0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(5):2106–2115
Copyright © 2007 by The Endocrine Society
doi: 10.1210/en.2006-1299

Characterization of Rainbow Trout Myostatin-2 Genes (rtMSTN-2a and -2b): Genomic Organization, Differential Expression, and Pseudogenization

Dilip K. Garikipati, Scott A. Gahr, Eric H. Roalson, and Buel D. Rodgers

School of Molecular Biosciences (D.K.G., B.D.R.), School of Biological Sciences (E.H.R.) and Department of Animal Sciences (B.D.R.), Washington State University, Pullman, Washington 99164; and United States Department of Agriculture/Agricultural Research Service (S.A.G.), National Center for Cool and Cold Water Aquaculture, Kearneysville, West Virginia 25430

Myostatin is an extremely potent negative regulator of vertebrate skeletal muscle development. A phylogenetic analysis suggests that salmonids should possess four distinct genes, although only MSTN-1 orthologs have been characterized. Described herein are the rainbow trout (rt) MSTN-2a and -2b genes and subsequence analysis of their promoters and their quantitative expression profiles. Both genes are similarly organized, contain several putative myogenic response elements, and are legitimate MSTN-2 orthologs based on Bayesian analyses. However, rtMSTN-2b contains two in-frame stop codons within the first exon and unspliced variants of both transcripts were expressed in a tissue-specific manner. Complete splicing of rtMSTN-2a occurred only in brain, where expression is highest, whereas rtMSTN-2b transcripts were mostly present in unspliced forms. The presence of stop codons in the rtMSTN-2b open reading frame and the expression of mostly unspliced transcripts indicate that this particular homolog is a pseudogene. These results confirm our previous phylogenetic analysis and suggest that all salmonids likely possess four distinct myostatin genes. The tissue-specific expression and differential processing of both rtMSTN-2 transcripts as well the pseudogenization of rtMSTN-2b may reflect compensatory and adaptive responses to tetraploidization and may help limit rtMSTN-2a's influences primarily to neural tissue. (*Endocrinology* 148: 2106–2115, 2007)

MYOSTATIN IS A MEMBER of the TGF-β superfamily and a potent negative regulator of skeletal muscle growth in mammals (1). Myostatin null phenotypes in rodents and cattle are characterized by extreme gains in muscle mass commonly referred to as double muscling (2–4). Indeed, extraordinary musculature has also been reported in a child with a 5′ splice site mutation in the myostatin gene's first intron (5), indicating that myostatin's function is widely conserved among mammals. Unlike mammals where myostatin expression is restricted primarily to skeletal muscle (4, 6) and to a much lesser extent in heart (7) and mammary glands (8), it is widely expressed in many different fish tissues (6, 9–13). Nevertheless, tissue-specific functions of myostatin have yet to be described and the existence of multiple myostatin genes (9, 12, 14–16) further complicates our understanding of myostatin biology in fish. Many salmonid species are highly prized for their commercial importance, and rainbow trout in particular are an emerging and alternative animal model for basic and biomedical research. Thus, improving muscle growth by controlling myostatin expression or bioactivity in these species could significantly impact aquaculture and academic communities alike.

An early genome duplication in the bony fish, before the teleost radiation, resulted in multiple copies of many genes (17, 18). A second duplication event occurred in the salmonids approximately 50–100 million years ago (19), although many paralogs may have been lost. Some of these genes have been characterized in rainbow trout and include homologs for Wilms' tumor gene (19), glutamate synthetase (20), the inhibitors of DNA binding/differentiation (ID) genes (21), and major histocompatibility genes (22). Our previous phylogenetic analysis of the myostatin gene family (9) identified two distinct fish clades, MSTN-1 and MSTN-2, and determined that all of the salmonid myostatin genes previously identified were actually MSTN-1 orthologs. These genes were therefore reclassified as either MSTN-1a or MSTN-1b. This study also predicted the existence of two additional myostatin paralogs, namely MSTN-2a and -2b, in most if not all salmonid species, although none of these genes have been identified to date.

Reported herein is the discovery of MSTN-2a and -2b genes in the rainbow trout, the genomic organization of both alleles, including their respective promoter regions, and the quantitative assessment of rtMSTN-2a gene expression using comprehensive RNA panels derived from multiple developmental stages and various adult tissues. These results are the first to confirm the existence of four myostatin genes in any salmonid species and reveal a strong conservation of genomic organization among all vertebrate homologs. In addition, we identified rtMSTN-2b as a pseudogene as well as unspliced transcripts of both rtMTSN-2a and -2b, which has never been described in any vertebrate.

First Published Online February 8, 2007

Abbreviations: F, Forward; gDNA, genomic DNA; PS, primer set; R, reverse; rt, rainbow trout; UTR, untranslated region.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

Materials and Methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Washington State University hatchery, a Center of Reproductive Biology core facility. Fish were reared and used according to protocols preapproved by the Institutional Animal Care and Use Committee. The RNA panels were generated from embryos and adult tissues of rainbow trout obtained from the National Center for Cool and Cold Water Aquaculture according to the guidelines approved by a separate Institutional Animal Care and Use Committee.

Cloning and characterizing rtMSTN-2a and -2b genes

Genomic DNA (gDNA) was isolated from fin clips (~50 mg) after incubating in 3 ml lysis buffer (30 mM Tris, 8 M urea, 4% wt/vol CHAPS, pH 8.0) containing 20 mg/ml proteinase K at 60 C. Three consecutive phenol/chloroform/isoamyl alcohol extractions were then performed, and gDNA quality was verified on a 1% agarose gel.

Degenerate primers (DegP-1 and DegP-2, Table 1) were designed to recognize conserved regions within the C-terminal domain of different fish MSTN-2 genes (shi drum, seabream, fugu, and zebrafish) and were used to amplify custom rainbow trout genomic libraries. Briefly, gDNA was digested with the blunt end restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I and subsequently ligated to adaptor linkers according to the kit manufacturer's protocol (BD GenomeWalker Universal kit; BD Biosciences, Franklin Lakes, NJ). Nested PCR was performed using degenerate primers and the adaptor primers (Table 1) with the Advantage 2 PCR kit (BD Biosciences). Cycling parameters were as follows and were used as default unless otherwise specified: an initial denaturation at 94 C for 1 min, 32 cycles of 94 C for 30 sec, 65 C for 3 min, and a final extension period of 4 min at 67 C. The PCR products were subcloned into the Topo TA vector (Invitrogen, Carlsbad, CA) and sequenced in the university's genomic core facility. This resulted in partial sequences of two new myostatin genes. Complete genomic clones were isolated using the gene-specific primers based on the partial sequences previously cloned. Whole gene sequences were then isolated by PCR using a high-fidelity polymerase (Pfu) (Stratagene, La Jolla, CA) and resequenced.

The 3′ untranslated region (UTR) of rainbow trout (rt)MSTN-2a was isolated using a 3′-RACE (rapid amplification of cDNA ends) kit (Invitrogen). Total RNA from juvenile skeletal muscle was extracted using Trizol and reverse transcribed using superscript reverse transcriptase, both according to the manufacturer's protocols (Invitrogen). cDNA was then amplified by PCR using a gene-specific forward primer (PS-I F/PS-II F; Table 1) and the universal adaptor primers provided in the kit. The PCR conditions were as follows: an initial denaturation at 94 C for 1 min followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 2 min and a final extension at 72 C for 3 min. Nested PCR was subsequently performed with the 3′-UTR2a primer and the abridged universal adaptor primer provided in the kit. The resulting PCR product was subcloned and sequenced as described.

Computational analysis

Putative cDNA sequences for each novel myostatin gene were constructed *in silico* from the predicted open reading frames using Genescan (www.genes.mit.edu/GENESCAN.html) and were confirmed by sequencing RT-PCR amplicons. The 2.4- and 1.5-kb regions upstream of the rtMSTN-2a and rtMSTN-2b coding regions, respectively, were also cloned and sequenced using similar protocols. Putative regulatory elements in the promoters were identified by subsequence analysis using Matinspector software (Genomatix Inc., www.genomatix.de), which searches for known consensus sequences of *cis* regulatory elements. Alignments of the putative rtMSTN proteins were performed using Vector NTI Align X (Invitrogen).

Phylogenetic analyses was performed using Bayesian inference as implemented in MrBayes version 3.1 (23) and the newly discovered myostatin genes, rtMSTN-2a and -2b, along with previously characterized myostatin genes from the following species: human (GenBank accession no. AF019627), chicken (AY448007), seabream (AF258448 and AY046314), shi drum (AF316881 and AY059386), fugu (AY445322 and AY445321), zebrafish (AY258034 and AY687474), striped bass (AF290910), white perch (AF290910), white bass (AF197194), tilapia (AF197193), king mackerel (AF317667), little tunny (AF317666), Atlantic salmon (AJ344158 and AJ297267), brook trout (AF313912 and AF247650), rainbow trout (AF273035 and AF273036), coho salmon (AY434465 and AF394687), blue catfish (AY540992) and channel catfish (AF396747). Amino acid alignments were created using Clustal X 1.83 (24) using default parameters. Bayesian analyses used a mixed amino acid model and 10 million generations were performed with four chains (Markov Chain Monte Carlo) sampling every 1000 generations, which resulted in a distribution of 10,000 trees. To test for the occurrence of stationarity, convergence, and mixing within the 10 million generations, multiple analyses were started from different random locations in tree space. The posterior probability distributions from these separate replicates were compared for convergence to the same posterior probabilities across branches. Majority-rule consensus trees of those sampled in Bayesian inference analyses yielded probabilities that the clades are monophyletic (25). Trees sampled during the burn-in of the chain, the first two million generations, were discarded to assure that only trees sampled after stationarity was established were included and the remaining trees were loaded into PAUP version 4.0 (26). Consensus trees were then created to display branches with posterior probabilities greater than 50%.

