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Two myostatin genes are differentially expressed in myotomal muscles of the trout (*Oncorhynchus mykiss*)

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Summary

Myostatin (GDF8) has been shown to be a major genetic determinant of skeletal muscle growth in mammals. In this study, we report the cloning of two trout cDNAs that encode two distinct myostatin-related proteins. The presence in this fish species of two myostatin genes (Tmyostatin 1 and Tmyostatin 2) probably results from the recent tetraploïdisation of the salmonid genome. A comparative reverse-transcriptase-linked polymerase chain reaction assay revealed that Tmyostatin 1 mRNA was present ubiquitously in trout tissues, while Tmyostatin 2 mRNA expression was restricted to muscle and brain. In developing muscle, Tmyostatin 1 expression was observed in eyed-stage embryos well before hatching, whereas Tmyostatin 2 was expressed only in free-swimming larvae. In myotomal muscle from adult animals, Tmyostatin 1 mRNA accumulation was similar in both slow- and fast-twitch fibres, and its concentration did not change during the muscle wasting associated with sexual maturation. In contrast, Tmyostatin 2 mRNA accumulated predominantly in slow-twitch fibres, and its concentration decreased dramatically in wasting muscles from maturing animals. This work shows that two distinct myostatin genes are present in the trout genome. Furthermore, it indicates that these two trout myostatin genes (i) exhibit a distinct expression pattern in muscle and non-muscle tissues and (ii) are not upregulated during the muscle wasting that accompanies sexual maturation.

Key words: myostatin, gene expression, myotomal muscle, rainbow trout, *Oncorhynchus mykiss*, sexual maturation, development.

Introduction

Myostatin (GDF8), a member of the transforming growth factor-β superfamily, is expressed predominantly in skeletal muscle and is a key regulator of skeletal muscle growth. Mice with null homozygous mutations of the myostatin gene have increased muscle mass compared with their heterozygous and wild-type littermates (MacPherron et al., 1997). Mutations in the myostatin gene in two breeds of cattle, Belgian Blue and Piedmonteise, are also associated with increased muscle development (MacPherron and Lee, 1997; Grobet et al., 1997; Kambadur et al., 1997; Grobet et al., 1998). In accordance with the role of myostatin as a negative regulator of muscle mass, myostatin expression is increased in the muscle of HIV-infected men with muscle wasting compared with healthy men (Gonzales-Cadavid et al., 1998).

In fish, the myotomal musculature presents an interesting problem of differentiation and development because of the presence of two major types of fibre (slow-oxidative *versus* fast-glycolytic) that are grouped into physically distinct areas: the slow muscle occurs as a thin continuous strip that lies external to the fast muscle, which constitutes the major part of the myotomal musculature. In addition, fish myotomal muscle grows by both fibre hyperplasia and hypertrophy (Koumans and Akster, 1995), whereas increases in the number of fibres in mammals and birds stop shortly after embryonic development (Goldspink, 1972). Fibre hyperplasia refers to the increase in muscle fibre number due to the formation of new fibres resulting from a continuous proliferation of satellite cells and their fusion into new myofibres (Rowlerson and Veggetti, 2001). This peculiar muscle growth pattern is associated with the continuing expression in muscle of the mitogen fibroblast growth factor 6 (FGF6) far into adulthood (Rescan, 1998). Thomas et al. (Thomas et al., 2000) have shown that the downstream function of myostatin is to prevent the progression of myogenic cells into the cell division cycle. So, we wondered in this study whether the muscle hyperplastic growth in fast-growing fish species that involves continuous division of myogenic cells for fibre recruitment correlates with an absence of myostatin gene expression in muscle.

During the sexual cycle of salmonids, a dramatic loss of muscle mass accompanies the maturation of the gonads and the release of ova or spermatozoa. This muscle atrophy results from the decreasing contents of fat and proteins that are used for energy metabolism and gonadal growth (Aksnes et al., 1986). In the present study, we demonstrate that two distinct myostatin genes exist in trout and that these are differentially expressed in various tissues. Taking advantage of the anatomical separation of slow and fast muscle fibres in fish, we report the expression of the two myostatin genes in these two

kinds of fibres in muscle of immature animals and in wasting muscle of maturing animals.

Materials and methods

Animals

Experiments were carried out on rainbow trout *Oncorhynchus mykiss* (Walbaum). Sixteen fish were rapidly anaesthetized with phenoxyethanol (Sigma; 4 ml per 10 l of fresh water) and then dissected. Immature males (N=5) weighed between 190 and 1500 g and exhibited a gonadosomatic index [GSI=100×(gonad mass/body mass)] that ranged from 0.04 to 0.07. Immature females (N=5) weighed between 160 and 1330 g and exhibited a GSI of 0.06–0.2. Spermiating (N=3; weighing between 1080 and 1300 g) and spawning (N=3; weighing between 1014 and 1150 g) trout exhibited a GSI greater than 3 and 18, respectively. All fish originated from the same strain (Mirwart), were reared at our experimental fish farm (SEDII, Sizun, France) under a natural photoperiod and water temperature and were fed to satiety with commercially prepared pellets (Aqualim, Nersac, France) at a rate recommended by the manufacturer.

