Molecular analysis of fiber type-specific expression of murine myostatin promoter

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Senna Salerno, Mônica, Mark Thomas, Davanea Forbes, Trevor Watson, Ravi Kambadur, and Mridula Sharma. Molecular analysis of fiber type-specific expression of murine myostatin promoter. Am J Physiol Cell Physiol 287: C1031–C1040, 2004. First published June 9, 2004; 10.1152/ajpcell.00492.2003.—Myostatin is a negative regulator of muscle growth, and absence of the functional myostatin protein leads to the heavy muscle phenotype in both mouse and cattle. Although the role of myostatin in controlling muscle mass is established, little is known of the mechanisms regulating the expression of the myostatin gene. In this study, we have characterized the murine myostatin promoter in vivo. Various constructs of the murine myostatin promoter were injected into the quadriceps muscle and the reporter luciferase activity was analyzed. The results indicate that of the seven E-boxes present in the 2.5-kb fragment of the murine myostatin promoter, the E5 E-box plays an important role in the regulation of promoter activity in vivo. Furthermore, the in vitro studies demonstrated that MyoD preferentially binds and upregulates the murine myostatin promoter activity. We also analyzed the activity of the bovine and murine promoters in murine skeletal muscle and showed that, despite displaying comparable levels of activity in murine myoblast cultures, bovine myostatin promoter activity is much weaker than murine myostatin promoter in mice. Finally, we demonstrate that in vivo, the 2.5-kb region of the murine myostatin promoter is sufficient to drive the activity of the reporter gene in a fiber type-specific manner.

Myostatin, a new member of the transforming growth factor-β superfamily, is predominantly expressed in developing and adult skeletal muscle. Myostatin-null mice display a two- to threefold increase in skeletal muscle mass that is due to both hyperplasia (i.e., increase in the number of fibers) and hypertrophy (i.e., increase in fiber thickness) (26). Naturally occurring mutations in the myostatin gene coding sequence in Belgian Blue and Piedmontese cattle breeds result in the heavy muscle phenotype (14, 22, 27). Hence, myostatin functions as a negative regulator of muscle growth.

Myostatin expression is detected in myogenic precursors during early embryogenesis, and the expression continues in postnatal skeletal muscle (22, 26). Changes in muscle mass have been shown to be related to changes in myostatin expression. Recently, Roth et al. (32) reported that myostatin mRNA levels are reduced in response to heavy-resistance strength training in humans. On the other hand, higher levels of circulatory and muscle myostatin have been observed in humans with acquired immunodeficiency syndrome-related muscle wasting or age-associated sarcopenia (13, 25). Furthermore, chronic underfeeding in sheep and hindlimb suspension in rats resulted in increased levels of myostatin (6, 21, 42). Collectively, these results and those described in other reports indicate that myostatin expression is regulated at the transcription level.

Although the functional role of myostatin in controlling muscle mass has been delineated, much remains to be learned about the regulation of the myostatin gene at the transcription level. Recently, two studies (24, 37) have analyzed the human and bovine myostatin promoter. The 5′ upstream regulatory and muscle myostatin have been observed in humans

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vivo experiments also show that E-box E5 of the murine myostatin promoter plays a crucial role in the regulation of promoter activity. Furthermore, we demonstrate that among the MRF, MyoD preferentially activates the murine myostatin promoter and that this overlaps with the expression of reporter activity in type IIB fibers in vivo.

MATERIALS AND METHODS

Cloning of the murine myostatin promoter fragments. A 2.5- and a 1.7-kb fragment of the myostatin promoter were amplified by PCR using genomic murine DNA as a template. Wild-type and mutated 5′ truncation constructs containing the various E-boxes were also generated by PCR using mouse genomic DNA as a template. Primer sequences and combinations are shown in Table 1. The amplification was performed using Taq polymerase (Invitrogen) in buffer containing 2 mM MgCl₂ and Q-solution (Qiagen) with the following conditions: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing at 50°C for 1 min, and extension for 2 min at 72°C. The final extension was at 72°C for 5 min. The amplified PCR products were cloned into a PGEM-T Easy vector (Promega), sequenced, and subsequently subcloned into pGL3-Basic vector (Promega). MyoD (pJM11) and Myf5 (pJM7) expression plasmids were described previously by Spiller et al. (37).

