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Organization and Functional Analysis of the 5′ Flanking Regions of Myostatin-1 and 2 Genes from *Larimichthys crocea*

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Myostatin (MSTN) is a negative regulator of skeletal muscle growth and development. There are two types of MSTNs in fish, but little is known about their gene regulation. Here, the 5′ flanking fragments of 1029 bp from *MSTN-1* and 643 bp from *MSTN-2* were cloned, sequenced, and analyzed in *Larimichthys crocea*. Both fragments contained CAAT box and several putative *cis*-regulatory elements. However, putative TATA box, MyoD, MEF3, SP1, USF, and GH-CSE sites were identified only in the *L. crocea MSTN-1* (*lcMSTN-1*) promoter. Transcriptional activities of four fragments (1013, 841, 514, and 261 bp) truncated from *lcMSTN-1* upstream region and two fragments (643 and 296 bp) from *lcMSTN-2* upstream region were examined *in vitro*, using transient transfection in CIK and L6 cells. In CIK cells, the promoter activity correlated positively with the length of truncated fragments in both *MSTN-1* and *2*. The *lcMSTN-2* promoter showed a higher activity than *lcMSTN-1* in the corresponding region, which was consistent with *MSTN* gene expression *in vivo*. In L6 cells, *lcMSTN-2* upstream showed an extremely high luciferase activity. These data indicated that both cloned 5′ flanking sequences contained functional promoters, and that transcription regulation of *lcMSTN-1* and *2* promoters was significantly different between mammalian and fish cells.

Introduction

Myostatin (MSTN), also known as growth and differentiation factor-8, was first identified in mice (McPherron *et al.*, 1997). It is a member of the transforming growth factor-beta superfamily, and a potent negative regulator of skeletal muscle growth and development in mammals (Lee, 2004). Both naturally occurring and experimentally induced mutations that result in lower MNST activity induce remarkable muscle mass increase in a number of vertebrates, including cattle (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron and Lee, 1997; Smith *et al.*, 2000; Marchitelli *et al.*, 2003), sheep (Clop *et al.*, 2006), dogs (Mosher *et al.*, 2007), mice (McPherron *et al.*, 1997; Szabo *et al.*, 1998; Zhu *et al.*, 2000), humans (Schuelke *et al.*, 2004), medaka (Sawatari *et al.*, 2010), and zebrafish (Xu *et al.*, 2003; Acosta *et al.*, 2005; Lee *et al.*, 2009). In cattle, because of its beneficial effects on meat production, several double-muscling breeds have been selectively bred for this trait (Rodgers and Garikipati, 2008). Conversely, *MSTN* overexpression in the skeletal muscle of the transgenic mice is associated with decreased body weight and skeletal muscle mass (Reisz-Porszasz *et al.*, 2003). Overexpression of *MSTN-2* in zebrafish reduces the expression of dystrophin associated protein complex, which leads to muscle dystrophy (Amali *et al.*, 2008). These growth-inhibitory actions have also been demonstrated *in vitro* using different mammalian cell lines (Thomas *et al.*, 2000; Rios *et al.*, 2001, 2002; Taylor *et al.*, 2001; Langley *et al.*, 2002; Joulia-Ekaza *et al.*, 2003; McCroskery *et al.*, 2003). In fish, soluble recombinant MSTN-1 prodomain can improve body growth (Lee *et al.*, 2010).

The key role of MSTN in skeletal muscle growth and its potential application prompted the study of MSTN from fishes with important commercial value. These studies have shown that fish *MSTN* is different in comparison with mammals. First, two or more types of *MSTNs* have been recently found in some teleosts (Garikipati *et al.*, 2006; Helterline *et al.*, 2007; Ostbye *et al.*, 2007). However, only one *MSTN* has been found so far in mammals. Second, fish *MSTN* has a wider expression range than mammals. In mammals, *MSTN* expression was observed in the skeletal muscle (McPherron *et al.*, 1997; Gonzalez-Cadavid *et al.*, 1998), and at a lower level, in the adipose tissue (McPherron *et al.*, 1997), cardiac muscle (Sharma *et al.*, 1999), and mammary gland (Ji *et al.*, 1998). In contrast to mammals, fish *MSTN* is differentially expressed in many tissues. In seabream, *MSTN-1* is expressed in the muscle, brain, eye, intestine, heat, and kidney, while *MSTN-2* is expressed mainly in the brain (Maccatrozzo *et al.*, 2001a, 2001b). In rainbow trout, *MSTN-1a* mRNA was present ubiquitously in trout tissues,

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while *MSTN-1b* mRNA expression was restricted to the muscle and brain (Rescan *et al.*, 2001). The expression of rainbow trout *MSTN-2a* was detected in all sampled tissues, including the brain, skin, gill, heart, kidney, spleen, intestine, stomach, liver, eye, and muscle (Garikipati *et al.*, 2007). Diverse expression patterns in fish suggest that the biological action of MSTN may not be restricted to the skeletal muscle, but may additionally influence other fish tissues as well (Kerr *et al.*, 2005).

In mammals, sequence alignment of the human, goat, sheep, pig, and cow *MSTN* promoters revealed five conserved sequences (Allen and Du, 2008). The regulation of *MSTN* expression under various physiological conditions has been extensively studied (Joulia-Ekaza and Cabello, 2006). Compared with higher vertebrates, the transcription regulation of *MSTN* promoters in teleosts is poorly understood. The *MSTN* promoters have been sequenced in some teleost species such as zebrafish (*Danio rerio*) (AY323521, DQ451548), largemouth bass (*Micropterus salmoides*) (EF071854), Atlantic salmon (*Salmo salar*) (EF392862), barramundi (*Lates calcarifer*) (EF672685), gilthead sea bream (*Sparus aurate*) (EU881511), olive flounder (*Paralichthys olivaceus*) (DQ997779), rainbow trout (*Oncorhynchus mykiss*) (DQ136028, DQ138301), brook trout (*Salvelinus fontinalis*) (AY227656), sea perch (*Lateolabrax japonicas*) (AY965685), and channel catfish (*Lctalurus punctatus*) (AF396747), but only gilthead sea bream *MSTN-1* promoter activity has been functionally analyzed (Funkenstein *et al.*, 2009). Functional analysis of the *MSTN-2* promoter has not been reported so far. Large yellow croaker (*Larimichthys crocea)* is a major marine-cultured species in China. We have cloned *MSTN-1* (Xue *et al.*, 2006) and *2* (EU571244) genes from *L. crocea*. To better understand their promoter structure and regulatory mechanisms, we cloned and functionally analyzed the promoters of *MSTN-1* and *2* from *L. crocea*.

