays (RPA) was generated by PCR using the bacterial artificial chromosome clone (RPCL-24-155M23) as a template. A 438-nt fragment included 226 nt of the 5′-flanking region and 79 nt of exon 1, and 133 nt of intron 1 of the MuSK gene was amplified using the primers 5′-TGGTC CCCCT CCACT CCTGATACAT (sense) and 5′-TCTTCT CTTCCT CATA CATTC ATCCCT TAC-3′ (antisense) and subcloned into pGEM-T (Promega). The plasmid was linearized with NcoI. Radiolabeled antisense RNA probe was produced with SP6 RNA polymerase and [α-32P]UTP using MAXiscript Kit (Ambion) following the manufacturer’s instructions. The labeled probe was mixed with 20 or 50 μg of total RNA of yeast, COS-7 cells, HEK293 cells, C2C12 myotubes (either control or treated), and mouse muscle and liver in RPA II hybridization buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, and 1 mM EDTA) at 42°C overnight. After digestion with ribonuclease, protected fragments were fractionated on 6% polyacrylamide sequencing gel next to a sequencing reaction with the same primer. Results and Discussion

Identification of the Transcription Initiation Sites of the MuSK Gene—The 5′-flanking region of the mouse MuSK gene is characterized by several unique restriction sites (Fig. 1A). To identify the transcription initiation sites, a 438-nt antisense RNA probe (complementary from −226 to +512 nt, Fig. 1A) was generated by PCR and incubated with total RNA from various tissues. Protection was observed in muscle cells including C2C12 myotubes and skeletal muscles but not in the liver, COS-7 cells, HEK293 cells, or yeast. These results demonstrate the muscle-specific expression of the MuSK gene in agreement with previous Northern analyses (15, 16). The protection fragments, ranging from −114 to +1 nt, varied in intensity, although all five protections were observed in both C2C12 myotubes and the skeletal muscles (Fig. 1, C and D). The transcription start site closest to the coding sequence, designated as nucleotide 1, appeared to be the thymidine 51 nt upstream from the translation start site (Fig. 1B, site A). Transcription may also start at −42 guanosine (Fig. 1B, site B), −60 cytidine (Fig. 1B, site C), −89 adenosine (Fig. 1B, site D), and −114 adenosine (Fig. 1B, site E). To further characterize the MuSK gene, we carried out primer extension analysis on RNA from skeletal muscle and C2C12 myotubes. Extended fragments were resolved on a 6% polyacrylamide sequencing gel next to a sequencing reaction with the same primer. Results depicted in Fig. 1E confirmed the five transcription start sites and suggested several additional sites located between A and B. These results demonstrate that transcription of the MuSK gene starts at a cluster of positions located between −114 and +1 in muscle cells consistent with the lack of an apparent TATA box within the vicinity of the transcription initiation sites.

Muscle-specific Activation and Regulation by Differentiation of the MuSK Promoter—To study the mechanisms regulating MuSK expression, we generated a reporter transgene containing the 4830-nt 5′-flanking region and the gene of firefly luciferase, M4830-Luc. The luciferase activity of the reporter, expressed as relative luciferase activity over cotransfected pRL-CMV, was similar to that of the empty vector pGL2-Basic in monkey kidney COS-7, HEK293 cells, or C2C12 myoblasts, suggesting that the promoter activity of MuSK was minimal in nonmuscle or undifferentiated muscle cells (Fig. 2A). Remarkably, the promoter activity increased 41-fold in transfected myotubes compared with myoblasts, revealing an up-regulation by muscle differentiation in agreement with previous reports of differentiation-dependent regulation of MuSK expression (15–17). To further characterize this phenomenon and as an initial step to scrutinize where the important cis-acting elements are located, we generated a battery of MuSK reporter constructs with sequential deletion in the 5′-flanking region (Fig. 2B). As with M4830-Luc, these reporters showed similar, but minimal, luciferase activity in C2C12 myoblasts. However, the promoter activity of M715-Luc and M405-Luc was higher in myotubes. The differentiation-dependent increase in promoter activity of these two reporters was similar to that of M4830-Luc, suggesting that the involved cis-elements may locate within the 465-nt 5′-flanking region. Further deletion in the 5′-flanking region decreased the responsiveness to differentiation. The promoter activity of M215-Luc increased 20-fold during the formation of the myotubes, and M37-Luc was unable to respond to muscle differentiation (Fig. 2B). These results demonstrate that the MuSK promoter is regulated by muscle differentiation and that the cis-elements essential for differentiation-dependent regulation exist between −465 and −37.

