

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 tissuespecific 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

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-B 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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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

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

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 $10 \times PCR$ buffer with 200 mM Tris-HCl (pH 8.4), 500 mM KCl, $1.5 \,\mu$ l of 50 mM MgCl₂, 1 μ l 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 $(5U \ \mu l^{-1})$ (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 CLUS-TAL 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 gapcontaining 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 SuperscriptTM 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

Organism	Genbank accession No.	Abbreviation	Protien	Reference
Mammalian				
Homo sapiens	AF019627	HoS-MSTN	375 aa	McPherron and Lee, 1997
1	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

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

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 MgSO₄, 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 \pm 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 $p \le 0.05$ was chosen.

 1
 tatcagtgtgggacattaatccaaactcagtccggtcgcgcattaggtccagcacacaccgagggatetttttaaaccaaactgcacactttagagaca

 100
 ATGCATCTCTCTCAGATTGTGCTGTATCTTGCTTGCTGATTGCTTTGGGTCCAGTAGTTTTGAGTGACCAAGAGGCGCACCAGCAGCCCCCGCACC

M H L S Q I V L Y L G L L I A L G P V V L S D Q E T H Q Q P S A T 199 AGCCCAGAAGACACGGAGCAGTGCGCAACCTGCGAGGGCCGGATCAAACCATGCGATTAAACGCGATTAAATCTCCAGATTCTGAGTAAACTG

595 aaaacatgtagcttctttgccaggctttataggcctattggaaatgtggatataataacattaccaagatttaaaactgtctttggggtggtgcattt 694 gaggcaggcgtgtggtcattaaagcgttattagcccacttccaaagttctaatctgccaaggaagttgatttacaccagtgtatttgtctgtaaattt

- 991 CCCCTGATOCCOGTCACAGACOGGAACAGGCACATACOCATCOCTCCCTGAAGATOGACGTGAATOCCGGGGTCAGCTCTTGGCAAAGTATAGACGTC R L M P V T D G N R H I R I R S L K I D V N A G V S S W Q S I D V 1090 AAACAAGTGTTGACTGTGTGGCTGCGGCAGCCGGGAGACCAACTGGGGAACTGAGCTTGGAGTTCAAGGGGAAATGACTTGGCGGTGACCTCC
- K Q V L T V W L R Q P E T N W G I E I N A F D S R G N D L A V T S GCAGAGCCTCGCAGAGGACGGACTGgtgagctgtacccttattttacattaaactaaaaccttatgatcaagtttttaatttaagtcataactagtaggc A E P G E D G L

1288 1387 atcctgtatgaaacaaatctaatgtccagtggtgcagtttctagttggttcataaaagcattttgaattctctattgtactgtccaaaggttttaact 1486 ttgggcttccagaggttttagtgtgcagagaggctttgcagagtcagcaggtggttaaatcaagaattaagacttttgaaagctgttaatcaacacaga 1585 ctccatgcaggcgcacacctgcatttggacaaacgcccggtccacaataacctcaaatcttattgcttgattacataaaagttcacctgccccacattcacatc1684 $ctta a agtattctgtga actgtg caaat caa agt a attge ctgc \underline{a caca a caca a caca a caca a caca a caca a caca caca a caca caca a caca ca$ 1783 1882 agtitcataatattattgtggcatgttgaaaaccagctgccaaaggtcgagggcatcagattaaagatgtacactgaatgttgcctcctttttgcaa accggctgtttttcaaagtattcacaccgctctgttgtttcagCAACCATTCATGGAGGTGAAGATCTCAGAGGGCCCCAGGCGTGTCAGGAGAGACTC 1981 Q P F M E V K I S E G P R <u>R V R R</u> D S AGGCCTGGACTGTGACGAGAACTCTCCAGAGTCCCCGGTGCTGCCGCTATCCCCTCACAGTGGACTTTGAAGACTTTGGCTGGGACTGGATTATTGCCCC 2080 G L D C D E N S P E S R C C R Y P L T V D F E D F G W D W I I A P 2179 K R Y K A N Y C S G E C E Y M H L O K Y P H T H L V N K A N P R G 2278 GACCGCAGGCCCCTCCTGCACCCACCAAGATGTCACCCCATCAACATGCTCTACTTTAACCGAAAAGAGCAGATCATCTATGGCAAGATCCCCTCCAT TAGPCCTPTKMSPINMLYFNRKEOIIYGKIPSM 2377 VVDRCGCS* 2476 agacttttttgacacaatccaatccaccagttccgatgctttcctgcagaacacggtgcatcagaaccagagtagaggccacaaacagcccgatcttcttg 2575 2674 2773 2872 gtgalaatcalttiticaagtlatgtillcagagtgaaaccaggaatccicatggacttillgaaagggcitggaaaaacacagccggagactalttaga 2971 gtcatatttcacactggtagaacatgttgtagcacacataggctcataggagttggtaaaaaatgtaaagcattcgtcacattcacattattactgcca 3070 tttattaaaatcaggccaaaaactgcaacattactaatgtggaaaattagacagatgtcattatttttcctgtattggatcaaactgtaaattctaatt 3169 tgggactaaaatcilaagtgcagctggtgacatggtggtlgatcaltcctgcgatcctactgacaaaaaagttctaccagggtgaagtaccactita 3268 3367 3466 gaataaaatgtgtttaagacagagaaaatggattgtcgagatgtatgaatgtlgagatcatgcttaaactctgtcttaagacaacaacagtttgcacta 3565 3664 taggagttgcctctttaaaccactgttggtaaatgtataaaactacaatctagcaagataaaagatgtaatacagcaactctataatcttgttttaaca 3763 aataaagtttctagcttgtt

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. 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 Gen-Bank with accession no. DQ493889.

