Original Article

MARINE BIOTECHNOLOGY

Molecular Cloning of Myostatin Gene and Characterization of Tissue-Specific and Developmental Stage-Specific Expression of the Gene in Orange Spotted Grouper, *Epinephelus coioides*

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Abstract

In this article we report the molecular cloning and characterization of a nonmammalian myostatin (growth and differentiation factor-8, MSTN) homolog from the orange spotted grouper (*Epinephelus coioides*) by polymerase chain reaction (PCR) cloning. The grouper MSTN gene consists of two introns [Intron I (363 bp) and Intron II (811 bp)] flanked by three exons [Exon I (379 bp), Exon II (371 bp) and Exon III (381 bp)]. A full-length cDNA clone (2608 bp) of the MSTN gene (GenBank DQ493889, nucleotide sequence in the coding region identical to GeneBank AY856860) was also isolated. This cDNA encodes a polypeptide of 376 amino acid residues that showed 25% to 96% homology with MSTNs of molluscan, teleostean, avian, and mammalian species. Phylogenetic analysis of the grouper MSTN polypeptide confirmed the evolutionary relationships of this MSTN with other known MSTNs. Results of reverse transcription (RT)-PCR analysis of the total RNA extracted from different tissues revealed that MSTN gene is expressed not only in the skeletal muscle, but also in other tissues. MSTN mRNA was also detected in different embryonic developmental and larval stages. Because the tissue-specific expression of MSTN gene in grouper is different from that in mammals, it might suggest that MSTN gene may possess additional functions other than regulating muscle growth in fish.

Keywords: *Epinephelus coioides* — GDF-8 — myostatin — orange spotted grouper — TGF-β

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Introduction

Myostatin (MSTN), originally identified as a growth differentiation factor 8 (GDF-8), is a recently discovered member of the transforming growth factor-β (TGF-β) superfamily (McPherron et al., 1997). The TGF-β superfamily includes a number of secreted factors that mediate key events in tissue growth and development through signal transduction cascades. In mammals, MSTN is expressed initially in the myotome compartment of the developing somites and continues to be expressed in the myogenic lineage throughout the development and in adult animals (McPherron and Lee, 1997; Lee and McPherron, 2001). The following two lines of evidence have led to the conclusion that MSTN functions as a negative regulator of skeletal muscle growth: (1) MSTN knockout mice display increased muscle mass (McPherron et al., 1997; Lee and McPherron, 2001); and (2) mutations of the MSTN gene in cattle are associated with muscle hyperplasia and hypertrophy (Grobet et al., 1997; McPherron and Lee, 1997).

MSTN gene has been cloned and identified from a wide variety of vertebrates including human (Gonzalez-Cadavid et al., 1998), mice (McPherron and Lee, 1997), cattle (McPherron and Lee, 1997), chicken (McPherron and Lee, 1997; Kocamis et al., 1999), and several fish species such as zebrafish (*Danio rerio*; Xu et al., 2003; Amali et al., 2004; Biga et al., 2005), Atlantic salmon (*Salmo salar*; Ostbye et al., 2001), rainbow trout (*Oncorhynchus mykiss*; Rescan et al., 2001), brook trout (*Salvelinus fontinalis*; Roberts and Goetz, 2001), striped bass (*Morone saxatilis*; Rodgers and Weber, 2001), tilapia (*Oreochromis mossambicus*; Rodgers et al., 2001), gilt-

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Table 1. Sequences of Degenerate and Gene Specific Primers Used in This Study

Primer	*Sequences*	*Code*	*Position*
Primers for RT-PCR			
Degenerate forward primer	5′-CGGTGYTGCMGSTAYCCNCTY-3′	MSTN-F	nt 940 ~ 960
Degenerate reverse primer	5′-TCABGAGCABCCRCADCGGTC-3′	MSTN-R	nt 1210 ~ 1230
Primers for 3′ RACE PCR			
5′ first primer	5′-CACCACCATGGAGGGGAT-3′	MSTN 3-IF	nt 1192 ~ 1209
5′ nested primer	5′-CTTGCCATAGATGATCTGCTC-3′	MSTN 3-2F	nt 1171 ~ 1191
GeneRacer™ Kit 3′ first primer	5′-GCTGTCAACGATACGCTACGTAACG-3′		
GeneRacer™ Kit 3′ nested primer	5′-CGCTACGTAACGGCATGACAGTG-3′		
Primers for 5′ RACE PCR			
GeneRacer™ Kit 5′ first primer	5′-CGACTGGAGCACGAGGACACTGA-3′		
GeneRacer™ Kit 5′ nested primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′		
3′ first primer	5′-ACAGTGGACTTTGAAGAC-3′	MSTN 5-1R	nt 961 ~ 978
3′ nested primer	5′-GGCTGGGACTGGATTATTGCC-3′	MSTN 5-2R	nt 982 ~ 1002
Primers for genomic DNA			
Genomic DNA forward primer	5′-CAGTGTGGGACATTAATCC-3′	MSTN-GF	nt 4 ~ 22
Genomic DNA reverse primer	5′-CTCACCAGGATCTCCGTCCC-3′	MSTN-GR	nt 2408 ~ 2427
Primers for Real-Time PCR			
MSTN 5′ primer	5′-GACGTGCTGGGAGATG-3′	MSTN RT-F	nt 388 ~ 403
MSTN 3′ primer	5′-AGCTGAGCTCGGACTA-3′	MSTN RT-R	nt 557 ~ 572

B, (C/G/T); D, (A/G/T); M, (A/C); N, (A/C/G/T); R, (A/G); S, (C/G); Y, (C/T).

head seabream (*Sparus aurata*; Maccatrozzo et al., 2001a,b), channel catfish (*Ictalurus punctatus*; Kocabas et al., 2002a), and European seabass (*Dicentrarchus labrax*; Terova et al., 2005). While MSTN gene is expressed primarily in myogenic linage cells in mammals, MSTN gene in fish is expressed in a variety of tissues including muscle, gill, eyes, tongue, spleen, heart, stomach, intestine, kidney, liver, ovaries, brain, and testes (Maccatrozzo et al., 2001a,b; Ostbye et al., 2001; Rescan, 2001; Rescan et al., 2001; Roberts and Goetz, 2001; Rodgers and Weber, 2001; Kocabas et al., 2002b; Radaelli et al., 2003; Gregory et al., 2004; Terova et al., 2005). Studies conducted by Amali et al. (2004) showed that downregulation of myostatin-1 gene in zebrafish embryos by antisense morpholino-specific to myostatin resulted in upregulation of muscle-specific transcription factors during embryonic somatogenesis. Because fish MSTN gene is expressed in many cell types other than myogenic linage in fish, this suggests that fish MSTN gene may be involved in additional functions other than regulating muscle development. In most salmonids and zebrafish, there are two MSTN transcripts that are products of separate genes. These two transcripts are differentially regulated, and tissues expressing these two MSTN genes appear to be paralog-dependent (Maccatrozzo et al., 2001a; Rescan et al., 2001; Roberts and Goetz, 2001; Xu et al., 2003; Amali et al., 2004; Biga et al., 2005).