TABLE 1. Primer sequences and annealing temperatures

Primer name	Sequence (5′–3′)	Annealing temperature (C)
DegP-1	TCNCCNGACCACTARTTGGGC	65
DegP-2	CGYTTGGGVGCRATKATCCAGTCCCA	65
β-Actin F	TCTGGCCGTACCACCGGTAT	60
β-Actin R	CGTGTTGGCGTACAGGTCCTT	60
PS-I F/PS-II F	TTGTCACTGTGTTATATAGGCCTGGAA	60
PS-I R	GCGGGAGATTTGCAAGAAGACAGTTG	60
PS-II R	TATGCGCGAGTTCCCTCAGCC	60
PS-III F	AACCTGCGGCCGGCTGAGGGAA	60
PS-III R	GGTTATCTTCCCATAGATGATCTGC	60
Adaptor primer 1	GTACTACGACTCACTATAGGGC	65
Adaptor primer 2	ACTATAGGGCACGCGTGGT	65
QMSTN-2a F	AATCTCCCCGCATAAAAGCAACCAC	66
QMSTN-2a R	CACCAGAAGCCACATCGATCTT	66
18s F	TGCGGCTTAATTTGACTCAACA	60
18s R	CAACTAAGAACGGCCATGCA	60
3′UTR2a	CTTAGTGAGGACATGAAACTGCCCATA	60
MSTN-1a F	CTTCACATATGCCAATACATATTA	60
MSTN-1a R	GCAACCATGAAACTGAGATAAA	60
MSTN-1b F	TTCACGCAAATACGTATTCAC	60
MSTN-1b R	GATAAATTAGAACCTGCATCAGATTC	60

N, A/T/G/C; R, A/G; Y, C/T; V, A/C/T; K, G/T.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

Qualitative analysis of rtMSTN-2a and -2b expression in adult tissues

Brain, skin, gill filaments, gill arches, heart, kidney, spleen, intestine, stomach, liver, eyes, and red and white skeletal muscle were collected from 2-yr-old male and female rainbow trout, snap frozen in liquid nitrogen, and stored at −80 C. Samples were powdered, and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with DNase (DNase RQ-1; Promega, Madison, WI) to remove contaminating gDNA. Samples were then reextracted with TRIzol reagent, and RNA quality was assessed by agarose gel electrophoresis. Assays were also performed using the RNA panel described below. Three different primer sets (PS), each spanning an intron, were used to amplify specifically rtMSTN-2a and -2b transcripts (see Fig. 5A). The PS-I reverse (R) primer (Table 1) is specific for rtMSTN-2a and primers PS-II R and PS-III forward (F) are specific for rtMSTN-2b gene transcripts. However, the other primers cross-hybridize to both rtMSTN-2 transcripts. Nevertheless, primer specificity was confirmed by sequencing amplicons. The PCR conditions were as follows: an initial denaturation at 94 C for 1 min followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 90 sec and a final extension at 72 C for 3 min. After the initial 30 cycles, an additional 30 cycles of PCR was performed using 5 μl of the PCR product as template. To confirm that the unspliced amplicons were not from gDNA contamination, equal quantities of RNA from gills were treated with or without RNase A following the manufacturer's protocol (Fermentas, Hanover, MD) and reextracted with TRIzol reagent. cDNA was then synthesized and amplified with primers specific to rtMSTN-2a (PS-I), rtMSTN-2b (PS-II/PS-III), or both rtMSTN-2 genes (PS-I F and PS-III R). The PCR protocol included an initial denaturation at 94 C for 1 min followed by 35 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 2 min and a final extension at 72 C for 3 min. An aliquot of 5 μl was then reamplified using the same protocol. Qualitative analysis of rtMSTN-1a, -1b, and -2a was performed in different sections of the brain with gene-specific primers (Table 1). The PCR protocol included an initial denaturation at 94 C for 1 min followed by 30 cycles of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 90 sec and a final extension at 72 C for 2 min.

Quantitative analysis of gene expression in developing embryos and in adult tissues

An RNA panel was generated from 5000 pooled eggs from multiple females (Trout Lodge, October 2004) that were fertilized by milt from two males. After fertilization, eggs were incubated at 13 C throughout embryonic development. Unfertilized eggs (d 0) and developing embryos were collected as whole egg samples daily for the first 14 d, every other day until hatch (d 24), and every third day thereafter. Each sample pool contained 18 eggs/embryos or nine post-hatched larvae that were pooled, and several samples were collected at each time point. Frozen samples were first powdered using a liquid nitrogen cooled Bessman tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA) and total RNA was extracted using TRI reagent (Sigma, St. Louis, MO) with the high-salt-solution modification to remove the excess glycosylated proteins and treated with DNase as described. Samples were then reextracted with TRI reagent, and RNA quality was assessed by agarose gel electrophoresis. The adult tissues were collected, and mRNA was extracted as previously described.

Total RNA (2 μg) was reverse transcribed with 1 μg oligo-dT primers (Promega) and 200 U Maloney murine leukemia virus reverse transcriptase (Promega). Subsequent real-time RT-PCR assays were conducted using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and gene-specific primers (QMSTN-2a F and QMSTN-2b R, Table 1). For each sample, 1 μl cDNA was combined with 5 μl of 2× SYBR Green PCR master mix (Applied Biosystems). For each reaction, 6 μl of this mixture was added to 9 μl of the primer mix containing 500 μM of each primer. The reactions were performed as follows: 50 C for 2 min, 95 C for 10 min, and then 40 cycles of 95 C for 15 sec and 60 C for 1 min. The cycling reaction was followed by a dissociation curve to verify amplification of a single product, and amplicons were also verified by DNA sequencing. The relative standard curve method was employed to quantify gene expression. A serial dilution of a mixed tissue cDNA was used to construct a standard curve for each assay plate. The standard curve was constructed by plotting the threshold cycle *vs.* the natural log of input RNA. This curve was then used to calculate the abundance of each transcript in each sample. Myostatin values were then normalized to those of 18s to control for differences in RNA and cDNA loading. Each sample was run in triplicate on a single plate, each plate was run in duplicate, and data are presented as normalized gene expression values.

Results

Genomic organization of rtMSTN-2a and -2b gene

Complete genomic clones for both rtMSTN-2a and -2b genes were isolated and sequenced (Fig. 1, A and B). This includes approximately 2.4 kb upstream from the translational start site for rtMSTN-2a and 1.5 kb for rtMSTN-2b. The annotated gene and promoter sequences of rtMSTN-2a and -2b were deposited into GenBank and assigned the accession numbers DQ138301 and DQ177320, respectively. The genomic organization of both genes was similar to all myostatin genes characterized to date (4, 9, 13, 27–30). The three exons of rtMSTN-2a are 340, 371, and 1063 bp in size, respectively, and separated by 158- and 255-bp introns (Fig. 1A). Similarly, the rtMSTN-2b is also organized into three exons with 346, 320, and 381 bp (partial), respectively, and are separated by 158- and 239-bp introns (Fig. 1A). The 3′-UTR of rtMSTN-2a was also cloned by 3′ RACE using RNA and was determined to be 782 bp, which is considerably shorter than the 3′-UTRs of the rtMSTN-1 genes (13). A partial 3′-UTR of rtMSTN-2b was also cloned and found to be indistinguishable from that of rtMSTN-2a. Therefore, the exact size of rtMSTN-2b's 3′-UTR could not be determined.

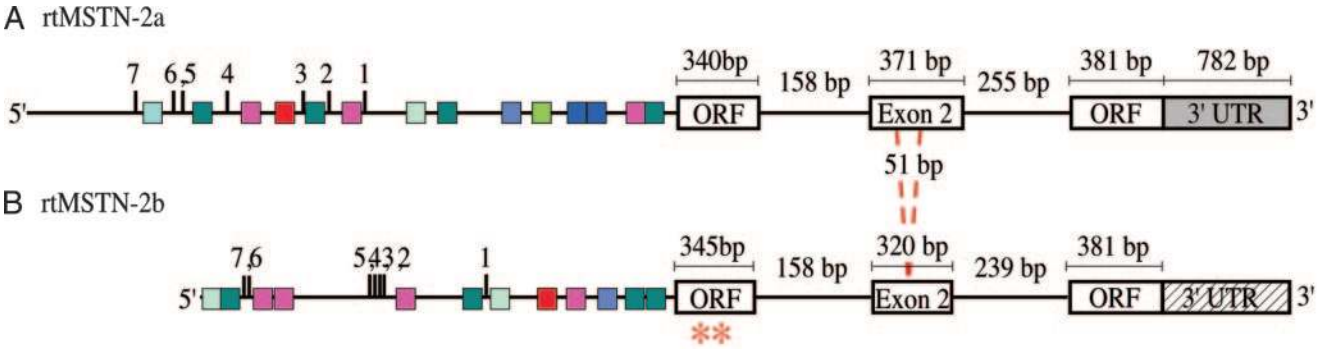
In silico analysis of rtMSTN-2a and -2b promoters

Subsequence analysis of the upstream 2.4- and 1.5-kb sequence for rtMSTN-2a and -2b, respectively, identified several putative muscle-specific transcriptional factor binding sites or *cis* regulatory elements. These regulatory elements include COMP1 (cooperates with myogenic proteins), MEF2 (myocyte enhancer factor), SRF (serum response factor), TEF-1 (transcriptional enhancer factor 1), MyoD (myogenic differentiation), and Muscle sp Mt site (Fig. 1). Each promoter also contained a muscle-specific TATA box and several putative E-boxes. Most importantly, a MyoD binding site and several MEF2 sites were identified, although the MyoD site was found only in the rtMSTN-2a promoter (−1658 and −1644). The presence of these putative sites is particularly intriguing because both regulate myostatin expression in different mammalian systems (31–33).

