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Molecular Cloning of Equine (Thoroughbred) Myostatin cDNA and Detection of Myostatin Precursor Proteins in the Serum

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Abstract. Growth/Differentiation Factor 8, or GDF-8, is a member of the TGF- β family and is expressed specifically in myogenic precursor cells of the myotome during development as well as in adult axial and paraxial muscles of the mouse. It acts as a negative regulator of skeletal muscle mass, and is known as myostatin. As a part of our studies to elucidate mechanisms underlying the growth of skeletal muscle mass due to physical training in mammals including horses and humans, we isolated and cloned myostatin cDNA from equine (Thoroughbred) skeletal muscles by an RT-PCR method. The base sequence of the entire coding region of equine myostatin cDNA (DDBJ accession no. AB033541) exhibited a high degree of homology to those of other species so far reported. In the deduced amino acid sequence, 4 amino acid positions were unique to the equine myostatin, i. e., Val¹⁵ instead of Met, Leu¹⁸ instead of Val or Ala, Ala²⁰¹ instead of Thr, Arg²⁴⁴ instead of Gly or Glu in other species. Western blot analysis of the equine skeletal muscles using anti-myostatin antibody, yielded 3 immunoreactive bands, i. e., 26, 45 and 50 kDa. The band at approximately 26 kDa probably represents mature myostatin and another at approximately 50 kDa corresponds in size to the unprocessed precursor myostatin proteins reported in the literature. Western blot analysis of equine sera of 2 independent breeds, Thoroughbred and Kiso-uma, revealed the possible presence of precursor myostatin proteins at relatively high levels. The molecular identity and the biological significance of serum myostatin immunoreactivities remain to be clarified.

Key words: myostatin, muscle, cDNA, Western blotting, serum, horse

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Growth/Differentiation Factor 8, or GDF-8, is a member of the TGF- β family and is expressed specifically in myogenic precursor cells of the

myotome compartment of developing somites as

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well as in adult axial and paraxial muscles of the mouse [1]. The targeted destruction of the gene coding for GDF-8 in mice revealed its function as a negative regulator of skeletal muscle mass; the GDF-8 null mice exhibited a dramatic and widespread increase in skeletal muscle mass causing them to weigh 2–3 times more than wild-type mice [1]. Hence, GDF-8 is named myostatin and the gene coding for it, *Mstn* [1]. Later a mouse strain with a phenotype of hypermuscularity, *Compact (Cmpt)*, was identified to carry a mutant *Mstn* gene which was mapped to Chromosome 1 [2].

Studies on the double-muscling phenotype in Belgian Blue and Piedmontese cattle, revealed that the bovine myostatin gene was mapped to the same chromosomal position as the muscular hypertrophy locus, *mh*, and that these cattle breeds had mutations in the myostatin coding sequence [3, 4]. Thus, Piedmontese double-muscling cattle had a G to A transition in exon 3, resulting in a cysteine to tyrosine substitution in the mature myostatin protein after processing. Belgian Blue cattle, on the other hand had a frame-shift in the C-terminal regions caused by an 11-nucleotide deletion in exon 3 [5–7].

The myostatin gene is highly conserved among the various vertebrate species so far examined, suggesting that the biological function of myostatin is crucial in skeletal muscle development of vertebrates [7, 8]. Lee and McPherron [9] proposed that myostatin may be responsible for the regulation of skeletal muscle mass as a putative negative regulator of tissue growth, i. e., chalone, the existence of which had been postulated originally by Bullough [10].

Myostatin shares several common structural features with other members of the TGF- β superfamily: 1) the presence of a hydrophobic core of amino acids near the N-terminus that functions as a secretory signal; 2) the formation of disulphide-linked dimers containing subunits of 110–140 amino acids as their biologically active forms; and 3) synthesis of its subunits as the C-terminal portion of a larger precursor which is cleaved at the proteolytic processing site [1].

Recently Wehling *et al.* [11] demonstrated that myostatin expression is modulated in fully differentiated, non-pathological hind limb muscles of Wistar rats in a manner inversely related to changes in the muscle mass. Thus, atrophy of

plantaris muscles induced by 10 days of unloading was accompanied by a 16% decrease in total mass of the muscle, a 110% increase in myostatin mRNA and a 37% increase in myostatin protein levels [11]. The elevated levels of myostatin expression, however, were not a sufficient cause for, but rather a result of muscle mass loss [11, 12].