The orange spotted grouper, a protogynous hermaphroditic fish, is of great interest for fisheries as well as aquaculture because of its excellent meat quality and its high commercial value. This fish species has become one of the most commercially important marine fish species, and is popularly cultured in Taiwan. One of the bottlenecks in commercial aquaculture of grouper is its slow somatic growth and prone to disease infection. To overcome these problems, development of strategies for acceleration of somatic growth and disease resistant would be desirable. As a step toward this direction, we have initiated studies to clone and characterize genes related to growth of this fish species. In this article we report cloning and characterization of MSTN gene in the orange spotted grouper and the tissue-specific and developmental stage-specific expression of the gene in 1-year-old fish. Because the only fish species that MSTN gene and its expression patterns have been studied is gilthead seabream (Maccatrozzo et al., 2001a,b) and European seabass (Terova et al., 2005), results of our study reported here for the orange spotted grouper will provide valuable information for comparative studies of MSTN gene in marine fish species.

Materials and Methods

Animal and Sample Collection. One-year-old orange spotted grouper, ranging from 250 to 350 g body weights and 20–25 cm body lengths, were obtained from a farm in Tainan, Taiwan, and acclimated in fiberglass containers with full strength seawater (35 ppt) for 3 days. The fish were fed daily with fish or shrimp meat at 10% of body weight. Various tissues for determining the distribution of MSTN mRNA were collected from 1-year-old fish. Unfertilized eggs, embryos, and larval grouper from

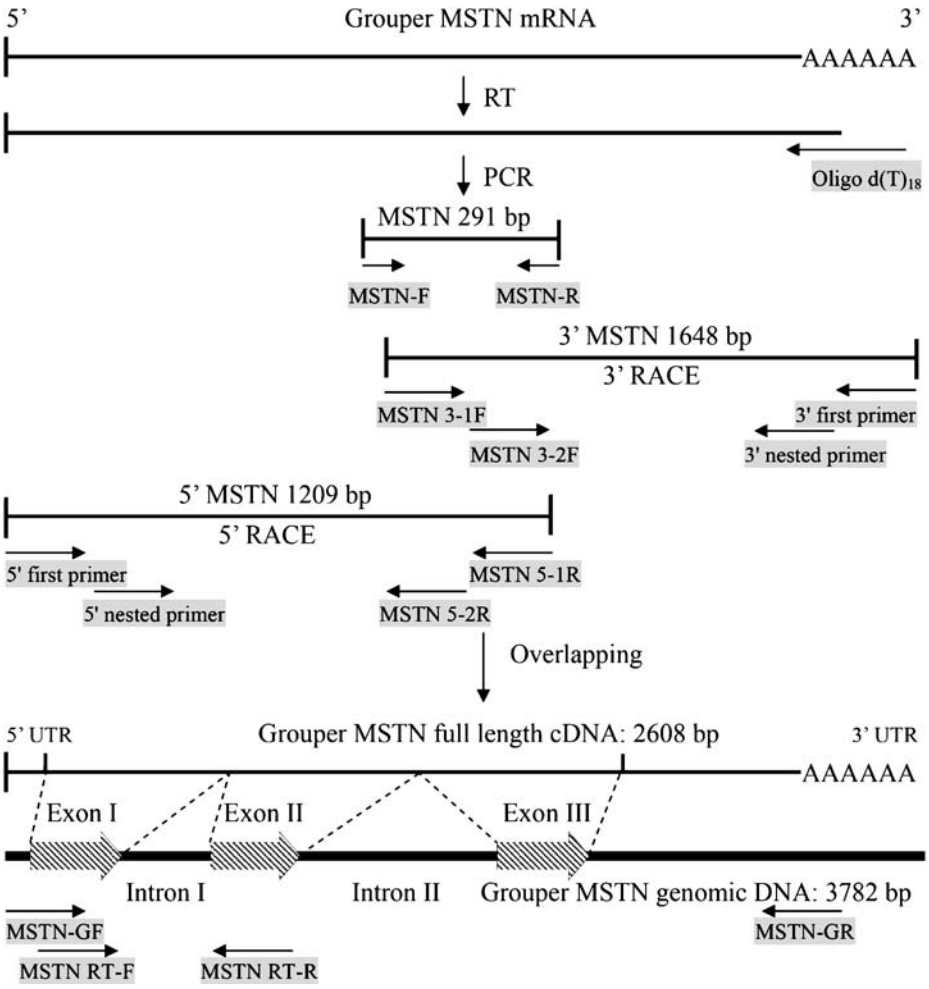

Fig. 1. Schematic diagram showing the strategy of PCR cloning of grouper MSTN gene. The names and relative positions of forward and reverse primers of grouper MSTN are shown in the diagram. Primers MSTN-F and MSTN-R were used to amplify genomic DNA. Primers MSTN RT-F and MSTN RT-R were used for quantitative real-time RT-PCR analysis.

1 to 60 days postfertilization (dpf) were collected during the reproductive season of 2005 (from May to June). All tissue samples were frozen immediately in liquid nitrogen and stored at –80°C until RNA extraction.

Isolation and Characterization of MSTN Gene. Total RNA was extracted from various tissues of adult grouper using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. The concentration of the total RNA was estimated by measuring the absorbance at 260 nm.

Degenerate primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of grouper MSTN cDNA are shown in Table 1. These primers were designed based on the highly conserved sequences of MSTN of zebrafish (AF019626, AY614000), brook trout (AF247650), tilapia (AF197193), white (AF197194), striped seabass (AF290910), white perch (AF290911), Atlantic salmon *S. salar* (ASA297267, ASA344158), rainbow trout (AF273035, AF273036), gilthead seabream (AF258447, AY046314), channel catfish (AF396747), blue catfish (AY540992), white catfish (AY540994), and European sea bass (AY839106) in the GenBank database (Benson et al., 1994).

For the isolation of a grouper MSTN cDNA fragment, total RNA (5 μg) was reverse transcribed using Superscript™ III RNase H^- reverse transcriptase (Invitrogen) and oligo $(T)_{18}$ primer to obtain first-strand cDNA. The generated cDNA was used to amplify MSTN gene using degenerative primers shown in Table 1. Initially, a partial cDNA fragment of 291 bp was amplified using a pair of degenerative primers: MSTN-F, 5′-CGGTGYTGCMGSTAY CCNCTY-3′ and MSTN-R, 5′-TCABGAG CABCCR CADCGGTC-3′. The resulting PCR products were ligated into pGEM-T Easy sequencing vector (Promega, Madison, WI) and transformed into *Escherichia coli* (JM109) competent cells (Promega). Positive clones were isolated by blue/white screening and were grown for plasmid preparation. The resulting cDNAs were sequenced using a modified dideoxy chain

termination method with Big Dye Terminator (Applied Biosystems, Foster City, CA). Sequencing reactions were precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems).