EpC-MSTN	(1)	MHLSQIVL	YLGLLIALGPVV	LSDQETHQQPSA	ISPEDIECOATCE	VRQQINTMRLN	AIKSQILSKI	RMKEAPNISRDIV	KQULEKAPPLOX	LDQYDVLGDDNKD	VVMEEDDEHATTETI	MMATEP
HoS-MSTN	(1)	-MQKLQLCVYI	YLFMLIVAGPVI	LNENSEQKE	NVEKEGLONACT	WRONTKSSNIE	AIKIQILSKI	RLETAPNISKDVI	RQUIEKAEPLRE	IDQYDVQRDDSSD	GSLEDDDYHATTETI	ITMPTES
PaH-MSTN	(1)	-MQKLQLCVYI	YLFMLIVAGPVE	LNENSEQKE	NVEKEGLONACT	WRONTKSSRIE	AIKIQILSKI.	RLETAPNI SKDA I	RQLIEKAPPLRI	IDQYDVQRDDSSD	GSLEDODYHATTETI	ITMPTES
BoT-MSTN	(1)	-MQKLQISVYI	YLFTLIVAGPVI	INENSEQKE	NVEKEGLONAGL	WEENTISSELE	AIKIQILSKI.	RLETAPNISKDAI	RQUEKAEPLLE	I DQFDVQRDASSD	GSLEDODYHARTETV	ITMPTES
SuS-MSTN	(1)	-MQKLQIYVYI	YLFMLIVAGPVI	LNENSEQKE	NVEKEGLONACH	MONTKSSELE	AIKIQILSKL	RLETAPNISKDAI	RQLIEKAEPLRE	IDQYDVQRDDSSD	GSLEDDDYHATTETI	ITMPTES
OvA-MSTN	(1)	-MOKLQIFVYI	YLFMLLVAGPVI	LNENSEQKE	NVEKKGLONAOL	WRONNESSELE	AIKIQILSKI	RLETAPNISKDAI	RQUEKAEPLRE	IDQYDVQRDDSSD	GSLEDDDYHVTTETV	ITMPTES
MuM-MSTN	(1)	MMQKLQMYVYI	YLFMLIAAGPVI	LNEGSEREE	NVEKEGLONAGA	WRONTRYSKIE	AIKIQILSKI.	RLETAPNISKDAI	RQUERAPLRE	IDQYDVQRDDSSD	GSLEDDDYHATTETI	ITMPTES
RaN-MSTN	(1)	MIQKPQMYVYI	YLFVLIAAGPVI	INEDSEREA	NVEKEGLONAGA	WRONTRYSKIE	AIKIQILSKI.	RLETAPNI SKDA I	RQUERAPLRI	I IDQYDVQRDDSSD	GSLEDODYHATTETT	I'IMPTES
GaG-MSTN	(1)	-MOKLAVYVYI	YLFMQIAVDPVA	LDGSSQPTE	-NAEKDGLONAGT	WRONTKSSNIE	AIKIQILSKI	RLEOAPNISRDVI	KQLLEKAEPLO	IDQYDVQRDDSSD	GSLEDDDYHATTETI	ITMPTES
MeG-MSTN	(1)		MOILVHPVA	LDGSSOPTE	-NAEKDGLONACT	WRONTKSSRIE	AIKIGILSKI	RLEOAPNISRDVI	KOLLEKAPPLO	IDQYDVQRDDSSD	GSLEDDDYHATTETI	ITMPTES
ArI-MSTN	(1)		MLNDTANST	YYYYE IEQVK-	NPKQ-QKQQMCT	INDEQURING	AIKNRI SHV	KLDVLGMPNIT	AKREEKVESFLE	REKYENAQMQSDS	PNSRKEEKLRYQDVQ	EEYGQPERTYS
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EpC-MSTN	(128)	ESVVQADG	EPKCCLFSFTON	FQANRIVRACLW	VELRPADEATTVF	LOISRLMP	-VIDGNRHIE	IRSLKIDVNAGVS	SWQSIDVKQVL	VALROPETNWCIEI	MEDSRENDLANTSA	FGED-GLOEF
HoS-MSTN	(126)	DFLMQVDG	KPKCCFFKFSSK	IQYNKVVKAQLW	IYLRPVETPTTVF	VQULRLIKP	-MKDGTRYTC	IRSLKLDMNPGTG	INQSIDVKTVLC	NMLKQPESNLCIE1	KALDENCHDUAWITFP	GPGED-GLNEF
PaH-MSTN	(126)	DFLMQVDG	KPKCCFFKFSSK	IQYNKVVKAQLW.	IYLRPVETPTTVF	VQULRLIKP	-MKDGTRYTC	IRSLKLDMNPGTG	INQSIDVKTVL	MULKOPESNLCIEI	KALDENCHULAWITFP	GPGED CLNEF
BoT-MSTN	(126)	DLETOVEG	KPKCCFFKFSSK	IQYNKLVKAQLW.	IYLRPVKTPATVF	VQULRLIKP	-MKDGTRYTG	IRSLKLDMNPGTG	INQSIDVKTVLO	NULKOPESNLEIEI	KALDENCHDUANTEPI	PCED - CLIEF
SuS-MSTN	(126)	DLEMQVEG	KPKCCFFKFSSk	IQYNKVVKAQLW	IYLRPVKTPTTVF	VQULRLIKP	-MKDGTRYTC	IRSLKLDMNPGTG	INQSIDVKTVL	NULKQPESNLCIEI	KALDENCHDUAWIFP	GPCED-CLNEF
OvA-MSTN	(126)	DLLAEVQE	KPKCCFFKFSSk	IQHNKVVKAQLW	IYLRPVKTPTTVF	VOILRLIKP	-MKDGTRYTO	IRSLKLDMNPGTG	INOSIEVKTVL	NULKOPESNLEIEI	KALDENCHDLAWITFP	PCEE CLNEF