Materials and Methods

Sample

L. crocea was collected at the Ningbo Bay Breeding Center of Aquatic Animals. Skeletal muscle tissue was dissected from living large yellow croakers, rapidly frozen in liquid nitrogen, and stored at −70°C.

Genomic DNA extraction

Genomic DNA was extracted from skeletal muscle tissue. Five hundred microliters lysis buffer (1% sodium dodecyl sulfate, 100 mM/L ethylenediaminetetraacetic acid, and 10 nM/L Tris, pH 8.0) was added to 0.15 g muscle tissue and incubated for 2 h with proteinase K (20 mg/mL) at 65°C. Three consecutive extractions, phenol, phenol: chloroform: isoamyl alcohol (25:24:1), and chloroform: isoamyl alcohol (24:1), were then performed. The precipitated DNA was washed with 70% ethanol, dried, resuspended in 100 μL ddH_2O, and stored at −20°C.

Cloning of L. crocea MSTN-1 and 2 promoters

Based on the cloned *MSTN* genes of *L. crocea* (AY842933, EU571244), six specific forward primers were designed to amplify the 5′ flanking regions of *MSTN-1* and 2 genes. Three forward primers, 1SP1, 1SP2, and 1SP3, were used in three round polymerase chain reaction (PCR) for *L. crocea MSTN-1* (*lcMSTN-1*) promoter cloning, respectively. The other three primers, 2SP1, 2SP2, and 2SP3, were used in three-round PCR for *lcMSTN-2* promoter cloning. The primer sequences were listed in Table 1. The nested PCR of three rounds was performed to isolate *lcMSTN-1* and *-2* promoters from genomic DNA using the Genome Walking Kit (TaKaRa Biotechnology) according to the manufacturer's instructions. The reverse primer was AP4 in all PCR reactions, which was supplied in the kit. For *lcMSTN-1* promoter cloning, the first round PCR conditions were 1 cycle (94°C 1 min, 98°C 1 min), 5 cycles (94°C 30 s, 65°C 1 min, and 72°C 2 min), 1 cycle (94°C 30 s, 25°C 3 min, and 72°C 2 min), and 15 cycles (94°C 30 s, 65°C 1 min, and 72°C 2 min; 94°C 30 s, 65°C 1 min, and 72°C 2 min; 94°C 30 s, 44°C1 min, and 72°C 2 min), and 1 cycle (72°C 10 min). The second-round PCR conditions were 15 cycles (94°C 30 s, 60°C 1 min, and 72 2 min; 94°C 30 s, 60°C 1 min, and 72°C 2 min; 94°C 30 s, 44°C 1 min, and 72°C 2 min), and 1 cycle (72°C 10 min). The third-round PCR conditions were 15 cycles (94°C 30 s, 60°C 1 min, and 72°C 2 min; 94°C 30 s, 60°C 1 min, and 72°C 2 min; 94°C 30 s, 44°C 1 min, and 72°C 2 min), and 1 cycle (72°C 10 min). The cloning protocol for the *lcMSTN-2* promoter was the same as the *lcMSTN-1* promoter. The detailed PCR conditions are listed in Supplementary Table S1. The PCR products were run on a 1% agarose gel, and then purified by EZ-10 Spin Column DNA Gel Extraction Kit (Shenggong).The purified PCR products were cloned into pMD18-T vector (TaKaRa Biotechnology), and the recombinant vector was transformed into competent DH5α cells. The recombinant plasmids were isolated from the transformed DH5α cells with EZ-10 Spin Column Plasmid Mini-Preps Kit (Shenggong). The inserted PCR fragments were sequenced on an ABIPRISM 3700 DNA sequencer.

Sequence analysis

Similarity searches of the sequenced *lcMSTN* promoters were done by Blastn (www.ncbi.nlm.nih.gov/blast/). The transcription factor binding sites were analyzed with TFSEARCH with a threshold score of 90 (Heinemeyer *et al.*, 1998), TESS (www.cbil.upenn.edu/cgi-bin/tess/tess), and MatInspector (Cartharius *et al.*, 2005). A multiple-sequence alignment was performed using ClustalW (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbor-joining tree of MEGA version 4.0 (Tamura *et al.*, 2007). The species in the sequence alignment include *D. rerio* (AY323521, DQ451548), *M. salmoides* (EF071854), *S. salar* (EF392862,), *L. calcarifer* (EF672685), *S. aurate* (EU881511, GQ379809), *P. olivaceus* (DQ997779), *O. mykiss* (DQ136028, DQ138301), *S. fontinalis* (AY227656), *L. japonicas* (AY965685), and *L. punctatus* (AF396747).

Construction of lcMSTN-1-Luc and lcMSTN-2-Luc transgenes

Four fragments, 1013, 841, 514, and 261 bp, of *lcMSTN-1* 5′ flanking sequence were amplified with primers 1F1, 1F2, 1F3, 1F4 (forward), and 1R (reverse), and two fragments, 643 and 296 bp, of *lcMSTN-2* 5′ flanking sequence with primers 2F1, 2F2, and 2R, respectively. The forward primers introduced a 5′ *Kpn*I restriction site, and the reverse primers, a 5′ *Hin*dIII restriction site (Table 1). The PCR condition was as follows: 94°C for 3 min followed by 35 cycles of 40 s at 94°C, 40 s at

Table 1. Nucleotide Sequences of Primers used in Polymerase Chain Reaction for *lcMSTN* Promoter Cloning and Characterization

Assigned name	*Sequence*	*Purpose*
1SP1	5′ CTGGTGCGTCTCTTGGTCAC 3′	Cloning of *lcMSTN-1* promoter
1SP2	5′ GAGACAGATGCATTGTCTCTC 3′	Cloning of *lcMSTN-1* promoter
1SP3	5′ GGACTGGGTTTGGATTAATGTC 3′	Cloning of *lcMSTN-1* promoter
2SP1	5′ AATGGTCTCTGTCGTAGCGTGGT 3′	Cloning of *lcMSTN-2* promoter
2SP2	5′ TTGATACTATGGAGCCGCATCTGTTT 3′	Cloning of *lcMSTN-2* promoter
2SP3	5′ CCCATTGAAAAGCCCGCAGAGAA 3′	Cloning of *lcMSTN-2* promoter
1F1	5′ GGGGTACCTGGTGTTCACACTTTAGAGT 3′	Construction of *lcMSTN-1* promoter reporter plasmids
1F2	5′ GGGGTACCCTAATGTGAATGACGTGAAC 3′	Construction of *lcMSTN-1* promoter reporter plasmids
1F3	5′ GGGGTACCGTCTGATGGATTTATTGTG 3′	Construction of *lcMSTN-1* promoter reporter plasmids
1F4	5′ GGGGTACCTCACAGTCTCCATCCCTTTAT 3′	Construction of *lcMSTN-1* promoter reporter plasmids
1R	5′ CCCAAGCTTTGTCTCTCAGGTGTGAAG 3′	Construction of lcMSTN-1 promoter reporter plasmids
2F1	5′ GGGGTACCGCAGTGCAGCACATCCA 3′	Construction of *lcMSTN-2* promoter reporter plasmids
2F2	5′ GGGGTACCGAAGTAAAATGCGAAC 3′	Construction of *lcMSTN-2* promoter reporter plasmids
2R	5′ CCCAAGCTTCCTGGAGCGATGAGGA 3′	Construction of *lcMSTN-2* promoter reporter plasmids

Restriction enzyme recognition sites introduced in some oligonucleotides are underlined.