To clone the entire cDNA of MSTN, 5 μg of total RNA were reverse transcribed using GeneRacer Kit (Invitrogen). To obtain the full-length MSTN cDNA, nested 3′- and 5′-rapid amplification of cDNA ends (RACE) PCR was performed. For 3′-RACE PCR, gene-specific sense primers (MSTN 3-1F and MSTN 3-2F, Table 1) and antisense primers (GeneRacer Kit 3′ primers, Table 1) were used as amplification primers and for 5′-RACE PCR, two specific antisense primers (MSTN 5-1R and MSTN 5-2R, Table 1) and GeneRacer Kit 5′ first primer were used as amplification primers. PCR was carried out in a 50-μl final volume that contains 5 μl of 10 × PCR buffer with 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 μl of 50 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 1 μl of 10 μM each primer, 36.3 μl of sterile deionized water and 0.2 μl of Platinum Taq DNA Polymerase (5U μl^{-1}) (Invitrogen), and 4 μl of a 1:100 dilution of RACE-ready first-strand cDNA as template. The amplification is: 2 min initial denaturation at 94°C for one cycle, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, followed by a 10-min extension at 72°C and cooling to 4°C. The PCR product of the predicted size was gel-separated, purified, and inserted into a pGEM-T Easy Vector, and positive clones were isolated and nucleotide sequence determined as described in the previous section.

DNA was extracted from muscle of orange spotted grouper using Gene-Spin™ Genomic DNA Isolation Kit following instruction provided by the supplier (Protech, Taiwan). The MSTN gene was amplified from the genomic DNA by PCR, using gene-specific oligomers, MSTN-GF (5′-CAGTGTG GGACATTAATCC-3′) and MSTN-GR (5′-CTCAC CAGGATCTCCGTCCC-3′) designed from the orange spotted grouper MSTN full-length cDNA as amplification primers. The positions of the primers used for the PCR are indicated by arrows in Figure 1. The amplified product was cloned into the pGEM-T Easy vector (Promega) and nucleic acid sequence determined.

Nucleotide Sequence Analysis. The cDNA sequence and the deduced amino acid sequence of grouper MSTN were compared with default settings on the complete nonredundant GenBank database using BLAST program available for the NCBI Internet Web site (http://www.ncbi.nlm.nih.gov/blast/). Sequences were translated into predicted amino acid sequences and both nucleotide and predicted protein sequences were aligned with various known MSTN sequences. Multiple alignments of cDNA sequences and amino acid sequences were performed using the programs of BioEdit and CLUSTAL W, and the same software was used to analyze similarity of the aligned sequences using a neighbor joining (NJ) algorithm. Based on these multiple sequence alignments, Poisson-corrected distances were estimated for all possible pairs. A phylogenetic tree was constructed using the neighbor-joining method based on the obtained distance matrix, and node robustness was assessed using the bootstrap method (N = 1000 replications). All phylogenetic analyses were conducted using the Phylip program and the phylogenetic tree of MSTN was constructed with the programs CLUSTAL W, Bioedit, Phylip, and Treeview. Unreliable alignment regions and gap-containing regions were excluded from the analysis. Pairwise comparisons between various MSTN sequences were conducted using CLUSTAL X 1.8 program (Thompson et al., 1997). The phylogenetic tree based on the obtained distance matrix was reconstructed by the neighbor-joining algorithm using Neighbor program in the Phylip 3.6 package (Saitou and Nei, 1987). The reliability of the branching was assessed by bootstrap resampling method using 1000 bootstrap replications (Felsenstein, 1985). A phylogenetic tree was depicted using Treeview 1.6.6 program (Page, 1996) (Table 2).

Quantitative Real-Time RT-PCR. Eye, brain, gill, heart, muscle, head kidney, stomach, intestine, spleen, and liver were dissected from 1-year-old fish, or embryos and larvae of different stages, and total RNA was extracted in the TRIzol reagent (Invitrogen) following the protocol provided by the supplier. The total RNA was digested by the addition of RNAse-free DNAse (Promrga) to remove any genomic DNA contaminant. Five micrograms of total RNA was used for synthesizing the first strand cDNA with Superscript™ III RNase H- reverse transcriptase (Invitrogen).

The standard curve of MSTN gene was prepared according to Bustin (2000). Synthetic MSTN RNA was prepared by in vitro transcription with T7 RNA polymerase (Promega) using a plasmid containing MSTN cDNA as the template, and the product was digested with RNase-free DNase (Promega) and the resulting synthetic RNA was quantified with a spectrophotometer. The synthetic RNA diluted to different concentrations with diethyl pyrocarbonate (DEPC)-treated water was used as RNA standards. The RNA was reverse transcribed as described in the

Table 2. Organisms, Genbank Accession Numbers, Abbreviations, Proteins, and References of MSTNs

Organism	*Genbank accession No.*	*Abbreviation*	*Protien*	*Reference*
Mammalian				
Homo sapiens	AF019627	HoS-MSTN	375 aa	McPherron and Lee, 1997
	AF104922			Gonzalez-Cadavid et al., 1998
Papio hamadryas	AF019619	PaH-MSTN	375 aa	McPherron and Lee, 1997
Bos taurus	AF019620	BoT-MSTN	375 aa	McPherron and Lee, 1997
	AF320998			Jeanplong et al., 2001
Sus scrofa	AF019623	SuS-MSTN	375 aa	McPherron and Lee, 1997
Ovis aries	AF019622	OvA-MSTN	375 aa	McPherron and Lee, 1997
Mus musculus	NM-010834	MuM-MSTN	376 aa	McPherron and Lee, 1997
Rattus norvegicus	AF019624	RaN-MSTN	376 aa	McPherron and Lee, 1997
Avian				
Gallus gallus	AF019621	GaG-MSTN	375 aa	McPherron and Lee, 1997
Meleagris gallopavo	AF019625	MeG-MSTN	362 aa	McPherron and Lee, 1997
Teleost				
Danio rerio	AF019626	DaR-MSTN-I	374 aa	McPherron and Lee, 1997
	AF540956			–
	AY323521			Xu et al., 2003
	AY614000	DaR-MSTN-II	366 aa	–
	AY693972			Biga et al., 2005
Salvelinus fontinalis	AF247650	SaF-MSTN	373 aa	Roberts and Goetz, 2001
Oreochromis mossambicus	AF197193	OrM-MSTN	376 aa	Rodgers et al., 2001
Morone chrysops	AF197194	MoC-MSTN	377 aa	Rodgers et al., 2001
Morone saxatilis	AF290910	MoS-MSTN	376 aa	Rodgers and Weber, 2001
Morone americana	AF290911	MoA-MSTN	376 aa	Rodgers and Weber, 2001
Salmo salar	ASA297267	SaS-MSTN-I	373 aa	Ostbye et al., 2001
	ASA344158	SaS-MSTN-II	373 aa	Ostbye et al., 2001
Oncorhynchus mykiss	AF273035	OnM-MSTN-I	373 aa	Rescan et al., 2001
	AF273036	OnM-MSTN-II	373 aa	Rescan et al., 2001
Sparus aurata	AF258447	SpA-MSTN-a	385 aa	Maccatrozzo et al., 2001a,b
	AY046314	SpA-MSTN-b	385 aa	Maccatrozzo et al., 2001a,b
Ictalurus punctatus	AF396747	IcP-MSTN	389 aa	Kocabas et al., 2002a
Ictalurus furcatus	AY540992	IcF-MSTN	390 aa	Gregory et al., 2004
Ameiurus catus	AY540994	AmC-MSTN	393 aa	Gregory et al., 2004
Dicentrarchus labrax	AY839106	DiL-MSTN	376 aa	Terova et al., 2005
Epinephelus coioides	DQ493889	EpC-MSTN	376 aa	present study
Molluscan				
Argopecten irradians	AY553362	ArL-MSTN	382 aa	Kim et al., 2004

preceding text and stored at –80°C. Two gene-specific primers, MSTN RT-F and MSTN RT-R, were used to amplify the MSTN transcript (Table 1) for quantitative real-time RT-PCR.