Cloning and sequencing of cDNAs

An embryonic trout cDNA library was screened at low stringency with a probe containing the coding region of bovine myostatin. After hybridisation for 16 h at 42 °C in 40 % formamide, 6× SSPE (0.9 mol l^{-1} NaCl, 6 mmol l^{-1} EDTA, 60 mmol l^{-1} NaH_2PO_4), 5× Denhardt's solution, 0.5 % sodium dodecylsulphate (SDS) and 0.1 mg ml^{-1} denatured calf thymus DNA, filters were washed twice in 2× SSPE, 0.5 % SDS at 50 °C for 30 min. One positive clone was purified, subcloned in Bluescript and sequenced using an automatic sequencing system (ABI Prism 310, PE Biosystems). Using primers designed from the sequence of the positive clone and RNA extracted from fry muscle, two distinct cDNAs were obtained by reverse transcriptase/polymerase chain reaction (RT-PCR). These two cDNAS were subsequently completed at their 5′ end by a rapid amplification of cDNA ends (RACE) protocol using the Gibco BRL kit (Version 2.0). Briefly, the first-strand cDNA was synthethised using the reverse primer 5′-CTTGTTCACC-AGGTGGGTGTG-3′ (nucleotides 1078–1098 in Tmyostatin 1 and 1072–1092 in Tmyostatin 2). After tailing of the cDNA using dCTP (final concentration 200 mmol l^{-1}) and terminal deoxynucleotidyl transferase, a PCR reaction was carried out using the reverse primer 5′-AGTCCCAGCCAAAGTCTTC-3′ (nucleotides 982–1000 in Tmyostatin 1 and 976–994 in Tmyostatin 2) and a poly(G)-containing primer. The 5′Race products were then subcloned in a pCRII vector (InVitrogen) and sequenced. To ensure that no mutation had been introduced during amplification, additional RT-PCR and sequencing were carried out using specific primers flanking the open reading frame of Tmyostatin 1 and 2.

Isolation of genomic clones encompassing the first intron

To further identify structural differences within the two trout myostatin genes, we generated genomic clones encompassing the first intron. For this, PCR experiments with trout genomic DNA were carried out using two sets of primers designed from the position of the intron/exon junction in the human myostatin gene (Gonzales-Cadavid et al., 1998). The primer sets used were 5′-CTTCACATATGCCAATACATATTA-3′ (nucleotides 51–74, see Fig. 1) and 5′-GCGGTTCACCTGAATCTTCGA-AC-3′ (nucleotides 545–567), to generate the Tmyostatin 1 genomic fragment, and 5′-TTCACGCAAATACGTATTCAC-3′ (nucleotides 50–70) and 5′-GTGCACCCATAGCTGT-GCCCG-3′ (nucleotides 568–588) to generate the Tmyostatin 2 genomic fragment. The genomic fragments obtained were subcloned in a pCRII vector (inVitrogen) and sequenced.

Reverse transcriptase/polymerase chain reaction

Total RNA was extracted following the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) using the TRIzol reagent (Gibco BRL). The RNAs were quantified on the basis of absorbance at 260 nm. Their integrity and quantity were checked by electrophoresis in ethidium-bromide-stained denaturing gel. The Access RT-PCR system (Promega) was used for cDNA synthesis and amplification in one tube. Briefly, the reverse transcription and PCR reaction conditions were as follows: 1 μg of total RNA, 0.2 mmol l^{-1} of each dNTP, 1 μmol l^{-1} sense primer, 1 μmol l^{-1} anti-sense primer (the two sets of primers used for the RT-PCR experiments were identical to those used to generate the genomic fragments encompassing the first intron; see above and Fig. 1), 1XAMV/Tfl buffer, 1 mmol l^{-1} $MgCl_2$, 5 units of AMV reverse transcriptase and 5 units of thermostable Tfl DNA polymerase. Amplification parameters were as follows: denaturation at 94 °C for 30 s, annealing for 1 min at 60 °C and extension at 68 °C for 1 min. We determined that, at 40 cycles, the PCR was in the linear range of amplification. The sequencing of the RT-PCR products consistently showed that the correct fragment was amplified. Samples of the reaction mixture were analysed on a 1.2 % agarose gel. Gels were blotted and then hybridised to verify the identity of the PCR product. Negative controls were performed by omitting RNA from the reaction buffer. They remained consistently negative.

Results

Identification of two distinct trout myostatin cDNAs.

The low-stringency screening of a trout embryonic cDNA library with full-length bovine myostatin cDNA yielded a 1.6 kilobase (kb) cDNA that included 1.2 kb of 3′ untranslated region and a partial open reading frame of 414 nucleotides encoding part of a protein closely related to the carboxy one-third of the mouse myostatin protein (90 % similarity). Using primers designed from this sequence and RNA extracted from fry muscle, two distinct cDNAs were generated by RT-PCR, indicating the presence of two myostatin genes in the trout genome. The whole coding region of these two cDNAs (Fig. 1) was completed by 5′Race PCR. An alignment of the predicted amino acid sequences of the two trout myostatin

A

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1     ACC TAT CAG TGT AGA GCC TGA TTT CAA ACA GAG GCA ACT CTG TAG   45
46    TCC GCC TTC ACA TAT GCC AAT ACA TAT TAC ATT TGG GAT TCA ATT   90
91    TTT ATA GCA AAC TCC GCA CCT TAG ATA ATG CAT CTG ACG CAG GTT   135
                                          M   H   L   T   Q   V     6

136   CTG ATT TAT CTC AGT TTC ATG GTT GCT TTC GGT CCA GTG GGT CTT   180
7      L   I   Y   L   S   F   M   V   A   F   G   P   V   G   L     21

181   GGT GAT CAA ACC GCG CAC CAC CAG CCC CCT GCC ACG GAT GAC GGC   225
22     G   D   Q   T   A   H   H   Q   P   P   A   T   D   D   G     36

226   GAG CAG TGC TCA ACA TGC GAG GTC CGA CAG CAG ATC AAA AAC ATG   270
37     E   Q   C   S   T   C   E   V   R   Q   Q   I   K   N   M     51