MuM-MSTN	(127)	DFLMQADG	KPKCCFFKFSSk	IQYNKVVKAQLW	IYLRPVKTPTTVF	VQILRLIKP	-MKDGTRYTC	IRSLKLDMSPGTG	IWQSIDVKTVL	NMLKQPESNLCIEI	KALDENCHULAWITFP	GPCED-CLNEF
RaN-MSTN	(127)	DFLMQADG	KPKCCFFKFSSk	IQYNKVVKAQLE	IYLRAVKTPTTVF	VQULRLIKP	-MKDGTRYTG	IRSLKLDMSPGTG	INQSIDVKTVL	NULKOPESNLE IEII	KALDENCHDUANTEP	GPCED-CLNEF
GaG-MSTN	(126)	DFLVQMEG	KPKCCFFKFSSK	IQYNKVVKAQLW.	IYLROVOKPTIVF	VQULRL1KP	-MKDGTRYTC	IRSLKLDMNPGTG	IWQSIDVKTVL	NGLKQPESNLCIEI	KAFDETCRDLAWITFP	GPGED - GLNEF
MeG-MSTN	(113)	DFL VQMEG	KPKCCFFKFSSK	IQYNKVVKHQLW	IYLROVOKPTIVF	VQULRLIKP	-MKDGTRYTC	IRSLKLDMNPGTG	INOSIDVKTVL	NULKOPESNICIEI	KAFDENCRDLAWITFP	GPGED CLNEF
ArI-MSTN	(117)	FARELPAEMDO	QFPNTIYFDMQD	SPEKETNKALLW	VY ISPDDIIDRNM	TELYVYTIDPP	GKFSKVPTKR	EIGRRKRHYMKAS	GMHHFD ILDEV	KWTYRTHLNLCLVVI	EAUDEICHNIMILPP	IFODDOYEEM
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EpC-MSTN	(254)	MEVKISEGPRE	- VRRDSCLDCDE	NSFESRCCRYPL	IVDFEDFGWDWIII	APERYKANYCS	GEOEYMHDOK	YPITTILVNKAN	BR GT/	GPCCIPIKMSPINM	YENRKEO I I YGK II.	SWWDRCGCS-
HoS-MSTN	(253)	LEVKVIDIPKR	- SRRDFGLDCDF	HSTESRCCRYPL	IVDFEAEGWDWI I	APERYKANYCS	HEOEFVFUQK	YPETHLVHQAN	R GS/	GPCCIPIKMSPINM	YENGKEQUI YOK II.	AMANDROGOS-
PaH-MSIN	(253)	LEVKVIDTPK	- SRRDFCLDCDE	HSTESRCCRYPL	IVDFEALGWDWI I	APKRYKANYCS	HEGEFVFILQK	YEWIHLVHQAN	GS/	GPCCIPIKMSPINM	YENGKEQ11 YCK III.	AMANDROGGS-
BoT-MSIN	(253)	LEVKVIDTPKR	- SRRDFGLDCDF	IISTESRCCRYPL	IVDPEARGWDWIII	APKRYKANYCS	GEOEFVELOK	YPETHLVHQAN	BRGS/	GPCCIPIKMSPINM	YENGECOLI YOK IL	AWWDRCGCS-
SuS-MSTN	(253)	LEVKVIDTPKK	- SRRDFCLDCDE	HSTESRCCRYPL	IVDFEARGWDWI I	ALKRYKENYCS	BEEFVFUCK	YPHTHLVHQAN	GS/	GRCCIPIKMSPINM	YENGKEQII I YCK II.	MINVDROGCS-
OvA-MSTN	(253)	LEVKVIDIPKE	-SNRDFGLLCDF	HSTESRCCRYPL	IVDFEARGWDWIII	AFRIMKANYOS	HE EFLELOK	YHETHLVHQAN	K GS/	GECCIPIKMSPINM	YENGKEQ, I YGK I	AWVDRCGCS-
MuM-MSTN	(254)	LEVKVTDTPKR	- SREDFCLDCDE	HSTESRCCRYPL	IVDFEAFGWDWIII	AFKRIKANYOS	HECEFVFDQK	YPHIIILVHQAN	GS/	GPCCIPIKMSPINM	YENGKEQ I I YCK II.	AMANDROGOS-
RaN-MSTN	(254)	LEVKVIDTPKR	- SNRDFGLUCDE	HSTESKCCRYPL	INDREAFGWDWIII	APKINYKANYOS	CHECEFVELOK	YHUTHLVHOMN	GS/	GECCIPIKMSPINM	YENGKECH I YOK IR	AMAYVDRCGCS-
GaG-MSTN	(253)	LEVRVIDTPR	-SERDFGLDCDE	HSTHSRCCRYPL	IVDFEARGWDWIII	AFKRYKANYCS	GECEFVEDQK	YHHIHLVHQAN	R GS/	GPCCIPIKMSPINM	YENGKEQ I I YGK II.	WINDROGOS-
MeG-MSIN	(240)	LEVRVIDTPRE	- SNRDFGLUCDF	HSTESRCCRYPL	IVDFEAFGWDW11	AFRICIKANYCS	ORCEFVELOK	YHUIHLVHON	<u>BR</u> <u>GS</u> /	GECCIPIKASPINA	MENGKEQUI YOK IL	WWWDRCGCS-
ArI-MSTN	(251)	LDLRTSLRKST	RSKRSTELYDDI	R-BITACCRYPL	SVDFVARGWDFV1	ALTNAMYYCA	GEOKGEQUDD	TLIAUVIQUPSP	TLSQUQSAISN	GPCCIPIKMSDLAM	FEDHNSNIALTRLE	WKVDRCGCA-
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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, A-F019627), *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

