Original Article

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Characterization of Muscle-Regulatory Gene, *MyoD*, from Flounder (*Paralichthys olivaceus*) and Analysis of Its Expression Patterns During Embryogenesis

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Abstract

Specification and differentiation of skeletal muscle cells are driven by the activity of genes encoding members of the myogenic regulatory factors (MRFs). In vertebrates, the MRF family includes *MyoD*, *Myf5*, *myogenin*, and *MRF4*. The MRFs are capable of converting a variety of nonmuscle cells into myoblasts and myotubes. To better understand their roles in fish muscle development, we isolated the *MyoD* gene from flounder (*Paralichthys olivaceus*) and analyzed its structure and patterns of expression. Sequence analysis showed that flounder *MyoD* shared a structure similar to that of vertebrate MRFs with three exons and two introns, and its protein contained a highly conserved basic helix–loop–helix domain (bHLH). Comparison of sequences revealed that flounder *MyoD* was highly conserved with other fish *MyoD* genes. Sequence alignment and phylogenetic analysis indicated that flounder *MyoD*, seabream (*Sparus aurata*) *MyoD1*, takifugu (*Takifugu rubripes*) *MyoD*, and tilapia (*Oreochromis aureus*) MyoD were more likely to be homologous genes. Flounder *MyoD* expression was first detected as two rows of presomitic cells in the segmental plate. From somitogenesis, *MyoD* transcripts were present in the adaxial cells that give rise to slow muscles and the lateral somitic cells that give rise to fast muscles. After 30 somites formed, *MyoD* expression decreased in the somites except the caudal somites, coincident with somite maturation. In the hatching stage, *MyoD* was expressed in other muscle cells and caudal somites. It was detected only in muscle in the growing fish.

Keywords: flounder — muscle — *MyoD* — myogenesis — somites

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Introduction

Members of the myogenic regulating factors (MRFs) family are basic helix–loop–helix (bHLH) transcription factors, and include *MyoD*, *Myf5*, *myogenin*, and *MRF4* (Buckingham, 1992). They exert a pivotal role in the determination and differentiation of vertebrate skeletal muscle. The MRF proteins contain several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, DNA binding, nuclear localization, and heterodimerization (Tapscott et al., 1988; Weintraub et al., 1991; Schwarz et al., 1992; Vandromme et al., 1995; Gerber et al., 1997). The important one is the bHLH domain which is highly conserved in all of the MRFs. The bHLH domain can dimerize with ubiquitously expressed E-proteins, such as E12, E47, HEB, and ITF (Murre et al., 1989; Sun and Baltimore, 1991; Lin and Konieczny, 1992; Langlands et al., 1997). This heterodimer binds to a consensus DNA sequence called E-box (CANNTG), present in the regulatory regions of many skeletal-muscle-specific genes (Lassar et al., 1989; Murre et al., 1989; Blackwell and Weintraub, 1990).

The MRFs are able to convert a wide range of cell types into the myogenic lineage when ectopically expressed (Edmonson and Olson, 1993). Gene targeting experiments indicated that *MyoD* and *Myf5* were required for myogenic determination, whereas *myogenin* and *MRF4* were important for terminal differentiation and lineage maintenance (Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995). Gene disruption in mice revealed the roles of MRFs in muscle development (Arnold and Winter, 1998). It appeared that MRFs exhibited distinct and partial redundant functions in regulating muscle formation (Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Wang et al., 1996). *MyoD* null mutant mice displayed normal

skeletal muscles but expressed about fourfold higher levels of *Myf5* (Rudnicki et al., 1992). In contrast to the *MyoD* mutant, mice lacking a functional *Myf5* gene died from severe rib abnormalities although there were no significant abnormalities in skeletal muscle (Braun et al., 1992, 1994). When both *MyoD* and *Myf5* are mutated, mice display a complete absence of skeletal myocytes or myofibers (Rudnicki et al., 1993). Inactivation of *MRF4* resulted in grossly normal muscle formation, and showed about a fourfold increase in expression of *myogenin* (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995).