271   AGA TTA CAC GCC ATC AAG TCC CAA ATT CTT AGC AAA CTG CGA CTC   315
52     R   L   H   A   I   K   S   Q   I   L   S   K   L   R   L     66

316   AAG CAA GCT CCC AAT ATC AGC AGA GAT GTT GTC AAG CAG CTC CTG   360
67     K   Q   A   P   N   I   S   R   D   V   V   K   Q   L   L     81

361   CCT AAG GCA CCA CCT TTG CAG CAA CTT CTT GAC CAG TAC GAT GTT   405
82     P   K   A   P   P   L   Q   Q   L   L   D   Q   Y   D   V     96

406   CTT GGA GAT GAC AAT AAG GAT GGA CTT ATG GAA GAA GAT GAT GAA   450
97     L   G   D   D   N   K   D   G   L   M   E   E   D   D   E    111

451   CAT GCC ATC ACA GAA ACA ATC ATG ACA ATG GCC ACT GAA CCC GAA   495
112    H   A   I   T   E   T   I   M   T   M   A   T   E   P   E    126

496   TCC ATC GTC CAA GTC GAT CGG AAA CCC AAG TGT TGC TTA TTC TCC   540
127    S   I   V   Q   V   D   R   K   P   K   C   C   L   F   S    141

541   TTC AGT TCG AAG ATT CAG GTG AAC CGC ATA GTT CAT GCG CAG TTA   585
142    F   S   S   K   I   Q   V   N   R   I   V   H   A   Q   L    156

586   TGG GTG CAC CTT TTG CCA GCT GAC GAA GTC ACC ACC GTG TTT CTG   630
157    W   V   H   L   L   P   A   D   E   V   T   T   V   F   L    171

631   CAA ATC TCC CGC CTG ATG CCT GTC ACG GAC GGG GGC AGG CAC ATA   675
172    Q   I   S   R   L   M   P   V   T   D   G   G   R   H   I    186

676   GGT ATC CGG TCT CTA AAG ATC GAC GTG AAT GCA GGA GTC AGC TCT   720
187    G   I   R   S   L   K   I   D   V   N   A   G   V   S   S    201

721   TGG CAA AGT ATC GAC GTG AAA CAA GTG CTG TCC GTA TGG CTG CGG   765
202    W   Q   S   I   D   V   K   Q   V   L   S   V   W   L   R    216

766   CAG CCG GAG ACG AAT TGG GGG ATC GAG ATA AAT GCG TTC GAC TCG   810
217    Q   P   E   T   N   W   G   I   E   I   N   A   F   D   S    231

811   AAG GGA AAT GAT CTG GCG GTT ACC TCA GCA GAA GCG GGA GAA GGA   855
232    K   G   N   D   L   A   V   T   S   A   E   A   G   E   G    246

856   CTG CAA CCC TTC ATG GAG GTG ACG ATT TCA GAG GGC CCA AAG CGC   900
247    L   Q   P   F   M   E   V   T   I   S   E   G   P   K   R    261

901   TTC AGG CGA GAC TCG GGC CTG GAC TGT GAC GAG AAC TCC CCC GAG   945
262    F   R   R   D   S   G   L   D   C   D   E   N   S   P   E    276

946   TCC CGC TGT TGC CGC TAC CCG CTC ACG GTA GAC TTT GAA GAC TTT   990
277    S   R   C   C   R   Y   P   L   T   V   D   F   E   D   F    291

991   GGC TGG GAC TGG ATT ATT GCC CCC AAG CGC TAC AAG GCC AAC TAC   1035
292    G   W   D   W   I   I   A   P   K   R   Y   K   A   N   Y    306

1036  TGC TCT GGT GAG TGC GAG TAC ATG CAC CTG CAG AAG TAC CCC CAC   1080
307    C   S   G   E   C   E   Y   M   H   L   Q   K   Y   P   H    321

1081  ACC CAC CTG GTG AAC AAG GCT AAC CCT CGC GGC ACT GCC GGG CCC   1125
322    T   H   L   V   N   K   A   N   P   R   G   T   A   G   P    336

1126  TGT TGC ACC CCC ACC AAG ATG TCC CCC ATC AAC ATG CTC TAC TTC   1170
337    C   C   T   P   T   K   M   S   P   I   N   M   L   Y   F    351

1171  AAC CGC AAA GAG CAG ATC ATC TAC GGC AAG ATC CCC TCC ATG GTG   1215
352    N   R   K   E   Q   I   I   Y   G   K   I   P   S   M   V    366

1216  GTG GAC CGA TGC GGC TGC TCG TGA GCG AGA GCT CTG CCG GTG AGG   1260
367    V   D   R   C   G   C   S   *
1261  GGG AGG GGC TCA GCA GGG TCT CCC CCC TGG ACT TTG GGA CAG ATC   1305
1306  CAT CCA CCA CTA CCA GTG CTT TCT GCA GAA CAC GGT GCA ATA GAG   1350
1351  CCA GAA TAG CGG CTA AAG AAA TGC CCG TTC ATT CGC TGA GCA GCG   1395
1396  CTT CAA CCA CGG ACA TGT   1413
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B

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1     CTA TCA GTG TAG AGC CTG ATT TTA AAC ACA GGC AAC TCT GTA GTC   45
46    CGC CTT CAC GCA AAT ACG TAT TCA CTT TTG GAT TTT TTT TTT ATA   90
91    GCA AAC TCC GCA CCT TAG ATA ATG AAT CTG ATG CAG GTT CTA ATT   135
                                  M   N   L   M   Q   V   L   I     8