As a part of our studies to elucidate mechanisms underlying the growth of skeletal muscle mass due to physical training in mammals including horses and humans, in order to determine a basis for developing the training methods optimal for the muscular development, we cloned equine myostatin cDNA and examined the expression of myostatin immunoreactivities in muscles and sera.

Materials and Methods

Collection of tissue and blood samples

Skeletal muscles were collected from fetal and adult Thoroughbred horses. Specimens of *M. rectus abdominis* were collected from fetuses whereas tissue samples of *M. semitendinosus* and *semimembranosus*, *M. splenius*, *M. gluteus medius* were obtained from adult horses. The obtained specimens were rapidly frozen in liquid nitrogen, and stored at -80°C until use. Blood samples were collected from the carotid arteries of Thoroughbred and Kiso-uma horses, centrifuged at 3,000 rpm at 4°C , and stored at -80°C until use.

Extraction of RNA and RT-PCR analysis

Total RNA was extracted from the muscles using an ISOGEN kit (Nippon Gene, Toyama-shi) and mRNA was purified from the total RNA using Oligotex dT30 super (Takara, Ohtsu-shi) according to the procedures described by the manufacturer. The mRNA (200 ng) was reverse-transcribed into first strand cDNA using Super Script (Gibco-BRL, NY) in a reaction volume of $20\ \mu\text{l}$ as recommended by the manufacturer. The reaction mixture contained $5 \times$ first strand buffer, 10 mM dNTP mixture, 0.1 M DTT, 0.5 mM oligo-dT primer and 200 U Super Script. For PCR amplification, 2 primer sets designed by Grobet *et al.* [4] for the bovine myostatin sequence were used. The primer sequences were as follows: 1) forward primers, 5'-TTT CAC TTG GCA TTA CTC AAA AG-3' and 5'-CAA TGC CTA AGT TGG ATT CAG GTT G-3', and 2) reverse primers, 5'-ACA GTG TTT GTG CAA

ATC CTG AGA C-3' and 5'-ATA CTC WAG GCC TAY AGC CTG TGG T-3'.

The PCR reaction mixture (50 μ l) contained 10 $\times$ LA PCR Buffer II, 2.5 mM MgCl₂, dNTP mixture, 2.5 U LA-Taq polymerase (Takara), 0.25 μ M each of the forward and reverse primers and 1 μ l of the RT reaction. PCR amplification was performed in a Perkin Elmer DNA thermal cycler (Model 2400), under the following conditions: denaturation at 94 C for 5 min was followed by 40 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 2 min, extension at 72 C for 2 min, and a final extension at 72 C for 7 min. After PCR amplification, an aliquot of the reaction mixture was loaded onto 1% TAE agarose gel and electrophoresed; the products were stained with ethidium bromide.

Sequencing and sequence analysis

The PCR-amplified equine myostatin cDNA fragments were cloned into pGEM-T vector (Promega, WI) and sequenced using a ThermoSequenase Cycle Sequencing kit (Amersham-Pharmacia, Kyoto) and a DSQ-2000L DNA sequencer (Shimadzu, Kyoto). Analyses of the obtained sequence were performed using DDBJ/Genbank databases and the BLAST search system as well as Genetyx software (Software Development, Tokyo).

Antibody

A custom-synthesized partial peptide of myostatin, QKYPHTLHVHQANPRGSAGPC (Sawady, Tokyo) (1 mg), was dissolved in 1 ml DMSO (Sigma) and mixed with key hole limpet hemocyanin (KLH) and a cross-linker, m-maleimidobenzoyl-N-hydroxy-succinamide (MB), under a nitrogen stream. After incubation for 3 h at room temperature, the mixture was frozen and stored at -20 C until use. The conjugate (100 mg) was emulsified with Freund's complete adjuvant (FCA) (1.5 ml; CAPPel) and injected into 20 subcutaneous parts of each of 3 rabbits used for the experiments. Subcutaneous injections of the antigen emulsified in Freund's incomplete adjuvant (FIA) (DIFCO) were given 3 weeks later. Booster injections of the FIA-emulsified antigen were repeated at an interval of 1 week. The antibody titers were checked regularly by ELISA assay method. When sufficiently high titers were obtained, the rabbits were exsanguinated and

serum was collected and stored at -20 C until use. The details of the antibody are described elsewhere [13].