60°C, 2 min at 72°C, and 72°C extended for 8 min. All PCR products were run on a 1% agarose gel and then gel purified by EZ-10 Spin Column DNA Gel Extraction Kit. Both the purified fragments and the promoterless reporter plasmid pGL3-Basic which contains a luciferase gene (Promega), were double digested with *Kpn*I and *Hin*dIII (TaKaRa Biotechnology). The resultant fragments were directly inserted into the pGL3-Basic, and the constructed plasmids were named pGL3-1-1013, pGL3-1-841, pGL3-1-514, pGL3-1-261, pGL3-2-643, and pGL3-2-296, respectively. All recombinant constructs were sequenced to verify the orientation of the promoter relative to the luciferase reporter gene.

Cell culture, transfection, and dual-luciferase reporter assay

The CIK cell line, derived from *Ctenopharyngodon idellus* kidney tissue, was provided by Zhejiang Institute of Freshwater Fisheries, and maintained in RPMI 1640 containing 10% fetal bovine serum at 26°C. CIK cells are heterogeneous cell culture, including fiber cell (98.3%), polygon cell (1.4%), and giant cell (0.3%). L6, a rat skeletal muscle cell line purchased from BOSTER, was maintained in DMEM with 10% fetal bovine serum in a 5% CO_2 incubator at 37°C.

For the transfection, 1.0×10^4 cells/well were plated in 96-well plates and grown to ~90% confluence. The cells were co-transfected at 26°C with 0.2 μg of the constructed reporter plasmid vector containing the *MSTN* promoter fragment and 1 ng of the *Renilla* luciferase reporter plasmid pRL-CMV (Promega) using the Lipofectamine 2000 system (Invitrogen) for each well according to the manufacturer's protocol. The cells were cultured for 6 h, and then, the transfection medium was replaced with fresh medium and incubation was continued. Cells were collected 48 h after transfection. Firefly and *Renilla* luciferase activities were measured in cell lysates using the Dual-Luciferase Assay System (Promega) in an SHG-D Luminometer (Shanghai Shangli Detecting Instrument Factory). The relative luciferase activities of promoters were normalized to the control reporter. The final data were from three independent experiments, and each experiment was performed in triplicate.

Mutation of MyoD binding site in the lcMSTN-1 *promoter*

The E-box6 (MyoD binding site) in the *lcMSTN-1* promoter was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the instruction manual. Two complementary primers containing the desired mutation to the MyoD binding site were used as primers for PCR to introduce mutations in pGL3-1-1013 using *Pfu* DNA polymerase. The two primers have the following sequences: P1 5′ CACT CACTCAGGTTGCCGCTTTGACGTCTCTAATG 3′, and P2 5′ CATTAGAGACGTCAAAGCGGCAACCTGAGTGAGTG 3′ (the mutated positions are underlined). The PCR product was then digested with the *Dpn* I restriction enzyme that specifically cuts the original methylated template plasmid but was unable to cut the newly synthesized DNA. The digested DNA was then transformed into XL-1 blue supercompetent cells. The white colonies were selected for sequencing analysis to identify the correct clone with desired mutations. The pGL3-1-1013 with MyoD binding site mutations were used in dual-luciferase reporter assay.

Expression analysis of lcMSTN-2

Total RNA was extracted using Trizol Reagent (Sangon) from ten different tissues and organs, spleen, heart, brain, adipose, kidney, gill, eye, intestine, liver, and skeletal muscle of large yellow adult croakers. Reverse transcription (RT)-PCR was used to analyze the tissue-specific expression of *lcMSTN-2*. Primers for amplifying *MSTN-2* are Myo2F 5′ CCATAGTAT CAAGTCCCAGATCC 3′, and Myo2R 5′ TGAACAGGCA GCAGGAGGACAAC 3′. β-actin gene served as a positive control. The primers of β-actin were 5′ CCAGATCATGT TCGAGACCTTC 3′, and 5′ GAACCTCTCATTGCCAATGG TG 3′. The RT-PCR products from each tissue were electrophoresed on a 1% agarose gel.

Results

Cloning of lcMSTN-1 *and* 2 *5′ flanking regions*

One thousand twenty-nine and 643 bp upstream sequences from the translation start codon of *lcMSTN-1* and *2* were cloned by the genome walking method. Their

nucleotide sequences are shown in Figure 1A and B. The upstream sequence of *lcMSTN-1* had a homology of more than 90% with the reported sequences of *Sparus aurata* and *L. japonicas* in the same region. A multiple-sequence alignment showed that the CAAT box and TATA box were conserved among teleost *MSTN-1* upstream sequences (Fig. 2). However, the upstream sequence of *lcMSTN-2* showed a low conservation compared with that of other fish *MSTN-2*. The conserved sequence could not be identified among *MSTN-2*, and between *MSTN-1* and *2* promoter sequences.

Sequence alignment revealed a big difference between 5′ flanking sequences of *lcMSTN-1* and *2*. More transcription factor recognition sites existed in *lcMSTN-1* 5′ flanking region than that of *lcMSTN-2* (Fig. 3A). A phylogenetic tree showed that 11 fish *MSTN-1* upstream sequences formed a subgroup, and three fish *MSTN-2* upstream sequences formed the other group (Fig. 3B).

Transcription factor binding sites in the 5′ flanking regions of lcMSTN-1 *and* 2 *genes*

One TATA box, one CAAT box, and six putative E-boxes were found in *lcMSTN-1*. The TATA box and CAAT box were located at -131 and -175 upstream from the ATG initiation codon, respectively. In the *lcMSTN-2*, two CAAT boxes and only one putative E box were found. The E box was located at -155, and two CAAT boxes at -76 and -146 bp upstream from the ATG initiation codon, respectively (Fig. 1A, B). However, no TATA box was found in the *lcMSTN-2* promoter.