136   TAT CTG AGT TTT ATG GTT GCT TTC GGT CCA ATG GGT CTT GGT GAT   180
9      Y   L   S   F   M   V   A   F   G   P   M   G   L   G   D    23

181   CAA ACG GCG CAC CAC CAA TCC CCG GCC ACG GAT GAC GGT GAG CAG   225
24     Q   T   A   H   H   Q   S   P   A   T   D   D   G   E   Q    38

226   TGC TCA ACA TGC GAG GTC CGA CAG CAG ATC AAA AAC ATG AGA TTA   270
39     C   S   T   C   E   V   R   Q   Q   I   K   N   M   R   L    53

271   CAC GCC ATC AAG TCA CAA ATT CTT AGC AAA CTG CGA CTC AAG CAC   315
54     H   A   I   K   S   Q   I   L   S   K   L   R   L   K   H    68

316   GCG CCC AAT ATT AGC CGA GAT GTT GTC AAG CAG CTC TTG CCC AAG   360
69     A   P   N   I   S   R   D   V   V   K   Q   L   L   P   K    83

361   GCA CCA CCT TTG CAG AAA CTT CTT GAC CAG TAT GAT GTA CTT GGA   405
84     A   P   P   L   Q   K   L   L   D   Q   Y   D   V   L   G    98

406   GAT GAC AAT AAG GAT GGA CTT ATG GAA GAA GAT GAT GAA CAT GCC   450
99     D   D   N   K   D   G   L   M   E   E   D   D   E   H   A    113

451   ATC ACA GAA ACA ATC ATG ACA ATG GCC ACT GAA CCC GAA TCC ATC   495
114    I   T   E   T   I   M   T   M   A   T   E   P   E   S   I    128

496   GTC CAA GTC GAT GGG AAA CCC AAG TGT TGC TTT TTC TCC TTC AAT   540
129    V   Q   V   D   G   K   P   K   C   C   F   F   S   F   N    143

541   TCG AAG ATT CAG GCG AAC CGC ATA GTT CGG GCA CAG CTA TGG GTG   585
144    S   K   I   Q   A   N   R   I   V   R   A   Q   L   W   V    158

586   CAC CTT CAG CCA CCT GAC GAA GTC ACC ACC GTG TTC CTG CAA ATC   630
159    H   L   Q   P   P   D   E   V   T   T   V   F   L   Q   I    173

631   TCC CGC CTG ATC CCT GTC ACG GAC GGG GGC AGG AAC ATA CAG ATC   675
174    S   R   L   I   P   V   T   D   G   G   R   N   I   Q   I    188

676   CGG TCT CTA AAG ATC GAC GTG AAT GCA GGA GTC AGC TCT TGG CAG   720
189    R   S   L   K   I   D   V   N   A   G   V   S   S   W   Q    203

721   AGT ATC GAC GTG AAA CAA GTG TTG TCG GTG TGG CTG CGG CAA CCG   765
204    S   I   D   V   K   Q   V   L   S   V   W   L   R   Q   P    218

766   GAC ACG AAT TGG GGG ATC GAG ATT AAT GCG TTG GAC TCA AAG GGA   810
219    D   T   N   W   G   I   E   I   N   A   L   D   S   K   G    233

811   AAT GAT CTG GCC GTT ACC TCA GCT GAA GCC GGA GAA GGA CTG CAA   855
234    N   D   L   A   V   T   S   A   E   A   G   E   G   L   Q    248

856   CCC TTC ATG GAG GTG AAG ATT TCG GAG GGC CCG AAG CGC TCC AGG   900
249    P   F   M   E   V   K   I   S   E   G   P   K   R   S   R    263

901   AGA GAT TCG GGC CTG GAC TGT GAT GAG AAC TCC CCC GAG TCC CGC   945
264    R   D   S   G   L   D   C   D   E   N   S   P   E   S   R    278

946   TGC TGC CGG TAC CCC CTC ACA GTG GAC TTT GAA GAC TTT GGC TGG   990
279    C   C   R   Y   P   L   T   V   D   F   E   D   F   G   W    293

991   GAC TGG ATT ATT GCC CCC AAG CGC TAC AAG GCC AAC TAC TGC TCT   1035
294    D   W   I   I   A   P   K   R   Y   K   A   N   Y   C   S    308

1036  GGT GAG TGC GAG TAC ATG CAC CTG CAG AAG TAC CCC CAC ACC CAC   1080
309    G   E   C   E   Y   M   H   L   Q   K   Y   P   H   T   H    323

1081  CTG GTG AAC AAG GCT AAC CCC CGG GGC ACC GCA GGG CCC TGC TGC   1125
324    L   V   N   K   A   N   P   R   G   T   A   G   P   C   C    338

1126  ACC CCC ACC AAG ATG TCC CCG ATC AAC ATG CTC TAC TTC AAC CGC   1170
339    T   P   T   K   M   S   P   I   N   M   L   Y   F   N   R    353

1171  AAA GAG CAG ATC ATC TAC GGC AAG ATC CCA TCC ATG GTG GTG GAC   1215
354    K   E   Q   I   I   Y   G   K   I   P   S   M   V   V   D    368