Western blotting

For Western blot analysis, 5 ml of horse blood serum were purified using Molcut-L LGC (Millipore) in an ice bath and were mixed vigorously with a buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 12% 2-mercaptoethanol and 20% glycerol. Skeletal muscles were homogenized with an Ultraturrex homogenizer (M. Zipper GmbH, Switzerland) in a buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 6% 2-mercaptoethanol, 10% glycerol and heated at 65 C for 10 min. The denatured protein extracts were separated on a 10% polyacrylamide-SDS gel by electrophoresis and transferred to a nitrocellulose membrane (Clear blot membrane-P; Atto, Tokyo). The membrane was soaked in 1% blocking buffer (Boehringer Mannheim, Tokyo) and then incubated with purified myostatin antibodies diluted with PBS (1:1000) at room temperature for 1 h. The membrane was washed 3 times for 5 min each with PBS and incubated with the 2nd antibody, goat anti-rabbit IgG conjugated to horse radish peroxidase (American Qualex, CA), for 1 h at room temperature. The membrane was washed for 15 min with PBS 3 times and HRP activity was detected using diaminobenzidine (DAB; Wako, Osaka).

Results and Discussion

We isolated and cloned myostatin cDNA from equine (Thoroughbred) skeletal muscles by an RT-PCR method (Fig. 1). The base sequence of the entire coding region was determined (Fig. 2). Alignment of the equine myostatin cDNA sequence to those of other species, confirmed the highly conserved nature of myostatin genes among the species so far examined (Fig. 3). In Fig. 4, the deduced amino acid sequence of the equine myostatin peptide is aligned with those of other species. Four amino acid positions were unique to the equine myostatin: Val¹⁵ instead of Met, Leu¹⁸ instead of Val or Ala, Ala²⁰¹ instead of Thr, Arg²⁴⁴ instead of Gly or Glu in other species (Fig. 4). Altogether equine myostatin exhibited a high degree of homology (94.7–97.3%) to myostatins of

other species so far reported in the literature (Table 1).

The equine myostatin possesses, like myostatins of other species, the TGF- β motif (298-313; IIAPKRYKANYCSGEC) and the EF-hand motif (99-111; DDSSDGSLEDDDY) (Fig. 4). A putative proteolytic processing site, R²⁶³SRR²⁶⁶, is indicated in Fig. 4. The myostatin precursor protein is cleaved at this site to give rise to a mature protein [1].

The hydropathy plots of myostatin revealed alternating hydrophobic and hydrophilic regions and exhibited an almost identical pattern to those of other species, indicating that the structural and functional features of myostatins are highly conserved among species (data not shown).

Western blot analysis under reducing conditions of the equine skeletal muscles yielded 3 immunoreactive bands at estimated molecular masses of 26, 45 and 50 kDa (Fig. 5). The 26 and 50 kDa bands corresponded to the molecular sizes of mature myostatin protein and unprocessed precursor myostatin proteins, respectively, so far reported in the literature (in sheep [14]; in humans [15]). The identity of the 45 kDa band remains to be clarified. However, on the basis of the amino acid sequence deduced from the cDNA base sequence, the estimated molecular mass of the precursor and mature myostatin proteins in different species are approximately 43,000 and 13,000, respectively (data not shown). The discrepancy between the molecular size calculated from the deduced amino acid sequence and that of the immunologically detected bands may be due to differences in the post-translational modifications such as glycosylation of myostatin proteins [11].

One possible explanation for the presence of 45 kDa band in the horse serum is that it might represent a non-glycosylated myostatin molecule. McPherron *et al.* [1] have detected a mature myostatin protein of 13 kDa on Western blot analysis of CHO cell extracts under reducing conditions. Wehling *et al.* [11] suggested that the difference between the 13 kDa and the 26 kDa immunoreactive protein may be accounted for by the difference in the post-translational modifications mentioned above [11].

Our results, therefore, strongly indicate that mature and unprocessed precursor myostatin proteins co-exist in adult equine skeletal muscles. Judging from the intensities of the bands, the

Fig. 1. PCR amplification of equine myostatin cDNA using two primer sets specific to the myostatin sequences. The PCR products were electrophoresed on 1.0% TAE agarose gel. Each band was excised from the gel, purified and ligated into pGEM-T vector (Promega) for subsequent sequencing.

expression levels of mature myostatin were much lower than those of the precursor protein in equine skeletal muscles.