Sequence analysis identified several putative muscle-specific transcriptional factor binding sites and *cis*-regulatory elements in both sequences. These regulatory elements include myocyte enhancer factor 2 (MEF2), activator protein 1 (AP1), cAMP response element (CRE), POU3F2 (N-Oct-3), POU1F1a (Pit1a), CCAAT/enhancer-binding protein, and nuclear transcription factor Y. However, the myoblast determining factor (MyoD), MEF3, SP1, USF, and growth hormone cell specific element (GH-CSE) sites were found only in the *lcMSTN-1* and SRF only in the *lcMSTN-2* promoter. The detailed distribution of transcriptional response elements on *lcMSTN-1* and *2* promoters is listed in Supplementary Table S2.

lcMSTN-1 *and* 2 *promoter activities in CIK and L6 cells*

To determine whether the genomic 5′ flanking regions of *lcMSTN-1* and *2* contain a functional promoter, six genomic

A lcMSTN-1

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-1029 CGGTATCGGAGTATTTTGGTGTTCACACTTTAGAGTACTGAGTTGATCTGAATGCACCAGGAATGAGAAA
-959  ACACGCAATCTTCGACTATTCCGAGCAGACTTGAAAGCATCTTTGATGTTTTATTTATGCATTTTTGTAT
-889  TTTACTTTTCAAATCCCCACACTCACTCAGGTTGCAGCTGTGACGTCTCTAATGTGAATGACGTGAACTC
                                       E-box6
-819  GCCACACGATGGCGCCATCTCCTCGTGTGCTACAATCTGGTTTCACCAACAAACGTTTGAACAATTTGGA
-749  CTAAATGTTTTATTTAGCTTTTGCTGTTTTTTTAATTCCTGTACAAGAAACGACGCATTTACATATTTGA
-679  TTGGAGGTGAGCGGAATCAAATCAAAAATGAATATGATCTATCCTGATGAGACGGACTTGATTTGCATTG
-609  GATTCAACTGTAACTGTTTTTTTTCCCTCATAAATGAAATCTATTTCCTTTAAACACACAGTTGCATCCA
       E-box5                                                    E-box4
-539  TGTACCTGCCACCAAAGACAATTCATGTCTGATGGATTTATTGTGGCCGCAGTGAAAAACGAATCTGAGG
-469  AAAAACTTTTTATTTTTAAAGTGCGTTATTTAAAAAAAAAGATTAGACGAGTGTTTCCTTATTAATGTGC
-399  ATTAAATATTCTTTAATCGCGATCACATAACTCATCAGAACATATAGCACAGACAGTCCGGTCTGCGTCT
-329  CTGCGCTCATTGCGCAACCACTGTAAGAAAAGTGAATTTATCCATGTGAGGACTCGTTCAGCACATGCTC
                                          E-box3                 E-box2
-259  ACAGTCTCCATCCCTTTATGGCTTGACAACGAAAAAAAGTTTTCATGTCAGTCGGTTAAAATTCATTGTT
-189  GCCTGTCCAGCCAATCATAGTTTTTGACGACACAAAAGAGGTTAAAGTTGGAGTATAAAAAGGTGCGCGC
                 CAAT box                                  TATA box
-119  TAATAAAGTATGATGCCTGTCAGTGTGGGACATTAATCCAAACCCAGTCCAGTCGCGCGTCAGGTCCAGC
                                                    +1
-49   ACAAACCAAGGGATCTTTTTTTCAAACCAAACTTCACACCTGAGAGACAATG
                                   E-box1
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B lcMSTN-2

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-643 GCAGTGCAGCACATCCACAAACATCACTACGCTGTAATATTTAACGGGGGAAACCTTCATTATATACATG
-573 TAGTATTTTGATGTCAATTATTTGACAAACCAGCGTTTATACTGATGAAAAAAAACATGGTAAAATATGT
-503 AAATAAAACACCAACTTTAACCATAAAAATCAACAGTACTAAGACCCTTTTTCTGCAATATTAGAAAAGG
-433 TTATACTGTATTTATTACAGCAAAATTCTTTATTTATACAGTATTTTAGGGAGATTTATACAGAATAAAG
-363 TTGTGTTTTAAATGACAGACAGTTAATTGTACAGTAATGGCAGAAGTTATTTTTGCTGACAAAAACTTAA
-293 GAAGTAAAATGCGAACCTTAAACGTGAAATCAACAGTACTACAATATCCTTTTATTGTAATATTGTAAAA
-223 AGTTATACCGTATGTATTACGGTAAAATTCTGGCATCCACAGCTTCCAGTTTTTTTTACTGTAAAAAACA
-153 AGTGAATCCAATATTGATCATAAAATATTGCTACCTCTTTAAATCTCCCTTCTGATTAAAAAAATTTCCT
      E-box  CAAT box
 -83 TGCAGCCAATCATAAAGTCCTGAGTCCTGGATACAGACTCATATCAGAGAGCGTCACAGCACTTACCTCC
          CAAT box +1
 -13 TCATCGCTCCAGGATG
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FIG. 1. Promoter sequences of *Larimichthys crocea MSTN-1* **(A)** and 2 **(B)**. The first nucleotide in the translation start codon ATG is designated +1. The TATA box and CAAT box are in boldface. E-box (CANNTG) is underlined. MSTN, myostatin.

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M. salmoides      TGTCAGTCGGTCAAAATTCATTGTTGC-CTGTCCAGCCAATCATAGTTTTTGACGACACA
S. aurata         TGTCAGTCGGTAAAAATTCATTGTTGC-CTGTCCAGCCAATCATAGTTTTTGACGACACA
L. japonicus      TGTCAGTCGGTTAAAATTCATTGTTCC-CTGTCCAGCCAATCATAGTTTTTGACGACACA
L. crocea         TGTCAGTCGGTTAAAATTCATTGTTGC-CTGTCCAGCCAATCATAGTTTTTGACGACACA
L. calcarifer     TGTCAGTCGGTTAAAATTCATTGTTGC-CGGTCCAGCCAATCATAGTTTTTGACGACACA
P. olivaceus      AGTCAGTCGGTTAAAATTCATTGTTGC-CTGTCCAGCCAATCATAGTTTTTGACGACACA
S. salar          TGTCTGTCTGTTAAA-TTCATTGTTGC-CAGCCCAACCAATCATAGATTTTGACGACACA
S. fontinalis     TGTCTGTCTGTTAAA-TTCATTGTTGC-CAGCCCAACCAATCATAGATTTTRACGACACA
O. mykiss         TGCCTGTCTGTTAAA-TTCATTGTTGC-CAGCCCAACCAATCATAGATTTTGACGACACA
D. rerio          GCTCTGTCCATTAGG-TTTATTGTTGC-CAGCTCAGCCAATCATTGAATCTTACGACACA
I. punctatus      AGTCCACCTCTGA---TTTATTGTTGCTCCGAGTAGCCAATCATAGATTTCGACGCCAGA
                     *   *  * *   ** ****** * * *   * ******** *  *   *** ** *

