Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin

Suzanne Reisz-Porszasz,1 Shalender Bhasin,1 Jorge N. Artaza,1 Ruoqing Shen,1 Indrani Sinha-Hikim,1 Aimee Hogue,1 Thomas J. Fielder,2 and Nestor F. Gonzalez-Cadavid1

1Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles 90059; and 2Transgenic Mouse Facility, University of California at Irvine, Irvine, California 92697

Submitted 14 March 2003; accepted in final form 15 June 2003

Reisz-Porszasz, Suzanne, Shalender Bhasin, Jorge N. Artaza, Ruoqing Shen, Indrani Sinha-Hikim, Aimee Hogue, Thomas J. Fielder, and Nestor F. Gonzalez-Cadavid. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. Am J Physiol Endocrinol Metab 285: E876–E888, 2003. —Mutations in the myostatin gene are associated with hypermuscularity, suggesting that myostatin inhibits skeletal muscle growth. We postulated that increased tissue-specific expression of myostatin protein in skeletal muscle would induce muscle loss. To investigate this hypothesis, we generated transgenic mice that overexpress myostatin protein selectively in the skeletal muscle, with or without ancillary expression in the heart, utilizing cDNA constructs in which a wild-type (MCK/Mst) or mutated muscle creatine kinase (MCK-3E/Mst) promoter was placed upstream of mouse myostatin cDNA. Transgenic mice harboring these MCK promoters linked to enhanced green fluorescent protein (EGFP) expressed the reporter protein only in skeletal and cardiac muscles (MCK) or in skeletal muscle alone (MCK-3E). Seven-week-old animals were genotyped by PCR of tail DNA or by Southern blot analysis of liver DNA. Myostatin mRNA and protein, measured by RT-PCR and Western blot, respectively, were significantly higher in gastrocnemius, quadriceps, and tibialis anterior of MCK/Mst-transgenic mice compared with wild-type mice. Male MCK/Mst-transgenic mice had 18–24% lower hind- and forelimb muscle mass and 18% reduction in quadriceps and gastrocnemius fiber cross-sectional area and myonuclear number (immunohistochemistry) than wild-type male mice. Male transgenic mice with mutated MCK-3E promoter showed similar effects on muscle mass. However, female transgenic mice with either type of MCK promoter did not differ from wild-type controls in either body weight or skeletal muscle mass. In conclusion, increased expression of myostatin in skeletal muscle is associated with lower muscle mass and decreased fiber size and myonuclear number, decreased cardiac muscle mass, and increased fat mass in male mice, consistent with its role as an inhibitor of skeletal muscle mass. The mechanism of gender specificity remains to be clarified.

A number of genetic factors, growth factors, hormones, and nutritional factors are important in the regulation of skeletal muscle mass; however, their precise role in the integrated, in vivo regulation of skeletal muscle homeostasis and muscle wasting associated with chronic illness and aging remains poorly understood. Considerable interest has focused recently on the role of myostatin, or growth differentiation factor (GDF) 8 (16, 35), a novel regulator of muscle mass that is produced predominantly in the skeletal muscle. A targeted deletion of the entire COOH terminus of myostatin (23) or a selected natural mutation leading to a truncated and inactive myostatin protein (38) causes a considerable increase in muscle mass in mice. Naturally occurring mutations in certain breeds of cattle that elicit an out-of-frame truncated protein or an inactive full-length protein are also associated with a hypermuscular phenotype (5, 6, 10, 24). Inhibition of myostatin expression in transgenic mice by other approaches, including dominant negative mutations engineered to affect the cleavage site in the myostatin precursor protein (27, 47), overexpression of the myostatin prodomain sequence (44), or overexpression of follistatin, which binds and inactivates myostatin (17), also results in increased skeletal muscle mass. Taken together, these observations indicate that myostatin is a negative regulator of the growth and/or replication of skeletal muscle fibers during embryological development.

A series of reports has shown that myostatin mRNA and/or protein concentrations are increased in skeletal muscle in conditions associated with loss of muscle mass in postnatal life, such as acquired immunodeficiency syndrome (4), sarcopenia of old age (21, 33, 45), and disuse atrophy in men under prolonged bed rest (46) and in rats during hindlimb unloading (2, 28, 42), after exposure to the microgravity environment of a spaceflight (12), and in association with glucocorticoid-induced muscle loss (13, 20). In contrast, recovery of muscle mass in the late stages of muscle regeneration...
is associated with a decrease of myostatin from the elevated levels seen immediately after injury (11, 31, 43). In a recent report, implantation of a cell line engineered to produce murine myostatin under the control of a Zn-inducible metallothionein promoter induced a considerable loss of muscle mass and body weight (48).

In the present work, we investigated the effects of an increase in myostatin expression in skeletal muscle by constructing two transgenic lines that express recombinant mouse myostatin under the control of a muscle-specific promoter. We hypothesized that increased tissue-specific expression of myostatin protein in skeletal muscle would be associated with lower muscle mass in transgenic mice. Animals were genotyped, and changes in body weight, epididymal and parametrial fat, and muscle mass were determined compared with wild-type littermates. These values were then related to the expression of myostatin RNA and protein in the muscle, muscle fiber size, and myonuclear number.