Sequence analysis

The coding sequences of both genes are similar (Figs. 2 and 3), although rtMSTN-2b's is smaller and lacks 51 bp from the second exon (Fig. 1), which corresponds to 17 amino acids present in the other rtMSTN proteins (Fig. 3). The putative rtMSTN-2 proteins possess all of the elements conserved in other myostatin proteins (Fig. 2). However, two in-frame stop codons were detected within the rtM-

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022



C rtMSTN-2a promoter

```
-2422                                          CA TTTCTATATT GCTACCCCAG
-2400 TATCTGCATG AACCATCAGG CCAAGTGTTT TTGGTTGACC TGCTGGACAG
-2350 AAAGAGAAGG AGGAATCGGA ACACTGCATT CAAATTCAGG TCTTAGTTAT
-2300 TCCATTGTAC TGGATCTAAT ACATATTAGC ATTTTACATA CATTCATAAG
-2250 CGTAATCATT TTTTATGACA TACTGTGAAA AACTCTCTTG GCTGCTGGCT
-2200 CTGTCACTTT CAGACATGCG CAGAGCAGAC TGAAAGGGGA AGGGTAGCTC
-2150 TTGACTTGAA CCACAGGGGT TCTCTGTAAA GAGAGAGTAA TCCAAAAGGC
-2100 ACAGACACAC CCCTTGACTT TCAATTGGCA CCCTTGACTT GTGTGTATTC
-2050 TCTCCTGATT GGTCTCTGTG GTGCTTAGTC AATGGGAACT CCGTTGCCGG
-2000 CCCCATCATA ACGTTTTTTA CACAGTCTGG CAGTCTGACT AAAGTGCCAG
-1950 AGGTATGAGG AGAGACATCC TCCTTTGTAT TTGTGGGAAC AAATATCTCC
-1900 TAACACTTTG GATCCTATTC TTGGACAGAT AGAAGGCACA ATGCGTCAAT
-1850 GCAAAGAAAA AAATACTAAT GACTGTCCAT GAGTAACCTC AACCTTGTGG
-1800 GTCTGTGGGT GTTTGGGGAA ATAAAGTGCC AACTAAATTC TACCAGTGAC
-1750 GAGTCTCTCA TGTTAAAAAA GAGAATAATT GAGTTAATAA AGCCGCATAC
-1700 AAACATGTCT CTTTTTTGCC TTCTTGAGTA AGGCAGCTCC AAAATGCAGG
-1650 TGTTCAAGCC TAGCTCAGAT CTTTCTGTGG TGGTGGGGCA GCCAGCGGAA
-1600 AATATGAAGG GTAGATGTTG GTGATATTCT CTAGTTGCGC CGTGGTTAGC
-1550 CCTGTGTTCT GTCACTCATG GGGAAGTCTA AGGGTAGACC TCGAAAATTC
-1500 AAATATAATA AAATCTAATT TTATTGGTCA CATACACATG GTTAGCAGAT
-1450 GATAGTGTGA ATGTAGTGAA ATGCTTGTGC TTTGACAATG CAGCAATATC
-1400 AACAAGTAAT CTAACAATTC CACAACAACT ACCTAATACA CACAAACCTA
-1350 AGTAAAGGGA TGGCTGGGCA CTTTAAACAC TCTGTGAAGT GCCACCTTCC
-1300 TGTTCAAGTG AGCACAGCAC AACAAGGTGA GTGCAAAAAT GTATTGTATG
-1250 CTGCTGGTAT AAATTATGTA ATATGCCAGG GATATATGTA CACTGTAGCT
-1200 AAGAAAGTAA TACTAAGTGT ATGTTGTGTA GTAAGATGTT AGTAGCCCAT
-1150 GTGCCTCACC CTAATAATTT GGTCTATGTA CCCCTCTTAA TTTTGCCTAC
-1100 TGTTCTGACT TGGTGGTACA CATGTAGCCT ATAACCTGTT TTAGAGAAAT
-1050 GTAATCATCA AATTTTGTAA GAGCTTTCAT TGTCTGCTTA TATGGCCCCT
-1000 TTATTTATCT GACGGTTCTG ACTTGGTGTA CAGGGAGAAC ACTGTAAGAA
-950 CGGCTCATGT TCTGAATTCT GTTGCTGTAC ATTTTAAAAG TGCTAAACAA
-900 ATAGTTAAAT TGACTACGTC CCTCCTAGTT CGCTCATTAA TGTCATTAAT
-850 TACGAATGGC CTCTTATTCG CTTGTCGTCC CTTTATACCA TAGTTTGTAC
-800 ATCTCATTTG TCATTAGAAA TCACATTTGT TTAAGCAAGT CAGCCATATC
-750 ACTTATGTTT TTTAAAAGGC AGTAAATGAG GCTGAATTAA CTGTTTCCCT
-700 GCCAGACAAG GATCTGCTGA TGACCAGTTG TAGCAGTGGT AAGATGTTAG
-650 GACTGCCATT GGGACAGCTT TATGTAGGCC CAAACAGTTT GTGGGGACAG
-600 TTTGTCACCG TTATAGTGCA ATTAATGTAT TGTTTAGTGT TGTGTTGTGT
-550 AATGGCTTTG CTGGCATGCA TCCCACATTT ATTTTATTTT TTTGCCCCAC
-500 CAAGATGTAC ATGCTAAAAT CGCCACTGCA CATTAGCTAT TTAATGTAAC
-450 AGTTTTTGTG ACAAAATTAC ACTAGTGTTG AAAATGCGGT GGAATAAAAA
-400 AACGTACGTG AAAAAGTAGG CGTACATTTT GTGTGCACCA CGTCATCGGG
-350 CACTGGTTTG TATCCACAAC CAGTCATTTT GGTGGAAACA CACCACAGGT
-300 GGAAAAATGT TATATTTTCT TTAAGTGGAG TCTAGAATAT TCGCATGAAA
-250 ATCTGTTGCC AATAGGATGG AAACATAGCT ACAGATAGCG CATGCTTCCA
-200 GTAATTGGCC TATGGCATTT TAAAGCTGTC TATCTCTTTA AAACGGAAAC
-150 AAAACATTCA TCTCTAATGC AGCCAATCAG AATCATAAAA GGGAGATGGG
-100 GAGATTATAA AAAATAGTAA TTAAAATATT TGTCACTGTG TTATATAGGC
-50  CTGGAATAGG CTATTCTGTC CAAAGTGGGA TTAAAAAGAC AAGTGACAGG
```

D rtMSTN-2b promoter

```
-1497   GGGGCGG AGGACGGAAT TTTCGGGACA TTTTATAAAT CCCGACAGAT
-1450 CATTTGTTCG ATTGCATAGT GGGATCTTTT TGTGTCAGTC AAATGAATAA
-1400 TGCGATAAAT GTTGGTGGAA ACGCCTTTAT GTGCAAATAT TGATATAATA
-1350 ACCATCATAT AAAGTAAACT TGGAGTCATG CGATGATATG GTGTGTGATG
-1300 GTCCTTCTAC TTCTACAAGT TTTAATGACT CCACTCCAAC CTAAGTGTAT
-1250 GTTAATTAAC TTACGACTTC AACTGTATGT ATACTGTAGC TAAGAAAGTA
-1200 ATACTAAGTG TATGTTGTGT AGTAAAATGT TAGTAGCCCA TGTGCCTCAC
-1150 CCTAATAATT TGGTGTATTT TCACCTCTTA ATTTCACCTA ATGTTCTGAC
-1100 TTGGTGGTAC ACACGTAGCC TATAACCTGT ATTTGAGAAA TGTAATCATC
-1050 CAATATTGTA AGAGCTTTCA TTATTTATTC TACGGTTGTG ACTTGGTGTA
-1000 CAGGGAGAAT ACTGTAAGAA CGGCTCATGT TCTGAATTTT GTTGCTGTAC
-950  ATTTTAAAAG TGCTAAACAA ATAGTTATAT GGACTACGTC CATCCTAGCT
-900  CACTCATTAA TGTCTTAATC GAAATTATGG ATTACCTCTA ATCCACTTGT
-850  CATCCCTTAT GTCACTTGTC ATCCCTTGTC ATCCCTTATG TACATCTCAA
-800  TTGTCATTAG AAACCACATT TGTTTAAGCA AGTCAGCCAT ATCAGCGATG
-750  ATTTTTTAAA AGGCAGTAAA TGAGGCTGAA TGAACTGTTC TGCTGCCAGA
-700  CAAGGCTCCG CTGATAGCTA GGTGTAGCAG TGGTAAGGTG TTGGGGACTG
-650  CTGTTAGGAC AGATTTATGT AGCCCCTATC AGTTTGTGGG GACCGTTTGT
-600  CACCATTATA GTGCAATTAA TGAATTGTTT AGTATTGTGT AGTAGCTTTG
-550  CTGGCATGCA TCCCACATTT TTTTTTTTTT TTTTGCCCCA ACAAGATTTC
-500  CATGCTAAAA TTGCCACAGA ACATTTGTA TTTAATGCAA CAGTTTTTGT
-450  GACAAAATTA CGGTAGAGTT GAAAATGAGA TGGAACCACA ATGGACTTTA
-400  GATTTTTATT CTGTACATAA AAAAACGTGC GTGAAAAAGT AGGCGTACAT
-350  TTTGTGTGCA CTATGTCATC AGGCACTGAT TTGTATTCAC AACAAGTCAT
-300  TTTGGTGGAA ACACACCCAA TGGAACATAA TATTTTCTTT ATGTGGATTC
-250  TACAATATTC CCATGAAATC TGTTGCCAAT AGGATGGAAA CATAGGCCAA
-200  TTACTGGAAG CATGTACTAT AGCATTTTAA AACAGCCTCT TTAAAACTGA
-150  ATCAAAACAT TCAACTCTAA TGCAGCCAAT CAGAATCATA AAAGGGAGA
-100  TTATTTAAAA AATTGTCATT CAAAATACTT GTCACTGATG TTATATAGAC
-50   CTGGAATATG CTATTCTGTA CAAAGTGGGG TTAAAAAGAC AAGTGACAGG
```