M. salmoides      A-------AAGAG--GCTAAA--GTT--GGAGTATAAAAAGGTGT---GCGCTAATAAAG
S. aurata         A-------AAGAG--GCTAAA--GTT--GGAGTATAAAAAGGTGC---GCGCTAATAAAG
L. japonicus      A-------AAGAG--GCTAAA--GTT--GGAGTATAAAAAGGTGT---GCGCTAATAAAG
L. crocea         A-------AAGAG--GTTAAA--GTT--GGAGTATAAAAAGGTGC---GCGCTAATAAAG
L. calcarifer     A-------AAGAG--GCTAAA--GTT--GGAGTATAAAAAGGTGT---GCGCTAATAAAG
P. olivaceus      A-------AAGAG--GCTAAA--GTT--GGAGTATAAAAAGGTGT---GCGCTAATAAAG
S. salar          A-------AAGAGAGGCCAAA--GTT--GCAATATAAAAAGGGCT---GCCGAATTAAAG
S. fontinalis     A-------AAGAGAGGCCAAA--GTT--GCAATATAAAAARGGCT---GCCGAATTAAAG
O. mykiss         A-------AAGAGAGGCCAAA--GTT--GCAATATAAAAAGGGCT---GCCGAATTAAAG
D. rerio          A-------TAGAGTGGCCAAA--GTT--GCAGTATAAAAAGCCTT---GCCGAATTTAAG
I. punctatus      GCCTAAATAAGAGCGGCGGAATAATTTGGCGGTATAAAAAGGCTTTTGGGCGAATTGAAG
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FIG. 2. The alignment of 11 teleost *MSTN-1* upstream sequences (partial). The conserved regions are CAAT box and TATA box. Consenus nucleotides in all aligned sequences are indicated with asterisks.

fragments were truncated from the 5′ ends of *lcMSTN-1* and *2*, 1013, 841, 514, and 261 bp for *lcMSTN-1* and 643, and 296 bp for *lcMSTN-2*. The fragments were then subcloned into the promoterless plasmid pGL3-Basic, respectively. The different fragments had a diverse effect on the luciferase activities in CIK cells (Fig. 4) and L6 cells (Fig. 5) *in vitro.* Both results from CIK and L6 cells indicate that the cloned sequences contain functional promoters.

In CIK cells, the 1013 bp fragment of the *lcMSTN-1* upstream sequence showed the strongest activity, 4.87-fold relative to pGL3-Basic. The lowest increase in luciferase activity was with the 261 bp fragment, 2.62-fold relative to pGL3-Basic. Luciferase activity rose as the fragment length and the number of E-boxes increased. The construct pGL3-1-261, which includes one CAAT box and one TATA box, exhibited about 54% of the promoter activity of pGL3-1-1013, indicating that these motifs alone are not sufficient to drive a significant amount of promoter activity. In the *lcMSTN-2* upstream sequence, the 296 bp fragment exhibited 4.29-fold, and 643 bp 4.53-fold relative to pGL3-Basic. There is no significant difference in promoter activity between the 296 and 643 bp of the *MSTN-2* 5′ flanking region. Notably, the *lcMSTN-2* promoter showed a higher activity than the *lcMSTN-1* promoter in CIK cells in the corresponding 5′ flanking region, which reflected the expression of *lcMSTN-1* and *2* in the kidney tissue of *L. crocea*.

Promoter activity was significantly different between *MSTN-1* and *2* in L6 cells and between CIK and L6 cells. In L6 cells, the 514 bp fragment of the *lcMSTN-1* upstream sequence showed the strongest activity, 10.29-fold relative to pGL3-Basic. The lowest increase in luciferase activity was with the 841 bp fragment, 1.93-fold relative to pGL3-Basic. The activities of the shortest construction pGL3-1-261 and the longest construction pGL3-1-1013 were in the middle, exhibiting 4.46- and 4.04-fold relative to pGL3-Basic, respectively. In the *lcMSTN-2* upstream sequence, the 643 bp fragment showed extremely strong activity, reaching 60.18-fold relative to pGL3-Basic, which is 5.85-fold of the activity of the *lcMSTN-1* 514 bp fragment. The short fragment construction pGL3-2-296 also exhibited a relatively high activity, 32.87-fold relative to pGL3-Basic.

In order to determine the effect of the MyoD binding site on promoter activity, the E-box6 (MyoD binding site) of the *lcMSTN-1* promoter was mutated. The results showed that the activity of the construction pGL3-1-1013 with the MyoD binding site mutation was 0.65-fold in L6 cells, and 0.93-fold in CIK cells, respectively, relative to the construction pGL3-1-1013. Mutation of E-box6 had an obvious effect on the