MATERIALS AND METHODS

Preparation of green fluorescent protein and mouse myostatin cDNA constructs. For the preparation of construct pEGFP-1/MCK1.3 driving the expression of the enhanced green fluorescent protein (EGFP) under the muscle-specific creatine kinase promoter MCK (15), the vector pEGFP-1 (Clontech Laboratories, Palo Alto, CA) was digested with HindIII. The construct, named pMCKG, containing the region from −1,354 to +1 bp of the MCK enhancer/promoter sequence (GenBank accession no. AF188002), was a gift from J. Nabmantougu (Dept. of Neurology, McGill University, Montreal, QC, Canada). The 1.3-kb MCK fragment was released from the plasmid by SpeI/HindIII. Both the vector and the MCK fragment were purified and their ends filled in with Klenow enzyme, and blunt-end ligation was performed. The orientation of the MCK fragment was assessed by restriction enzyme digestion and DNA sequencing. The 2.2-kb fragment containing MCK enhancer/promoter, EGFP, and SV40 poly(A) was released with XhoI/AflII digest and used for pronuclear injection (8). A 1.26-kb form of this promoter with a triplication in the E-boxes that abolishes expression in the cardiac muscle (36) was obtained from p12563E mut) MCK/CAT plasmid (a generous gift from S. D. Hauschka, Univ. of Washington School of Medicine, Seattle, WA) and subcloned as above. It was linked to EGFP, and the construct was named pEGFP-1/MCK-3E.

The construct pmMst/MCK1.3 driving the expression of the mouse myostatin protein under MCK promoter was prepared as follows. The full-length mouse myostatin cDNA (mMst) was cloned from mouse quadriceps muscle mRNA by RT-PCR. Total mRNAs were isolated by using an oligotex direct mRNA minikit (Qiagen, Valencia, CA). After RT-PCR, the 1.2-kb mouse myostatin cDNA was cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced. This sequence was identical to the mMst DNA sequence at GenBank (accession no. NM_010834). To verify the ability of the construct to generate myostatin protein, the mMst sequence was subcloned into pcDNA 3.0 vector and used as a template in the TNT T7-coupled in vitro transcription/translation reaction (Promega). Apal and XbaI restriction sites were introduced at the 5'- and 3'-ends of the mMst sequence by PCR using pcDNA 3.0/Mst construct as template. The PCR fragment and the pEGFP-1 vector were digested with Apal/XbaI enzymes to replace the EGFP sequence with the mMst sequence. The 1.2-kb mMst sequence and the remaining 3.5-kb vector were ligated, resulting in a pmMst construct. To introduce the MCK promoter, the pmMst construct was opened with SacII, and the pMCKG plasmid was digested with SpeI/HindIII to release the 1.3-kb MCK promoter. After DNA purification and Klenow enzyme reaction, the pmMst/MCK1.3 plasmid was generated by blunt-end ligation and then sequenced. The 2.6-kb-length MCK-mMst-SV40 poly(A) fragment was released with KpnI/AflII digest and used for pronuclear injection. A similar approach was utilized to prepare the construct with the mutated MCK promoter, resulting in the pmMst/MCK-3E plasmid.

In vitro expression of EGFP and myostatin. The murine myoblast cell line C3H C2C12 (1, 19, 39) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4 mM glutamine, 4.5 g/l glucose, 100 IU/ml penicillin, and 100 µg/ml streptomycin serum (Life Technologies, Grand Island, NY) and incubated at 37°C in 5% CO2 at 10–50% confluence. Cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA) by use of 1 µg of all four constructs: pEGFP-1/MCK1.3, pmMst/MCK1.3, pEGFP-1/MCK-3E, and pmMst/MCK-3E plasmid, respectively (39). The pEGFP-1 plasmid driven by the CMV promoter was used as a control. For differentiation into myotubes, myoblasts were allowed to grow to ~90–100% confluence, and after 2 days the medium was changed to DMEM containing 5% horse serum. Myotubes began to form after 3–4 days, when lipofectamine transfection was performed the same way. EGFP-transfected cells were monitored under fluorescent microscope after 24, 48, and 72 h of transfection. The EGFP expression reached maximum level at 48 h after transfection in both myoblast and myotubes.

Generation of transgenic mice and genotyping. The gel-purified 2.2-kb MCK1.3-EGFP-SV40 poly(A) and 2.6-kb MCK1.3-mMst-SV40 poly(A) fragments and the respective forms of the mutated promoter MCK-3E were used for the generation of transgenic mice by pronuclear injection (8). Eggs were generated for injection by mating superovulated female CB6F1 mice with CB6F1 males. All mice used to generate eggs were obtained from Harlan Sprague Dawley (Indianapolis, IN). Eggs that survived injection were implanted into the oviducts of outbred pseudopregnant females (ICR strain; Harlan Sprague Dawley). Animal care and use were approved by the Institutional Laboratory Animal Use and Care Committee, following National Institutes of Health guidelines.

DNA was extracted from the tails of 14-day-old pups. Genotyping was performed using PCR primers specific to the constructs that recognize the sequences at the junction of MCK promoter and the EGFP or Mst sequence. Primer sequences for the MCK1.3/EGFP construct were as follows: forward primer 5'-AGTGAGCAAGTCAGCCCTTG and reverse primer 5'-GAACCTCAGGTTACGTGTC. Primer sequences for the MCK1.3/mMst construct were: forward primer same as for MCK1.3/EGFP construct, reverse primer 5'-GCCAGCAGCATAC. These primers were also applied for the MCK-3E constructs.