Growth rate of cultured fish is one of the most important factors in the success of aquaculture. The normal stimulus for muscle growth in growing fish is not well understood. Understanding the regulation of muscle growth in fish is of particular importance for aquaculture. Fish meat, consisting of most skeletal muscles, provides high value proteins in the diet. Evidence indicates that muscle growth is regulated positively and negatively by a variety of growth and transcription factors. These factors include growth hormone (Du et al., 1992; Devlin et al., 1994; Mommsen and Moon, 2001), fibroblast growth factors (Du, 2004; Rescan, 2005), insulin-like growth factors (Rescan, 2005), and transforming growth factor-β (TGF-β)(Xu et al., 2003; Rescan, 2005) and directly or indirectly act on MRFs. Several MRF genes have been studied in fish (Rescan et al., 1995, 1999; Weinberg et al., 1996; Kobiyama et al., 1998; Delalande and Rescan, 1999; Chen et al., 2000, 2001; Coutelle et al., 2001; Tan and Du, 2002; Tan et al., 2002) and were shown to be muscle specific. In rainbow trout, it has shown that the myogensis expressions levels related to the myoblast hyperplasia and hypertrophy (Johansen and Overturf, 2005). These data indicate that MRFs play important roles in regulating muscle development and growth in fish.

Flounder is an economically important fish in the Asian region. Although flounder has been cultured for many years, there is little information about its skeletal muscle formation. To understand the muscle formation and the functions of factors that regulate muscle growth in flounder, herein we report the isolation and characterization of the flounder *MyoD* gene and determination of its expression pattern during embryonic development.

Materials and Methods

Fish and Embryos Culture. Flounder were cultured at the Institute of Oceanology, Chinese Academy of Sciences and a fish farm in Rongcheng city, Shandong Province under natural or controlled conditions (photoperiod, 14 h light: 10 h dark; temperature, 15 ± 1°C; seawater; aeration). Fish were fed a commercial particle diet twice a day. The fertilized eggs were obtained by mixing sperm and eggs collected from matured males and females by artificial gently stripping, respectively. The embryos were cultured at 15 ± 1°C in $1m^3$ tank under the same condition as the fish culture.

Isolation of Flounder MyoD Gene. The flounder *MyoD* genomic gene was isolated as overlapping DNA fragments from flounder Genome Walker libraries. Briefly stated, flounder genomic DNA was completely digested with restriction enzymes (*Dra*I, *Eco*RV, *Hpa*I, *Pvu*II, *Sca*I, *Sma*I, and *Stu*I) to yield blunt-ended DNA fragments. Then the digested

Table 1. Genomic Walker Primers and RT-PCR Primers

Primer	*Primer sequence(5′–3′)*
MRF1	TT(T/C)CT(C/G)AGGATCTCCACCTTGGG(C/A)AG
MRF2	CCACCTTGGG(C/A)AG(T/C/A)C(T/G)CTG(A/G)(T/C)TGGGGTT
Ap1	GTAATACGACTCACTATAGGGC
Ap2	ACTATAGGGCACGCGTGGT
MyoD1	CTGCAAGGCCTGCAAGTGGAAG
MyoD2	CTGCAAGTGGAAGACCACCAAC
MyoD3	GTCATAGGGGAATCAACAGTTTCG
MyoD4	CACATCACACAGTCTAAAGTAGC
MyoD5	ATCTGCAGGTGGTCAGAAGAGTGAC
MyoD6	AAGAGTGACCGCAGATCGGTGGTC
MyoDR1	GGGAAAGACATATCCGACAACTCC
MyoDR3	GACAACTCCATAGTTCAGTCCTTGG
FLMDc-1	AGACTCCTCTGTGTCTTCCACTG
FLMDc-2	TATGAGTGGACTGTGTGAGAGG
Act-5	AGAGCAAGAGAGGCATCCTGAC
Act-3	CGATGGGTGATGACCTGTCC

DNA was ligated with a DNA adaptor (Clontech, USA). The resulting DNA fragments were used as templates for PCR amplification of *MyoD* using two adaptor-specific primers together with two MRF-consensus primers. Specifically, the promoter sequence and part of the first exon I of the flounder *MyoD* gene were isolated by two rounds of polymerase chain reaction (PCR) using MRF consensus primers (MRF1 and MRF2) and the adapter primers (Ap1 and Ap2). The remaining part of *MyoD* genomic sequences were cloned by several rounds of PCR using *MyoD*-specific primers together with the adapter primers. The *MyoD* gene-specific primers were MyoDR1 and MyoDR3, MyoD1 and MyoD2, MyoD3 and MyoD4, and MyoD5 and MyoD6 (Table 1). All of the fragments were cloned into pUCm-T vector (Sangon, Shanghai) and sequenced.