E Promoter elements

Elements	Location (bp) rtMSTN2a	rtMSTN2b
Comp1	-879/-899	-302/-323
MEF 2	-5/27,-392/414,-772/794, -1315/1337	-4/27,-82/105,-490/503, -1453/1475
Muscle sp Mt Site	-458/-466	-466/-475,-1477/-1486
Muscle TATA	-169/-185	-161/-178
Myo D	-1644/-1658	
SRF	-17/35,-685/703,-1001/1019	-249/268,-690/709 -1305/1323,-1354/1372
TATA box	-82/-98,-104/-120	
TEF-1	-136/-152	

FIG. 1. Genomic structure and organization of the rtMSTN-2a and -2b genes. A and B, Maps of rtMSTN-2a and -2b genes and putative myogenic *cis* regulatory elements within the promoter regions starting from the initiator. Exons are boxed with the open reading frame (ORF) in *white*, the UTR of rtMSTN-2a in *gray*, and the putative 3′-UTR of rtMSTN-2b *hatched*. Each individual *cis* element is placed relative to its position within the promoter and is color-coded as indicated in E. Putative E-boxes are numbered. The 51-bp cassette missing from the second exon of rtMSTN-2b is indicated by *red lines*. The *asterisks* below the first exon in B indicate in-frame stop codons. C and D, Sequence of the promoter regions for rtMSTN-2a and -2b, respectively, with color-coded *cis* elements. *Boxed* are consensus sequences [CAN(T/A)TG] for E-boxes. Nucleotide positions correspond to the start codon. E, Key to the color-coded promoter elements in A–D and their corresponding nucleotide positions.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

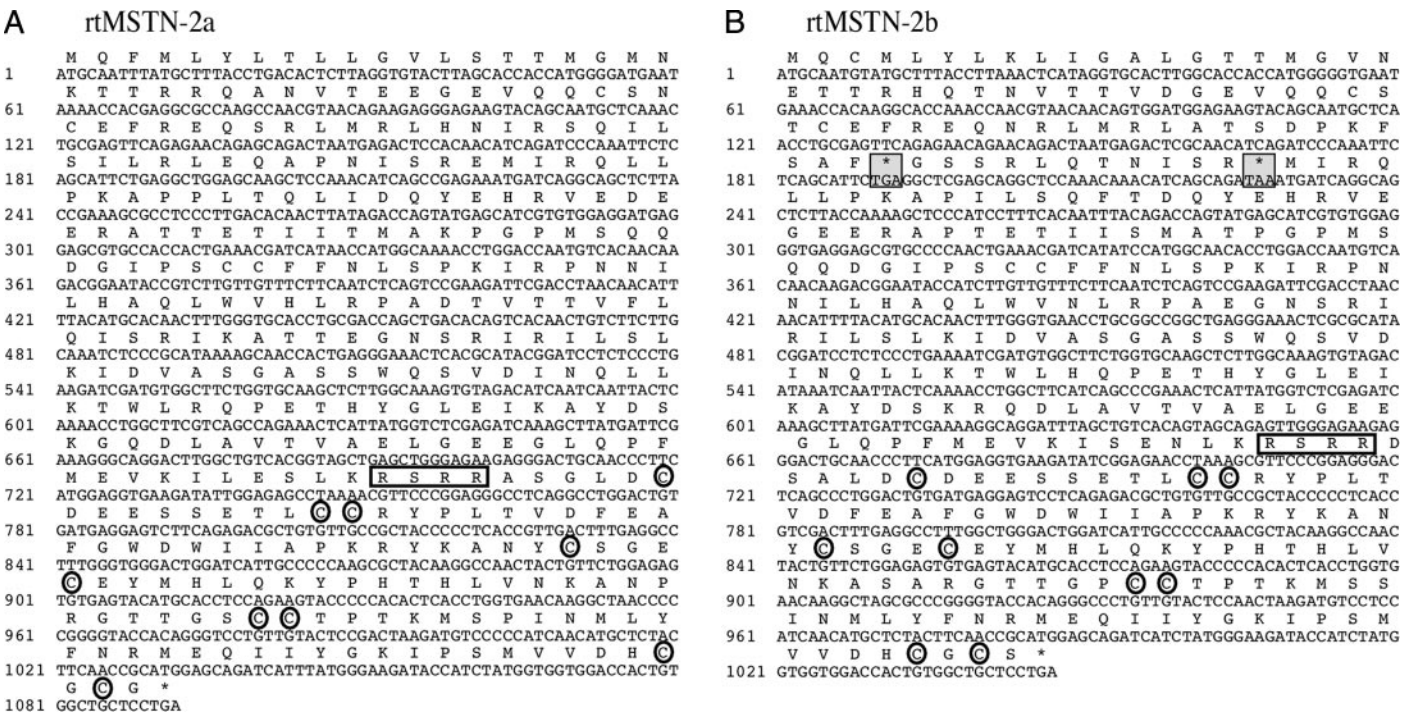


FIG. 2. Annotated cDNA sequences of rtMSTN-2a and -2b. Nucleotide positions are *numbered*, conserved cysteine residues in the C-terminal regions are *circled*, and the proteolytic processing sites are *boxed*. The in-frame stop codons in rtMSTN-2b are *highlighted* by *gray boxes*.

STN-2b ORF (Fig. 2B), which were coded by the first exon (Fig. 1B). A multiple sequence alignment of the four rtMSTN amino acid sequences, including the stop codons in rtMSTN-2b, revealed several unique regions including a nine-residue epitope that is missing from the rtMSTN-2 sequences (Fig. 3). Individual comparisons indicate that rtMSTN-2a and -2b are 81% identical overall and 94% in the conserved C-terminal domain, whereas rtMSTN-2a is 66 and 91%, respectively, to the rtMSTN-1 proteins. However, the C-terminal domains of the rtMSTN-2 sequences contained two notable differences: a methionine substitution for lysine 354 (rtMSTN-1a) and a histidine substitu-

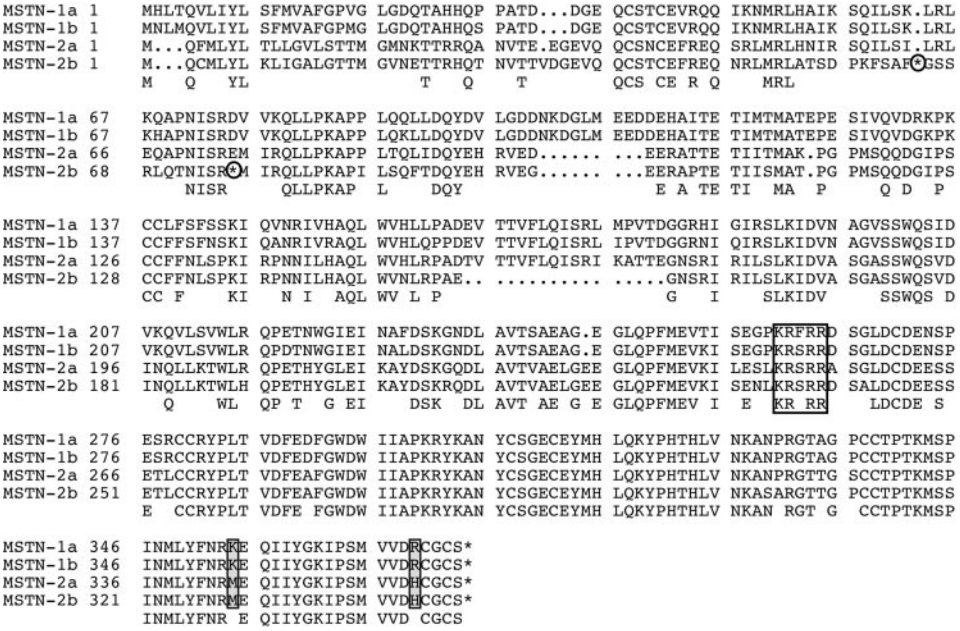


FIG. 3. Alignment of rtMSTN-1a, -1b, -2a, and -2b amino acid sequences. Alignments were performed using VECTOR NTI Align X. Identities between proteins are shown in the consensus whereas gaps (. . .) and stop codons (*) are indicated in the individual sequences. The in-frame stop codons in rtMSTN-2b are also *circled*. The conserved proteolytic RXXR site is *boxed*, and the residues *highlighted* in *gray* indicate notable differences in the conserved carboxy-terminal bioactive domain.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

tion for arginine 369 (Fig. 3). This particular arginine is replaced by either histidine (seabream, shi drum, and fugu) or leucine (zebrafish) in other MSTN-2 proteins.