Identification of an E-box Element That Is Required and Sufficient for MuSK Promoter Activity—Higher MuSK promoter activity in myotubes than in myoblasts indicates that the MuSK 5′-flanking region contains cis-elements that are regulated by transcription factors of muscle differentiation. Studies of AChR subunit genes have demonstrated that their expression is regulated by myogenic factors acting on E-box cis-elements (18). Analyzing the sequence of the MuSK 5′-flanking region between −465 and −37, we identified four putative E-box elements characteristic of CANNTG (where N is any nucleotide) (Fig. 1B). To determine whether any of these E-box elements is involved in regulating the MuSK promoter activity, we generated site-specific mutants in which cytidine was mutated to thymidine and guanosine to adenosine in M715-Luc as the parental vector. Their relative luciferase activity was assayed in transfected myotubes. As shown in Fig. 3B, mutation of E-box2, -3, or -4 (as in E134, E265, or E123, respectively) did not appear to alter the promoter activity, suggesting that these elements may not be essential for regulation. In contrast, the mutation of E-box1 alone (as in E234) dramatically decreased the promoter activity in C2C12 myotubes, suggesting a requirement of E-box1 for the MuSK promoter activity. To determine whether E-box1 is sufficient to mediate differentiation-dependent regulation, we generated M715-Luc mutants that contained either one or a combination of other E-box elements. Not surprisingly, E0, in which four E-box elements were all mutated, revealed a suppressed promoter activity. Remarkably, the addition of E-box1 restored the ability of the reporter gene to respond to differentiation, suggesting that this element is
sufficient (Fig. 3B). As a control, the addition of E-box4 or of both E-box3 and E-box4 failed to rescue the promoter activity.

Expression of muscle-specific genes, such as the AChR, is regulated by myogenic factors (21, 22). The finding that the MuSK promoter activity is increased by differentiation suggests the possibility that myogenic factors participate in the regulation. To test this hypothesis, we analyzed the report activity of M4830-Luc in muscle cells cotransfected with or without MyoD. As shown in Fig. 3C, the reporter activity was increased mildly by MyoD in COS-7 cells and in C2C12 myoblasts. The activity, although significantly higher than the control, was lower than that when the reporter gene was expressed in C2C12 myotubes, suggesting that other factors intrinsic to differentiated muscle cells may be necessary (Fig. 3C). On the other hand, MyoD co-transfection further increased the promoter activity in myotubes, which may suggest that myogenic factors may be available in limited amount. Studies of the deletion mutants indicated that the increase in the MuSK promoter activity by MyoD had a similar structure requirement to the differentiation-dependent regulation (data not shown). To identify the cis-elements for the regulation by myogenic factors, we characterized promoter activity of the E-box deletion and “add-back” mutants of M715-Luc (which were described in Fig. 3A) in myotubes cotransfected with or without MyoD. E-box1 appeared to be necessary and sufficient for MyoD-mediated increase in the MuSK promoter activity (data not shown). This notion was confirmed in studies with M4830(mE1)-Luc in which E-box1 (and only E-box1) was mutated. This mutation abolished the increase in the promoter activity by co-transfected MyoD in C2C12 myotubes (Fig. 3C), suggesting that the up-regulation of the MuSK promoter activity is mediated by E-box1.

No Effect of Neuregulin on MuSK Expression—Synapse-specific transcription of the AChR genes is mediated by complex mechanisms. In addition to myogenic factors that regulate AChR gene expression during differentiation, electrical activity suppresses AChR gene transcription in all nuclei in the skeletal muscle (18, 23–25). The transcription of AChR genes in the synaptic region is believed to be mediated by neuregulin, a polypeptide synthesized in motoneurons and enriched in the synaptic basal lamina (19, 26). By counteracting the inhibitory effect of electrical activity, neuregulin increases local AChR transcription in the synaptic region. In light of the MuSK mRNA concentration around synaptic nuclei in the skeletal muscle (15), we determined whether the MuSK promoter was regulated by neuregulin. In control experiments, we studied the neuregulin regulation of the promoter activity of e416-Luc.
Neuregulin stimulation caused a 100% increase in Renilla luciferase activity (firefly/luciferase) in myoblasts (24 h after transfection) and in myotubes (72 h after being switched to differentiation medium). Both panels show relative luciferase activity (firefly/Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. MB, myoblasts; MT, myotubes. Putative cis-elements (E-box, squares; MEF2, ovals) are indicated.