A

```
P.olivaceus     MELSDMSFPIPADDDFYDDPCFPPSDMHFFEDLDSRLVHVGLLKPDDSSSLSSSSPSS-S 59
O.aureus        MELPDISFPIPTADDFYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDDSSSSSSSPSS-S 59
O.mykiss1       MELPDIPFPITSPDDFYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDD------------- 47
O.mykiss2       MELSDISFPVTSADDFYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDD------------- 47
T.rubripes      MELSEISFSIPAADDFYDDPCFSTSDMHFFEDMDPRLVHAGLLKPDDCCSSSSLSPSS-S 59
D.rerio         MELSDIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLLKPDE------------- 47
C.carpio        MELSDIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLLKPDE------------- 47
S.aurata1       MELSDISFPIPAADDFYDDPCFNTSDMHFFEDLDPWLVHVGLLKPDDSSSSVSPSPSSSA 60
S.aurata2       MDLSDLPFPLSSADDLYDDPCFSTSDMNFFDDLDARLMHAGLLKPED------------- 47
                *:*.::.*.:.: **:****** ..**:**:*:*. *:*..****::

P.olivaceus     SSSPSSLLHLHHHAEV----EDDEHVRAPSGHHQAGRCLLWACKACKWKTTNADRRKAAT 115
O.aureus        SSSPSSLLHLHHHAEV----EDDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAAT 115
O.mykiss1       ----------HHHKE-------DEHIRAPSGHHQAGRCLLWACKACKRKTTNADRRKAAT 90
O.mykiss2       ----------HHYNE-------DEHIRAPSGHHQAGRCLLWACKACKRKTTNSDRRKAAT 90
T.rubripes      SASPSSLLHIHHHTEA----EDDEHIRAPSGHHHAGRCLLWACKACKRKTTNVDRRKAAT 115
D.rerio         ----------HHHIE-------DEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAAT 90
C.carpio        ----------HHHLE-------DEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAAT 90
S.aurata1       SSSPSSLLHLHHHAEG----EDDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAAT 116
S.aurata2       ------HLHHHHHYHVPIAEEEDEHVRAPGGLHQAGHCLLWACKACKRKTTHADRRKAAT 101
                      **: .       ***:***.* *:**:********** ***: *******

P.olivaceus     MRERRRLSKVNDAFETLKRCTSANPNQRLPKVEILRNAISYIESLQALLRG-GQD-DGFY 173
O.aureus        LRERRRLSKVNDAFETLKRCTTANPNQRLPKVEILRNAISYIESLQALLRG-GQE-DGFY 173
O.mykiss1       MRERRRLSKVNDAFETLKRCTSTNPNQRLPKVDILRNAISYIESLQGLLRGAGQE-GNYY 149
O.mykiss2       MRERRRLGKVNDAFENLKRCTSNNPNQRLPKVEILRNAISYIESLQSLLR--GQDGENYY 148
T.rubripes      LRERRRLSKVNEAFETLKRCTNTNPNQRLPKVEILRNAISYIESLQALLRG-GQD-EAFY 173
D.rerio         MRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLR--SQE-DNYY 147
C.carpio        MRERRRLSKVNDAFETLKRCTSNNPNQRLPKVEILRNAISYIESLQALLR--GQE-ENYY 147
S.aurata1       LRERRRLSKVNDAFETLKRCTSANPNQRLPKVEILRNAISYIESPQALLRG-GQD-DGYY 174
S.aurata2       MRERRRLSRVNDAFETLKRCTASSPNQRLPKVDILRNAISYIESLQALLRT-GRD-ESFY 159
                :******.:**:***.*****  .********:********** *.***  .::   :*

P.olivaceus     PVLEHYSGDSDASSPRSNCSDGMTDFNGPTCQSTRRGSYESSSYFSQTPNGGQKSDRRSV 233
O.aureus        PVLEHYSGDSDASSPRSNCSDGMTDFNGPTCQTTRRGSYDSSSYFSETPNGGLKSERSSV 233