EpC-MSTN	(1)	MHLSQIVLYLGLLIA	LEPVVLSDQETHOQ	PSATSPE	DIEQCATOEVRO	IKIMRLNAI	KSQILSULRAKEAF	NISRDIVKQULI	PKAPPLOCLLDO	DVLGDDNK	DVVMEEDDEHA
DaR-MSTN-1	(1)	MHFTQVLISLSVLIA	CEPVGYGD1TAHOQP-	STATE	ESELCSTOEFRON	ISKLARLHAI	KSQILSULREKCAP	NISRDVVKQLLI	PKAPPLOOLLDOY	DVLGDDSK	DGAVEEDDEHA
DaR-MSTN-11	(1)	MFLLFYLSFWGV	LESONONLSTITTTT	QAFVTPGD	DNOQCTTEQFRQ	SKLLRLHSI	KSQILSILREEQAF	NISEDTVKLLL	PKAPPERELLDO	QNCGISE	DEEQA
SaF-MSTN	(1)	MHLIQVLIYMGEMVA	FTPLGLGDQTAHHQS-	PATD	DGEQCSTOEVRQC	IKNMRI HAI	KSQILSULREKHA F	NISRDVVKQLL	PKAPPLQKLLDQ	EVLGDDNK	DOVMEEDDDHA
OrM-MSTN	(1)	MILSQIVLYLSLLIA	LEPVVLSDQEAIIQQ	PSVSTPV	DIDQCATOEVROO	IKTMRLNAI	KSQILSBLRAKEAF	MISREIVKQULI	PKAPPLOCLLDO	DVLGDDNR	EEVLEDDDEHA
MoC-MSTN	(1)	MHLSQIVLYLSLLIA	L2PVVLSDQETHQQ	PSATSPE	DIEQCAICEVRQ	IKIMRLNAI	KSQ1LSQLRWKEAF	NISRDI VKQLLI	PKAPPLOQLLDOY	DVLGDDNR	DVVMEDDDEHA
MoS-MSTN	(1)	MHLSQIALYLSLLIA	L2PVVLSDQETHQQ	PSATSPE	DIEQCATOEVROO	IKTMRLNAI	KSQILSØLRÆREAF	NISRDI VKQUL	PKAPPLQQLLDQ	DVLGDDNR	DVVMEDDDEHA
MoA-MSTN	(1)	MHLSQIGLYLSLLIA	LEPV+LSDQETHQQ	PSATSPE	DIEQCATOEVROO	IKTMRLNAI	KSQILSØLRAKEAF	NISRDI VKQUL	PKAPPLOQLLDQ	DVLGDDNR	DVVMEDDDEHA
SaS-MSTN-I	(1)	MHVMQVLISLSFMVA	FESMGLGDQTAILQS-	PATD	DGEQCSTREVEQ	IKNWRLHAI	KSQILSØLRUKHAF	AISRDVVKQUU	PKAPPLOKLLDOY	DVLGDDNK	DOVMEDDDEHA
SaS-MSTN-II	(1)	MHLTQVLIYLSFMVA	FCPVGLGDQTAILIQP-	PATD	DGEQCPTCEVRQC	INNRLHAI	KSQILSULREKÇAF	AISRDVVKQLL	PKAPPLOCLLDO	DVLGDDNK	DGVMEEDDEHA
OnM-MSTN-I	(1)	MHLTQVLIYLSFMVA	FCPVGLGDQTAHHQP-	PATD	DGEQCSTREVRQ	IKNMRLHAI	KSQILSØLREXCAF	AISRDVVKQLL	PKAPPLCCLLDOY	DVLGDDNK	DGLMEEDDEHA
OnM-MSTN-11	(1)	MNLMQVLIYLSFMVA	FEPMGLODOTAIL OS-	PATD	DGEQCSTREVEQU	IKNMRI HAI	KQULSALRIAHAF	AISRDVVKQLL	PKAPPLOKLLDO	DVLGDDNK	DGLMEEDDEHA
SpA-MSIN-a	(1)	MHPSQIVLYLSLLIV	LEPWILSEDETQOQQQ	QQQQQQQPSATSPE	DIELCAIGENRO	IKIMRUNAI	KSQILSØLRAKEAF	AISRDIVKQLL	PKAPPLOOLLDUY	UVLGDDNR	D/VMEEDDEHA
SpA-MSIN-b	(1)	MLNFLGLIVILS	ASSVEMNUTSKLLAE		SGEOCSAUDHEEF	SKOMRUHS	KSQILSILKLEQAF	NISRDMIRQUL	PKAPPLTULLDUY	PRVEEED	HAT
ICP-MSIN	(1)	MHLAQVLISEGEVVA	PEPMARIDICAPEOQQ	QQPTAVIEEREA	QCSAASACAFRON	SKOLRUQA	KSQILSNLRLKQAF	AVSRDVVKQLL	KAPPVOOLLDL	DVLGDDGKPGTAL	QUEEEDDEEHA