1216  CGC TGC GGC TGC TCG TGA GCG AGA GTT CTG CTG GGG AAG GGG AGG   1260
369    R   C   G   C   S   *

1261  GGC TTA GCA GGG TCT CCA CCC TGG ACT TTG GGA CAG ATC CAT CCA   1305
1306  TCA CTA CCA GTG CTT TCT GCA GAA CAC AGT GCA ATA GAG CCA CAA   1350
1351  TAG CGG CTA AAG AAA CAC CTG TCC ATT CGC TGA GCA GGC TTC AAC   1395
1396  CAC GGA CAT GTC   1407
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Fig. 1. Nucleotide and deduced amino acid sequences of Tmyostatin 1 (A) and Tmyostatin 2 (B). The stop codon is indicated by a star. The sequences underlined correspond to the sense and antisense primers used to generate the intron-1-containing genomic fragment and for semi-quantitative RT-PCR. Accession numbers: AF273035 (Tmyostatin 1); AF273036 (Tmyostatin 2).

proteins with those of zebrafish, chicken and mouse homologues is shown in Fig. 2. The Tmyostatin 1 and Tmyostatin 2 proteins exhibit 94 % similarity and appear to be more closely related to zebrafish (84.2 and 82.3 %, respectively) than to avian (64.3 and 63.8 %) and mouse (62.2 and 63 %) myostatin proteins. The alignment identity is greater in the C-terminal domain following the proteolytic processing site (amino acid residues 267–270). In this region, which corresponds to the functional domain of protein, the two trout sequences are identical and exhibit 95 % similarity with the zebrafish sequence and 89 % similarity with the chicken and mouse sequences.

Characterisation of the first intron in trout myostatin 1 and 2 genes

To further identify structural differences between the two trout myostatin genes, we examined the sequence of the first intron of the two genes. To this end, we generated PCR products

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                10        20        30        40        50        60
        ....|....|....|....|....|....|....|....|....|....|....|....|
1       M--HLTQVL-IYLSFMVAFGPVGLGDQTAHHQP-PATDDGEQCSTCEVRQQIKNMRLHAI  Tmyostatin 1
1       M--NLMQVL-IYLSFMVAFGPMGLGDQTAHHQS-PATDDGEQCSTCEVRQQIKNMRLHAI  Tmyostatin 2
1       M--HFTQVL-ISLSVLIACGPVGYGDITAHQQPSTATEESELCSTCEFRQHSKLMRLHAI  Zebrafish mstn
1       M-QKLAVYVYIYLFMQIAVDPVALDGSSQPTEN---AEKDGLCNACTWRQNTKSSRIEAI  Chicken mstn
1       MMQKLQMYVYIYLFMLIAAGPVDLNEGSEREEN---VEKEGLCNACAWRQNTRYSRIEAI  Murine mstn

                70        80        90       100       110       120
        ....|....|....|....|....|....|....|....|....|....|....|....|
57      KSQILSKLRLKQAPNISRDVVKQLLPKAPPLQQLLDQYDVLGDDNKDGLMEEDDEHAITE  Tmyostatin 1
57      KSQILSKLRLKHAPNISRDVVKQLLPKAPPLQKLLDQYDVLGDDNKDGLMEEDDEHAITE  Tmyostatin 2
58      KSQILSKLRLKQAPNISRDVVKQLLPKAPPLQQLLDQYDVLGDDSKDGAVEEDDEHATTE  Zebrafish mstn
57      KIQILSKLRLEQAPNISRDVIKQLLPKAPPLQELIDQYDVQRDDSSDGSLEDDDYHATTE  Chicken mstn
58      KIQILSKLRLETAPNISKDAIRQLLPRAPPLRELIDQYDVQRDDSSDGSLEDDDYHATTE  Murine mstn

               130       140       150       160       170       180
        ....|....|....|....|....|....|....|....|....|....|....|....|
117     TIMTMATEPESIVQVDRKPKCCLFSFSSKIQVNRIVHAQLWVHLLPADEVTTVFLQISRL  Tmyostatin 1
117     TIMTMATEPESIVQVDGKPKCCFFSFNSKIQANRIVRAQLWVHLQPPDEVTTVFLQISRL  Tmyostatin 2
118     TIMTMATEPDPIVQVDRKPKCCFFSFSPKIQANRIVRAQLWVHLRPAEEATTVFLQISRL  Zebrafish mstn
117     TIITMPTESDFLVQMEGKPKCCFFKFSSKIQYNKVVKAQLWIYLRQVQKPTTVFVQILRL  Chicken mstn
118     TIITMPTESDFLMQADGKPKCCFFKFSSKIQYNKVVKAQLWIYLRPVKTPTTVFVQILRL  Murine mstn

               190       200       210       220       230       240
        ....|....|....|....|....|....|....|....|....|....|....|....|
177     M-PVTDGGRHIGIRSLKIDVNAGVSSWQSIDVKQVLSVWLRQPETNWGIEINAFDSKGND  Tmyostatin 1
177     I-PVTDGGRNIQIRSLKIDVNAGVSSWQSIDVKQVLSVWLRQPDTNWGIEINALDSKGND  Tmyostatin 2
178     M-PVKDGGRHR-IRSLKIDVNAGVTSWQSIDVKQVLTVWLKQPETNRGIEINAYDAKGND  Zebrafish mstn
177     IKPMKDGTRYTGIRSLKLDMNPGTGIWQSIDVKTVLQNWLKQPESNLGIEIKAFDETGRD  Chicken mstn
178     IKPMKDGTRYTGIRSLKLDMSPGTGIWQSIDVKTVLQNWLKQPESNLGIEIKALDENGHD  Murine mstn