All experiments were done in 7-wk-old mice. The genotypes of mice were further confirmed by Southern blot analysis of liver DNA obtained at the time they were killed. Genomic DNA (30 µg) was digested with EcoRI enzymes and electrophoresed on 0.7% gels. After DNA transfer, membranes were used for hybridization using a DIG High Prime DNA Labeling and Detection kit (Roche Molecular Biochemicals, Indianapolis, IN). The probe was generated by PCR
using the aforementioned primers. We used EcoRI-digested pmSt/MCKL1.3 plasmid as the control.

**Tissue specimens and EGFP expression.** Tail-genotyped animals with or without the recombinant EGFP or mSt aggregates were killed at 7 wk of age. The following tissues were collected for the present study: 1) skeletal muscles (tibialis anterior, gastrocnemius, quadriceps, biceps femoris, and forelimb); and 2) other tissues (liver, lung, small intestine, and heart). In the case of the myostatin-transgenic mice, one piece of each tissue was stored at −80°C for protein isolation, and another piece was placed into RNA/later solution (Ambion, Austin, TX) for RNA isolation. A piece of each muscle was fixed in formaldehyde for histomorphometric analysis.

Muscles and tissues collected from EGFP-expressing animals were rinsed in PBS and fixed in N-ethylmaleimide-ammonium sulfate in citrate buffer (Zeus Scientific, Raritan, NJ) for fluorescence analysis (3). Skeletal muscle, heart, liver, and lung samples from the EGFP-transgenic mice were cryosectioned (cryostats; Mikron Instruments, San Diego, CA), and the 6-μm-thick sections were stored at −80°C and rinsed in acetone before fluorescence microscopic analysis (Leica DMLB; McBain Instruments, Chatsworth, CA).

**Determination of myostatin mRNA.** Total RNA was extracted from skeletal muscle by applying TRIzol reagent (Life Technologies). To check the quality of the RNA, sample aliquots were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The RNA concentration was measured spectrophotometrically. Aliquots containing 1 μg of RNA were submitted to reverse transcription (RT) using a 16-mer oligo(dT) primer, as previously described (4). The resultant cDNA was amplified using PCR in a total volume of 25 μl. The locations of the primers utilized for the quantitative estimation of mouse myostatin mRNA were nt 136–156 (forward) and 648–667 (reverse), numbering from the translation initiation codon (later called F2/R2) as previously described (19). These primers and the ones for the housekeeping gene GAPDH were employed in a multiplex reaction. The thermal amplification of the 531-bp myostatin DNA fragment was conducted after a 5-min step of denaturation at 94°C, followed by 35 cycles of 94°C (35 s), 56°C (45 s), and 72°C (80 s), and a final step at 72°C for 8 min. In another type of experiment, primers amplifying the whole 1.2-kb myostatin region were used (forward nt 1–15; reverse nt 1117–1131, later called F1/R1).

For Northern blot analysis, total RNA (20 μg) was separated using 1.2% agarose-formaldehyde denaturing gels, transferred overnight onto Hybond-N+ membranes (Amer-sham International), and fixed by exposure to ultraviolet light (4). Blots were hybridized at 60°C overnight in an ExpressHyb (Clontech) solution containing [32P]dCTP-labeled, random-primed, 531-bp-long myostatin fragment. This sequence was specifically chosen to avoid possible cross hybridization with the closely related GDF11 gene. The GAPDH probe was based on a region of the published rat GAPDH probe was based on a region of the published rat sequence (20). The GAPDH probe was based on a region of the published rat sequence (20). Denaturation and hybridization were performed at 50°C and 65°C, respectively, overnight. The hybridized membranes were washed at 60°C for 5 min in a loading buffer containing 0.1% SDS, Tris-HCl, and a 1:20 dilution of β-mercaptoethanol, as described (4, 39). Protein concentrations were measured using the Micro BCA Protein assay reagent (Pierce), and protein samples (30 μg) were heat denatured (95°C for 5 min) in loading buffer containing β-mercaptoethanol and electrophoretically separated using 12% Tris-glycine polyacrylamide gels (ReadyGel; Bio-Rad, Hercules, CA), and the proteins were visualized using Coomassie brilliant blue staining. The electrophoretically separated samples were transferred to a nitrocellulose membrane and immunodetected using the previously described procedure (4). The antibody employed for myostatin was a polyclonal antibody elicited against a synthetic peptide within the sequence of the 110 carboxy terminus amino acids of human myostatin, which was named antibody B. This antibody has been extensively validated by its ability to detect the recombinant 375-amino acid myostatin precursor and the processed 110-amino acid protein (12, 39) as well as a 30- to 32-kDa band in mouse, rat, and human skeletal extracts (4), which is considered to be the dimer of the 110-amino acid protein cleaved from the myostatin precursor. An anti-rabbit IgG secondary antibody linked to horseradish peroxidase (HRP) was used. In addition, a mouse monoclonal purified IgG against rabbit GAPDH from skeletal muscle (Chemicon International, Temecula, CA) was utilized for normalization (1). Blots were developed with an enhanced chemiluminescent substrate for HRP and exposed to film (ECL Hyperfilm; Amersham).
RESULTS

In vitro testing of the myostatin-transgenic construct. A schematic representation of the DNA fragment utilized for generating the myostatin-transgenic animal harboring the MCK promoter is presented in Fig. 1A. The 1.2-kb myostatin sequence of this fragment was subcloned in an appropriate vector and tested in the cell-free reticulocyte assay to verify the expression of myostatin protein. Figure 1B shows an autoradiogram of the polyacrylamide gel electrophoresis (PAGE) of the 35S-labeled products of in vitro transcription and translation of the mouse myostatin DNA fragment. The presence of the expected 52-kDa band for the 375-amino acid myostatin monomer indicates that this construct is translated in this cell-free reticulocyte system (lane 3). A similar 52-kDa myostatin band was observed after transcription and translation of the human myostatin cDNA (lane 2) (4).