ICF-MSIN	(1)	MHLAQVL I SLOPVVP	PERMARIDIGAPEDQQ	QQQPTAVIEEREA	QCSAASACAPROP	SKULKLOAT	NSQILSSLREAHAP	NISRDVVKQLL	PKAPPVUQLLDL	DVLODIOKPOTAL	ODEEEDDEEHA
Amc -MSIN	(1)	MHEAQVETSEOPVVP	PERMARTUTOAPERQU	HIQQQQPTAV TEEKEA	UCSAASACAPROF	SAULKI QAT	N QILSALREAHAP	NUSRDVVKQLL	PKAPPVOOLLIDI	DVLODIONIO TAL	DOLEED DEENA
DIL-MSIN	(1)	MINSQUALITALITY	TTERM TSDOETHOU	P3A15PE	DIEGGAIGEVROA	INTERLINAT	Veolit 2011 2011 Out - Date	NISRUIVAQLL	AAPPLOOLLDO	VLGDDNK	DA MAEDUDERA
THE METRI	11161	THE ISPACE OF THE PARTY OF	A REAL PROPERTY AND INCOMENTS	TYNAND TUTO AND SUITE	DADE ATT ATT	THE REAL PROPERTY OF	STRUTT OF VIDAS	LOUG CHARTER	TANK THE PART		CADI VETCETTE
DeD MCTN I	(110)	THETHOMATERESY	A A A A A A A A A A A A A A A A A A A	I CAN DI VICIO INVILIO	PADEAT INFLOT	TOPAIL VILLA	CHID DESERTION	-AUVSSIGSTD	NAQUE I VICEQUE		CADLANT SAFA
DaR-MSTN-1	(113)	SCHOLITHATEPOAL	THE VENERAL SE	IT DOST VALUE IN MARK	DACEDTINVIOLS	THE CCCCCC	NAUSDIK OF TOWN	ADTISUCIAL	NAME OF TAXABLE OF	SNECTED VASEAN	CADLAUTSALS
SoF_MSTN-11	(114)	TTEDIATANTEDESI	VITYTYPE	VIOANDIVENT WHI	PADEVTIMELOIS	DI I DVIDO	CENTOTESI KIDAN	ACVSSICSID	KOWI SWWI FORT	INWCIETNALDAR	GNDLAUTSTEA
OrM_MSTN	(116)	TTETTVWWWTEPOPA	VIVICOPROCESSIT	FOASRVVRA I WHI R	PSEEVITIMELOIS	RE IPVTT	NRHIRSESIKIDVA	PEPASHOSID	KOWLTWILLOP	TINUCTEDNALEDSE	CNDL ANTSAHD
MoC-MSTN	(116)	TTETT MANATEPEST	VIVICEPEOGESETU	FOANRIVRACEWHER	OSDEATTIMET OTS	RIMPVITY	NRHIRINSI FOR IF	WPUSASWOSID	KOW SWWI BOR	TNWGTETNALDSP	CNDI AUTSAEP
MoS-MSTN	(116)	ITEDIMMATEPESI	VAVOGEPROOF	FOANRIVRAOLWVHIR	OSDEATTWELOIS	RIMPVTD	NRHIRINSLKIELN	AGVSSIOSID	KOMI SWWI ROPE	TINUCTIETNAEDSE	GNDLAWTSAEP
MoA-MSTN	(116)	ITERIMMATEPESI	VAVDGEPROCHESETO	FOANRIVRACLWVHIR	OSDEATTIVELOIS	MIMPVITTE-	NRHIRISSIKIFIN	-AGVSSIOSID	KOMLSWULKOPE	INWGIETNAEDSE	GNDLAWTSAEP
SaS-MSTN-I	(114)	ITET INTRATEPEST	VOIDGKPKCOFUSESS	IOANRILRAOLEVHLO	PADEVTTVLLOIS	RIPVID	GRNIOISSLKIDVN	AGVSSIGSID	NOVE SWILL ROPE	TNWGIEINALDSK	GNDLAWTSTEA
SaS-MSTN-II	(114)	ITET INTRATEPOSI	VEVDRKPKCCLESFSS	IOVNRIVHAOLWVHUL	PADEVITIMFLOIS	RIMPVILC-	CRHICIESLKIDVN	-AGVSSICSID	KOVLSVWLROPE	TINWGIEINAFDSK	GNDLAWTSAEA