Levels of MSTN mRNA expression in adult tissues or in embryos of different developmental stages were determined by quantitative real-time RT-PCR in the LightCycler 1.0 Continuous Fluorescence Detection System (Roche Diagnostics, Indianapolis, IN). The amplification primers for MSTN mRNA are MSTN RT-F (5′-GACGTGCTGG GAGATG-3′) and MSTN RT-R (5′-AGCTGAGCTC GGACTA-3′) which were designed across an exon: intron boundary to ensure that any contaminating DNA present was not amplified. Both reverse transcription and PCR amplification were carried out in one tube of 20 μl as the final reaction volume, which contained 0.2 μM forward primer, 0.2 μM reverse primer, 5 mM $MgSO_4$, and 0.5 μg of total RNA. The reaction programs were as follows: initial RT incubation at 50°C for 30 min, initial denaturation step for 5 min at 94°C, 40 cycles of denaturing (94°C for 5 s), annealing (60°C for 10 s), and extending (72°C for 20 s). All samples were run in duplicate with a standard curve of serially diluted synthetic RNA. For all real-time assays, ΔC_t values were converted to relative values based on their respective standard curves and were normalized to the corresponding β-actin RNA values. Data analysis was performed via LightCycler software V3.5 (Roche, Indianapolis, IN). Data are presented as means ± SD ($n = 3$).

Statistical Analysis. A multiple comparison (Tukey) test was conducted to compare the significant differences among levels of MSTN gene expression in different tissues and different developmental stages using SAS software (SAS Institute, Cary, NC). A significance level of $p \leq 0.05$ was chosen.

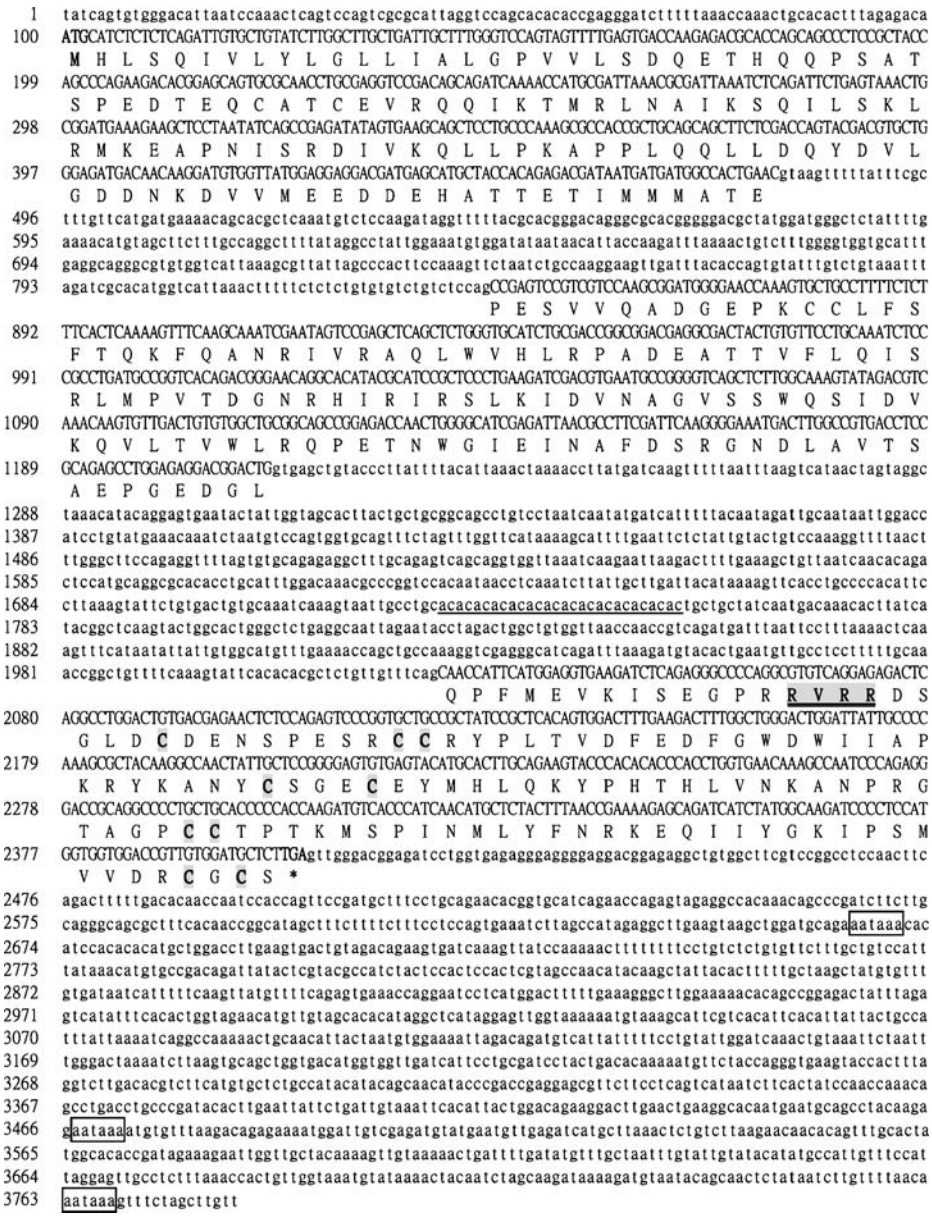

Fig. 2. Nucleotide sequence of grouper MSTN gene with the deduced amino acid. The deduced amino acid sequence of grouper MSTN is reported below the corresponding coding region (upper case), which is delimited by the putative start and stop codons (in boldface). Nucleotides are numbered from the first base at the 5′ end (transcription start site). Amino acids are numbered from the initiating methionine. The noncoding sequences are typed in lower case. The (AC) repeat region (see text) is underlined. The proteolytic processing site (RVRR) is indicated double underlined with gray shading. The nine conserved cysteine residues, characteristic of MSTN, are indicated in bold with gray shading. The asterisk indicates the stop codon. The polyadenylation signals are enclosed in solid lines at the 3′ downstream region. The sequence was submitted to GenBank with accession no. DQ493889.