MCK promoter was effective in directing tissue-specific expression. The efficacy of both the MCK promoter and its mutated version (MCK-3E) was assessed by placing them upstream of the coding region of EGFP. C3C12 myoblasts were transfected in vitro with the MCK/EGFP reporter construct, and cells were collected while they were still undifferentiated as mononucleated myoblasts (Fig. 2A) or after differentiation into myotubes (Fig. 2B). Both cultures exhibited the expected green fluorescence in most of the cells, indicating good transfection efficiency and activation of both MCK promoters.

Further confirmation of the selectivity of the MCK and MCK-3E promoters was obtained in vivo. Cryostat tissue sections from the gastrocnemius, quadriceps, and tibialis anterior (Fig. 2D, E, and F, respectively) from EGFP-transgenic mice, in which the expression of EGFP was directed by the MCK promoter, showed an intense fluorescence in all skeletal muscle fibers and in the heart muscle, as expected, but not in other tissues (not shown). The green fluorescence was absent in the corresponding sections from a wild-type mouse (Fig. 2C). These data indicate that the MCK promoter was effective in restricting the expression of EGFP to the skeletal muscle and heart. Transgenic mice harboring the MCK-3E promoter expressed green fluorescence only in the skeletal muscle, not in the cardiac muscle.

Confirmation of the genotype of myostatin-transgenic mice. Tail DNA from twenty 14-day-old mice obtained from pronuclear injection was subjected to genotyping by PCR. Figure 3A shows the ethidium bromide staining of the PCR products of genomic DNA separated by agarose gel electrophoresis. In the transgenic mice, PCR amplification should occur only in the DNA from mice that carry the transgene (MCK/Mst); because the primers used for PCR were located at the junction of the 5’-end region of the mouse myostatin cDNA and an upstream sequence present only in the recombinant DNA used to generate the transgenic mice. The expected 290-bp PCR product should be absent in the wild-type mice. In Fig. 3A, this band is present in five males and six females (myostatin-positive transgenic mice) and absent in five males and four females (wild-type) in this series. An additional 29 mice, progeny of the first-generation transgenic mice, were also genotyped; thus a total of 15 transgenic males, 12 transgenic females, 10 wild-type males, and 10 wild-type females were subjected to PCR.

The Southern blotting assay was conducted on DNA extracted from liver at the time the animals were killed at 7 wk of age. It was based on detection of the mouse cDNA sequence (no introns) in EcoRI-digested genomic DNA by utilization of the 290-bp PCR fragment as a probe. This sequence is present in DNA from the remaining tissue sections from the gastrocnemius, quadriceps, and tibialis anterior (Fig. 2D, E, and F, respectively) from EGFP-transgenic mice, in which the expression of EGFP was directed by the MCK promoter, showed an intense fluorescence in all skeletal muscle fibers and in the heart muscle, as expected, but not in other tissues (not shown). The green fluorescence was absent in the corresponding sections from a wild-type mouse (Fig. 2C). These data indicate that the MCK promoter was effective in restricting the expression of EGFP to the skeletal muscle and heart. Transgenic mice harboring the MCK-3E promoter expressed green fluorescence only in the skeletal muscle, not in the cardiac muscle.

MCK promoter was effective in directing tissue-specific expression. The efficacy of both the MCK promoter and its mutated version (MCK-3E) was assessed by placing them upstream of the coding region of EGFP. C3C12 myoblasts were transfected in vitro with the MCK/EGFP reporter construct, and cells were collected while they were still undifferentiated as mononucleated myoblasts (Fig. 2A) or after differentiation into myotubes (Fig. 2B). Both cultures exhibited the expected green fluorescence in most of the cells, indicating good transfection efficiency and activation of both MCK promoters.

Further confirmation of the selectivity of the MCK and MCK-3E promoters was obtained in vivo. Cryostat tissue sections from the gastrocnemius, quadriceps, and tibialis anterior (Fig. 2D, E, and F, respectively) from EGFP-transgenic mice, in which the expression of EGFP was directed by the MCK promoter, showed an intense fluorescence in all skeletal muscle fibers and in the heart muscle, as expected, but not in other tissues (not shown). The green fluorescence was absent in the corresponding sections from a wild-type mouse (Fig. 2C). These data indicate that the MCK promoter was effective in restricting the expression of EGFP to the skeletal muscle and heart. Transgenic mice harboring the MCK-3E promoter expressed green fluorescence only in the skeletal muscle, not in the cardiac muscle.

Confirmation of the genotype of myostatin-transgenic mice. Tail DNA from twenty 14-day-old mice obtained from pronuclear injection was subjected to genotyping by PCR. Figure 3A shows the ethidium bromide staining of the PCR products of genomic DNA separated by agarose gel electrophoresis. In the transgenic mice, PCR amplification should occur only in the DNA from mice that carry the transgene (MCK/Mst); because the primers used for PCR were located at the junction of the 5’-end region of the mouse myostatin cDNA and an upstream sequence present only in the recombinant DNA used to generate the transgenic mice. The expected 290-bp PCR product should be absent in the wild-type mice. In Fig. 3A, this band is present in five males and six females (myostatin-positive transgenic mice) and absent in five males and four females (wild-type) in this series. An additional 29 mice, progeny of the first-generation transgenic mice, were also genotyped; thus a total of 15 transgenic males, 12 transgenic females, 10 wild-type males, and 10 wild-type females were subjected to PCR.