               250       260       270       280       290       300
        ....|....|....|....|....|....|....|*...|....**...|....|....|
236     LAVTSAEAG-EGLQPFMEVTISEGPKRFRRDSGLDCDENSPESRCCRYPLTVDFEDFGWD  Tmyostatin 1
236     LAVTSAEAG-EGLQPFMEVKISEGPKRSRRDSGLDCDENSPESRCCRYPLTVDFEDFGWD  Tmyostatin 2
236     LAVTSTETGEDGLLPFMEVKISEGPKRIRRDSGLDCDENSSESRCCRYPLTVDFEDFGWD  Zebrafish mstn
237     LAVTFPGPGEDGLNPFLEVRVTDTPKRSRRDFGLDCDEHSTESRCCRYPLTVDFEAFGWD  Chicken mstn
238     LAVTFPGPGEDGLNPFLEVKVTDTPKRSRRDFGLDCDEHSTESRCCRYPLTVDFEAFGWD  Murine mstn

               310       320       330       340       350       360
        ....|....|..*.|.*..|....|....|....|....|..**|....|....|....|
295     WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTAGPCCTPTKMSPINMLYFNRK  Tmyostatin 1
295     WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTAGPCCTPTKMSPINMLYFNRK  Tmyostatin 2
296     WIIAPKRYKANYCSGECDYMYLQKYPHTHLVNKASPRGTAGPCCTPTKMSPINMLYFNGK  Zebrafish mstn
297     WIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQANPRGSAGPCCTPTKMSPINMLYFNGK  Chicken mstn
298     WIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQANPRGSAGPCCTPTKMSPINMLYFNGK  Murine mstn

               370
        ....|....|....|*.*.
355     EQIIYGKIPSMVVDRCGCS                                           Tmyostatin 1
355     EQIIYGKIPSMVVDRCGCS                                           Tmyostatin 2
356     EQIIYGKIPSMVVDRCGCS                                           Zebrafish mstn
357     EQIIYGKIPAMVVDRCGCS                                           Chicken mstn
358     EQIIYGKIPAMVVDRCGCS                                           Murine mstn
```

Fig. 2. Comparison of the trout, zebrafish, chicken and mouse myostatin (mstn) proteins. The predicted proteolytic processing site is underlined in bold type, and the conserved cysteine residues in the C-terminal region found in all TGF-β family members are marked with an asterisk. The shading indicates identity.

using primers designed to cross one intron. As expected, we found that the PCR fragments generated from genomic DNA were much larger (approximately 1600 nucleotides for Tmyostatin 1 and 1100 nucleotides for Tmyostatin 2) than those generated from the cDNA sequences (approximately 500 nucleotides), indicating the presence of one intron in this region. The sequence analysis revealed that, for both genomic fragments, the positions of the splice donor and acceptor sites were perfectly analogous to those described for mammalian species. DNA sequencing of the two introns revealed conservative features, with the exception of the noteworthy insertion of a 509-nucleotide element in Tmyostatin 1 that is lacking in Tmyostatin 2 (Fig. 3). Using the Blast program, we found that this additional element exhibited numerous regions of similarity with the 3′ untranslated region of the trout natural-resistance-associated macrophage protein alpha cDNA (GenBank accession number AF054808).

Differential expression of the two trout myostatin genes in various tissues

A comparative RT-PCR assay was performed to assess the relative expression of the two trout myostatin genes in various tissues. For this, the same primers that generated intron-1-containing DNA fragments (see above) were used,