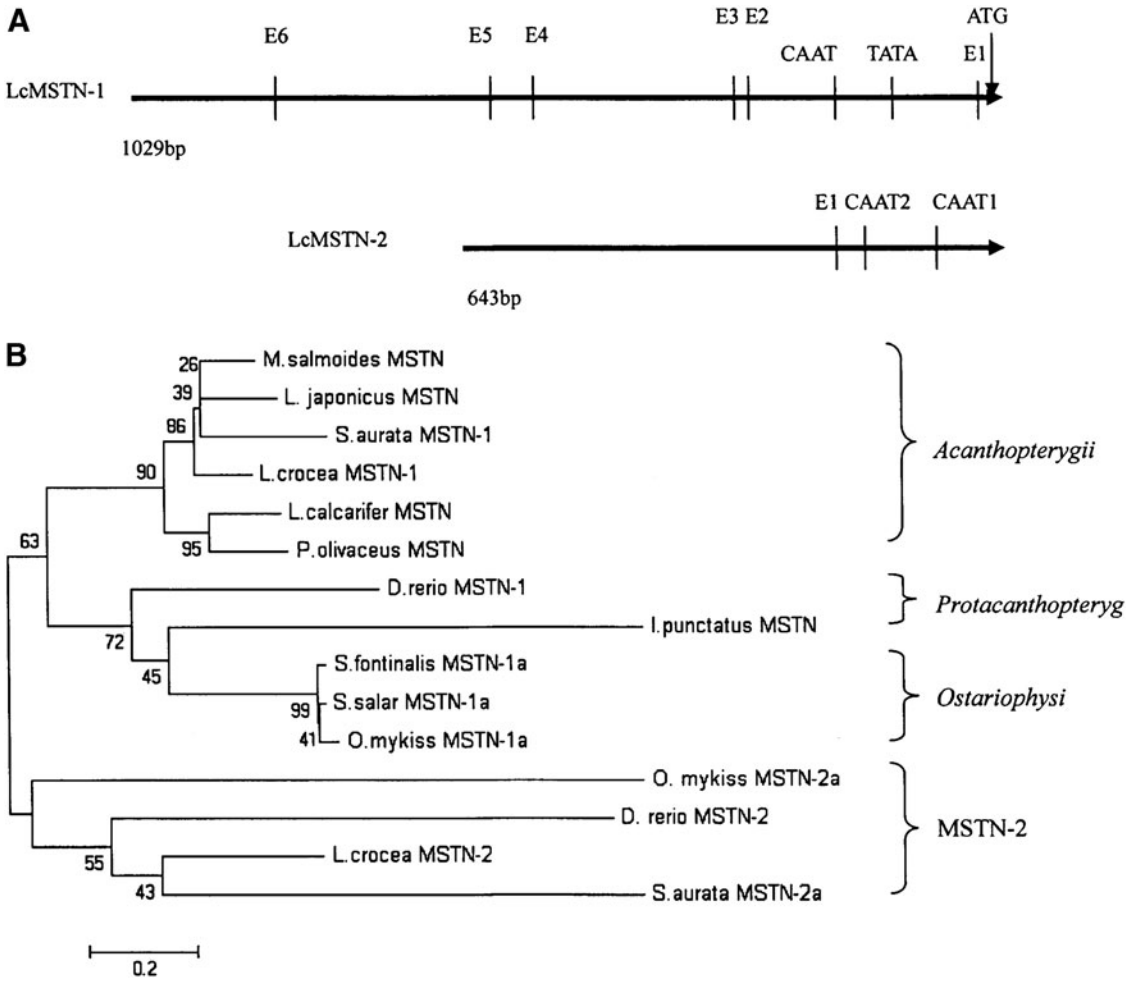

FIG. 3. Comparative analysis of major elements in 5′ flanking regions of *L. crocea MSTN-1* (*lcMSTN-1*) and 2 **(A)** and neighbor-joining tree based on 5′ flanking regions of *MSTN* genes **(B)**. Numbers at tree nodes refer to the percentage bootstrap values after 1000 replicates.

expression of the *GFP* reporter gene in L6 cells, and had little or no effect on the expression of *GFP* in CIK cells.

Tissue-specific expression of lcMSTN-2

Previous studies identified *MSTN-1* expression in the liver, kidney, brain, intestine, and skeletal muscle of large yellow croakers. *MSTN-2* also had a wide distribution of expression, and was expressed in the spleen, brain, adipose, kidney, gill, eye, intestine, and liver. The strongest expression was detected in the brain and adipose, and weak in the kidney (Fig. 6). However, *MSTN-2* mRNA transcript was not detected in the heart and skeletal muscle. No alternative splicing variant of *MSTN-2* was found in all tested tissues.

Discussion

Blast searches indicated that *lcMSTN-1* 5′ flanking sequence had a high homology with the reported sequences in Perciformes. In this region, putative TATA box, CAAT box, and several putative transcriptional factor binding sites such as CRE, MEF2, MEF3, POU3F2, GH-CSE, and E-box were identified. These putative *cis*-regulatory elements also existed in the *MSTN-1* promoter regions of other fishes, including brook trout (Roberts and Goetz, 2003), zebrafish (Xu *et al.*, 2003; Kerr *et al.*, 2005), rainbow trout (Garikipati *et al.*, 2006), Atlantic salmon (Ostbye *et al.*, 2007), sea perch (Ye *et al.*, 2007), and gilthead sea bream (Funkenstein *et al.*, 2009), of which some elements, such as MEF2 and MyoD, are muscle-specific transcription factor binding sites. CAAT box and TATA box were highly conserved among teleost *MSTN-1* upstream sequences. MyoD and MEF2 are critical to the differentiation of skeletal muscle and the regulation of *MSTN* gene expression in mammals (Cornelison *et al.*, 2000; Ma *et al.*, 2001; Spiller *et al.*, 2002; Salerno *et al.*, 2004; Du *et al.*, 2005).

The alignment of *MSTN-2* promoter sequences among large yellow croakers, zebrafish (DQ451548), and rainbow trout (DQ138301) revealed a very low conservation. The cloned fragment of the *lcMSTN-2* promoter contains only one E-box, two CAAT boxes, and several putative transcriptional factor binding sites such as CRE, MEF2, POU3F2, and SRF. No putative TATA box was found when 90 was set as a threshold score of TFSEARCH. However, a putative TATA box can be found when a threshold score is 85. Two TATA boxes were found in rainbow trout *MSTN-2a* (Garikipati *et al.*, 2007) and one TATA box in zebrafish *MSTN-2* (Kerr