OnM-MSTN-I	(114)	ITER INTRATEPEST	VOVDRKPKCOLI SFSS	IOWNRIVHACLEVHLL	PADEVTTWFLOIS	RUMPVTEC	CRHICIESLKIDVN	AGVSSICSID	KOVLSVWLROPE	TINWGIEINAFDSK	GNDLAWTSAEA
OnM-MSTN-11	(114)	ITET INTRATEPESI	VOVDGKPKCOFFSFNS	I QANRI VRACLWVHLO	PPDEVTTMFLOIS	RI I PVIDC-	CRNIQUESLKIDVN	-AGVSSWQSID	KOVLSVWLROPI	INWGIEINALDSK	GNDLAWTSAEA
SpA-MSTN-a	(125)	ITET IMMATEPEPV	VOVDGEPROOF SFTQ	IQANRIVRACLWVHUR	ASDEANIMFLOIS	REMPVILL	NGHIHINSLKIDVN	-AGVGSWQSID	KOVLSVWLROPH	INWGICINAEDSE	GNDLAWTSAEP
SpA-MSTN-b	(100)	-TETI I TMATKHNPI	ACDELTS-COLLESLEP	NIQPKNILRACLWVHLR	PADIVTSMELOIS	RUKPGKECN	NTRIRVESLKIDTE	-AGAGSWOSID	IKSLLQAWLROP	INYGIEINAYDSS	GEDLANTSAEP
IcP-MSTN	(129)	TTETVMSMAEPNPD	WWWDOKPKCOFFSFSP	IQASRIVRACLWVHLR	PADEATTWFLOIS	RUMPIKDC-	RRHVRIKSLKIDVI	-AGVSSNQSID	WKOWLAWWLROPH	TINWGIEINAFDSK	SNDLA ITSAEP
IcF-MSTN	(130)	TTEIVMSMAEPNPD	WWWDOKPKCCLESFSF	IQASRIVRAQLWVHL R	PADEATTMFLOIS	QUMPIKDC-	KSNEQIESLKIDVI	-AGVNSWCSID	VIKQVILAVWILROPH	TINNGLEINAFDSK	SNDLAITSAEP
AmC-MSTN	(133)	TTETVMSMAEPNPD	VOVDOKPKCOFFSFSF	NIQASRIVRAQLWVHUR	PADEATTWFLOIS	RUMP I KDG -	RRHVRIKSLKIDVI	-AGVNSWCSID	VKQVLAVWLROPE	TINNGLEINAFDSK	SNDLAITSAEP
DiL-MSTN	(116)	TTET IMMATEPEST	VQVDGEPRCOHESETQ	FQANR I VRAQLWVHUR	QSDEATTMFLQ15	REMPVIDE-	NRHIRIESLKIELN	-AGVS <mark>SNO</mark> SID	WROMESVWEROPH	TNWGIEINAFDSR	GNDLAWTSAEP
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EpC-MSTN	(246)	GEDGLOPFMENKISE	GPF RVRF DSGLDCDEN	SPESRCCRYPLTVDFED	FGWDWIIIAPKRYR	ANYCSGEC	YMHLQXYPHIIHLVN	KANPRGIAGPCO	TIPTKMSPINMLY	FNRSEQIIYGKIF	SMVVDRCGCS-
DaR-MSTN-1	(244)	GEDGLLPFMENKISE	GPF RIRFDSGLDCDEN	SESECCRYPLIVDFED	FGWDWIIIAPKRYB	ANYCSGEO	YMYLQKYPHIHHVY	KASPRGIAGPCO	THTEMSPIRMLY	FNGREQIIYGKIF	SMVVDRCGCS-
DaR-MSIN-11	(240)	GEFERROPH PANALSI	TGERSREDTCLDCDER	STESRCCRYPLIVDEED	FGWDWIIIAPKRYS	ANYCSGEC	VERYPHISH IV	KANFIFFAGPO	TUTKMSPINMLY	ENDREQITYGKIF	SMWDLCGCS-
SaF-MSIN	(244)	C - EGDQERMEAKI SE	GPARSREDSCEDCDEN	SEERCCRYFLIVDFED	FGWDWITAPKRYS	ANYCSGEO	YMHDOXYPHILHUVY	KANFRGIACECO	THE TRANSPORT	PNRKEQITYGKTF	SMWVDRCGCS-