```
                          Exon 1 ↓ Intron 1
Tmyostatin 1  5′ATGGCCACTGAACGTAAGTTTGACAGACTATTATTCAATGTAAC[....509 nt.....]AATAACGTTAAAAA 3′
                 ||||||||||||||||||||  | |  ||||||||||||| |||                 ||||||| || ||||
Tmyostatin 2  5′ATGGCCACTGAACGTAAGTTGTATATGCTATTATTCAATGCAACACTATGCGTCTCAATATAATAACTTTGAAAA 3′
```

Fig. 3. Nucleotide alignment of the 5′ end of the first intron of Tmyostatin 1 and Tmyostatin 2 showing an additional insertion (in brackets) of a 509-nucleotide (nt) fragment within the first intron of the Tmyostatin 1 gene. Identity is indicated by a vertical line. The arrow indicates the exon/intron boundary. Accession numbers are AF278606 for intron 1 of Tmyostatin 1 and AF278607 for intron 1 of Tmyostatin 2.

such that any products derived from contaminating genomic DNA could be distinguished from messenger templates. Using Tmyostatin 1 sense and antisense primers, a PCR product of the expected size was specifically generated with RNA from brain, intestine, testis, ovary and fast and slow muscles and, to a lesser extent, with RNA from heart, kidney, gills and liver (Fig. 4A). In contrast, using Tmyostatin 2 sense and antisense primers, a PCR product was only generated with RNA from brain and slow muscle (Fig. 4B). Collectively, these observations demonstrate differential expression of the two myostatin genes in trout tissues. Tmyostatin 1 is ubiquitously expressed, whereas Tmyostatin 2 exhibits a more restricted expression pattern, as observed for myostatin in mammals.

Differential expression of Tmyostatin 1 and Tmyostatin 2 in developing myotomal musculature in trout

To determine whether the myostatin genes may regulate early myogenesis in trout, we analysed their expression in developing muscle using RT-PCR assays. Tmyostatin 1 mRNA was present in equal amounts in the trunk of embryos at the eyed stage (when somite development is completed), at hatching and in the myotomal musculature of fry (Fig. 5A). In contrast, the expression of Tmyostatin 2 mRNA was detected only in the myotomal musculature of fry (Fig. 5B). Therefore, the two myostatin genes are subject to different developmental control in trout embryos.

Tmyostatin 1 and Tmyostatin 2 mRNA in slow- and fast-twitch muscle in immature trout

To evaluate the accumulation of Tmyostatin 1 and Tmyostatin 2 mRNAs in muscle fibres with differing phenotypes in post-larval trout, we carried out RT-PCR assays with RNA from fast- and slow-twitch muscles of immature male and female trout. The amount of Tmyostatin 1 mRNA was found to be similar in the fast-twitch and slow-twitch muscle of both male (Fig. 6A, upper panel, lanes 1–10) and female (Fig. 6B, upper panel, lanes 1–10) immature trout. In contrast, Tmyostatin 2 mRNA was always seen to accumulate predominantly in slow-twitch muscle, little expression, if any, was detected in fast-twitch muscle (Fig. 6A,B, lower panel, lanes 1–10).

Differential expression of Tmyostatin 1 and Tmyostatin 2 during the muscle wasting that accompanies sexual maturation

To analyse the relationships between the muscle atrophy that occurs in maturing trout and myostatin expression, we also assessed the level of myostatin transcripts in both slow- and fast-twitch muscles in spermiating males and spawning females using the RT-PCR assay. Our results reveal that Tmyostatin 1 mRNA levels, in both slow- and fast-twitch muscles, were similar in immature and mature animals (Fig. 6A,B, upper panel). In contrast, the amount of Tmyostatin 2 mRNA was found to decrease dramatically in both slow- and fast-twitch atrophied muscles from spermiating or spawning trout (Fig. 6A,B, lower panel).

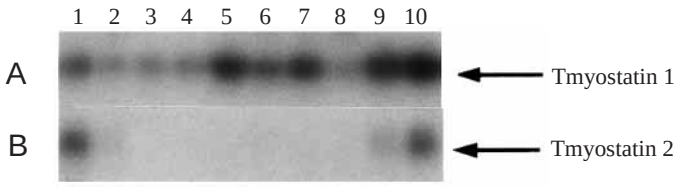

Fig. 4. Expression of Tmyostatin 1 (A) and Tmyostatin 2 (B) mRNA in trout adult tissues. Comparative RT-PCR of total RNA (1 μg) from, brain, heart, kidney, gills, intestine, testis, ovary, liver, fast muscle and slow muscle (lanes 1–10, respectively).

Discussion

In this study, we report the identification in trout of two cDNAs that encode distinct myostatin proteins. Several lines of evidence demonstrate that these two myostatin cDNAs are not the products of true alleles but originate from two distinct loci. First, these two cDNA diverge substantially, particularly in the untranslated domain. Second, we observed a differential rearrangement of the first intron of the genes from which these

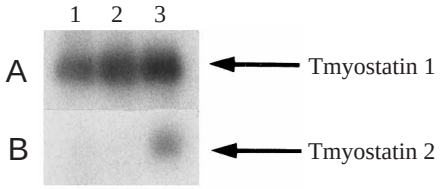

Fig. 5. Expression of Tmyostatin 1 (A) and Tmyostatin 2 (B) mRNA in the myotomal muscle of developing rainbow trout embryos. Comparative RT-PCR of total RNA (1 μg) from the trunk of trout embryos at the eyed stage (lane 1), at hatching (lane 2) and in the myotomal musculature of a 5 g alevin (lane 3).

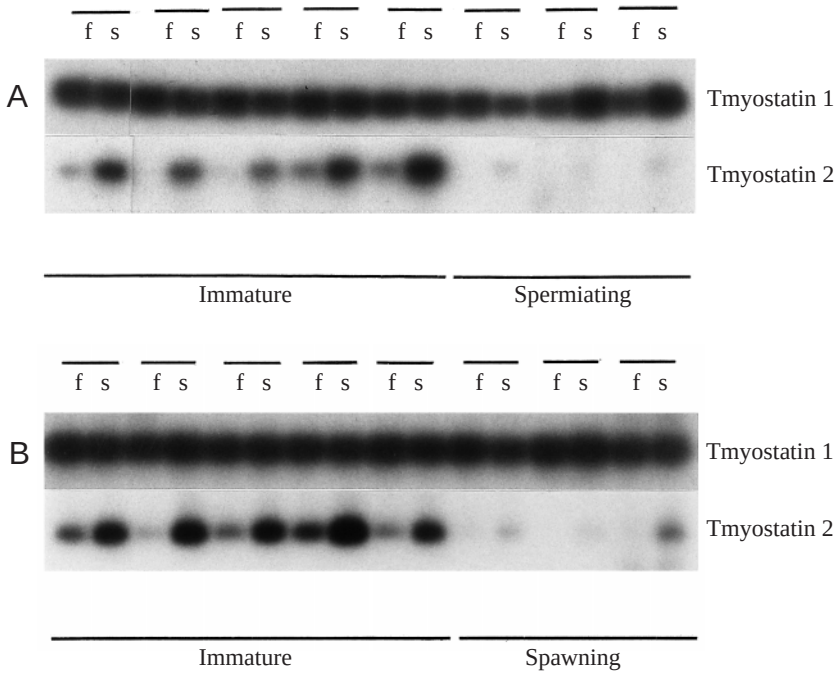

Fig. 6. (A) Expression of Tmyostatin 1 (upper panel) and Tmyostatin 2 (lower panel) mRNA in myotomal muscle of male rainbow trout. Comparative RT-PCR of total RNA (1 μg) from fast-glycolytic (f) and slow-oxidative (s) muscles from five immature (lanes 1–10 from the left) and three spermiating (lanes 11–16 from the left) males. (B) Expression of Tmyostatin 1 (upper panel) and Tmyostatin 2 (lower panel) mRNA in myotomal muscle of female rainbow trout. Comparative RT-PCR of total RNA (1 μg) from fast-glycolytic (f) and slow-oxidative (s) muscles from five immature (lanes 1–10 from the left) and three spawning (lanes 11–16 from the left) females.

two cDNAs were derived. Third, the two myostatin transcripts are differentially expressed.