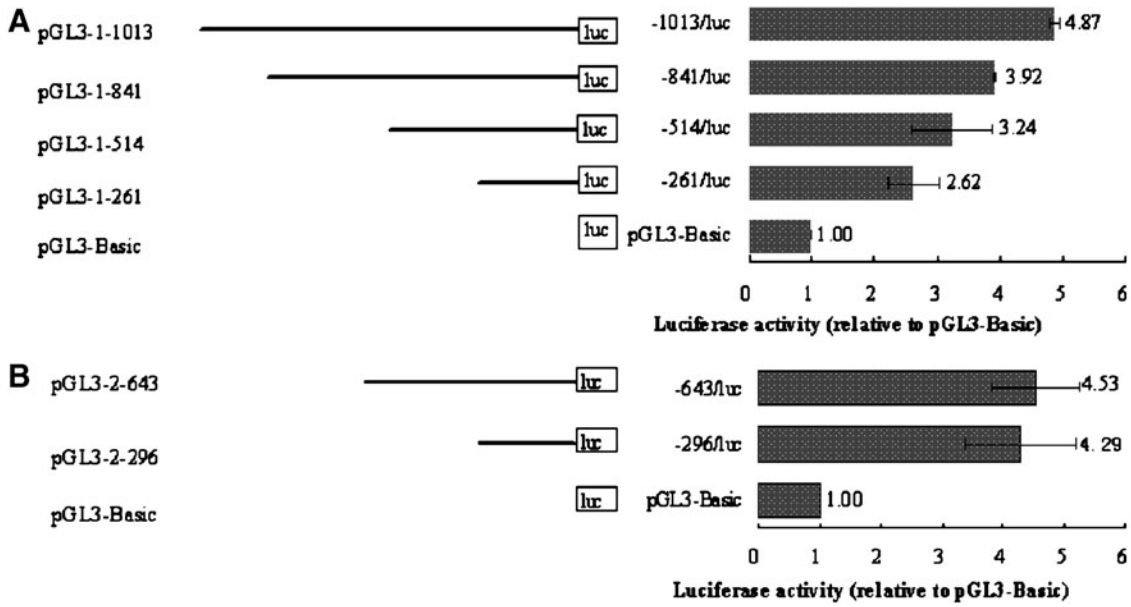

FIG. 4. Promoter activity analysis of the 5′ flanking region of *lcMSTN-1* **(A)** and 2 **(B)** in CIK cells. The promoterless plasmid, pGL3-Basic, and plasmids containing 5′ flanking fragments were shown on the left. The relative luciferase activities (fold-increase) expressed as mean ± SE of three experiments were shown in the right, which was normalized against the activity of the pGL3-Basic plasmid. SE, standard error.

et al., 2005). These results may mean that the *MSTN-2* promoter region is more variable than that of *MSTN-1*.

Previous studies demonstrated that differences between two types of MSTNs existed in both coding sequence and gene expression patterns (Rodgers *et al.*, 2007). In addition, their 5′ flanking regions are different. Three *MSTN-2* promoter sequences formed a subgroup, and all *MSTN-1* promoter sequences formed the other subgroup in the phylogenetic tree (Fig. 2B). A sequence analysis of their respective promoter regions revealed significant differences in the quantity and functional nature of the putative *cis* regulatory elements. The *MSTN-1* promoter had five E-boxes, and the *MSTN-2* promoter had only one in the corresponding region. Some important elements, such as MyoD, MEF3, SP1, USF, and GH-CSE sites, were found only in the *lcMSTN-1* promoter. These results suggest that lcMSTN-1 might play a more important role in the skeletal muscle development of *L. crocea* than lcMSTN-2. Some putative *cis* regulatory elements, such as MEF2, POU1F1a, POU3F2, CRE, and AP1, were indentified in both promoters of *lcMSTN-1* and *2*, but their locations were different. The divergence of two *MSTN* promoters supports the idea that there was early genome duplication during the fish lineage evolution.

Recently, several fish *MSTN* promoters were reported (Roberts and Goetz, 2003; Xu *et al.*, 2003; Garikipati *et al.*, 2006; Ostbye *et al.*, 2007; Ye *et al.*, 2007; Funkenstein *et al.*, 2009). The transient expression of GFP in zebrafish muscle fibers could be directed by zebrafish and sea perch *MSTN* promoters (Xu *et al.*, 2003; Ye *et al.*, 2007). However, a quantitative assay for functional analysis of fish *MSTN-1* promoter activity has been done only in gilthead sea bream, using the reporter gene luciferase and A204 cell line derived from a human rhabdomyosarcoma (Funkenstein *et al.*, 2009). Our previous works showed that the *lcMSTN-1* gene expressed in both the skeletal muscle and kidney (Xue *et al.*, 2006), and *lcMSTN-2* expressed in the kidney, but not in the skeletal muscle of *L. crocea*. In this study, CIK, a grass carp kidney cell line, and L6, a rat skeletal muscle cell line, were used to analyze the promoter activity of *lcMSTN-1* and *2*. In CIK cells, the shortest 261 bp fragment of the *lcMSTN-1* 5′ flanking region exhibited the lowest luciferase activity, and the longest 1013 bp fragment exhibited the highest luciferase activity. Luciferase activity correlated positively with the length of *lcMSTN-1* 5′ flanking fragment. These results confirm that the *lcMSTN-1* 5′ flanking region within the 1013 bp upstream from the translation initiation codon ATG contains a functional promoter. It also suggests that negative regulatory elements might not exist in the 1013 bp upstream. Similar results were obtained in the gilthead sea bream *MSTN-1* promoter, in which negative regulatory elements were found between 1127 and 1369 bp upstream (Funkenstein *et al.*, 2009). In 5′ flanking region of *lcMSTN-2*, the 643 bp fragment had a little higher luciferase activity than 296 bp, and both were over fourfold relative to pGL3-Basic, which indicates that a functional promoter exists in this region. In the corresponding 5′ flanking region, the *lcMSTN-2* promoter showed a higher activity than the *lcMSTN-1* promoter in the CIK cells, which was consistent with the *MSTN* gene expression *in vivo*. In the kidney tissue of *L. crocea*, the quantity of *lcMSTN-2* mRNA was larger than the *lcMSTN-1*.

E-boxes are involved in regulating the muscle-specific expression of genes. In our study, mutation of E-box6 (MyoD binding site) had a greater effect on the expression of the GFP reporter gene in L6 cells than in CIK cells. In zebrafish embryos, mutation of a single E-box (E1), MEF2, or MEF3 site alone had little or no effect on the muscle-specific expression of GFP reporter genes. However, when both E-boxes in the myogenin promoter were mutated, there was a significant reduction in the muscle-specific expression of GFP reporter