Results

Cloning and Characterization of the Orange Spotted Grouper MSTN cDNA. To obtain the sequence of a full-length MSTN cDNA from the orange spotted grouper, RACE PCR was performed in the 3′ and 5′ directions. The 3′ RACE PCR generated a single cDNA fragment of 1648 bp, including a partial ORF (open reading frame) and the entire portion of the 3′-untranslated region (Figure 1). The 5′-RACE PCR extended the cDNA sequence in the 5′ direction with a single cDNA fragment of 1209 bp and provided the complete precursor protein sequence (Figure 1). The MSTN cDNA obtained from grouper is 2608 bp in length and has an ORF of 1128 bp encoding a prepro-MSTN of 376 amino acid residues (Figure 2). The 5′-untranslated region is 99 bp and the 3′-untranslated region is 1381 bp with three consensus AATAAA polyadenylation signals. Like other members of the TGF-β superfamily, grouper MSTN peptide has nine conserved cysteine residues and a RVRR proteolytic cleavage site (Figure 2). The grouper MSTN cDNA sequence and the deduced amino acid sequence have been submitted to the NCBI GenBank (accession no. DQ493889).

Two gene specific primers (MSTN-GF and MSTN-GR) were used to amplify a DNA fragment of 3598 bp from the genomic DNA. Nucleotide sequence analysis revealed that this DNA fragment contains 2 introns (Intron I, 363 bp; Intron II, 811 bp)

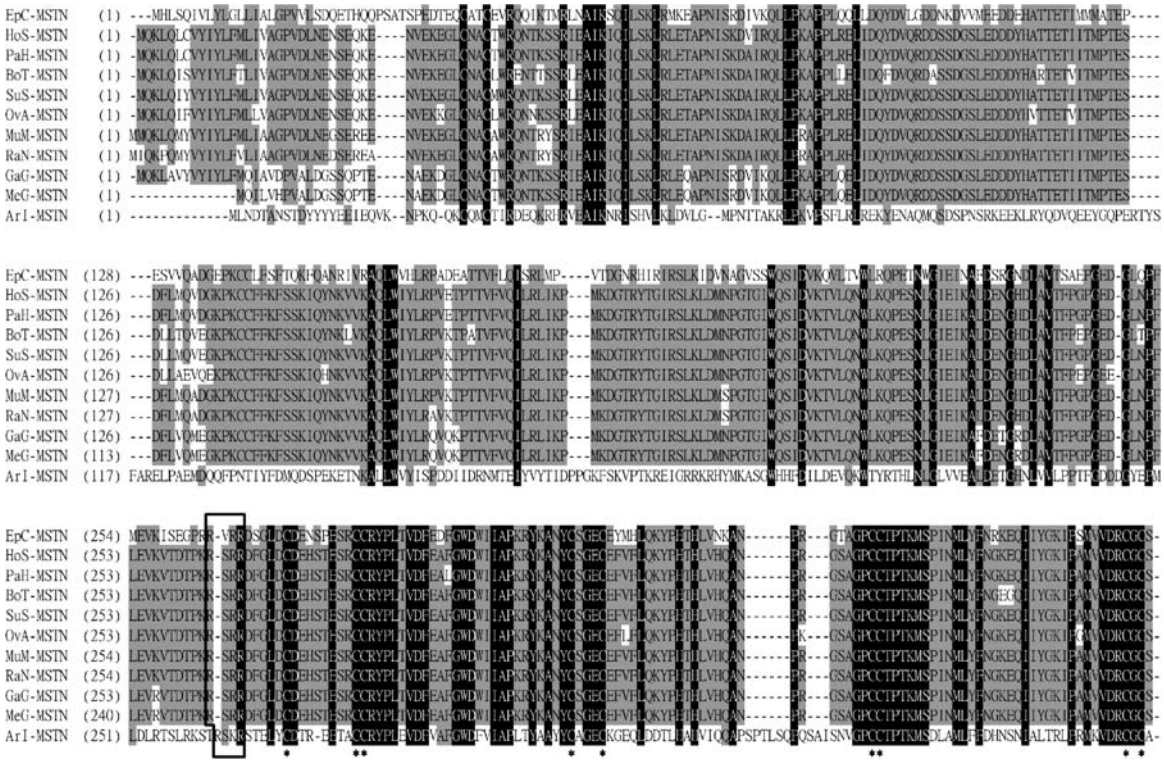

Fig. 3. Multiple alignment of the predicted amino acid sequence of grouper MSTN with amino acid sequences of MSTN in mammalian and avian species. MSTN proteins compared by CLUSTAL W multiple sequence alignments and their associated accession numbers are as follows: *Epinephelus coioides* (EpC-MSTN, DQ493889), *Homo sapiens* (HoS-MSTN, AF019627), *Papio hamadryas* (PaH-MSTN, AF019619), *Bos taurus* (BoT-MSTN, AF019761), *Sus scrofa* (SuS-MSTN, AF019623), *Ovis aries* (OvA-MSTN, AF019622), *Mus musculus* (MuM-MSTN, NM_010834), *Rattus norvegicus* (RaN-MSTN, AF019624), *Gallus gallus* (GaG-MSTN, AF019621), *Meleagris gallopavo* (MeG-MSTN, AF019625), and *Argopecten irradians* (ArI-MSTN, AY553362). Dashes indicate insertion–deletions; shading refers to different degree of overall conservation for each site (black, identical; gray, conservative). The putative RXXR proteolytic processing site of MSTN is enclosed in solid lines. The nine conserved cysteine residues in MSTN C-terminus are indicated with an asterisk.

and three exons (Exon I, 379 bp. Exon II, 371 bp and Exon III, 381 bp). In comparison with the MSTN gene in mammals, the sizes of Intron I and Intron II of grouper MSTN gene are much shorter than those of the mammalian MSTN gene.

Comparison of MSTN Amino Acid Sequences Among Molluscs, Fish, Chicken, and Mammals. Results presented in Figures 3 and 4 and Table 3 show that the amino acid sequence of the predicted grouper prepro-MSTN shared a high degree of homology with other MSTNs reported to date. Grouper MSTN shared about 96% homology with that of *Morone saxatilis*, *Morone americana*, and *Dicentrarchus labrax*, and had a lower homology with *Danio rerio* MSTN-II (62%), *Sparus aurata* MSTN-b (66%), *Ictalurus punctatus* (75%), *Ictalurus furcatus* (74%) or *Ameiurus catus* (74%). With *Homo sapiens*, *Papio hamadryas*, *Bos taurus*, *Sus scrofa*, *Ovis aries*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Meleagris gallopavo*, or *Argopecten irradians*, grouper prepro-MSTN shares a homology of 64%, 64%, 62%, 64%, 63%, 63%, 63%, 64%, and 63% with each other, respectively (Table 3). Figure 5 presents the phylogenetic relationship of prepro-MSTN with molluscs, fish, chicken, and mammals. As shown in Figure 5, the grouper MSTN belongs to the cluster of teleostean MSTN and was closely related to *Morone saxatilis*, *Morone americana*, and *Dicentrarchus labrax*.