Transfections and luciferase assays. C2C12 cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY) and buffered with NaHCO₃ (41.9 mmol/l; Sigma, St. Louis, MO) and gaseous CO₂. Phenol red (7.22 nmol/l; Gibco-BRL, Grand Island, NY) was used as a pH indicator. Penicillin (100 IU/ml; Sigma), streptomycin (100 mg/l; Sigma), and fetal bovine serum (FBS, 10%; GIBCO-BRL) were routinely added to the medium. For transfections, cotransfection experiments were done by subcloning a Myf5 fragment from the expression plasmid pJM7 into the KpnI and EcoRI sites of pRSET A vector (Invitrogen).

Table 1. Oligonucleotides used to generate various murine myostatin promoter constructs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>2.5FWD</td>
<td>5′-GGTACCCCGCTTTTTTAAGTCCAAGTCAAGACGG-3′</td>
</tr>
<tr>
<td>1.7FWD</td>
<td>5′-GGTACCCCGCTTTTTTAAGTCCAAGTCAAGACGG-3′</td>
</tr>
<tr>
<td>E3wt</td>
<td>5′-GGATTTATTTTTATTATTTGAGATGTTACAACTGAGCAGGTGC-3′</td>
</tr>
<tr>
<td>E3mut</td>
<td>5′-GGATTTATTTTTATTATTTGAGATGTTACAACTGAGGTC-3′</td>
</tr>
<tr>
<td>E5wt</td>
<td>5′-GCCGATCGGCCTGGCTGAGCTGTGCCCCCATCATCCACAGTGACCT-3′</td>
</tr>
<tr>
<td>E5mut</td>
<td>5′-GCCGATCGGCCTGGCTGAGCTGTGCCCCCATCATCCACAGGTTGGC-3′</td>
</tr>
<tr>
<td>E4mut</td>
<td>5′-GCCGATCGGCCTGGCTGAGCTGTGCCCCCATCATCCACAGTCTGAC-3′</td>
</tr>
<tr>
<td>MstnR</td>
<td>5′-GGTACCCCGCTTTTTTAAGTCCAAGTGACGG-3′</td>
</tr>
</tbody>
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Mutated primer sequences are shown in italics. E3wt, wild-type E-box E3; E4wt, wild-type E-box E4; E5wt, wild-type E-box E5; E3mut, mutated E-box E3; E4mut, mutated E-box E4; E5mut, mutated E-box E5; MstnR, myostatin promoter reverse primer.

Individual luciferase value for each assay was normalized against total protein value.

**Gene mobility shift assays.** Recombinant MyoD, Myf5, and E47 were expressed and purified from Escherichia coli as described previously (37). The pQ7-MyoD and pQ7-E47 plasmids were kind gifts from Stephen F. Konieczny (Dept. of Biological Sciences, Purdue University, West Lafayette, IN) and Kyung-Sup Kim (Institute of Genetic Science, Dept. of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, Korea). The pRSET-Myf5 plasmid was obtained by subcloning a Myf5 fragment from the expression plasmid pJM7 into the KpnI and EcoRI sites of pRSET A vector (Invitrogen).

One microgram of MyoD or Myf5 protein was mixed with 100 ng of E47 protein and equilibrated at room temperature to form heterodimers for 10 min in binding buffer containing 10 mM HEPES (pH 7.9), 10% glycerol, 75 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.5 μg of poly(dI-dC) (Roche), and 0.5% fetal calf serum.