Phylogenetic analyses and comparative exon mapping

The current Bayesian inference analysis of different fish myostatin sequences produced only minor differences from the previous maximum likelihood analysis of the entire myostatin gene family (9). Multiple independent analyses converged on the same posterior probability values for branches, suggesting that convergence and mixing are occurring. Most of the major internal branches are well supported, and the consensus tree clearly supports the placement of the newly discovered proteins within the MSTN-2 clade. This not only confirms the identity of rtMSTN-2a and -2b as legitimate MSTN-2 orthologs but also validates our previous phylogenetic analysis and suggests that other salmonid MSTN-2 genes likely exist as well. Comparative mapping of coding regions of different myostatin genes reveals a similar organization in all species. Codons flanking the first and the second exon are highly conserved among the fishes despite minor differences in rtMSTN-2a and -2b, whereas the codons flanking the second and third exon are highly conserved among all vertebrate species (Fig. 4B). The previously identified consensus sequence (13) for the codons flanking the first and second exon, MAT(E/K) | PXXI (X = any amino acid), was slightly different in the rtMSTN-2 proteins [MAT(E/K) *vs.* MA(T/K)], whereas the consensus between the second and third exons, (G/E)(E/D)GL | XPFϕ (ϕ + hydrophobic amino acid), was found in both rtMSTN-2 coding frames. This further indicates a high level of conservation of myostatin genes as reflected in both gene sequences and organization.

Qualitative analysis of rtMSTN-2a and -2b expression

Amplification of rtMSTN-2 transcripts in different adult tissues was performed using three primer sets. A schematic of hybridization sites and amplicon sizes are shown in Fig. 5A. In most tissues, both transcripts were expressed at low or undetectable levels except for brain where rtMSTN-2a was readily detected (Fig. 5, B and C). However, amplicons corresponding to both spliced and unspliced transcripts were detected for both rtMSTN-2a and -2b and were confirmed by sequencing. PS-I, which is specific for rtMSTN-2a, amplified both spliced and unspliced transcripts in most tissues, although the amplified variant appeared to be more abundant. However, only the spliced variant was detected in brain. By contrast, only unspliced rtMSTN-2b transcripts were detected regardless of the tissue. This was confirmed using two gene-specific primer sets that spanned either the first (PS-II) or second (PS-III) intron. To rule out the possibility of gDNA contamination of our cDNA, we amplified β-actin using gDNA or gill cDNA as template and intron spanning primers (Fig. 6A). However, the larger amplicons produced with gDNA template were not produced with

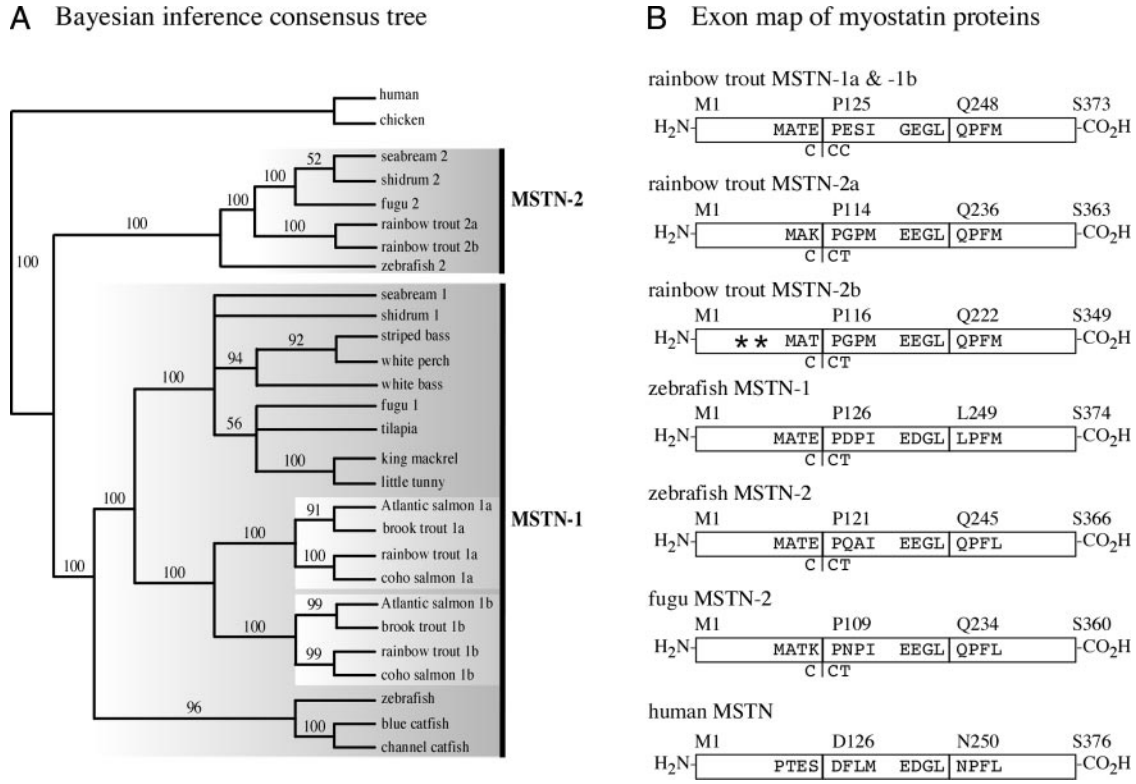


FIG. 4. Phylogenetic analysis and comparative exon mapping of the myostatin proteins. A, Bayesian inference analysis was performed on a single matrix composed of homologous myostatin sequences from the indicated species. Two independent analyses were performed and produced identical results. Bayesian posterior probability values are shown above each branch when greater than 50%. The individual clades are *shaded* and *labeled*. B, Comparative exon mapping of the myostatin proteins. All the vertebrate myostatin genes characterized to date are organized into three exons. The first amino acid coded by each exon and the last amino acid of each protein (sequential *white boxes*) are shown above. Amino acids coded by adjacent regions to each exonic boundary are shown inside the *boxes*. In all the fish genes, the codon of the proline residue located at the first exonic boundary is partially coded by nucleotides in the first and second exons as shown. The in-frame stop codons in rtMSTN-2b are indicated with *asterisks*.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