OrM-MSIN	(246)	GEEGEOPENENKI SE	GPF RARED SCLUCDEN	SEESROCKYPLIVDEED	FGWDWHTAPKRYB	ANYCSGEO	YMHLQXYPHIHLVY	KANERGIACEC	JIETKMSPINML)	PNRREQUIYGKTF	SMWURCUCS-
MOC-MSIN	(247)	GEEGBOPFMENKISE	GPP RAREDSELDCDEN	SEE RCCRYPLIVDFED	FGWDWATAPKRY	ANYCSGEO	YMHIOXYPHILLUY	KANPRGIAGPO	TKMSPINML	FNRREQITYGKTF	SMVVDRCGCS-
MOS-MSIN	(240)	CHECK OPPNENNISE	OPP RAREDS CLUCDEN	STESRUCKYPLIVDFED	PGWDWHTAPKR II	ANTCSUEU	MINHIDONY PHILED VN	KARPROHAGPU	TELEVISION NUL	ENREQUINGNIE	SMVVDRCUCS-
MOA-MOIN	(240)	CHEGLOPPHEVINISE	OPERARED SOLDCORN	SPECKUCKIPLIVDFED	POWDWITAPKK IN	ANTCSUEU	MINING AN THERE AND	KARPROTACPO	TELEVISION NUL	PNREQUITONIE ENDECOTIVCE IE	SMV VLRCUCS-
SaS-MSIN-1	(244)	C - FOLCPINEVALSE	CPAROREDSOLDCDAY	SPESECCKIPLIVDPED	POWDWITAPKRIE	ANTCSUEU	MALL OF THE LAS	RANPROTACEVY	THE INSPIRATE	ENDEDITIONI	SMVVDBCUCS-
OWNERS I	(244)	C-ECHODRADA TISE	CITE CORPOSED CODEN	SEESACCAIPLIVDPED	DOWDWII IAPKKIS	ANTCSCEC	VALUE OF STREET, STREE	MANTRODACION	TUNCTUNIL I	TAREQUINCLU	SMADACOCS
OnM-MSTN-11	(244)	C. ECHOPEMENT SE	CPLESPEDSEL DODA	SEESPCCRVDI TVDEED	FGWDWIITAPKRY	ANVCSGEO	VMHI ON CHIERON	KANEROTACEO	TREEMSPERMEN	ENREGITION	SHUTHOUS
SnA_MSTN-4	(255)	GED GLOPPAEWINS	CPERVICE DISCLOCOPIN	SEESROCRVPI TVDEP	FGWDWIIIAPYPW	ANVCSGEO	WHILD SYPHOLINY	KANPRESACEO	TETKISPINI	ENREGITYCKIE	SINTECTOS
SpA-MSTN-h	(200)	GEEGLOPE IEWIT	NEERSREDSCI NCDER	AFTROCRYPT TYDEE	FGWDWIIIAPEPV	ANYCSGEC	TMHI OCYPITALITY	KANPROTACICO	TETEMSPINAL	ENRAFOLIYCKUP	SHVTHCCCS
IcP-MSTN	(259)	GEEGI I PELEVITISE	VE RTREESCUTCHEN	SESRCCRYPI TVDEE	FGWDWILLAPKRVS	ANYCSGEC	WVHI ONYPHIEND	KANERGTAGEO	TETKASPINAL	ENCREOILYCKIE	SMVUDBCCCS
IcF-MSTN	(260)	GEEGLI PELEVITISE	VENETREESGLICDEN	SESRCCRYPL TVDEED	FGWDWILLAPKRYS	ANYCSGEO	YVHI OXYPHILITY	KANERGTAGEO	TETKASPINAL	ENGREOUTYCKUP	SMVVDBCCCS
AmC-MSTN	(263)	GEEGLI PELEVICISE	VERTRESCUTCHEN	SESROCRYPLTVDEED	FGWDWHIAPKRY	ANYCSGEO	YVHIOSYPHIERVY	KANERGTAGEO	TETKMSPINMLY	FNGREOITYGKIE	SMWDRCCCS
DiL-MSTN	(246)	GEEGLOPENENKI SE	GPERARE DSCLOCDEN	SHESRCCRYPLTVDFFD	FGWDWI LAPKRY	ANYCSGEO	YMHLONYPHITHINN	KANERGIAGEO	TETKMSPINMLY	FNRKEOI LYGK IP	SMVVDSCCCS-
								Contraction of the local division of the loc			