Tissue-Specific and Developmental Stage-Specific Expression of Grouper MSTN Gene. To examine tissue-specific expression of grouper MSTN gene in 1-year-old fish, central nervous system and various tissues (eyes, brain, gill, heart, muscle, head kidney, stomach, intestine, spleen, and liver) were collected from 1-year-old fish and

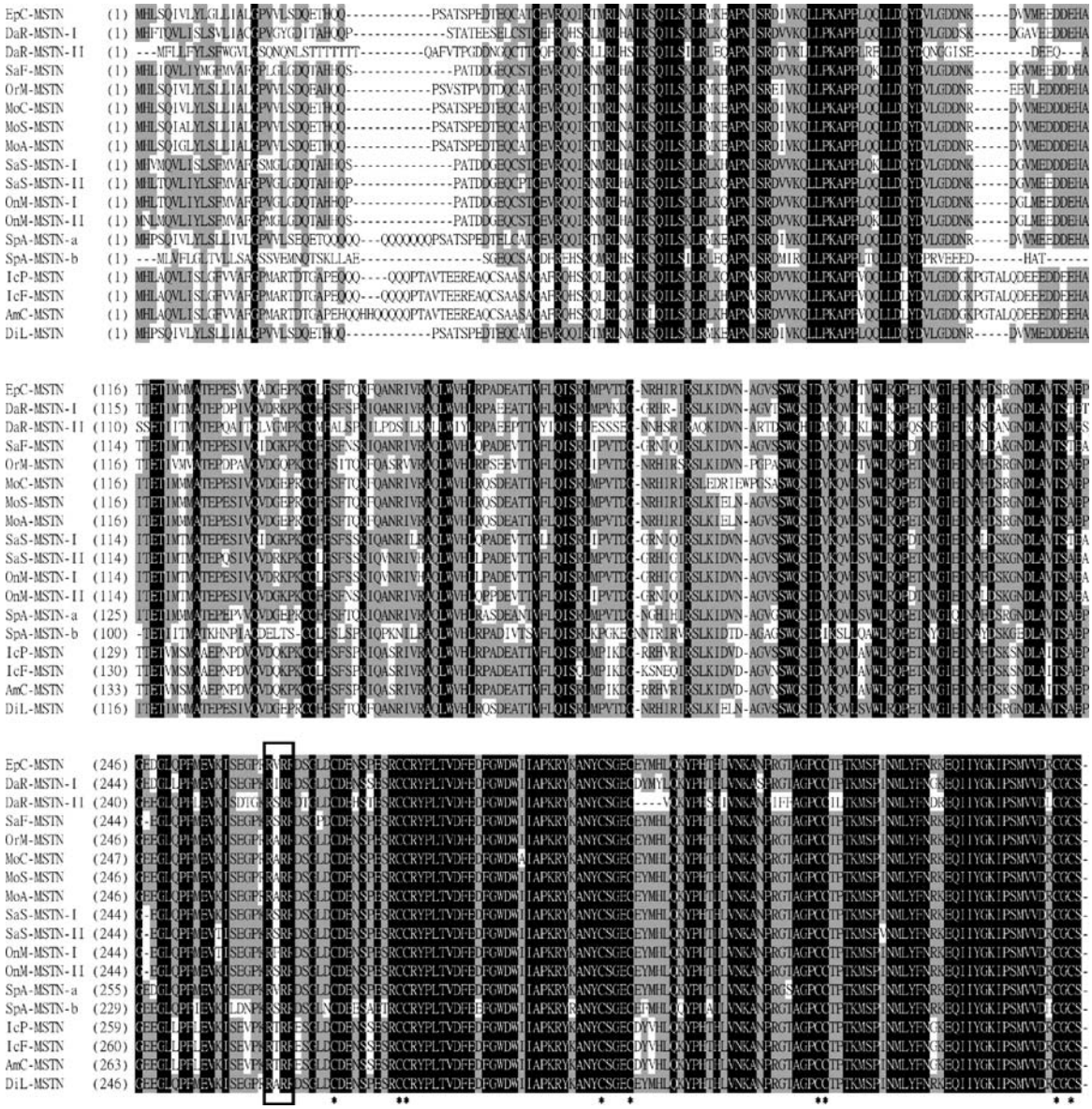

Fig. 4. Multiple alignment of the predicted amino acid sequence of grouper (*Epinephelus coioides*) MSTN with other piscine MSTN amino acid sequences. Piscine MSTN proteins compared by CLUSTAL W multiple sequence alignments and their associated accession numbers are as follows: *Danio rerio* (DaR-MSTN-I, AF019626; DaR-MSTN-II, AY693972), *Salvelinus fontinalis* (SaF-MSTN, AF247650), *Oreochromis mossambicus* (OrM-MSTN, AF197193), *Morone chrysops* (MoC-MSTN, AF197194), *Morone saxatilis* (MoS-MSTN, AF290910), *Morone americana* (MoA-MSTN, AF290911), *Salmo salar* (SaS-MSTN-I, ASA297267;SaS-MSTN-II, ASA344158), *Oncorhynchus mykiss* (OnM-MSTN-I, AF273035;OnM-MSTN-II, AF273036), *Sparus aurata* (SpA-MSTN-a, AF258447;SpA-MSTN-b, AY046314), *Ictalurus punctatus* (IcP-MSTN, AF396747), *Ictalurus furcatus* (IcF-MSTN, AY540992), *Ameiurus catus* (AmC-MSTN, AY540994), and *Dicentrarchus labrax* (DiL-MSTN, AY839106). Dashes indicate insertion–deletions; shading refers to different degree of overall conservation for each site (black, identical; gray, conservative). The putative RXXR proteolytic processing site of MSTN is enclosed in solid lines. The nine conserved cysteine residues in MSTN C-terminus are indicated with an asterisk.

RNA samples extracted from these tissues for measuring levels of MSTN mRNA by quantitative real-time RT-PCR analysis. As shown in Figure 6, MSTN mRNA was detectable in all of these tissues. The highest level of MSTN mRNA was observed in the gill and muscle tissues, medium levels in eyes, brain, heart, head kidney, stomach, and intestine tissues, and low levels in spleen and liver tissues.

Table 3. Amino Acid Sequence Percent Similarity (%) of Orange Spotted Grouper Prepro-MSTN Compared to Different Molluscan, Teleostean, Avian and Mammalian Species