Serial twofold dilutions of MyoD/Myf5 or Myf5/E47 heterodimers, from 600 ng to 33 pg, were mixed with DNA probe (10 pmol) in a final volume of 20 μl in binding buffer. After 30-min incubation at room temperature, samples were subjected to electrophoresis on a native 5% polyacrylamide gel in 0.5× Tris-borate-EDTA at 35 mA at room temperature. Gels were dried and exposed either to a film (Kodak X-Omat) at −80°C or to a PhosphorImager screen (Imaging Screen K-HD; Bio-Rad) for 3 h at room temperature and scanned on a PhosphorImager (Molecular Image FX; Bio-Rad). The relative optical density of bands corresponding to free and bound DNA was measured using Quantity One 4.2.2 software (Bio-Rad). The binding affinity was calculated as described elsewhere (7, 39). Because the protein concentrations did not take into account the fraction of inactive proteins, the data are referred to as apparent dissociation constants [Kd(app)].

**Animals.** Wild-type mouse strain C57BL/10 was bred at Ruakura Small Animal Colony (Hamilton, NZ). Animal handling and care was performed according to the specifications of the Ruakura Animal Ethics Committee.

**Injection of naked DNA into muscles.** Four- to 5-week-old C57BL/10 mice were anesthetized with intraperitoneal injections of 0.1 ml/10 g body wt of Hypnorm (5 ml/Hypnovel (2 ml) mix. A small incision (≈4 mm) was made in the hindlimb, and the quadriiceps femoris muscle group exposed. Each quadriiceps muscle was injected (i.e., the left and right quadriiceps of each animal) with 50 μl (1 μg/μl DNA in sterile PBS) of either the bovine myostatin promoter construct 1.6b or the murine myostatin promoter constructs 2.5P, 1.7P, tE5, tE4, tE3, mE5, mE4, or mE3 or the control vector pGL3-B. For immunochemistry purposes, 4-wk-old mice were injected with 60 μl/muscle (1 μg/μl DNA in sterile PBS) of either the murine constructs 2.5P or tE5 or the control vector pGL3-B, mixed with a small amount of India ink to facilitate the localization of the injected DNA (3). The incisions were closed using stainless steel clips. Six animals
per DNA construct underwent injection. For the experiment involving the murine 1.7P and the bovine 1.6b constructs, half of the mice were killed at day 3 and the remaining mice were killed at day 7 after the naked DNA injection, and the muscles (6 muscles/day/construct) were then processed for total luciferase activity. For the remaining experiments, all of the mice were killed at day 7 after naked DNA injection, and the muscles (12 muscles/construct) were then processed for total luciferase activity.

**Total muscle luciferase and protein assay.** Each muscle was frozen in liquid nitrogen and ground to a fine powder. The material was resuspended in 1 ml of cell lysis buffer [25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (Sigma), 10% glycerol, and 1% Triton X-100], and processed as described for cell lysates. Ten microliters of undiluted muscle extract were used for luciferase assay. Five microliters (1:100 dilution in lysis buffer) of muscle extract were used for estimating total protein concentration by Bio-Rad protein assay reagent. The protein estimates were used to normalize the luciferase readings.

**Immunocytochemistry.** The muscles from mice injected with 2.5P, tE5, or pGL3-B DNA were collected and frozen in isopentane chilled in liquid nitrogen. Cryosections were cut at 10 μm, and the slides were frozen at −20°C until used.

The sections were permeabilized in PBS and 0.1% Triton X-100 for 30 min at room temperature and then incubated with primary anti-luciferase antibody (Promega) at 1:50 dilution in PBS, 5% normal goat serum, 1% BSA, and 0.1% Triton X-100 overnight at 4°C. After washing three times for 3 min each in PBS, the slides were incubated in PBS, 3% normal rabbit serum, 1% BSA, and 0.1% Triton X-100 for 1 h to reduce nonspecific binding of the secondary antibody. The sections were incubated with biotinylated secondary antibody (Amersham, Little Chalfont, UK) at 1:300 dilution in 1 h at room temperature. After being washed in PBS, the sections were finally incubated in streptavidin-conjugated fluorescein-labeled tertiary antibody (Alexa Fluor streptavidin; Molecular Probes, Eugene, OR) for 1 h at room temperature, washed in PBS, counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes), mounted in fluorescent mounting medium (DAKO, Copenhagen, Denmark), and analyzed for reporter gene expression. For fiber typing, permeabilized serial sections were incubated with undiluted anti-fast type II fiber antibody N1.551 or anti-slow type I fiber antibody A4.840 (17, 18, 41) for 2 h at room temperature. Slides were washed in PBS and then blocked in PBS, 0.1% Triton X-100, and 5% normal sheep serum for 30 min at room temperature. Slides were then incubated with biotinylated secondary antibody (Amersham) and treated as described above. Control sections were incubated with either no primary antibody or mouse control IgG and then processed as described above.