In fish, and this is particularly true of salmonid species, genes often occur in pairs as the result of ancient genome duplication. The presence of two prolactin-encoding genes (Yasuda et al., 1986), two somatostatin-encoding genes (Moore et al., 1999) and two MyoD-encoding genes (Rescan and Gauvry, 1996) has been reported in salmonids. This genetic redundancy is believed to have created new structural proteins and/or to have modified their expression pattern (Meyer and Schartl, 1999; Shimeld, 1999). In the present study, we show that, after the duplication that led to their emergence, the two trout myostatin genes also evolved separately, acquiring distinct expression patterns. While Tmyostatin 1 is ubiquitously expressed, suggesting a role in growth regulation of many tissues in the trout, Tmyostatin 2 is preferentially expressed in muscle and brain.

It would be of great interest to determine whether the additional insertion of a 509-nucleotide fragment in the first intron of Tmyostatin 1 contributes to the differences observed in the expression pattern of the two myostatin genes. Surprisingly, in addition to the differential expression of the two trout myostatin genes in non-muscle tissues, major differences are also observed in their expression in muscles: while the synthesis of Tmyostatin 1 mRNA appears to be constitutive, that of Tmyostatin 2 mRNA is subject to regulation depending on the fibre phenotype (slow *versus* fast) and on the physiological status of the animal (immature *versus* mature). The constitutive expression of Tmyostatin 1 raises the question of its role in the homeostasis of muscles. In this regard, further studies are needed to ascertain whether the two proteins are processed in a similar manner and with the same kinetics in muscles, and it would be of interest to define the relative contribution of each of the two proteins to global myostatin activity.

In mammalian muscle, hyperplasia is restricted to the pre- and perinatal periods. In contrast, in some teleosts, including salmonids, hyperplasia continues, together with hypertrophic growth, far into adulthood (Rowlerson and Veggetti, 2001; Stickland, 1983). In this peculiar context, we expected to correlate the continuous proliferation of myogenic cells that leads to the formation of new myofibres in teleosts with an absence of myostatin expression. Surprisingly, we clearly demonstrated the presence of Tmyostatin 1 transcript in both slow and fast muscle and of Tmyostatin 2 transcript in slow muscle. Therefore, hyperplastic growth of muscle in trout cannot be attributed to the absence of myostatin gene transcription. However, the sequencing analysis showed that there is no obvious mutation within the coding region of the myostatin gene that would disrupt myostatin function.

The targeted inactivation of the myostatin gene in mice and the identification of mutations in the myostatin gene in double-muscled cattle provide strong evidence that myostatin is a negative regulator of muscle mass (MacPherron et al., 1997; Grobet et al., 1997; MacPherron and Lee, 1997; Kambadur et al., 1997; Grobet et al., 1998). In accordance with such a regulatory function, wasting muscle in HIV-infected men has been shown to express higher levels of myostatin than non-pathological muscle (Gonzales-Cadavid et al., 1998). Since dramatic muscle atrophy occurs during the sexual cycle of salmonids, we examined whether the increase in myostatin expression might mediate the loss of muscle mass in maturing fish. We observed that the amount of Tmyostatin 1 transcript was similar in muscles from immature and mature trout and that the amount of Tmyostatin 2 mRNA decreased dramatically in muscles from spawning and spermiating trout. These observations, which preclude an association between an upregulation of myostatin and muscle atrophy in maturing

trout, are reminiscent of previous studies that have either reported the lack of a strong correlation between mouse muscle atrophy induced by unloading and over-expression of myostatin (Carlson et al., 1999) or shown that, in unloaded muscle subjected to intermittent loading, over-expression of myostatin may occur without causing muscle mass loss (Wehling et al., 2000). Therefore, we believe that myostatin is not a mediator of muscle atrophy in vertebrates.

Recently, with the intention of elucidating the molecular mechanisms by which myostatin exerts its activity, Thomas et al. (Thomas et al., 2000) have shown that myostatin specifically upregulates p21, a cyclin-dependent kinase inhibitor, and decreases the level of Cdk2 proteins, thus preventing the progression of myoblasts into the cell cycle. Although these data do not totally exclude additional roles for myostatin in the homeostasis of differentiated cells, they reinforce the idea that myostatin exerts its effect on myogenic mononucleated cells rather than on already existing muscle fibres.

In summary, rainbow trout possess two myostatin-encoding genes that have evolved separately. One (Tmyostatin 1) is ubiquitously expressed in trout tissues and appears to be constitutively expressed in muscle. The other (Tmyostatin 2) is preferentially expressed in muscle and is subject to considerable regulation, depending on the fibre phenotype and the physiological status of the animal. Finally, neither Tmyostatin 1 nor Tmyostatin 2 is upregulated during the muscle wasting that accompanies sexual maturation.

During the reviewing of this paper, Roberts and Goetz (Roberts and Goetz, 2001) reported the isolation of two cDNAs encoding myostatin homologues in another species of trout (*Salvelinus fontinalis*). These authors have isolated one cDNA from muscle and brain and a second cDNA from ovarian tissue. The isoform they isolated from muscle and brain is very probably homologous to the Tmyostatin 2 described here. Unfortunately, Roberts and Goetz (Roberts and Goetz, 2001) did not give the full sequence of the ‘ovarian’ myostatin cDNA or the expression pattern of the ‘ovarian’ isoform in various tissues, particularly in muscle. So, the homology between the ‘ovarian’ isoform and the Tmyostatin 1 gene remains to be established.

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