A surprising finding was made when we carried out Western blot analysis of equine sera of 2 independent breeds, Thoroughbred (a typical imported breed) and Kiso-uma (a breed native to Japan), for the possible presence of myostatin. As shown in Fig. 6, in the equine sera, a 50 kDa band matching the size and immunoreactivities of unprocessed precursor myostatin in the skeletal muscle was detected (Fig. 5), but no band was seen in the 26 kDa regions representing mature myostatin.

Previously, the presence of myostatin-like protein as detected by Western blot analysis in the sera of HIV-infected men was reported by Gonzalez-Cadavid [15]. This was the first report describing the expression of myostatin or myostatin-related protein in the serum. However, recently Wehling *et al.* [11] questioned the

AAAGTAAAAG	GAAGAAATAA	GAGCAAGGAA	AAAGATTGAA	CTGATTTTAA	AATC ATGCAA	60
AAACTGCAAA	TCTCTGTTTA	TATTTACCTG	TTTGTGCTGA	TTCTTGCTGG	TCCAGTGGAT	120
CTAAATGAGA	ACAGCGAGCA	AAAAGAAAAT	GTGGAAAAAG	AGGGGCTGTG	CAATGCATGT	180
ACTTGAGAC	AAAACACTAA	ATCTTCAAGA	ATAGAAGCCA	TAAAAATTCA	GATCCTCAGT	240
AAACTGCGCC	TGGAAACAGC	TCCTAACATC	AGCAAAGATG	CTATTAGACA	ACTTTTGCCC	300
AAAGCTCCTC	CACTCCGGGA	ACTGATTGAT	CAGTACGATG	TCCAGAGAGA	TGACAGCAGT	360
GATGGCTCTT	TGGAAGATGA	TGATTACCAC	GCGACGACGG	AAACAATCAT	TACCATGCCT	420
ACAGAGTCTG	ATCTTCTAAT	GCAAGTGGAA	GGAAAACCCA	AATGTTGCTT	CTTTAAATTT	480
AGCTCTAAAA	TACAATACAA	TAAAGTAGTA	AAGGCCCAAC	TGTGGATATA	TCTGAGACCC	540
GTCAAGACTC	CTACAACAGT	GTTTGTGCAA	ATCCTGAGAC	TCATCAAACC	CATGAAAGAC	600
GGTACAAGGT	ATACTGGAAT	CCGATCTCTG	AAACTTGACA	TGAACCCAGG	CGCTGGTATT	660
TGGCAGAGCA	TTGATGTGAA	GACAGTGTTG	CAAAATTGGC	TCAAACAGCC	TGAATCCAAC	720
TTAGGCATTG	AAATCAAAGC	TTTAGATGAG	AATGGTCATG	ATCTTGCTGT	AACCTTCCCA	780
AGACCAGGAG	AAGATGGGCT	GAATCCATTT	TTAGAAGTTA	AGGTAACAGA	CACACCAAAA	840
CGATCCAGAA	GAGATTTTGG	ACTTGACTGT	GATGAGCACT	CCACAGAATC	TCGATGCTGT	900
CGTTACCCTC	TAAGTGTGGA	TTTTGAAGCT	TTTGGATGGG	ATTGGATTAT	TGCACCCAAA	960
AGATATAAGG	CCAATTACTG	CTCTGGAGAG	TGTGAATTTG	TATTTTTTACA	AAAATATCCT	1020
CACACTCATC	TTGTACACCA	AGCAAACCCC	AGAGGTTTCA	CAGGCCCTTG	CTGTACTCCC	1080
ACAAAGATGT	CTCCAATTAA	TATGCTATAT	TTTAATGGCA	AAGAACAAAT	AATATATGGG	1140
AAAATTCCAG	CCATGGTAGT	AGATCGCTGT	GGGTGCTCAT	GAGATTTTACA	TGTGGTTCAT	1200
AACTTCCTAA	AACATGGAAG	GCCTTCCCCT	CACAATTGTG	AACTGTGAA	ATTATGT	1257

Fig. 2. Nucleotide sequence of equine myostatin cDNA. The Open Reading Frame sequence (Hosoyama *et al.*, DDBJ accession no. AB033541) is indicated by bold capital letters and the untranslated regions are represented by plain capital letters. Translation initiation (ATG) and stop codons (TGA) are indicated by bold italics. Arrowheads indicate exon-exon boundaries.

authenticity of the myostatin-immunoreactivities described by Gonzalez-Cadavid [15], on the basis of the discrepancy in the molecular mass.