**Histochemical fiber typing of the muscle sections.** For histochemical fiber typing of the muscle, a standard alkanil ATPase method was used, as described by Guth and Samaha (15, 16). In summary, serial sections were fixed in a solution of 5.5% formaldehyde, 200 mM sodium cacodylate, 68 mM CaCl₂, 340 mM sucrose for 5 min, and washed in Tris rinse solution (100 mM Tris, pH 7.4, and 18 mM CaCl₂). The slides were then incubated in alkaline solution [100 mM 2-amino-2 methyl-1 propanol (AMIP; BDH Laboratory Supplies, Poole, UK), and 70 mM CaCl₂, pH 10.4] for 15 min at room temperature, followed by washes in Tris rinse solution. The slides were transferred to staining reaction solution containing 100 mM AMIP, 18 mM CaCl₂, and 2.7 mM ATP disodium salt (Boehringer Mannheim, Mannheim, Germany), preheated to 37°C, and incubated for 20 min. The slides were washed in 70 mM CaCl₂, incubated in 2% CoCl₂ for 3 min at room temperature, and washed in 100 mM AMIP. They were then transferred to a solution of 1% ammonium polysulhide [(NH₄)₂S; BDH] and incubated for 3 min at room temperature, followed by vigorous washing in water. The slides were counterstained with DAPI and mounted in DAKO faramount aqueous mounting medium.

**RESULTS**

**Transcription factor binding sites in the murine myostatin gene promoter.** Sequence analysis of the 2.5-kb fragment of the murine myostatin promoter using MatInspector identified a series of muscle-specific transcription factor binding sites. As shown in Fig. 1, one myocyte enhancer factor 2 site is present within 1.3 kb of the promoter and seven E-box motifs are present in the 2.5-kb region. The E-boxes in the murine promoter are arranged in clusters: the first cluster contains two E-boxes with an identical CACCTG sequence (E1 and E2), and the second cluster contains another two E-boxes with the sequence CAATG (E3 and E4). E-box E5 does not appear to be a part of any cluster, while the last cluster contains E-boxes E6 and E7. Figure 1 also depicts the various 5′ truncation and mutation E-box constructs used in the experiments. Furthermore, closer analysis of the promoter region showed the presence of two polyA tracts, A₁₄ and A₃₀̇₅, at positions 1,661 and 705, respectively.

**Analysis of the murine myostatin promoter activity in myoblasts.** An extensive in vitro analysis of the murine myostatin promoter activity was performed before the in vivo characterization of the promoter. A series of truncation constructs containing different numbers of E-boxes were generated and transfected into C₂C₁₂ cells. As shown in Fig. 2, the effect of the E-boxes seems to be additive because the activity of the promoter increased with the number of E-boxes present in the construct. Moreover, inclusion of E-box E5 increased the reporter gene activity to the same level observed with construct 2.5P, which includes E-boxes E6 and E7. This suggests that the presence of the first five E-boxes, and in particular E-box E5, is sufficient to render maximal promoter activity in C₂C₁₂ cells.

Because MRF are known to bind to E-boxes and induce promoter activity (1, 2, 8), the murine 1.7P promoter construct, which contains the first five E-boxes, was cotransfected with either MyoD or Myf5 expression vector into C₂C₁₂ cells and the reporter gene activity was determined. The results show that both factors induced the activation of the myostatin promoter (Fig. 3). However, while MyoD increased the promoter activity more than sixfold, Myf5 cotransfection led to only a twofold increase, suggesting that MyoD preferentially regulates myostatin promoter activity.