The Southern blotting assay was conducted on DNA extracted from liver at the time the animals were killed at 7 wk of age. It was based on detection of the mouse cDNA sequence (no introns) in EcoRI-digested genomic DNA by utilization of the 290-bp PCR fragment as a probe. This sequence is present in DNA from the re-
combinant myostatin-positive transgenic mice but is absent in the wild-type animals. Figure 3B shows that the expected band was found in all animals identified as positive by PCR and was absent in all animals that were negative by PCR. The 20 animals represented in Fig. 3 were selected for further characterization by the RT-PCR, Northern blot, and Western blot analyses presented below. Southern blotting of DNA from the remaining 29 animals (not shown) also confirmed the results of the PCR genotyping.

In the case of the transgenic animals harboring the MCK-3E promoter driving myostatin expression, determined by PCR and Southern blotting assays, we identified 13 male and 8 female mice as positive (transgenic) and 7 male and 9 female mice in the same litters as wild type. Representative PCR results of these animals' genotyping are shown in Fig. 3C.

Overexpression of myostatin mRNA and protein in skeletal muscle of transgenic mice harboring the recombinant myostatin gene driven by the MCK promoter. To determine whether the myostatin-transgenic mice harboring the MCK promoter overexpressed myostatin in skeletal muscle, we evaluated the expression of myostatin mRNA and protein. RNA was isolated from the gastrocnemius muscle, and myostatin mRNA concentration was measured by RT-PCR using a set of primers designed to avoid amplification of the homologous GDF11 gene or any contaminant DNA in the RNA preparation. Figure 4A shows that myostatin mRNA was expressed at a higher level in all of the specimens analyzed from the transgenic mice compared with the wild-type animals, as assessed by ethidium bromide staining of the PCR bands separated by agarose gel electrophoresis. Expression of the reference gene...
GAPDH mRNA was not significantly different between the transgenic and wild-type mice. Densitometric quantitation of the myostatin cDNA band intensities corrected by the respective GAPDH values (Fig. 4C, RT-PCR) confirmed the significant increases in myostatin mRNA expression in the male and female transgenic mice compared with the wild-type animals. There was considerable heterogeneity in the expression of myostatin mRNA among different skeletal muscle groups; the myostatin mRNA concentrations in the quadriceps of both transgenic and wild-type mice was lower than in the tibialis and gastrocnemius.

The myostatin protein levels in extracts from the gastrocnemius muscles were quantitated by Western blot assays utilizing a previously validated antibody against a synthetic peptide representing a sequence of the myostatin carboxy-terminal protein (4). We (39) have previously demonstrated that this antibody recognizes recombinant full-length 375-amino acid myostatin protein and the 111-amino acid carboxy-terminal fragment. Western blot analysis using this antibody revealed higher intensity of the 30-kDa band in gastrocnemius of the male and female MCK/Mst-transgenic animals compared with wild-type controls (Fig. 4B). When the densitometric quantitation was performed and corrected by the levels of the housekeeping GAPDH gene (Fig. 4C, Western blot), the relative myostatin protein concentrations were significantly higher in the transgenic mice.

Fig. 3. Genotyping of transgenic (TG) mice by PCR and Southern blot analysis. A and C: genotyping was initially conducted by PCR (290 bp) on DNA extracted from the tail of 14-day-old pups by use of primers anchoring on the MCK or mutant (triple mutation in the E-box, MCK-3E) and myostatin sequences and analyzed by agarose gel electrophoresis with ethidium bromide staining. B: further confirmation of the genotype for MCK/Mst-transgenic mice was obtained by Southern blotting of EcoRI fragments from liver DNA, utilizing a digoxigenin-labeled probe and detection with NBT/BCIP colorimetric reaction. TG, mice harboring the expected MCK/Mst 2.4-kb DNA sequence; WT, mice without the MCK/Mst sequence. C: representative genotyping of MCK-3E/Mst-transgenic mice, performed as in A. TG mice showed the expected 290-bp PCR fragment. c, Control; f, female; m, male. Genotyping was performed in all mice, although the results from only a few representative mice are shown.

Fig. 4. Increase of myostatin mRNA and protein expression in gastrocnemius muscle of TG mice harboring the MCK/Mst DNA. A: myostatin mRNA was identified by RT-PCR followed by agarose gel electrophoresis and ethidium bromide staining. GAPDH, reference gene. B: myostatin protein was identified by Western blot. C: RT-PCR: densitometric analysis of intensities of the myostatin band referred to the GAPDH band; Western blot: densitometric analysis of intensities of the myostatin band corrected by intensities of the GAPDH band. Open bars, WT animals; gray bars, recombinant myostatin-TG animals. Both groups of mice were 7 wk old. Values are means ± SE, *P< 0.05.
Western blot analysis was performed with our previously validated antibody B (1, 4, 12, 39). This antibody was tested (Fig. 5B) against an extract from wild-type mouse skeletal muscle (MM), where the major band corresponded to the 30-kDa protein, and against two recombinant human myostatin proteins: 1) the full-length precursor, and 2) the carboxy-end 110-amino acid protein putatively arising by cleavage from the precursor. The major bands detected were for proteins running as expected: 52 and 15 kDa for the precursor monomer and the carboxy-end protein, respectively. The 30-kDa band was absent in the protein homogenates from the skeletal muscles from the myostatin-null mice but was present in the wild-type and MCK/Mst-transgenic mice (Fig. 5C). These data provide further evidence of the antibody specificity. The expression of the housekeeping gene GAPDH was not significantly different between the myostatin-null, wild-type, and myostatin-transgenic mice at the RNA and protein levels (Fig. 5, A and C).