The 50 kDa band presently detected in equine sera coincided with the molecular mass of the band detected in the equine skeletal muscles and also with that of precursor myostatin proteins in sheep reported by Sharma *et al.* [14]. Therefore, we tentatively concluded that myostatin precursor proteins are present in adult equine serum at sufficiently high levels as detected by Western blot analysis.

Interestingly, before the myostatin gene was identified, Gerrard and Judge [16] had demonstrated that in murine L6 myoblasts, the induction of cell proliferation took place in media supplemented with serum from double-muscle cattle [16], suggesting that in the control serum of normal cattle, a factor suppressing proliferation might be present.

The molecular identity and the biological significance of serum myostatin immunoreactivities remain to be clarified. However, they might reflect

the physiological status of skeletal muscles under varying degrees of loading in physical exercises. Further characterization of the presently-described serum myostatin immunoreactivities is currently in progress at our laboratory.

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equine	1:ATGCAAAAGCTGCAATCTCTGTTTATATTTACCTGTTTGTGCTGATTCTTGCTGGTCCAGTGGATCTAAATGAGAACAGCGACCAAAAGAAAATGTGG
bovine	1:.....A...A.....G.....C.....G.....G.....G.....G.....
baboon	1:.....C...G.....A.....G.....T.....
human	1:.....C...G.....A.....G.....T.....
equine	101:AAAAAGAGGGCTGTGCAATGCATGTACTTGGAGACAAACACTAAATCTTCAAGATAGAAGCCATAAAATTCAGATCCTCAGTAACTGCCCTGGA
bovine	101:.....T.....TTG.....GG.....C...C.....C.....C...A.....T.....
baboon	101:.....T.....T.....A...A.....T...T....
human	101:.....T.....T.....T...G...A...A.....T...T....
equine	201:AACAGCTCCTAACATCAGCAAAGATGCTATTAGACAACCTTTGCCAAAGCTCCTCCACTCCGGAACTGATTGATCAGTACGATGCCAGAGAGATGAC
bovine	201:.....C.....G.....T.....T.....C...
baboon	201:.....A.....A.....T.....G.....
human	201:.....T...A.....A.....T.....G.....
equine	301:AGCAGTGATGGCTCTTTGGAAGATGATGATTACCAGCGACGACGGAACAATCATTACCATGCCACAGAGTCTGATCTTCTAATGCAAGTGAAGGAA
bovine	301:.....C.....C.....C.....C.....C.....G.....C.....G.....C.....
baboon	301:....C.....C.....T...T...A.....T...T.....T...
human	301:....C.....C.....T...T...A.....T.....T.....
equine	401:AACCCAAATGTTGCTTCTTTAAATTAGCTCTAAATACAATAACAAGTAGTAAAGCCCAACTGTGGATATATCTGAGACCCGCTCAAGACTCCTAC
bovine	401:.....G.....C.....G...T.....G...
baboon	401:.....G.....A.....T.....G.....
human	401:.....A.....T.....G.....
equine	501:AACAGTGTGTGCAATCCTGAGACTCATCAACCCATGAAAGACGGTACAAGGTATACTGGAATCCGATCTCTGAAACTTGACATGAACCCAGGCGCT
bovine	501:G.....A...
baboon	501:.....T.....A...
human	501:.....T.....A...
equine	601:GGTATTTGCCAGAGCATTGATGTGAAGACAGTGTGCAAAATTGGCTCAACAGCCTGAATCCAACCTAGGCATTGAAATCAAGCTTTAGATGAGAATG
bovine	601:.....G...C.....A.....
baboon	601:.....A.....A.....
human	601:.....A.....A.....
equine	701:GTCATGATCTTGCTGTAACTTCCCAAGACCAGGAGAAGATGGGCTGAATCCATTTTGTAGAAGTTAAGGTAACAGACACACCAAAACGATCCAGAAGAGA
bovine	701:..C.....GA.....A...C...T.....C.....A...T...G....
baboon	701:.....G.....C.....G...C.....A.....G...
human	701:.....G.....G.....G...C.....A.....G...
equine	801:TTTTGGACTTGACTGTGATGAGCACTCCACAGAATCTCGATGCTGCTTACCTCTAAGTGGATTGGAAGCTTTGGATGGGATTGGATTATTGCA
bovine	801:....G...T.....A.....
baboon	801:....T.....A.....G.....C.....C...T
human	801:....T.....A.....A.....C.....C...T
equine	901:CCCAAAAGATATAAGCCAATTACTGCTCTGGAGAGTGAATTTGTATTTTACAAAAATATCCTCACACTCATCTTGTACACCAAGCAAAACCCAGAG
bovine	901:..T.....A.....G...G.....T...C.....G.....
baboon	901:..T.....T.....G.....
human	901:..T.....T.....G.....
equine	1001:GTTCAGCAGGCCCTGCTGTACTCCCAAGATGTCTCCAATTAATATGCTATATTTTAATGGCAAGAACAAATAATATATGGGAAAAATCCAGCCAT
bovine	1001:.....C.....T.....G...G.....C...G.....
baboon	1001:.....T.....
human	1001:.....T.....G...
equine	1101:GGTAGTAGATCGCTGTGGGTGCTCATGA
bovine	1101:.....T.....
baboon	1101:.....C...C.....
human	1101:.....C.....