**Binding of MyoD/E47 and Myf5/E47 to E-box E5 in vitro.** To confirm that MyoD and Myf5 bind to E-boxes in vitro, we performed gel shift assays using the sequences containing the wild-type (E5wt) or mutated (E5mut) E-box E5 as probes. Although in vitro both MyoD and Myf5 can bind to E-boxes as homodimers, in vivo they form heterodimers with E proteins such as E12 or E47. As shown in Fig. 4A, MyoD alone was able to form a complex with E5wt (lane 1), but a higher intensity of complex formation was observed when the MyoD/ E47 heterodimer was present (Fig. 4A, lane 2). On the other hand, no complex was formed with the E5wt probe and Myf5 alone (Fig. 4A, lane 3), but the addition of E47 led to the formation of a complex (lane 4). These results suggest that
while MyoD can bind to an E-box as a homodimer in vitro, Myf5 is able to bind only as a heterodimer. Significantly lesser complexes were formed when the E5mut probe was used (Fig. 4A, lanes 5–8), suggesting that the binding of MyoD and Myf5 to E-box E5 is dependent on its sequence integrity.

Fig. 1. Schematic representation of the different murine myostatin promoter constructs. Constructs containing different fragments of the murine myostatin promoter were generated by PCR and cloned into the luciferase (LUC) reporter vector pGL3-Basic. A: 2.5P construct contains seven E-boxes (squares), five of which are present in the 1.7P construct. A myocyte enhancer factor 2 (MEF2) site is also present in the 1.7P construct (triangles). Truncation constructs spanning up to E-boxes E3 (tE3), E4 (tE4), and E5 (tE5) are also depicted. B: mutation constructs were also generated in which individual E-boxes E3 (mE3), E4 (mE4), and E5 (mE5) were mutated (crossed squares).

Fig. 2. In vitro analysis of the murine myostatin promoter constructs. Construct 2.5P, as well as truncation constructs tE3, tE4, and tE5, were transfected into C2C12 cells, and luciferase activity was measured. Results reflect relative induction over the promoterless vector pGL3-Basic (pGL3-B). Bars indicate means ± SD of 4 independent experiments. ***P < 0.01, *P < 0.05 vs. 2.5P construct.

Fig. 3. Stimulation of myostatin promoter activity by MyoD and Myf5. Two micrograms of each expression plasmid (pJM11, pJM7, and pcDNA3) were cotransfected with 2 μg of the 1.7P construct. Relative stimulation of myostatin promoter activity by MyoD (1.7P + MyoD) or Myf5 (1.7P + Myf5) over that of 1.7P construct (1.7P + pcDNA3) is shown. Bars indicate means ± SD of 3 independent experiments. ***P < 0.01, *P < 0.05 vs. 1.7P + pcDNA3.
To investigate the binding affinity of MyoD/E47 and Myf5/E47 further, we performed EMSA in which a limiting amount of E5wt probe was incubated with decreasing concentrations of MyoD/E47 or Myf5/E47 (600 ng–33 pg). A representative autoradiogram of the results for MyoD/E47 and Myf5/E47 is shown in Fig. 5, A and B, respectively. The relative optical density of free DNA was measured and subtracted from the protein-bound DNA and the values used for the calculation of the $K_d$ as described in MATERIALS AND METHODS. The $K_d$ values for the binding of MyoD/E47 and Myf5/E47 to the probe were 0.1 and 0.2 μM, respectively.