**Fig. 2.** Muscle-specific expression and regulation by differentiation. A, promoter activity of M4830-Luc in various cell lines. Cells were transfected with M4830-Luc and pRL-CMV. Firefly and Renilla luciferase activity was assayed as described under “Experimental Procedures.” B, higher MuSK promoter activity in myotubes than in myoblasts. C2C12 myoblasts were transfected with indicated vectors plus pRL-CMV. Luciferase activities were assayed as in A in myoblasts (24 h after transfection) and in myotubes (72 h after being switched to differentiation medium). Both panels show relative luciferase activity (firefly/Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. MB, myoblasts; MT, myotubes. Putative cis-elements (E-box, squares; MEF2, ovals) are indicated.

A transgene reporter that contains the 416-nt AChR ϵ-subunit 5′-flanking region, which drives the expression of luciferase (27). Neuregulin stimulation caused a 100% increase in e416-Luc expression in transfected C2C12 myotubes (Fig. 4A) in agreement with previous observations (20, 28–30). However, under the same conditions, neuregulin did not appear to increase the promoter activity of M4830-Luc in transfected C2C12 myotubes (data not shown). Neuregulin regulation is believed to be mediated by N-box, a cis-element that is required for synapse-specific transcription (31–33). N-box elements usually locate upstream of the transcription initiation site in the AChR ϵ- or ω-subunit genes. However, the 4830-nt 5′-flanking region of mouse MuSK did not appear to contain an N-box element. Rather, sequence analysis identified a putative N-box element (CCGGAA, nt +805 to +811) in the intron between exon 1 and exon 2. Thus we created the M4830-Luc(N) reporter transgene, which contains the 999-nt intron sequence after the luciferase (see “Experimental Procedures” for details). As shown in Fig. 4A, M4830-Luc(N) promoter activity did not increase in C2C12 myotubes challenged by neuregulin, suggesting that the transcription of the MuSK gene may not be regulated by neuregulin, confirming an earlier report that MuSK mRNA was not increased in C2C12 myotubes in response to neuregulin stimulation (34). These results are in line with recent observations that MuSK expression remains unchanged in transgenic mice expressing a general Ets dominant-negative mutant specifically in skeletal muscle (35).

Neuregulin acts by activating its receptor, the 180-kDa ErbB receptor tyrosine kinases. Several intracellular kinases are implicated in mediating neuregulin regulation of AChR gene expression including mitogen-activated protein kinases Erk (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) and Cdk5 (20, 28, 30, 36, 37). In particular the Ras-Raf-Erk pathway activation appeared to be required and sufficient for the neuregulin effect and for synapse-specific expression of the ϵ-transgene in vivo (20, 34, 36). To investigate further whether neuregulin regulates MuSK expression, we studied the effect of active- or dominant-negative Ras mutant. Expression of these mutants alone has a profound effect on AChR promoter activity (20, 36, 37). As shown in Fig. 4A, however, the promoter activity of M4830-Luc(N) was not increased in C2C12 myotubes cotransfected with active Ras, unlike e416-Luc in which luciferase activity was increased. These results corroborate with those with neuregulin and support the notion that neuregulin may play a limited role in regulating MuSK expression.

**Increase in MuSK Expression by Wnt**—Wnt signaling is known to play a role in the induction of myogenesis (38) and has been implicated in the formation of the neuromuscular junction (17, 39). We asked whether Wnt signaling was involved in regulating MuSK expression. To test this hypothesis, transfected C2C12 myotubes were stimulated with Wnt1 at a concentration that increases β-catenin in the cytosol (17) and were assayed for the promoter activity of M4830-Luc. As shown in Fig. 4B, the promoter activity was increased by 75% (29, n = 6, p < 0.05), suggesting a possible involvement of Wnt signaling in MuSK expression. In the Wnt canonical pathway, Wnt binds to and activates its receptor, the Frizzled family of seven transmembrane-domain proteins (40–42), which probably, via Dishevelled, causes the inhibition of β-catenin phosphorylation...
(or Arm for armadillo) by glycogen synthase kinase \(3\beta\) (GSK3 or Zw-3). The net result is the up-regulation of \(\beta\)-catenin and its translocation into the nucleus (41, 43–46). To further characterize the involvement of Wnt signaling in this event, we investigated whether the promoter activity was altered by LiCl, an inhibitor of GSK3\(\beta\). As shown in Fig. 4B, treatment of C2C12 myotubes with LiCl (40 mM for 24 h) increased the MuSK promoter activity further supporting the involvement of Wnt signaling. We next asked whether Wnt regulates the expression of the endogenous MuSK gene in nontransfected muscle cells. C2C12 myotubes were stimulated without (control) or with Wnt1 for 24 h. Cells were lysed and total RNA isolated, which was subjected to RPA. The intensity of each radioactive band was normalized by \(\alpha\)-actin in a respective sample.