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*
1	EpC-MSTN	100	64	63	61	64	62	63	63	64	63	82	62	85	91	94	96	96	84	86	86	85	92	66	76	75	74	96	25
2	HoS-MSTN	–	100	99	94	98	94	96	95	92	90	67	57	65	64	62	63	63	64	65	65	65	63	59	61	60	60	63	29
3	PaH-MSTN	–	–	100	94	98	94	96	95	91	89	67	57	64	64	62	63	63	64	64	64	65	63	59	60	59	60	63	29
4	BoT-MSTN	–	–	–	100	95	93	92	91	88	85	65	56	63	61	61	61	61	62	63	63	63	62	58	59	58	58	61	29
5	SuS-MSTN	–	–	–	–	100	95	96	95	91	89	67	57	65	64	62	63	63	64	64	64	65	63	60	60	60	60	63	29
6	OvA-MSTN	–	–	–	–	–	100	92	91	88	85	65	57	64	63	61	62	62	63	64	64	64	62	59	60	59	59	62	29
7	MuM-MSTN	–	–	–	–	–	–	100	98	91	88	66	56	64	63	62	62	62	64	63	63	64	61	59	60	59	60	62	28
8	RaN-MSTN	–	–	–	–	–	–	–	100	90	87	66	56	63	62	62	62	62	63	63	63	64	62	59	60	59	59	62	28
9	GaG-METN	–	–	–	–	–	–	–	–	100	96	67	56	65	64	63	64	64	64	66	66	65	62	59	61	60	61	64	28
10	MeG-MSTN	–	–	–	–	–	–	–	–	–	100	66	57	64	63	62	63	63	64	65	65	64	62	59	61	60	60	63	28
11	DaR-MSTN-I	–	–	–	–	–	–	–	–	–	–	100	64	84	79	79	80	80	84	85	86	84	78	67	78	77	77	80	25
12	DaR-MSTN-II	–	–	–	–	–	–	–	–	–	–	–	100	63	60	59	61	61	63	63	63	63	59	61	59	58	58	61	24
13	SaF-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	100	81	81	83	83	96	92	93	97	79	66	75	75	74	83	25
14	OrM-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	100	91	91	91	81	82	82	82	86	64	74	72	72	91	25
15	MoC-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	97	97	82	83	83	83	90	64	73	72	72	97	24
16	MoS-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	99	84	85	85	85	92	65	75	73	73	99	25
17	MoA-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	84	85	85	85	92	65	75	73	73	99	25
18	SaS-MSTN-I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	93	93	97	79	67	76	75	75	84	26
19	SaS-MSTN-II	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	99	94	81	67	77	75	75	85	25
20	OnM-MSTN-I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	95	81	67	77	76	75	85	26
21	OnM-MSTN-II	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	81	67	76	76	75	85	26
22	SpA-MSTN-a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	63	74	73	74	92	25
23	SpA-MSTN-b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	64	63	62	65	26
24	IcP-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	97	98	75	24
25	IcF-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	97	73	24
26	AmC-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	73	24
27	DiL-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100	25
28	ArI-MSTN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100

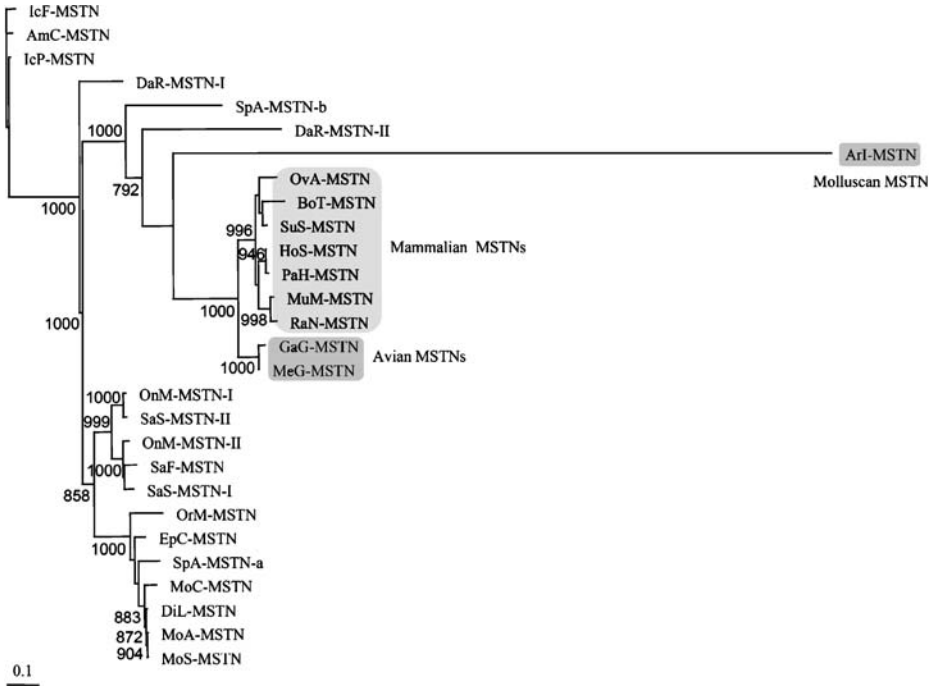

Fig. 5. Neighbor-joining phylogetic tree of MSTN amino acid sequences of molluscan, teleostean, avian, and mammalian species, based on Poisson-corrected protein distances. Phylogenetic tree of MSTNs obtained using Phylip software via the neighbor-joining method. The tree was generated via CLUSTAL X1.8 and depicted visually via TreeView1.6.6. Positions containing gaps were excluded from the analysis. Numbers at tree nodes refer to bootstrap values after 1000 replicates. The scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site.

These results showed that grouper MSTN gene is expressed in many tissues other than gill and muscle in 1-year-old fish.

The expression of grouper MSTN gene in different embryonic stages and larval stages was also assessed by quantitative real-time RT-PCR analysis. MSTN mRNA was detected in the unfertilized eggs, the newly fertilized eggs, 16-cell stage, and morula stage embryos (Figure 7a). Although lower levels of MSTN mRNA were detected in the blastula, gastrula, and neurula stages, higher levels of mRNA were detected during the lens formation stage and then gradually decreased to lower levels again in day 60 larval stage (Figure 7b).

Discussion

In this article we report the isolation of a full-length cDNA encoding the MSTN precursor from the muscle of orange spotted grouper using RACE-PCR. The grouper MSTN cDNA sequence is 2608 bp in length and has an ORF of 1128 bp encoding a prepro-MSTN with 376 amino acid residues. The deduced amino acid sequence contains a potential proteolytic site (RVRR), matching with the RXRR consensus site, and nine conserved cysteine residues at the carboxyl-terminus, like all of the previously described MSTN orthologues (Hu et al., 1998). It is believed that these nine cysteine residues found in the MSTN orthologues of all TGF-β superfamily members participated in the formation of the characteristic cysteine knot through intramolecular disulfide linkages (Rodgers et al., 2001). Comparison of the amino acid sequence of grouper MSTN with those of known MSTN reported to date revealed that grouper MSTN shared a higher degree of homology with those of

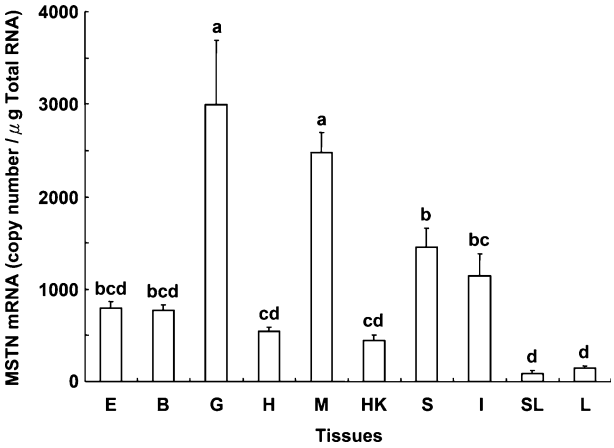

Fig. 6. Levels of MSTN mRNA in various tissues of adult grouper. RNA samples were isolated from adult tissues and levels of MSTN mRNA were measured by real-time RT-PCR analysis following procedures described in Materials and Methods. Each data point is the average of three independent determinations and each sample contains tissues from three animals. The data points are expressed as mean ± SD, and were analyzed by one-way analysis of variance (ANOVA). Different letters indicate means with significant differences ($P < 0.05$). E, eyes; B, brain; G, gills, H, heart; M, muscle; HK, head kidney; S, stomach; I, intestine, SL, spleen; and L, liver.