In vivo activity of the myostatin promoter. To analyze the activity of the myostatin promoter in vivo, two constructs of approximately the same length, the murine 1.7P and the bovine 1.6b construct, were injected into the quadriceps femoral muscle of mice. Control mice were injected with the promoterless reporter gene vector pGL3-B. The mice were killed on day 3 or 7 after injection, and the luciferase activity was determined. The results demonstrate that the preferred time to obtain maximal expression was at day 7 after injection, as the average expression was over 3.5-fold higher than that obtained at day 3 (Fig. 6). Therefore, for all subsequent in vivo experiments, the muscles were recovered 7 days after naked DNA injection.
Myostatin promoter (2.5 kb) drives the expression of the reporter gene in a fiber type-specific manner. Myostatin expression has been associated with fast-twitch muscle fibers (23), in particular type IIb fibers (6). Hence, to determine whether 2.5P and tE5 were also able to drive reporter gene expression in a fiber type-specific manner, the quadriceps muscles of C57BL/10 mice were injected with construct 2.5P, or tE5 or pGL3-B control vector. The 2.5P and tE5 constructs were chosen for this experiment because they demonstrate similar levels of luciferase reporter activity both in vivo and in vitro.

The muscles were recovered 7 days after the DNA injection, and the tissues were processed as described in MATERIALS AND METHODS. The resulting tissue sections were immunostained with various primary antibodies. Sections derived from the mice injected with the 2.5P construct were positive for luciferase expression (Fig. 8A), while sections from muscles injected with pGL3-B displayed no fluorescence when incubated with anti-luciferase antibody (data not shown). Serial sections from mice injected with the 2.5P construct were also immunostained with anti-slow type I fiber and anti-fast type II fiber antibodies, and the results were compared with those for luciferase antibody. The fibers that were positive for luciferase expression (Fig. 8A) colocalized with the fibers that were reactive with anti-fast type II fiber antibody (Fig. 8B) but not with fibers that were reactive with anti-slow type I antibody (Fig. 8C). This indicates that the myostatin promoter was restricting the expression of the reporter gene to fast type muscle fibers. However, the anti-fast type II antibody N1.551 was described as anti-fast type IIa in rats (41). To determine whether any of the fibers detected by N1.551 were type IIb,
histochemical fiber typing was performed. The results show that most of the fibers positive for luciferase expression are darkly stained fibers, expected to be type IIb fibers (Fig. 8D). Control sections treated with no primary antibody or with mouse IgG did not display any fluorescence (data not shown). Interestingly, tE5 did not display fiber type-specific expression when injected into the muscle. As seen in Fig. 9A, a diffuse pattern of expression was observed when anti-luciferase antibody was used, and the luciferase-positive fibers colocalized with fibers reactive with anti-slow type I (Fig. 9B) as well as anti-fast type II (Fig. 9C) antibodies. Although able to express the level of reporter gene activity similar to wild-type 2.5P construct both in vivo and in vitro, tE5 is unable to confer fiber type specificity.

DISCUSSION

Myostatin is predominately expressed in skeletal muscle and is a known negative regulator of skeletal muscle development and growth. Although the transcriptional regulation of myostatin has been demonstrated in the myoblast cell lines (24, 37), no information is available on the regulatory elements controlling myostatin gene expression in skeletal muscle in vivo. In the present study, the activity of the myostatin promoter was analyzed in vivo via somatic gene transfer to determine the species-specific and fiber type-specific expression of the myostatin gene.

Murine vs. bovine myostatin promoter activity in murine skeletal muscle. To date, the 5′ upstream sequences of the myostatin gene have been isolated from cattle, pig, mouse, and human (GenBank accession nos. AF348479, AF093798, AX139025, and AX058992, respectively). In the present study, the activity of promoter constructs from two different species, mouse and cattle, were examined in murine skeletal muscle. Interestingly, a comparison of the activity of the bovine and the murine myostatin promoter in vivo revealed that bovine promoter activity is significantly weaker than that of the murine promoter (Fig. 6). However, similar levels of reporter gene activity for these constructs were seen in C2C12 myoblasts. Previously, it was reported that 1.6 kb of bovine promoter is sufficient for the maximal promoter activity (37). The murine and bovine promoters share 68% homology within this region. However, the two promoters also display some distinct features, such as the number and arrangement of the E-boxes, the consensus binding sites for MRF, which are critical for muscle-specific gene expression. Because the in vivo system is more sensitive, these genetic differences between the bovine and
murine promoters become more relevant. Furthermore, the murine sequence has a polyA tract (A20) between E-boxes E4 and E5 that is not present in the bovine sequence. PolyA sequences have been associated with DNA spatial conformation, and it has been argued that DNA curvature between motifs can lead to more efficient dimerization of proteins by bringing factors into proximity with each other (40). The presence of a polyA tract in the murine sequence could lead to a specific DNA conformation and therefore contribute to the level of promoter activity in vivo. Together, these differences might be sufficient to impair the ability of the bovine promoter to induce the reporter gene expression in murine skeletal muscle to the same level as that in C2C12 cells. Thus species specificity is a factor to be considered when generating transgenic mice for a particular gene promoter.