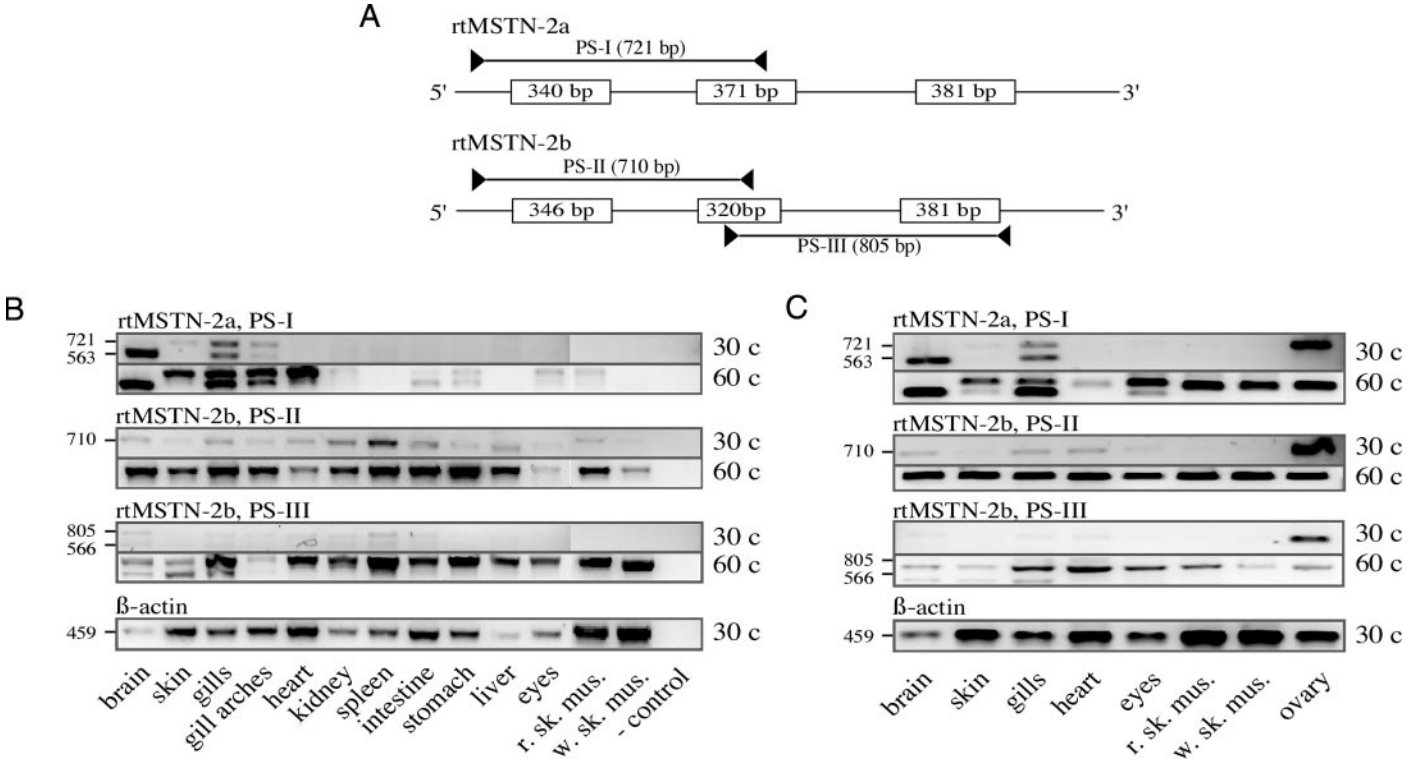


FIG. 5. Expression of rtMSTN-2 transcripts. A, Primer annealing locations (*triangles*) on rtMSTN-2 gene maps used for expression analysis. Exons are *boxed* and amplicon sizes (*lines* connecting primers), resulting from the amplification of gDNA or unprocessed transcripts are indicated. B, Expression analysis of rtMSTN-2a and -2b and β-actin in different male rainbow trout tissues using the primer sets shown above. Initial amplification for 30 cycles was performed, and 5 μl of the product was then used for another 30 cycles of PCR. Exact amplicon sizes are indicated and were verified by sequencing. C, Gene expression in different female tissues. r. sk. mus., Red skeletal muscle; w. sk. mus., white skeletal muscle.

cDNA even after 60 cycles. Amplifying gill cDNA with primers that recognize both rtMSTN-2 transcripts produced unspliced amplicons for both genes and spliced for rtMSTN-2a (verified by sequencing). To further illustrate that these amplicons originated from RNA, samples were treated with or without RNase before cDNA synthesis and were eventually amplified with primers specific to rtMSTN-2a or -2b or both transcripts (Fig. 6B). Regardless of primers, all of the amplicons produced in the control samples either disappeared or were significantly reduced with RNase treatment. Taken together, these results indicate that the detection of unspliced transcripts for both rtMSTN-2 genes in different tissues is not due to gDNA contamination. The expression pattern shown represents a single male fish; however, it was additionally confirmed in a female fish and by using the RNA panel described herein. Only unspliced transcripts were present in ovaries (Fig. 5C) and testes (data not shown), and no evidence of sexually dimorphic mRNA processing was detected in any tissue. Nevertheless,

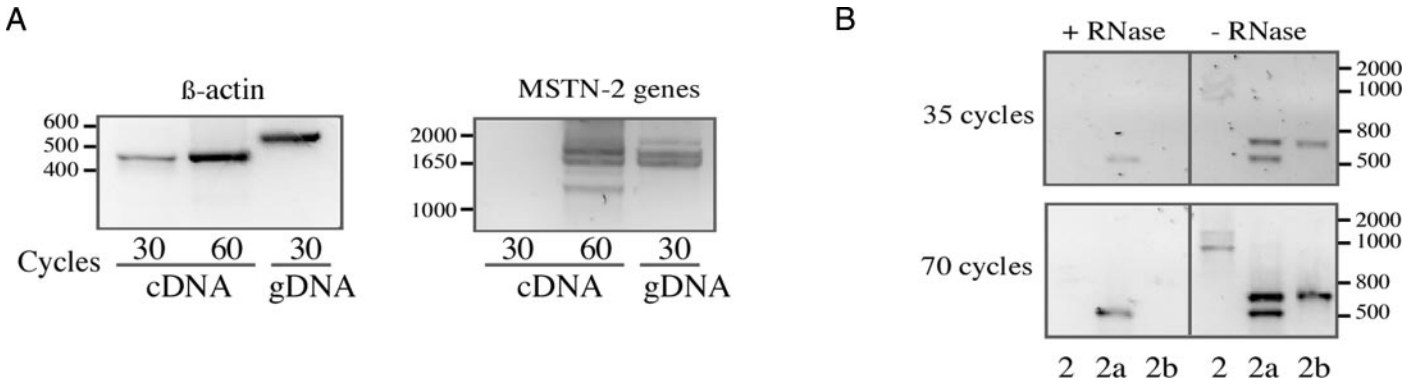


FIG. 6. Pre-mRNA processing of rtMSTN-2 transcripts. A, Amplification of β-actin and rtMSTN-2 genes from gill cDNA and gDNA. Total RNA was treated twice with DNase, reextracted, and used to synthesize cDNA. Primers for β-actin and those that recognize both rtMSTN-2 genes (forward primer of PS-I and reverse primer of PS-III) were then used for 30 and 60 cycles of PCR. The same primers were also used to amplify gDNA. B, Expression of MSTN-2 transcripts at 35 and 70 cycles using cDNA synthesized from RNase-treated and nontreated gill RNA. Separate but equal RNA pools were treated with RNase and nuclease-free water, reextracted, and used for cDNA synthesis before PCR (2, all MSTN-2 primers; 2a and 2b, gene-specific primers).

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

this is the first report of myostatin splice variants for any species and possibly reflects a novel mechanism for limiting rtMSTN-2a influences to the brain and for further silencing rtMSTN-2b.

Embryonic and adult tissue expression

Embryonic expression of rtMSTN-2a remained very low throughout development and did not differ significantly between sample times (data not shown). In adults, expression of rtMSTN-2a was detected in all tissues sampled and was highest in brain, which was 10-fold higher than in testis, spleen, and red and white skeletal muscle and at least 100-fold higher than all other tissues (Fig. 7A). Qualitative analysis of rtMSTN-1a, -1b, and -2a expression in different brain regions revealed gene-specific expression patterns (Fig. 7B). Although rtMSTN-1b was similarly expressed in all regions (myelencephalon, cerebellum, proencephalon, and optic lobe), rtMSTN-1a and -2a expression was barely detected in the proencephalon and appeared highest in the cerebellum, albeit using a qualitative assay. Comparing the normalized expression of all the three genes in selected tissues revealed that rtMSTN-1b expression was consistently highest (Fig. 7C). In the brain, it was 12-fold higher than rtMSTN-2a expression, which in turn was 8-fold higher than that of rtMSTN-1a. In red and white muscle, however, rtMSTN-2a expression was 6- to 10-fold lower than rtMSTN-1a expression and over 100-fold lower in eye.

Discussion

An early genome duplication event before the teleost radiation, but after the divergence of ray- and lobe-finned fishes, resulted in multiple copies of many genes (17, 18). A second event specifically in the salmonids produced additional copies of some genes (19) in this family. To date, two myostatin genes have been identified in zebrafish (9, 27), brook trout (34), rainbow trout (15), Atlantic salmon (12), coho salmon (35), seabream (16, 36), shi drum (37), and fugu (Fernandas J. M., J. R. Kinghorn, and I. A. Johnston, GenBank accession nos. AY445321 and AY445322). However, all the salmonid genes are actually MSTN-1 paralogs because no MSTN-2 genes have been identified in these species until now. Indeed, a phylogenetic analysis (Fig. 4A) and sequence alignment (Fig. 3) clearly indicate that rtMSTN-2a and -2b are legitimate MSTN-2 paralogs. Organization of the newly discovered rtMSTN-2 genes (Fig. 1, A and B) is highly conserved with three exons, identical to rtMSTN-1 genes and other vertebrate genes characterized to date. The second exon/intron boundary, which separates the coding regions for the latency-associated peptide from that of the bioactive domain of both rtMSTN-2 genes, is also highly conserved (Fig. 4B). However, comparable introns in both rtMSTN-2 genes have similar sequences but are significantly smaller than their rtMSTN-1 counterparts (13), which is further indicative of their orthologous relationship. These data validate our previous phylogenetic analysis (9) and strongly suggest the presence of additional myostatin genes in other salmonid species. Whether or not all genes are functionally active, similarly expressed, or lost/silenced to pseudogenization in all salmonids remains to be determined. These studies are dependent upon the characterization of genes from additional salmonid species and possibly from other basal fish groups. Thus, comparative mapping of different myostatin genes (Fig. 4B) and the identification of consensus sequences for exon boundaries should prove invaluable.