O.mykiss1       PVMDHYSGDSDASSPRSNCSDGMMDFNGQSCPPRRRNKYDST-YFNEAPN-DSRHKKNSV 207
O.mykiss2       PVLEHYSGDSDASSPQSNCSDGMMDYNAPTCTSARRSNYDSS-YFAETPNADSRSNKNAA 207
T.rubripes      TVLEHYSGDSDASSPRSNCSDGMTDFNGPTCQSNRRGSYYSS-YFSQTPKGSLKAERN-- 230
D.rerio         PVLEHYSGDSDASSPRSNCSDGIMDFMGPTCQTRRRNSYDSS-YFNDTPNADARNNKNSV 206
C.carpio        PVLEHYSGDSDASSPRSNCSDGMMDFMGPTCQSRRRNSYDSS-YFNDTPNADARNTKSSV 206
S.aurata1       PVLEHYSGDSDASSPRSNCSDGMTDFNGPSCQSNRRGSYDSSSYFSETPNGGLKSERSSV 234
S.aurata2       PPLEHYSGDSDASSPRSNCSDGMMDFISP-CSSTSENS-DGS--FSNQTAYESRRSKRSL 215
                ::***********:******: *: .  * .  ...  .:  * : .    :  :

P.olivaceus     VSSLDCLSSIVERISTDNSSLMPAVDGPVSPPT----DQTGETAAPGPLQVPSPTAS--- 286
O.aureus        VSSLDCLSSIVERISTDNSSLLPPADGPGSPTT----TTT------VPMQFADPTRR--- 280
O.mykiss1       ISSLDCLSNIVERITTDTSACPAVQ--DGSEGSSPCSPGDGSIASENGAPIPSPINCVPA 265
O.mykiss2       VSSLDCLSNIVERISTDTSACTVLSGQEGSEGS-PCSPQEGSILSRNGGTVPSPTNC-PQ 265
T.rubripes      -SSLDCLSSIVERISTATSSGPPPVDGRGSP--------------GPLQASSPRSS--- 271
D.rerio         VSSLDCLSSIVERISTETPACPVLSVPEGHEES-PCSPHEGSVLSDTGTTAPSPTSC-PQ 264
C.carpio        VSSLDCLSSIVERISTETPACPVLSVPEGHEGS-PCSPQEGSVLSETGAPAPSPTTC-PQ 264
S.aurata1       VSSLDCLSSIVERISTDTSSLLPAADGPASPTT----PPTGEAAAPGPVQIPSPTAS--- 287
S.aurata2       VSSLDCLSSIVERISTDPAVAPPGDSVVPQGPG----------SPQNSPTGSSPAGS-SH 264
                 *******.*****:*  .                                  ..*

P.olivaceus     -QDPNLIYQVL 296
O.aureus        -R--------- 281
O.mykiss1       LHDPNTIYQVL 276
O.mykiss2       P-SHDPIYQVL 275
T.rubripes      -REPNLIYQVL 281
D.rerio         QQAQETIYQVL 275
C.carpio        QQARDPIYQVL 275
S.aurata1       -QDPNLIYQVL 297
S.aurata2       PAEPNSIYEPL 275
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Fig. 1. (**A**) Comparison of deduced amino acid sequences of flounder *MyoD* with those of other vertebrate. The highly conserved basic helix-loop-helix domains are underlined, and the basic region is indicated by shading. *Represents identical amino acid. The GenBank accession numbers for these fish *MyoD* genes are: flounder *MyoD* (*Paralichthys olivaceus*, DQ184914); seabream *MyoD1* (*Sparus aurata*, AF478568); seabream *MyoD2* (*Sparus aurata*, AF478569); tilapia *MyoD* (*Oreochromis aureus*, AF270790); trout *MyoD1* (*Oncorhynchus mykiss*, X75798); trout *MyoD2* (*Oncorhynchus mykiss*, Z46924); takifugu *MyoD* (*Takifugu rubripes*, T007049); zebrafish *MyoD* (*Danio rerio*, AF318503); carp *MyoD* (*Cyprinus carpio*, AB012882). (**B**) Putative muscle specific transcription factor binding sites in the 0.6-kb promoter region of flounder *MyoD* gene. Numbers indicated the nucleotide position relative to the translation start code (ATG). (**C**) The identification of conserved region in the *MyoD* promoters. The sequence comparison of a 187-bp highly conserved region in the promoters of flounder *MyoD* and seabream *MyoD1* (GenBank accession no. AF478568) genes. Numbers indicate the nucleotide position relative to the translation start code (ATG). **Continues.**