MyoD and Myf5 activate myostatin promoter differentially. MRF, in particular MyoD and Myf5, regulate their target genes by binding to E-boxes in the promoter region (1, 2, 8). Sequence analysis of the 2.5-kb fragment of the murine promoter revealed the presence of seven E-box motifs (Fig. 1). However, analysis of the murine myostatin promoter activity in C2C12 cells indicated that the first five E-boxes, in particular E-box E5, are sufficient for its maximal activity (Fig. 2). Furthermore, analysis of the induction of the murine promoter activity by these two MRF revealed that Myf5 is a weak activator, while MyoD appears to be a potent activator, of promoter activity (Fig. 3). Importantly, analysis of E-box activity in vivo confirmed the results obtained in vitro, where the presence of E-box E5 was sufficient to restore maximal promoter activity (Fig. 2 and 7). Moreover, mutation of E-box E5 decreased promoter activity (by 83%) to levels comparable to those observed in constructs lacking this E-box (Fig. 7), confirming the importance of E-box E5 for the activity of the murine promoter.

The MRF have been shown to bind to an E-box in complex with ubiquitous helix-loop-helix E-proteins, including E12 and E47. It has been suggested that MyoD/E47 heterodimers cooperate in binding to promoters containing multiple E-boxes (4) and that MyoD/E12 heterodimer binding affinity to DNA targets containing multiple E-boxes is much higher than that observed in interactions with targets containing a single E-box (10). On the other hand, under certain experimental conditions, a single E-box motif is capable of binding a specific MRF and confer activity (12, 35).

The results reported here indicate that MyoD preferentially activates myostatin promoter activity, which is consistent with findings of the previous study by Spiller et al. (37). To further investigate the basis of higher activation of the promoter by MyoD compared with Myf5, we examined the DNA binding of MyoD/E47 and Myf5/E47 to E-box E5 of the murine myostatin promoter in vitro. The E-box E5 was chosen for the EMSA experiments on the basis of its crucial nature in the activation of the myostatin promoter (Fig. 2 and 7). Our in vitro results indicate a difference, although small, between the binding affinity of MyoD/E47 to the DNA compared with that of Myf5/E47. The relatively higher binding affinity of MyoD could be contributing to the stronger activation of the myostatin promoter by MyoD than that by Myf5 in the transient transfections (Fig. 3). Furthermore, the presence of more than one E-box in the transfected constructs may also help the activation by MyoD. Previously, the murine sarcoma virus

Fig. 9. Expression pattern of the tE5 construct in quadriceps muscle. Construct tE5 was injected into the quadriceps muscle of 4-wk-old mice. Seven days after injection, the muscles were recovered and processed as described in MATERIALS AND METHODS. Serial sections of injected quadriceps muscle were immunostained with anti-luciferase antibody (A), anti-slow fiber antibody A4.840 (B), or anti-fast fiber antibody N1.551 (C). Arrowheads indicate location of fibers reactive with anti-fast type II antibody. Asterisks indicate fibers reactive with anti-slow type I fiber antibody. Bar in C, 10 μm.
(MSV) promoter-enhancer containing six E-boxes was tested for MyoD-E12 and myogenin-E12 interactions. The report demonstrated that DNA sequences containing multiple E-boxes have higher binding affinity to MyoD/E12 and Myogenin/E12 DNA than DNA fragments containing a single E-box (10). Furthermore, it was shown that MyoD-E12 has higher affinity for the MSV enhancer and bound to the DNA with higher cooperativity than did myogenin-E12. On the basis of these results, we postulated that multiple E-boxes in the promoter region could not only contribute to high-affinity DNA binding but also confer MyoD and myogenin DNA recognition specificity (10).