Presence of recombinant myostatin mRNA in skeletal muscle of transgenic mice harboring the recombinant myostatin gene driven by the MCK promoter. Because the myostatin mRNA concentrations presented in the previous section reflect the total concentration of myostatin mRNA, we wished to determine whether the transgene was being transcribed. To clarify this question, PCR reactions were performed on the cDNA from the RT of the gastrocnemius muscle RNA by utilizing a set of primers called F3/R3 (Fig. 6A), designed to amplify the 3'-end of the myostatin-coding region and the 5'-end of the SV40 poly(A) signal present in the DNA construct utilized for making the MCK-transgenic mouse but absent in the transcribed region of the endogenous myostatin gene.

Figure 6B shows that the gastrocnemius cDNA from two representative male transgenic mice generates the 1.2-kb full-length myostatin-coding region (P1/R1 primers) as well as the 531-bp-long sequence (P2/R2 primers) used in the preceding experiments to quantitate mRNA. These two sets of primers do not distinguish between the recombinant and the endogenous myostatin. The absence of a second, 2.3-kb band with F2/R2 primers in these cDNAs that would originate from the 1.8-kb intron-1 rules out any potential genomic DNA contamination. When we applied the F3/R3 transgene-specific set of primers, the expected band for the fragment (174 bp) was generated, thus clearly detecting the recombinant myostatin mRNA. To confirm these results, the same set of primers was used for amplifying cDNAs used previously to quantify myostatin mRNA. Figure 6C shows that all transgenic animals expressed the recombinant myostatin, whereas none of the wild-type animals had the myostatin band, irrespective of gender.

Further confirmation was obtained by Northern blot analysis on some representative total RNA samples, which demonstrated the presence of the 1.2-kb recombinant myostatin mRNA in quadriceps from the MCK-transgenic mice and its absence in the wild-type controls (not shown). This band was smaller than the

Fig. 5. Validation of the anti-myostatin antibody by use of skeletal muscle from 7-wk-old mice with different degrees of expression of the myostatin gene. A: RT-PCR performed with total RNA from gastrocnemius (G), quadriceps femoris (Q), and tibialis anterior (T) of myostatin knockout (KO), WT, and TG mice harboring MCK/Mst DNA. B: revalidation of the custom-made antibody B (4) performed by Western blot analysis on 7.5% gels against a mouse skeletal muscle extract (MM, 30 μg protein), recombinant full-length human myostatin precursor (P, 100 ng), and recombinant 110-amino acid carboxy-end of the human myostatin protein (110, 1 ng). C: Western blot as in B, with sequential reblotting with GAPDH antibody, performed with protein extracts from the skeletal muscles depicted in A.
2.6-kb endogenous myostatin mRNA, because the cDNA sequence utilized to generate the transgenic animals lacked the long untranslated 3' region and the shorter 5' region normally transcribed in the skeletal muscle.

**Skeletal muscle mass in myostatin-transgenic mice.**

The presence of the recombinant myostatin gene in the male transgenic mice (n = 15) was associated with a significant 20–22% lower skeletal muscle mass, as represented by the wet weights of the gastrocnemius, quadriceps, and tibialis anterior muscle groups compared with those of the wild-type mice (n = 10; Fig. 7A). The body weights of transgenic male mice were, on average, 10% lower than those of wild-type mice (22.3 ± 2.4 vs. 24.5 ± 2.2 g), due mostly to the decrease in skeletal muscle mass. That the general reduction in body mass is predominant, but not entirely, due to muscle loss is apparent when the muscle weights are expressed as a percentage of total body weight (e.g., muscle loss relative to body weight is 10.9%, whereas total muscle loss is 19%). The transgenic mice still had a significantly lower relative mass of the quadriceps and tibialis anterior muscle groups, although the differences between wild-type and transgenic mice were of a lesser magnitude. The mean wet weights of gastrocnemius, quadriceps, and tibialis anterior muscle groups were not significantly different between female wild-type (n = 10) and transgenic (n = 12) mice with (Fig. 7B) or without (not shown) adjustment for total body weight.

In some animals, the forelimb muscles were also excised and weighed, showing that the male MCK/Mst-transgenic mice had lower muscle mass (25.8% biceps and 17.6% forearm) compared with wild-type animals (Table 1.). As in the case of the hindlimb muscles, there was no decrease of forelimb muscle mass in the female transgenic animals compared with the wild-type mice.

The results obtained with the MCK/Mst-transgenic mice were reproduced in the MCK-3E/Mst-transgenic mice, where protein expression directed by this promoter is abolished in the heart. The reduction of 19–22% in the mass of the three hindlimb muscles in the male MCK-3E/Mst-transgenic (n = 13) vs. the wild-type animals (n = 7; Fig. 7C) was nearly identical to that observed in the male MCK/Mst-transgenic animals. In contrast to the animal harboring the nonmutated promoter, no significant weight reduction occurred in the forelimb muscles in the male MCK-3E/Mst-transgenic mice compared with the respective wild-type mice (Table 1). The skeletal muscle mass in the female MCK-3E/Mst-transgenic mice (n = 8) was not significantly different from that in the wild-type mice (n = 9) either in the hindlimb (Fig. 7D) or in the forelimb muscles (Table 1).