Fig. 3. Alignment of the nucleotide sequences of equine, bovine (McPherron and Lee, 1997, GenBank no.AF019620), baboon (McPherron and Lee, 1997, GenBank no.AF019619) and human (Gonzalez-Cadavid *et al.*, 1998, GenBank no. AF104922) myostatin ORFs. Dots denote identical nucleotides among the species.

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equine 1:-MQKLQISVYIYLFVLILAGPVDLNENSEQKENVEKEGLCNACTWRQNTKSSRIEAIKIQLSKLRLETAPNISKDAIRQLLPKAPPLRELIDQYDVQRD
bovine 1:-.....M..V.....L..E..T..L.....L....F....
mouse 1:M.....MY.....M..A.....G..RE.....A.....RY.....R.....
baboon 1:-.....LC.....M..V.....
human 1:-.....LC.....M..V.....V.....

equine 101:DSSDGSLEDDDYHATTETITITMPTESDLLMQVEGPKCCFFKFSKIQYNKVVKAKLWIYLRPVKTPTTVFVQILRLIKPMKDGTRYTGIRSLKLDMPNG
bovine 101:A.....R...V.....T.....L.....A.....
mouse 101:.....F...AD.....S..
baboon 101:.....F...D.....E.....
human 101:.....F...D.....E.....

equine 201:AGIWQSIDVKTVLQNWLKQPEISNLGIEIKALDENGHDLAFTFPRPGEDGLNPFLEVKVTDTPKRSRDFGLDCDEHSTESRCCRYPLTVDFEAFGWDWII
bovine 201:T.....E.....T.....
mouse 201:T.....G.....
baboon 201:T.....G.....L.....
human 201:T.....G.....

equine 301:APKRYKANYCSGCECFVFLQKYPHTHLVHQANPRGSAGPCC'TPTKMSPINMLYFNGKEQIIYGKIPAMVVDRCGCS
bovine 301:.....EG.....
mouse 301:.....
baboon 301:.....
human 301:.....

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Fig. 4. Alignment of amino acid sequences of equine, bovine, murine, baboon and human myostatins. Dots denote identical amino acids among the species. TGF- β and EF-hand motifs are underlined. A putative proteolytic processing site is framed.

Table 1. Homology scores of myostatin protein among different mammalian species

equine	100%				
bovine	94.9%	100%			
mouse	95.5%	92.5%	100%		
baboon	97.3%	94.1%	96.3%	100%	
human	97.3%	94.1%	96.3%	99.5%	100%
	equine	bovine	mouse	baboon	human

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Fig. 5. Western blot analysis of the soluble fractions of equine skeletal muscle homogenates. After electrophoresis, the membranes were blotted, and probed with anti-myostatin antibodies. The positions of myostatin precursor protein (50 kDa) and mature myostatin (26 kDa) are indicated by arrows. The immunochemically stained bands coincide in size to unprocessed and processed myostatin proteins, respectively. Two bands could be resolved in the high molecular weight precursor proteins of equine myostatin.

Fig. 6. Western blot analysis of the equine serum. The collected sera were electrophoresed, blotted, and probed with anti-myostatin antibodies. The position of myostatin unprocessed precursor protein is indicated by an arrow (50 kDa). Immunoreactivities were detected only in the region corresponding in size to the unprocessed myostatin protein. The same myostatin immunoreactivities were detected in both Thoroughbred(A) and Kiso-uma (B) horses.

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