Subsequence analysis of rtMSTN-2a and -2b promoter regions identified several putative *cis* regulatory elements that could contribute to the myogenic process. Some of these promoter elements were also found in other fish myostatin genes including brook trout (14) and zebrafish (9). However, a putative MyoD binding site was found only in promoters for zebrafish MSTN-2 and rtMSTN-2a. In mammals, MyoD and

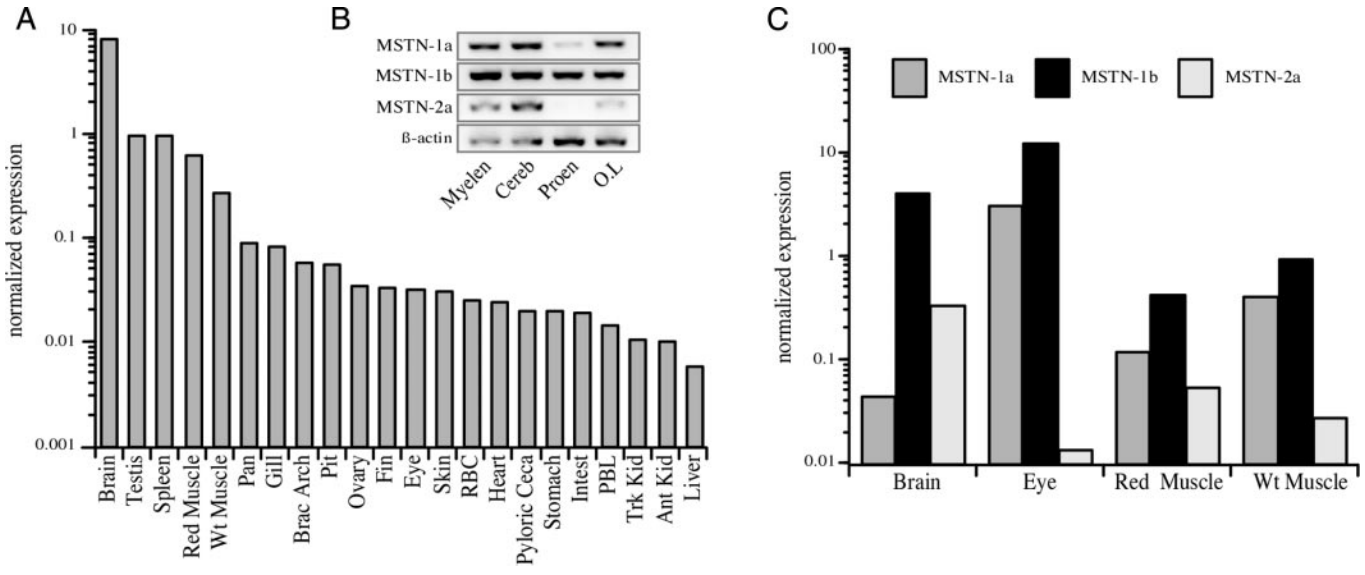


FIG. 7. Comparative expression analysis of different rainbow trout myostatin genes in different adult tissues. A, Real-time RT-PCR was used to quantify rtMSTN-2a expression in different adult tissues. Assays were repeated twice and run in triplicate from pooled tissue samples. Ant kid, Anterior/head kidney; PBL, peripheral blood lymphocytes; Pit, pituitary; Trk, trunk; Intest, whole intestine; RBS, red blood cells; Wt, white. B, Qualitative analysis of rtMSTN-1a, -1b, and -2a expression in different brain sections. Cereb, Cerebellum; Myelen, myelencephalon; O.L., optic lobe; Proen, proencephalon. C, Real time RT-PCR was used to quantify and compare normalized expression of rtMSTN-1a, -1b, and -2a in brain, eye, and red and white skeletal muscle using the pooled tissue samples in A.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

MEF2 are critical to the differentiation of skeletal muscle and induce myostatin expression (31–33). However, rtMSTN-2a expression was relatively low in skeletal muscle when compared with brain expression (Fig. 7), which is consistent with tissue expression levels of other MSTN-2 genes (16, 38). MyoD is also expressed in brain and helps in the early differentiation of neurons (39). Nevertheless, MyoD binding sites alone cannot explain the possible preferential expression of MSTN-2 genes in brain because MSTN-1 gene expression also occurs in the tissue. Indeed, MSTN-1a and -1b are both differentially expressed in brook trout (34) and in rainbow trout brains (Fig. 7B). A recent computational analysis of the structural changes that occurred throughout the evolution of the myostatin gene family failed to identify markers of positive selection in any taxonomic group (40). Thus, diversity in muscle mass among vertebrates may have evolved from changes in the *cis* regulation of myostatin gene expression rather than from changes in the protein structure *per se* (40).

The rtMSTN-2 genes are highly similar to each other, and their coding sequences are shorter than that of the rtMSTN-1 genes, which is not necessarily true in other species (9, 16, 37). Like other myostatin genes, the bioactive domains of both are entirely coded by the third exons, and each coding sequence contains the modular architecture common to other TGF-β and myostatin gene family members. A prominent difference between the two genes is that rtMSTN-2b lacks a 51-bp cassette found in rtMSTN-2a (corresponding to 17 amino acids) and contains two in-frame stop codons within the first exon. Qualitative expression analysis of rtMSTN-2b indicates that neither intron is spliced from the primary transcript regardless of tissue (Fig. 5). Both introns contain in-frame stop codons, which prohibits the production of functional protein even in the presence of alternative translation start sites. The presence of stop codons in the coding sequence and in the unspliced intronic regions indicates that the rtMSTN-2b gene is a pseudogene. Both spliced and unspliced variants of rtMSTN-2a were also detected in all tissues surveyed except brain, which expressed only the correctly spliced transcript. The pre-mRNA splice sites for both rtMSTN-2 transcripts are identical; thus, the presence of correctly spliced rtMSTN-2a in some tissues is likely due to higher levels of rtMSTN-2a expression rather than differential processing. Whether or not MSTN-2b orthologs from other salmonid species are also pseudogenes remains to be determined. Furthermore, the exact timing of rtMSTN-2b pseudogenization or of any other salmonid MSTN cannot be estimated until other MSTN-2 genes are isolated from these species.

The bioactive C-terminal domain of myostatin is highly conserved in all species. In fact, the amino acid identity shared between most fish and mammalian species is 88% (6). These comparisons were made with MSTN-1 orthologs, and although the bioactive domains of MSTN-2 proteins are also well conserved, notable differences exist. These include methionine for lysine and histidine for arginine substitutions in the C terminus (Fig. 3). The latter conserved substitution is also seen in other fish MSTN-2 proteins (fugu, seabream, and shi drum) and may be unique to many MSTN-2 orthologs. The former, however, occurs in a region hypothesized to have contributed to enhanced musculature in domesticated and wild bovids (4, 41, 42). Myostatin's actions are mediated by binding to the activin receptor ActRIIb (43, 44) because in fact any perturbation in ActRIIb signaling increases muscle mass (44–46). The carboxy terminus of activin is nearly identical to that of myostatin and is critical to ActRIIb signaling (47). Any change in myostatin's structure could potentially impact the activation of ActRIIb. Future functional studies are therefore needed to determine whether MSTN-1 and -2 proteins bind ActRIIb equally or to other receptors.

Quantitative analysis of rtMSTN-2a expression in different stages of embryonic development revealed no significant differences during any stage (data not shown), suggesting that rtMSTN-2a may not play a significant role throughout development. This is consistent with the expression of MSTN-2 orthologs in other fish species including seabream (16) and zebrafish (9, 38), although MSTN-2 expression did appear to rise slightly during early somitogenesis in zebrafish. Nevertheless, this is in stark contrast to the developmental expression patterns of other fish MSTN-1 orthologs (13, 14, 38, 48), particularly rtMSTN-1a and -1b, which increased significantly during somitogenesis and after hatching and yolk sac absorption (13). In adult tissues, expression of rtMSTN-2a was comparable to that of MSTN-1a and -1b; however, it was at least 10-fold higher in brain than in any other tissue. In addition, brain was the sole tissue that expressed only the correctly spliced transcript. Teleost fish exhibit an enormous capacity to replace damaged neurons in the central nervous system, a process that involves a subdivision of the cerebellum (49, 50). Each myostatin gene was differentially expressed in different brain sections, although rtMSTN-2a levels were highest in the cerebellum (Fig. 7B). Qualitative analysis of MSTN-2 expression in seabream tissues indicates that it occurs primarily in brain (16), which is consistent with its expression in zebrafish, although more sensitive and quantitative assays detected low levels of MSTN-2 expression in other zebrafish tissues as well (51). These data together suggest that the biological actions of rtMSTN-2a, and possibly other MSTN-2 orthologs, may be limited primarily to the brain and may help regulate neurogenesis.

The rainbow trout is a unique model organism and representative of the salmonidae family. A better understanding of myostatin genes in this fish will add significantly to our understanding of the gene family's evolution. Although similar to other myostatin genes in many ways, both subtle and obvious differences distinguish the trout genes. Whether or not pseudogenization or alternative splicing also occurs among MSTN-2 genes of other salmonids or whether their expression occurs primarily in neural tissue remains to be determined. These studies will nevertheless be aided by the characterization of MSTN-2 genes from other species and by additional computational and functional analysis.

Acknowledgments

We acknowledge Dr. Caird Rexroad III, Kristy Anderson, and Roseanna Athey for their assistance with embryonic sampling and tissue collection.

Received September 22, 2006. Accepted January 26, 2007.

Address all correspondence and requests for reprints to: Buel D. Rodgers, Ph.D., Department of Animal Sciences, 124 ASLB, Washington State University, Pullman, Washington 99164-6351. E-mail: danrodgers@wsu.edu.

This work was supported by a grant from the United States Department of Agriculture (2004-34468-15199) to B.D.R.

Disclosure Statement: The authors have nothing to disclose.