The cardiac muscle mass was significantly lower in male MCK/Mst myostatin-transgenic mice compared with wild-type controls (17.1% lower; Table 1). There was no significant difference in mean cardiac muscle mass between female myostatin-transgenic and wild-type mice (Table 1).

The mean weights of the epididymal fat pad were significantly higher in myostatin-transgenic male mice compared with wild-type controls (216.5 ± 33.4 vs. 130.2 ± 27.5 mg). However, in female transgenic mice, there were no significant differences in the mean weights of the parametral fat between female transgenic and wild-type mice (94 ± 29.6 vs. 110.2 ± 32.7 mg).
Effect of myostatin overexpression on fiber size, typing, and myonuclear number. To determine whether the observed decrease in skeletal muscle mass in the transgenic animals overexpressing myostatin was the result of decreased myofiber size, we measured the fiber cross-sectional areas in two muscle groups, gastrocnemius and quadriceps, from the MCK/Mst-transgenic mice. Figure 8A shows that the mean muscle fiber cross-sectional area for the gastrocnemius muscle group was 18% lower in the male transgenic mice than in wild-type mice, which agrees well with the 20.6% lower muscle mass in the transgenic mice (n/H11005 5/group). These observations suggest that the reduction in muscle fiber area accounts for most of the decrease in muscle mass. Most of the muscle fibers in gastrocnemius and quadriceps muscle groups were type II fibers, and no significant differences were found in fiber composition between transgenic and wild-type mice. The number of myonuclei was also significantly lower in male myostatin-transgenic mice compared with controls (n = 5/group; Fig. 8B).

DISCUSSION

To our knowledge, this is the first direct demonstration that overexpression of myostatin mRNA and protein in skeletal muscle during embryological development is associated with a moderate but significant gender-dependent decrease of skeletal muscle mass and a decrease of muscle fiber size. These changes obtained in our myostatin-overexpressing transgenic mice are detectable during postnatal life until after completion of sexual maturation and are not accompanied by noticeable impairment of their appearance, activity, or other general indicators of health. Previous inference of myostatin negative regulation of skeletal muscle mass was based on loss-of-function models, where myostatin expression and/or activity is blocked.

Table 1. Effects of myostatin overexpression on forelimb and heart muscle weights in 7-wk-old MCK/Mst- and MCK-3E/Mst-transgenic and WT mice

<table>
<thead>
<tr>
<th>Construct</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Biceps</td>
</tr>
<tr>
<td>WT</td>
<td>4</td>
<td>27.5 ± 2.1</td>
</tr>
<tr>
<td>MCK/Mst</td>
<td>10</td>
<td>20.4 ± 1.2*</td>
</tr>
<tr>
<td>WT</td>
<td>7</td>
<td>26.4 ± 1.5</td>
</tr>
<tr>
<td>MCK-3E/Mst</td>
<td>13</td>
<td>24.7 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals in the given group. MCK, muscle-specific creatine kinase; MCK-3E, mutated muscle creatine kinase MCK; Mst, myostatin; WT, wild type. *P < 0.05.
from gestation all throughout life by spontaneous or experimental mutations (5, 6, 10, 16, 23, 24, 35, 38). Additional insights have emerged from experiments published while this work was in progress in which short-term overexpression of myostatin protein was achieved outside the skeletal muscle tissue by implantation of a nonmuscle cell line engineered with a recombinant myostatin cDNA construct in adult mice (48). Overexpression of myostatin achieved by implantation of these cell lines was associated with significant loss of muscle and decreased body weights. However, this model of myostatin overexpression differs from that in our present work in several salient respects: 1) the mice bearing the cell line that overexpressed myostatin cDNA demonstrated extreme reductions in body weight, more indicative of generalized severe cachexia than of sarcopenia; the weight loss was so drastic that in some cases the mice died within 2 wk; 2) the myostatin overexpression was systemic in the former study, whereas in our transgenic mice the MCK promoter restricted the expression to the skeletal and cardiac muscle; 3) systemic myostatin overexpression in the former study was associated with a loss of fat, opposite to what would be expected from known effects of myostatin on adipogenesis (18, 25); and 4) the secretion of factors other than myostatin by the implanted engineered cells that might have contributed to profound cachexia cannot be completely ruled out. Thus it remains unclear whether the extreme cachexia observed in the report by Zimmers et al. (48) can be attributed solely to myostatin overexpression.

We utilized a transgenic model, with which we demonstrated that myostatin overexpression during the embryonic period and postnatal life is associated with significantly lower skeletal muscle mass and muscle fiber size. The myostatin-overexpressing transgenic mouse that we have generated may serve as a useful model to study the role of myostatin protein in skeletal muscle aging and skeletal muscle remodeling in response to exercise, injury, unloading, and other anabolic and catabolic stimuli that may affect muscle mass and fiber size.

The use of a muscle-specific promoter allowed us to restrict the expression of the recombinant myostatin to the skeletal muscle; muscle-specific overexpression of myostatin in these transgenic mice reduced muscle mass but did not compromise viability or produce cachexia. The selection of the widely used wild-type MCK promoter (15) rather than the myostatin promoter (19, 31) was dictated by the objective of achieving constitutive overexpression in the muscle independent of regulatory factors that might control myostatin synthesis in vivo.