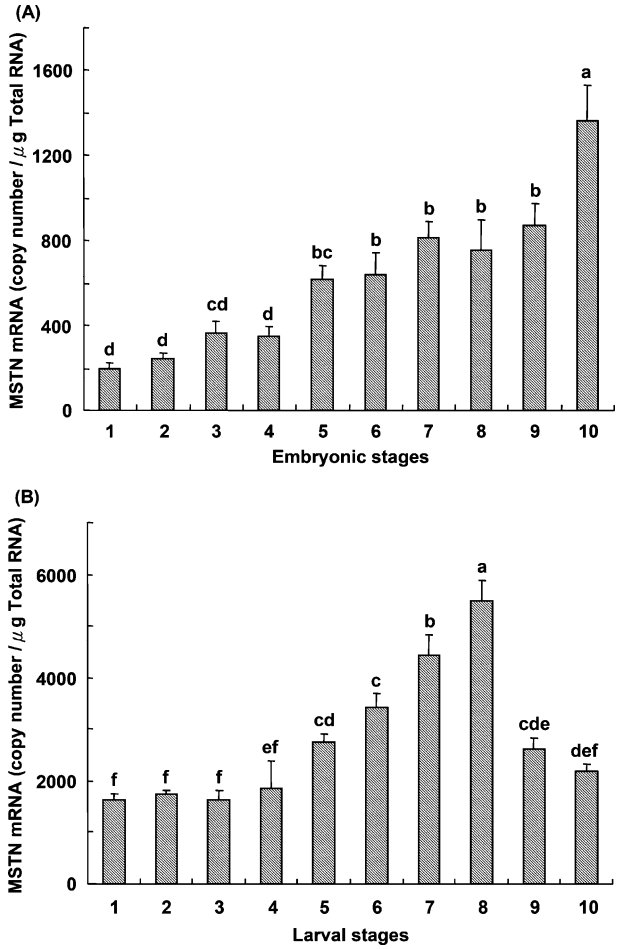

Fig. 7. Levels of MSTN mRNA in grouper of different embryonic (**A**) and larval (**B**) stages. RNA samples were isolated from grouper of different developmental stages and levels of MSTN were determined by real-time RT-PCR as described in Materials and Methods. The data points are expressed as mean ± SD, and were analyzed by one-way analysis of variance (ANOVA). Different letters indicate means with significant differences ($P < 0.05$). (**A**) 1, unfertilized eggs; 2, fertilized eggs; 3, 16 cells stage; 4, morula stage; 5, blastula stage; 6, gastrula stage; 7, neurula stage; 8, lens formation stage; 9, somite stage; and 10, hatching. (**B**) 1, 1-day-old larvae (newly hatched larvae with yolk); 2, 2-day-old larvae (fry); 3, 5-day-old larvae (mouth formation); 4, 10-day-old larvae (pigment formation); 5, 15-day-old larvae (fin rays formation); 6, 20-day-old larvae (fin rays maximum); 7, 25-day-old larvae (fin rays reduction); 8, 30-day-old larvae (5 to 25 mm total length (TL)); 9, 45-day-old larvae (25 to 35 mm TL); and 10, 60-day-old larvae (35 to 45 mm TL).

other fish MSTN, but a lower degree of homology with those of mammalian MSTN. Results of phylogenetic analysis showing that grouper MSTN is closest to *Morone saxatilis*, *Morone americana*, and *Dicentrarchus labrax* in the dandrogram further support the conclusion that grouper MSTN is closer to MSTN of other fish than to higher vertebrates (Kerr et al., 2005). Because grouper MSTN is well conserved through evolution, it suggests that this peptide may be important in controlling growth and development, and the biological action may be conserved as well.

While the function of MSTN in controlling muscle development and growth has been well documented in higher mammals (McPherron and Lee, 1997; McPherron et al., 1997; Lee and McPherron, 2001), the function of fish MSTN is awaiting elucidation. Two recent studies have been attempted unsuccessfully to reproduce the enhanced muscle growth phenotype in zebrafish by overexpressing the prodomain of MSTN which will inhibit the function of MSTN (Xu et al., 2003) or by reducing the level of MSTN mRNA production with an antisense morpholinos (Amali et al., 2004). In both studies, only an increased number of myofibers in skeletal muscles was observed, suggesting that fish MSTN may control only the hypertrophy of the skeletal muscle. Therefore, detail studies on the tissue-specific and developmental stage-specific expression of MSTN gene in different fish species may shed insight on the understanding of the biological function(s) of MSTN in fish. Quantitative real-time RT-PCR analysis on the tissue distribution of MSTN mRNA in 1-year-old grouper revealed that several tissues other than myogenic lineage cells expressed MSTN gene. Although the highest level of grouper MSTN mRNA was detected in the gill and muscle, lower levels in the eyes, brain, heat, head kidney, stomach, and intestine, and much lower levels in the spleen and liver. These results are in good agreement with those reported for other fish species (Maccatrozzo et al., 2001a,b; Roberts and Goetz, 2001; Rodgers and Weber, 2001; Rodgers et al., 2001; Radaelli et al., 2003), even though we did not determine the presence of myostatin protein in these tissues. In contrast to fish, MSTN mRNA is predominantly expressed in skeletal muscle, though there have been reports of MSTN protein detected in cardiomyocytes, Purkinje fibers of the heart, adipose tissue (Sharma et al., 1999), and in the mammary gland (Ji et al., 1998) in higher vertebrates.

In this study, the expression of MSTN gene in grouper during embryonic and larval development was also determined by quantitative real-time PCR analysis. The results of the study showed that low levels of MSTN mRNA were detected in the unfertilized eggs, the newly fertilized eggs, 16-cell stage, and morula stage, while higher levels were detected in the blastula stage, gastrula stage, neurula stage, lens formation stage, and somite stage. Further, the levels of MSTN mRNA continue to increase during development and peaks at 30-day-old larvae. These

results are somewhat consistent with the expression pattern of MSTN in mammals in which MSTN is expressed in the myotome compartment of the somite during embryogenesis and in limb muscle during fetal development (McPherron et al., 1997). Variation on the ontogeny of MSTN gene expression in fish has been observed in catfish [detected at 1 day postfertilization (Kocabas et al., 2002b)], and in zebrafish [detected at 4 days postfertilization via in situ hybridization (Xu et al., 2003)]. However, one distinct difference between the expression of MSTN gene in grouper and in mammals is that grouper MSTN mRNA is found as a maternal mRNA and is also detectable in very early stages of embryogenesis. Because grouper MSTN gene is expressed in early embryogenesis as well as in multiple tissues, we, therefore, believe that, in addition to controlling skeletal muscle growth and differentiation, MSTN gene might also play other roles in fish. Recently Mitchell et al. (2006) reported that treatment of human term placental explants with myostatin protein resulted in an increase in deoxyglucose uptake compared with controls. Our findings of MSTN mRNA in mature oocytes as well as during early stages of embryogenesis might suggest its involvement in the mobilization of carbohydrates and proteins in these stages of development. Further in-depth studies are required to unveil the biological function(s) of MSTN in fish.

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