Myostatin promoter activity is fiber type specific. Because myostatin expression has been described in association with fast-twitch muscle fibers (6, 23), we assessed the capacity of myostatin promoter to drive expression of reporter gene in vivo. Corin et al. (9) showed the expression of injected troponin I slow isoform (TnIs)-luciferase constructs in slow fibers and inferred that chromosomal integration was not a primary requirement to achieve appropriate fiber type-specific gene regulation. It was postulated that the DNA sequence of the injected promoter conveyed the necessary information for fiber type-specific TnIs gene expression in the adult skeletal muscle. Similarly, Swoap (38) reported the fast fiber type-specific expression of the MHC type IIB promoter in vivo using somatic gene transfer. The results of our study demonstrate that the 2.5P and tE5 to render fiber-type specificity to reporter gene expression in vivo. Corin et al. (9) showed the expression of injected troponin I slow isoform (TnIs)-luciferase constructs in slow fibers and inferred that chromosomal integration was not a primary requirement to achieve appropriate fiber type-specific gene regulation. It was postulated that the DNA sequence of the injected promoter conveyed the necessary information for fiber type-specific TnIs gene expression in the adult skeletal muscle. Similarly, Swoap (38) reported the fast fiber type-specific expression of the MHC type IIB promoter in vivo using somatic gene transfer. The results of our study demonstrate that the 2.5P fragment of the murine myostatin promoter is sufficient to drive the expression of the reporter gene in a fiber type-specific manner in 4-wk-old mice. Interestingly, MyoD has also been shown to be expressed preferentially in fast-twitch muscle fibers (19, 20), and myostatin and MyoD expression overlap during embryogenesis (30). Furthermore, although the levels of MyoD decrease in the adult muscle, it is still highly expressed in 4-wk-old mice (28), coinciding with the manifestation of fiber type-specific properties, mainly the switch from embryonic and neonatal MHC to MHC types IIA, IIB, and IX that develop by 3–4 wk of age in mice (5). Also, Wheeler et al. (43) demonstrated that the presence of a functional E-box within the MHC type IIB promoter is essential for the expression of the reporter gene in vivo in a fiber type-specific manner. These results also support our finding that the absence of the sequences upstream of E-box E5 in tE5 leads to loss of fiber-specific expression, as is evident in the expression of luciferase activity in both types I and II fibers (Fig. 9). It is noteworthy that the sequences upstream of E5 E-box contain two additional E-boxes, E6 and E7, that are present in the 2.5P construct (Fig. 1A).

Yan et al. (44) demonstrated that an intragenic E-box present in the myoglobin gene restricted the expression of the reporter gene to slow oxidative fibers. Mutation of this E-box increased the expression of the reporter gene in the mainly fast glycolytic extensor digitorum longus muscle threefold compared with the wild-type construct. Interestingly, the same mutation did not significantly alter the activity of the promoter in the slow oxidative soleus muscle. They postulated that basic helix-loop-helix proteins such as MyoD can form complexes with corepressors to negatively regulate gene expression in certain cell backgrounds. Similarly, mutation of three different E-boxes in the promoter region of the muscle creatine kinase gene led to distinct expression patterns in slow and fast skeletal, cardiac, and tongue muscles of transgenic mice (11, 29, 34).

Although other factors may also be involved in conferring fiber-type specificity to the murine myostatin promoter, in vivo results together with the results obtained in vitro (EMSA and cotransfection experiments) make it attractive to propose that MyoD could control the muscle-specific and fiber type-specific transcription of myostatin in skeletal muscle.

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