The effectiveness of the MCK promoter in restricting gene expression to the skeletal muscle was also demonstrated in transgenic mice expressing the EGFP reporter gene controlled by either the nonmutated MCK or the mutated MCK-3E promoters. In the MCK/EGFP-transgenic mice, no green fluorescence was detected in tissues other than the skeletal muscle and the heart, and in the MCK-3E-transgenic animals it was completely restricted to the skeletal muscle, establishing the tissue specificity of gene expression. This pair of constructs allowed us to discriminate between autocrine and paracrine effects on the skeletal muscle from those exerted in the heart because of the differential promoter activation. Work in progress has shown that the heart weight is significantly lower in the animals in which myostatin is overexpressed under the control of the MCK promoter but not in mice harboring the mutated MCK-3E promoter, suggesting that myostatin expression in the cardiac muscle may affect this organ (34).

The increase of myostatin protein expression in the skeletal muscle groups of our MCK/Mst-transgenic mice was clearly shown by Western blot with an antibody that detects a 30-kDa band that is absent in the myostatin knockout mouse. When taken together with our previous validation experiments using the recombinant myostatin proteins (39), these data collectively support the view that the 30-kDa immunoreactive band represents the processed form of myostatin protein, likely a dimer of the 111-amino acid carboxy-terminal fragment resistant to dissociation under the conditions of the PAGE run. The Western blot data establish that myostatin protein expression was significantly higher in the transgenic mice than in the wild-type controls; this demonstration is important, because
genotyping by itself is not an indicator of overexpression. The observed increase in the relative intramuscular myostatin protein concentrations agrees well with the reduction in fiber size and myonuclear number in transgenic mice and with the loss of muscle mass seen in the males. The higher myostatin mRNA concentrations in transgenic mice are also in good agreement with the Western blot results.

There was good agreement in the relative reductions of hindlimb muscle mass seen in the male transgenic mice harboring the nonmutated MCK and the mutated MCK-3E promoters, confirming previous observations that both promoters are activated in the skeletal muscle (36). The fact that the effects on the forelimb muscles are much lower in the MCK-3E/Mst male animals is consistent with the fact that the triple mutation in the E-box of the MCK-3E promoter sequence not only abolishes activation in the heart but also differentially affects activation in some skeletal muscles (36).

It is intriguing that the female transgenic mice did not differ significantly from wild-type mice in either body weight or skeletal muscle mass even though the expression of myostatin protein was increased in the skeletal muscle. Although the precise mechanisms that account for these gender differences in myostatin effects are unknown, these data suggest that there may be gender-specific mechanisms that can override the effects of myostatin on muscle mass in female transgenic mice. This interpretation is supported by the recent demonstration that decreased abundance of processed myostatin in young wild-type mice is associated with increased body mass and skeletal muscle mass in male compared with female animals (22). It is possible that association with other proteins, more abundant in females than in males, may partially inactivate myostatin in females despite the higher levels seen in this sex, as has been shown to occur in transgenic mice overexpressing follistatin (17). Although follistatin levels are similar in both sexes in the human (26), we do not know whether male and female mice differ in follistatin expression in the skeletal muscle, where follistatin is abundantly expressed (41). Other possibilities include differences in circulating levels of estrogens that exert anabolic effects on the skeletal muscle (9, 32) and androgens, expression of myostatin receptors in the male and female mice, or differences in postreceptor signaling mechanisms. Because myostatin mRNA and protein expression was not lower in the skeletal muscles of female mice compared with male mice, differences in transcriptional or translational regulation of the myostatin gene are unlikely to account for these sex differences in phenotype. The molecular mechanisms by which myostatin inhibits skeletal muscle mass are not fully understood. However, previous studies by our group (39) and others (29, 40) suggest that myostatin inhibits muscle cell replication by restraining the entry of myoblasts into the cell cycle. Recombinant myostatin protein also inhibits protein synthesis in C2C12 muscle cells in vitro (39). In addition, myostatin has been shown to inhibit MyoD expression in myoblasts and their differentiation into myotubes (14, 30). However, myostatin protein has opposite effects on muscle and fat mass; it decreases muscle mass but increases fat mass (25), so that myostatin may actually affect stages prior to myogenesis itself. One plausible hypothesis that could provide a unifying explanation for the reciprocal effects of myostatin on muscle and fat mass is that myostatin acts on a mesenchymal pluripotent precursor cell (7) and inhibits the commitment of these cells into the myogenic lineage and promotes their differentiation into the adipogenic lineage.

In conclusion, myostatin overexpression in the skeletal muscle of the adult male transgenic mice is associated with lower body weight, skeletal muscle mass, heart muscle mass, and myonuclear number, smaller fiber cross-sectional area, and increased epididymal fat mass. The usefulness of this myostatin-transgenic model in elucidating the functional role of myostatin in skeletal muscle response to injury, aging, exercise, and other anabolic and catabolic stimuli should be further explored.

We thank Dr. Se-Jin Lee for providing the myostatin knockout mice, Dr. J. Nalbantoglu for the MCK promoter sequence, and Dr. S. D. Hausekha for the mutated MCK promoter sequence.

DISCLOSURES

This study was supported by National Institutes of Health Grants RO1 AG-14369–01, 2R01 DK-49296–06, and 1RO DK-59627–01, Federal Drug Administration Grant ODP-00001397, Research Centers in Minority Institutions (RCMI) Clinical Research Initiative (PI0 RR-11145), RCMI Grants G12 RR-03026 and U54 RR-14616, and the University of California AIDS Research Program Drew Cares HIV Center.

REFERENCES

Kambadur R, Sharma M, Smith TP, and Bass JJ.


Lee SJ and McPherron AC.

Ma K, Mallidis C, Artaza J, Taylor W, Gonzalez-Cadavid N, and Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadavid NF.