B

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AAACGACATGCTATCTTATAAAAAACACACATCTAACACAAGTGTATACATGTGAAAAAA  -549
                                        USF E-box  E-box
ATTGTAGCATTAATAATGAAAATAGGATGTATTAATCAACAAGTACATTTACCTTTACCC  -489
              OCT-1
CAATTTCCCCTGGGATGAATAAACTATTTGTGATTCTGAAGAATGTATTTCAGATGTATC  -429
   NF-AT                                        OCT-1    E-box
AGAGAAATCTACACGTTAAGACAGACACAGTGTTGTAATGTGCTGTGATTGTAACCAGCA  -369

CGTAATGTGTCCTCAGTATGAAGCAGTCCACATGTGTGTGCCCCCCCCCCCCTGCAGCTCT  -309
                                E-Box USF       SP1
GTTAGGGGTAATTGTACACTAATTAGCGTGAAGTTGTAAACCCCTCCCGCTGGCTGCTGA  -249
                                                        NF-Y
TTGGTCAGACCCCAGTGGACACGGTCGCCCGGCCCCCGCCCCCCCCGGGTGTCCCAGGTA  -189
              USF             SP1             USF
TAAGTTGGTCCAACTAGACAGCTGAGGGGACACACCAATTTGTGACAGGACTCTACATTC  -129
TATA-box        E-box
CCGCTGAAAACACTCAGACTGCAACCACTGTCTCCTTCCAAATCTTCCTGCGTTTGTTTT   -69
                                                    MEF-2
TAGCTCCAGACTCCTCTGTGTCTTCCACTGGGATTTGTCTTCTCGCTCGCTGGGCCAAGG    -9
                          USF
ACTGAACTATG                                                     +3
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C

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P.olivaceus     CTCTGTTAGGGGTAATTGTACACTAATTAGCGTGAAGTTGTAAACCCCTCCCGCTGGCTG -253
S. aurata 1     CTCTCTTTGGGGTAATTGTACACTAATTACCGTGAAGTTGTAAACCCCTCCTGCTGCCAG -214
                **** ** ********************** ********************** **** * *

P.olivaceus     CTGATTGGTCAGACCCCAGTGGACACGGTCGCCCGGCCCCCGCCCCCC-CGGGTGTCCC -194
S. aurata 1     CTGATTGGTCAGCCTTCAGCGGACCTCATCACCCTGACCCCGCCCCCCGGCGTGTGTCCC -154
                *********** *  *** ****    ** *** * ***********  ** *******
                     NF-Y                          SP1            USF
P.olivaceus     AGGTATAAGTTGGTCCAACTAGACAGCTGAGGGGACACACCAATTTGTGACAGGACTCTA -134
S. aurata 1     AGGTATAAGAGGCTCCAGGTCAGCAGCTGAGGGGACAGAACAGTTTGTGACAGGACTCTA -98
                ********  * ****  *   ************** * ** ******************
                   TATA              E-box

P.olivaceus     CATTCCC                                                     -127
S. aurata 1     CATTCCC                                                     -91
                *******