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022

References

1. **Lee SJ** 2004 Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol 20:61–86
2. **McPherron AC, Lee SJ** 1997 Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA 94:12457–12461
3. **Kambadur R, Sharma M, Smith TP, Bass JJ** 1997 Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 7:910–916
4. **McPherron AC, Lawler AM, Lee SJ** 1997 Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387:83–90
5. **Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ** 2004 Myostatin mutation associated with gross muscle hypertrophy in a child. N Engl J Med 350:2682–2688
6. **Rodgers BD, Weber GM, Sullivan CV, Levine MA** 2001 Isolation and characterization of myostatin complementary deoxyribonucleic acid clones from two commercially important fish: *Oreochromis mossambicus* and *Morone chrysops*. Endocrinology 142:1412–1418
7. **Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ, Bass JJ** 1999 Myostatin, a transforming growth factor-β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol 180:1–9
8. **Ji S, Losinski RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FF, Spurlock ME** 1998 Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am J Physiol 275:R1265–R1273
9. **Kerr T, Roalson EH, Rodgers BD** 2005 Phylogenetic analysis of the myostatin gene sub-family and the differential expression of a novel member in zebrafish. Evol Dev 7:390–400
10. **Radaelli G, Rowlerson A, Mascarello F, Patruno M, Funkenstein B** 2003 Myostatin precursor is present in several tissues in teleost fish: a comparative immunolocalization study. Cell Tissue Res 311:239–250
11. **Amali AA, Lin CJ, Chen YH, Wang WL, Gong HY, Lee CY, Ko YL, Lu JK, Her GM, Chen TT, Wu JL** 2004 Up-regulation of muscle-specific transcription factors during embryonic somitogenesis of zebrafish (*Danio rerio*) by knockdown of myostatin-1. Dev Dyn [Erratum (2005) 234:1064] 229:847–856
12. **Ostbye TK, Galloway TF, Nielsen C, Gabestad I, Bardal T, Andersen O** 2001 The two myostatin genes of Atlantic salmon (*Salmo salar*) are expressed in a variety of tissues. Eur J Biochem 268:5249–5257
13. **Garikipati DK, Gahr SA, Rodgers BD** 2006 Identification, characterization, and quantitative expression analysis of rainbow trout myostatin-1a and myostatin-1b genes. J Endocrinol 190:879–888
14. **Roberts SB, Goetz FW** 2003 Myostatin protein and RNA transcript levels in adult and developing brook trout. Mol Cell Endocrinol 210:9–20
15. **Rescan PY, Jutel I, Ralliere C** 2001 Two myostatin genes are differentially expressed in myotomal muscles of the trout (*Oncorhynchus mykiss*). J Exp Biol 204:3523–3529
16. **Maccatrozzo L, Bargelloni L, Cardazzo B, Rizzo G, Patarnello T** 2001 A novel second myostatin gene is present in teleost fish. FEBS Lett 509:36–40
17. **Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH** 1998 Zebrafish hox clusters and vertebrate genome evolution. Science 282:1711–1714
18. **Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly JS, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL, Talbot WS** 1998 Vertebrate genome evolution and the zebrafish gene map. Nat Genet 18:345–349
19. **Brunelli JP, Robison BD, Thorgaard GH** 2001 Ancient and recent duplications of the rainbow trout Wilms' tumor gene. Genome 44:455–462
20. **Essex-Fraser PA, Steele SL, Bernier NJ, Murray BW, Stevens ED, Wright PA** 2005 Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. J Biol Chem 280:20268–20273
21. **Gahr SA, Rodriguez MF, Rexroad 3rd CE** 2005 Identification and expression profile of the ID gene family in the rainbow trout (*Oncorhynchus mykiss*). Biochim Biophys Acta 1729:64–73
22. **Phillips RB, Zimmerman A, Noakes MA, Palti Y, Morasch MR, Eiben L, Ristow SS, Thorgaard GH, Hansen JD** 2003 Physical and genetic mapping of the rainbow trout major histocompatibility regions: evidence for duplication of the class I region. Immunogenetics 55:561–569
23. **Huelsenbeck JP, Ronquist F** 2001 MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755
24. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG** 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
25. **Lewis PO** 2001 Phylogenetic systematics turns over a new leaf. Trends Ecol Evol 16:30–37
26. **Swofford DL** 2001 PAUP: phylogenetic analysis using parsimony, version 4.0b6. Laboratory of Molecular Systematics, Smithsonian Institution, Washington, D.C. Sunderland, MA: Sinauer
27. **Xu C, Wu G, Zohar Y, Du SJ** 2003 Analysis of myostatin gene structure, expression and function in zebrafish. J Exp Biol 206:4067–4079
28. **Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S** 1998 Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci USA 95:14938–14943
29. **Jeanplong F, Sharma M, Somers WG, Bass JJ, Kambadur R** 2001 Genomic organization and neonatal expression of the bovine myostatin gene. Mol Cell Biochem 220:31–37
30. **Stratil A, Kopecny M** 1999 Genomic organization, sequence and polymorphism of the porcine myostatin (GDF8; MSTN) gene. Anim Genet 30:468–470
31. **Spiller MP, Kambadur R, Jeanplong F, Thomas M, Martyn JK, Bass JJ, Sharma M** 2002 The myostatin gene is a downstream target gene of basic helix-loop-helix transcription factor MyoD. Mol Cell Biol 22:7066–7082
32. **Ma K, Mallidis C, Artaza J, Taylor W, Gonzalez-Cadavid N, Bhasin S** 2001 Characterization of 5′-regulatory region of human myostatin gene: regulation by dexamethasone in vitro. Am J Physiol Endocrinol Metab 281:E1128–E1136
33. **Salerno MS, Thomas M, Forbes D, Watson T, Kambadur R, Sharma M** 2004 Molecular analysis of fiber type-specific expression of murine myostatin promoter. Am J Physiol Cell Physiol 287:C1031–C1040
34. **Roberts SB, Goetz FW** 2001 Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. FEBS Lett 491:212–216
35. **Roberts SB, McCauley LA, Devlin RH, Goetz FW** 2004 Transgenic salmon overexpressing growth hormone exhibit decreased myostatin transcript and protein expression. J Exp Biol 207:3741–3748
36. **Maccatrozzo L, Bargelloni L, Radaelli G, Mascarello F, Patarnello T** 2001 Characterization of the myostatin gene in the gilthead seabream (*Sparus aurata*): sequence, genomic structure, and expression pattern. Mar Biotechnol (NY) 3:224–230
37. **Maccatrozzo L, Bargelloni L, Patarnello P, Radaelli G, Mascarello F, Patarnello T** 2002 Characterization of the myostatin gene and a linked microsatellite marker in shi drum (*Umbrina cirrosa*, *Sciaenidae*). Aquaculture 205:49–60
38. **Helterline DLI, Garikipati D, Stenkamp DL, Rodgers BD** 2007 Embryonic and tissue-specific regulation of myostatin-1 and -2 gene expression in zebrafish. Gen Comp Endocrinol 151:90–97
39. **Kablar B** 2002 Different regulatory elements within the MyoD promoter control its expression in the brain and inhibit its functional consequences in neurogenesis. Tissue Cell 34:164–169
40. **Pie MR, Alvares LE** 2006 Evolution of myostatin in vertebrates: is there evidence for positive selection? Mol Phylogenet Evol 41:730–734
41. **Rodgers BD, Weber GM** 2001 Sequence conservation among fish myostatin orthologues and the characterization of two additional cDNA clones from *Morone saxatilis* and *Morone americana*. Comp Biochem Physiol B Biochem Mol Biol 129:597–603
42. **Tellgren A, Berglund AC, Savolainen P, Janis CM, Liberles DA** 2004 Myostatin rapid sequence evolution in ruminants predates domestication. Mol Phylogenet Evol 33:782–790
43. **Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L** 2003 Myostatin signals through a transforming growth factor β-like signaling pathway to block adipogenesis. Mol Cell Biol 23:7230–7242
44. **Lee SJ, McPherron AC** 2001 Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98:9306–9311
45. **Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, Wright JF, Barker C, Ehrmantraut G, Holmstrom J, Trowell B, Gertz B, Jiang MS, Sebald SM, Matzuk M, Li E, Liang LF, Quattlebaum E, Stotish RL, Wolfman NM** 2005 Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc Natl Acad Sci USA 102:18117–18122
46. **Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ** 2002 Induction of cachexia in mice by systemically administered myostatin. Science 296:1486–1488
47. **Harrison CA, Gray PC, Fischer WH, Donaldson C, Choe S, Vale W** 2004 An activin mutant with disrupted ALK4 binding blocks signaling via type II receptors. J Biol Chem 279:28036–28044
48. **Johansen KA, Overturf K** 2005 Quantitative expression analysis of genes affecting muscle growth during development of rainbow trout (*Oncorhynchus mykiss*). Mar Biotechnol (NY) 7:576–587
49. **Zupanc MM, Zupanc GK** 2006 Upregulation of calbindin-D28k expression during regeneration in the adult fish cerebellum. Brain Res 1095:26–34
50. **Zupanc GK, Clint SC, Takimoto N, Hughes AT, Wellbrock UM, Meissner D** 2003 Spatio-temporal distribution of microglia/macrophages during regeneration in the cerebellum of adult teleost fish, *Apteronotus leptorhynchus*: a quantitative analysis. Brain Behav Evol 62:31–42
51. **Biga PR, Roberts SB, Iliev DB, McCauley LA, Moon JS, Collodi P, Goetz FW** 2005 The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, *Danio rerio*. Comp Biochem Physiol B Biochem Mol Biol 141:218–230

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/endo/article/148/5/2106/2501788 by guest on 21 August 2022