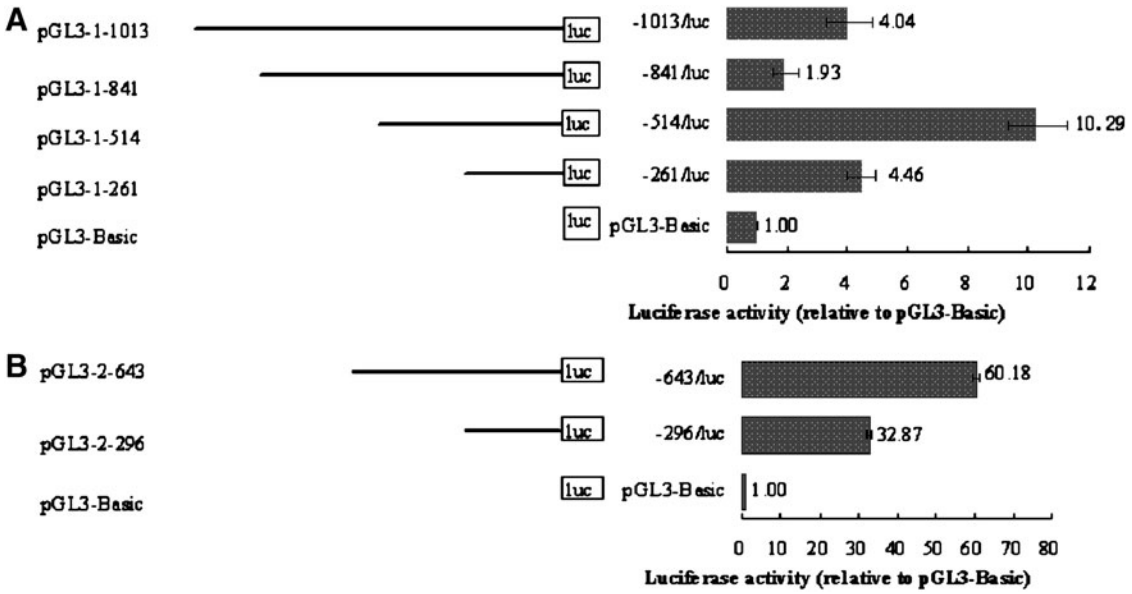

FIG. 5. Promoter activity analysis of the 5′ flanking region of *lcMSTN-1* **(A)** and 2 **(B)** in L6 cells. The promoterless plasmid, pGL3-Basic, and plasmids containing 5′ fragments were shown on the left. The relative luciferase activities (fold-increase) expressed as mean ± SE of three experiments were shown in the right, which was normalized against the activity of the pGL3-Basic plasmid.

genes. Mutation of the MEF2 binding site together with MEF3 binding sites or with E-boxes could reduce the activity of the promoter (Du *et al.*, 2003). In the *lcMSTN-1* upstream sequence, there are five E-boxes, one EMF2 and one EMF3 sites. These sites may play an important role together in regulating MSTN-1 expression in the skeletal muscle of large yellow croakers.

Surprisingly, there is a significant difference between CIK and L6 cells. In L6 cells, the fragments from the *lcMSTN-2* 5′ flanking region showed an extremely high luciferase activity, of which the 643 bp fragment reached 60.18-fold relative to pGL3-Basic, and was 13.28-fold in CIK cells. *In vivo*, the expression of *MSTN-2* was significantly lower than that of *MSTN-1*, and hardly detectable in the skeletal muscle of *L. crocea*. The *MSTN-2* gene has not been found in mammals, and functional analysis of other fish *MSTN-2* promoters has not been reported so far. The reason that the *MSTN-2* promoter has such a high activity in L6 cells is unknown. For the *lcMSTN-1* 5′ flanking region, the highest activity was found with the fragment of 514 bp, while the lowest activity was with the fragment of 841 bp. It suggests that the upstream sequence of *lcMSTN-1* might have a negative regulatory element between −514 and −841 in L6 cell experiments. These data indicated that transcription regulation of *lcMSTN-1* and *2* promoters was significantly different between mammalian and fish cells or *in vivo* and *in vitro*. In gilthead sea bream, no reporter gene activity was found on transient transfection of the recombinant plasmids into the murine myoblast C2 cell line and a mammalian nonmuscle cell line CHO-K1 (Funkenstein *et al.*, 2009). These results showed that it was important to choose an appropriate cell line for a quantitative assay of the fish *MSTN* promoter function.

Our work revealed that both *lcMSTN-1* and *2* promoters contained numerous potential transcription factor binding sites, and had the function of transcription regulation. It lays a foundation for the functional analysis of fish *MSTN-1* and *2* promoters. Further studies are needed to understand the regulation of fish *MSTN-1* and *2* gene expression under various physiological conditions as well as the effect of transcription factors and hormones.

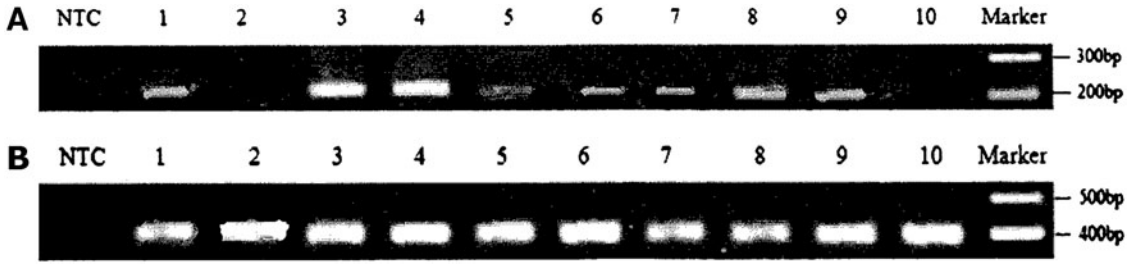

FIG. 6. The expression pattern of *MSTN-2* in the tissues of *L. crocea*. **(A)** *MSTN-2*; **(B)** β-actin. Lines from 1 to 10 represent spleen, heart, brain, adipose, kidney, gill, eye, intestine, liver, and skeletal muscle, respectively, and NTC represents negative control.

Conclusion

The 5′ flanking fragments of 1029 bp from *lcMSTN-1* and 643 bp from *lcMSTN-2* were cloned. The upstream sequence of *lcMSTN-1* had a homology of more than 90% with the reported sequences of *S. aurata* and *L. japonicas*, while the upstream sequence of *lcMSTN-2* showed low conservation. Both fragments contained a CAAT box and several putative *cis*-regulatory elements. However, some elements such as a putative TATA box, MyoD, MEF3, USF, and GH-CSE sites were identified only in the *lcMSTN-1* upstream region. Functional analysis of promoters revealed that the different fragments had diverse effects on luciferase activities in CIK and L6 cells. In CIK cells, luciferase activity rose as the length of truncated fragments and the number of E-boxes increased. The *lcMSTN-2* promoter showed a higher activity than *lcMSTN-1* in the corresponding region, which was consistent with the expression of *lcMSTN-1* and *2* genes in the kidney tissue of *L. crocea*. In L6 cells, functional analysis of promoters suggested that the upstream sequence of *lcMSTN-1* might have a negative regulatory element between -514 and -841. The *lcMSTN-2* upstream showed an extremely high luciferase activity. Mutation of E-box6 (MyoD binding site) had a greater effect on GFP expression in L6 cells than in CIK cells. These results indicated that both cloned fragments contained functional promoters, and that the transcription regulation mechanism of two promoters differed between mammalian and fish cells. It suggests that a cell line, derived from the tissue in which both *MSTN-1* and *2* genes are expressed, may be appropriate for a quantitative assay for fish *MSTN* promoter activity.

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Disclosure Statement

No competing financial interests exist.

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