Wnt signaling. We next asked whether Wnt regulates the expression of the endogenous MuSK gene in nontransfected muscle cells. C2C12 myotubes were stimulated without (control) or with Wnt1 for 24 h. Cells were lysed and total RNA isolated, which was subjected to RPA. The intensity of each radioactive band was normalized by \(\alpha\)-actin in a respective sample.

**Fig. 3.** E-box1 was required for differentiation-dependent and MyoD-mediated regulation of the MuSK promoter activity. A, schematic diagrams of E-box mutants in M715-Luc. The first (cytidine) and sixth (guanosine) in the consensus CANNTG sequence were mutated to thymidine and adenosine, respectively. B, dependence of the MuSK promoter activity on E-box1. C2C12 myoblasts were transfected with the indicated M715-Luc mutants and pRL-CMV. Luciferase activities were assayed as in Fig. 2A in myotubes (72 h after being switched to differentiation medium). Shown is the relative luciferase activity (firefly\(\div\)Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. C, MyoD regulation of the MuSK promoter activity requires E-box1. COS-7 cells and C2C12 myoblasts were transfected with pRL-CMV, M4830-Luc, and M4830(mE1)-Luc (E-box1 of which was mutated). Luciferase activities were assayed as in Fig. 2A in COS-7 or myoblasts (24 h after transfection) or in myotubes (72 h after being switched to differentiation medium). Shown is the relative luciferase activity (firefly\(\div\)Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. MB, myoblasts; MT, myotubes.

**Fig. 4.** MuSK expression was increased by Wnt. A, the MuSK promoter was not activated by neuregulin. C2C12 myoblasts were transfected with pRL-CMV and e416-Luc or M4830-Luc(N) (which contained the 999 nt in intron1) with or without active Ras (Ras-ac). Myotubes, which formed 48 h after being switched to differentiation medium, were stimulated with neuregulin (10 nM, 24 h). Luciferase activities in myotubes were assayed as in Fig. 2A. Shown is the relative luciferase activity (firefly\(\div\)Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. B, increase in M4830-Luc activity by Wnt1. C2C12 myotubes transfected with pRL-CMV and M4830-Luc were stimulated with Wnt1-conditioned media or 40 mM LiCl for 24 h. Luciferase activities in myotubes were assayed as in Fig. 2A. Shown is the relative luciferase activity (firefly\(\div\)Renilla, mean ± S.E.) from a representative experiment in duplicate that was repeated three times with similar results. C, MuSK mRNA was increased in Wnt1-stimulated muscle cells. C2C12 myotubes were stimulated with or without Wnt1 for 24 h. Cells were lysed and total RNA isolated, which was subjected to RPA. The intensity of each radioactive band was normalized by \(\alpha\)-actin in a respective sample.
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with Wnt1 and assayed for the MuSK mRNA by RNA protection using a 184-nt probe spanning exon1 and exon2. As shown in Fig. 4C, Wnt1 stimulation increased the MuSK mRNA by 70%. These results were in agreement with those with the MuSK transgene, supporting the notion that the MuSK gene transcription is regulated by Wnt signaling. Interestingly, the extracellular regions of MuSK and Frizzled show homology (47–49), although it remains to be determined whether MuSK is able to interact with Wnt1 directly. However, stimulation of muscle cells with Wnt1 does not activate MuSK (17). It is possible that the regulation of MuSK gene expression by Wnt1 is via a mechanism independent of MuSK kinase activation.

In summary, we have characterized the MuSK promoter in this study. The transcription of MuSK starts at multiple sites. We have identified an E-box-like cis-element that is both required and sufficient for differentiation-dependent transcription. Interestingly, the promoter activity of the MuSK gene did not respond to neuregulin, a factor believed to mediate the synapse-specific transcription of AChR subunit genes. Rather, MuSK expression is increased in muscle cells stimulated with Wnt or under conditions where the Wnt signaling was activated. These results suggest a novel mechanism for the MuSK synapse-specific expression.

Acknowledgments—We are grateful to Dr. M. Sliwkowski and Dr. Xi He for valuable reagents.

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