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. <i>⊥</i> Mammal	Amino Ac ian Specie	id Ser	duenc	te Per	cent	Simi	larity	(%)	of Or	ange	Spott	ed Gr	oupe	r Prep	ro-M	STN	Comj	pared	to Di	ffere	nt Mo	llusc	an, T	eleos	tean,	Avia	n and	
		1	0	3	4	5	9	7	8	6	10	11 1	2 1	3 1	11 t	5 10	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 9	5 16	14 5	6 9	6 8,	4 86	86	85	92	66	76	75	74	96	25
2 HoS-	MSTN	I	100	66	94	98	94	96	95	92	90	67	57	65	54	52	3 6	9 3	4 65	65	65	63	59	61	60	60	63	29
3 PaH-	MSTN	I	I	100	94	98	94	96	95	91	89	67	57	64	54	52 6	3 6	9 3	4 62	- 64	. 65	63	59	60	59	60	63	29
4 BoT-Ì	MSTN	I	Ι	I	100	95	93	92	91	88	85	65	56	63	51 6	51 6	1 6	1 6	50	63	63	62	58	59	58	58	61	29
5 SuS-I	ASTN	I	I	Т	I	100	95	96	95	91	89	67	57	65	54	52 6	3 6	9 3	4 62	- 64	. 65	63	60	60	60	60	63	29
6 OvA-	MSTN	Ι	I	I	Ι	I	100	92	91	88	85	65	57	64	33	51 6	2 6	5 5	3	+ 64	. 64	62	59	60	59	59	62	29
7 MuN	-MSTN	I	Ι	I	Ι	I	I	100	98	91	88	99	56	64	33	52 6	2 6	6 7	4	63	64	61	59	60	59	60	62	28
8 RaN-	MSTN	I	I	I	Ι	I	Ι	I	100	90	87	66	56	63	52	52	2 6	5 5	80	63	64	62	59	60	59	59	62	28
9 GaG-	METN	I	Ι	I	Ι	I	I	I	I	100	96	67	56	65	54	33 6	4 6	4 6	4 66	99	65	62	59	61	60	61	64	28
10 MeG	MSTN.	I	Ι	I	I	I	I	I	I	I	100	99	57	64	33	52 6	3 6	و م	4 65	65	64	62	59	61	60	60	63	28
11 DaR-	MSTN-I	I	Ι	I	Ι	I	I	I	I			100	64	84	62	3	8 0	× 0	4 8 9	86	84	78	67	78	77	77	80	25
12 DaR-	MSTN-II	I	I	Ι	Ι	I	Ι	I	I	·		-	8	63	50	69	1 6	1	80	63	63	59	61	59	58	58	61	24
13 SaF-A	4STN	Ι	I	I	Ι	I	I	I	I	·		1	Г	8	81 8	31 8	3 8	39	5 92	93	97	79	66	75	75	74	83	25
14 OrM.	MSTN	I	I	I	I	I	I	I	I				I	1	8	10	1 9	1 8	1 82	8	82	86	64	74	72	72	91	25
15 MoC	-MSTN	I	I	I	I	I	Ι	I	I				I	I	Ξ	00	5	× 8	8	83	83	90	64	73	72	72	97	24
16 MoS-	MSTN	I	I	I	Ι	I	Ι	I	I	·		1	I	Ι	Ι	10	6 0	8 8	4 85	85	85	92	65	75	73	73	66	25
17 MoA	MSTN	I	I	I	Ι	I	Ι	I	I	·			I	Ι	Ι	Ι	10	× 0	4 85	85	85	92	65	75	73	73	66	25
18 SaS-N	1-NTSI	I	I	I	I	I	I	I	I				I	I	I	I	I	10	66	93	97	79	67	76	75	75	84	26
19 SaS-N	II-NTSI	I	I	I	I	I	I	I	I				I	I	Ι	I	I	I	100	56 (94	81	67	77	75	75	85	25
20 OnM	I-NLSM-	I	I	I	I	Ι	I	I	I			1	I	Ι	Ι	I	I	I	I	100	95	81	67	77	76	75	85	26
21 OnM	II-NTSM-	ا 	I	I	I	I	I	I	I				I	I	I	I	I	I	I	I	100	81	67	76	76	75	85	26
22 SpA-	MSTN-a	I	I	I	I	Ι	I	I	I			1	I	Ι	Ι	I	I	I	I	I	I	100	63	74	73	74	92	25
23 SpA-	MSTN-b	I	I	I	I	I	I	I	I			1	I	I	I	Ι	I	I	I	I	I	I	100	64	63	62	65	26
24 IcP-N	ISTN	I	I	I	Ι	I	Ι	I	I	·			I	Ι	Ι	Ι	I	I	I	I	I	I	I	100	97	98	75	24
25 IcF-N	ISTN	I	I	I	I	I	Ι	I	I				I	I	Ι	Ι	I	I	I	I	I	I	I	I	100	97	73	24
26 AmC	-MSTN	I	I	Т	Ι	I	I	I	I	·			I	I	Ι	Ι	I	T	I	I	I	I	I	I	I	100	73	24
27 DiL-l	ASTN	I	I	I	Ι	I	Ι	I	I			1	I	I	Ι	Ι	I	I	I	I	I	I	I	I	I	I	100	25
28 ArI-N	ISTN	I	I	I	I	I	I	I	I				I	Ι	I	Ι	I	I	I	I	I	I	I	I	I	I	I	100



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

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 neighborjoining method. The tree was generated via CLUSTAL X1.8 and depicted visually via Tree-View1.6.6. 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.

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 \pm 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.

formation); 5, 15-day-old larvae (fin rays formation); 6, 20day-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

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 \pm 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

(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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