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Fig. 1. Continued.

Reverse Transcriptase-PCR (RT-PCR). To determine the intron–exon boundary, flounder *MyoD* cDNA was isolated by RT-PCR. Total RNAs were extracted from flounder embryos using Trizol (Invitrogen, USA). cDNA was synthesized using the first strand cDNA synthesis kit (Promega, USA). PCR was performed using *Pfu* enzyme (Promega, USA) and specific primers (FLMDc-1 and FLMDc-2) (Table 1). The fragments were cloned into pBluescript II SK (Stratagene, USA) *Sma* I site and sequenced.

To determine if the *MyoD* exhibited distinct pattern of expression in different tissue of growing fish, total RNAs were extracted from muscle, kidney, liver, spleen, and heart of growing flounder about 10 cm in length. The expression of *MyoD* was analyzed by RT-PCR using specific primers (MyoD1 and FLMDc-2) (Table 1). *Actin* was used as the control. The specific primers for flounder *actin* were Act-5 and Act-3 (Table 1).

Protein Alignment and Phylogenetic Analysis. Protein alignment and phylogenetic analysis was done by using program Clustal W (http://www.ebi.ac.uk/clustalw/). During phylogenetic analysis, full protein sequences were used, no amino acid was deleted, gaps were not ignored, and the distances were corrected using Kimura Correction of distances method according to Clustal W program (Thompson et al., 1994). The phylogenetic tree was constructed using the neighbor-joining method according to Clustal W program (Thompson et al., 1994).

Transcriptional Factor Binding Site Prediction. Transcriptional factor binding sites were predicted by using Match program (http://www.gene-regulation.com/pub/programs.html#match). The database is TRANSFAC® 6.0 (Kel-Margoulis et al., 2002).

Whole Mount in Situ Hybridization. The plasmid clone containing the 5′ UTR and part of the exon-1 sequence of flounder *MyoD* was used as a template to generate the sense and antisense digoxigenin-labeled RNA probes using T7 RNA polymerase. RNA probes were made by in vitro transcription in the presence of digoxigenin-11-UTP* (Roche Applied Science, Germany). Hatching embryos were anesthetized with 0.2% MS222 (3-aminobenzoic acid ethyl ester) before fixation. Embryos were fixed overnight at 4°C with 4%

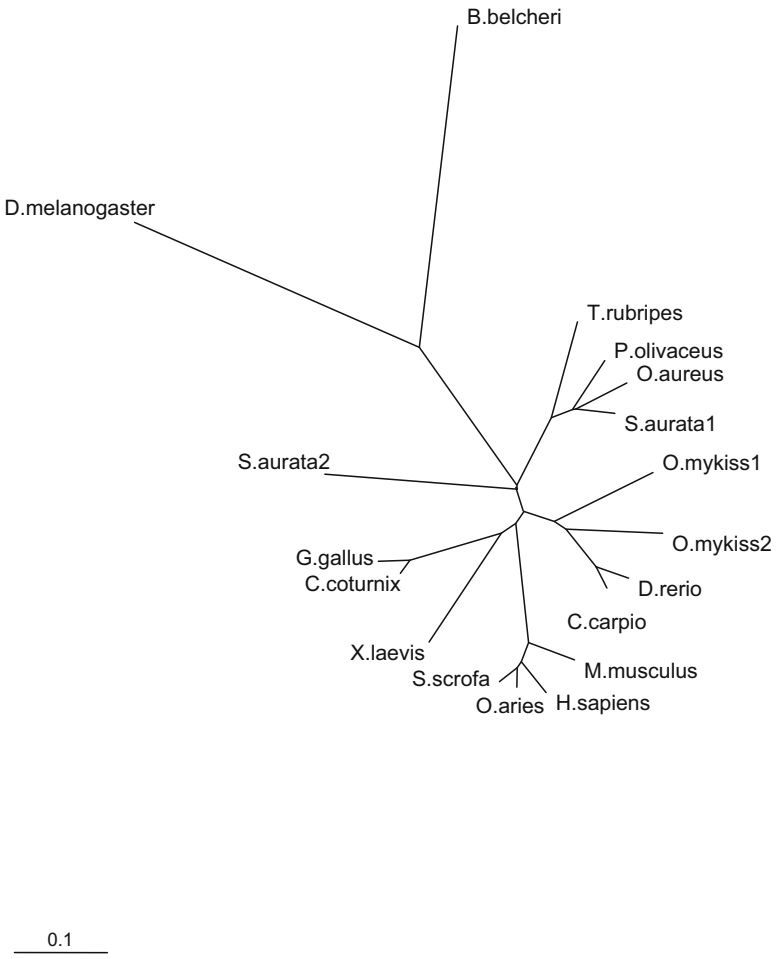

Fig. 2. Phylogenetic analysis of flounder *MyoD* gene sequences relative to *MyoD* genes of other fish species and vertebrates. The deduced protein sequences were used in the analysis using Clustal W sequence alignment program. Note flounder *MyoD*, sea bream *MyoD1*, and takifugu and tilapia *MyoD* are in the same branch. The tree was constructed from the set of aligned sequences shown in Figure 1, plus *Drosophila MyoD* (*Drosophila melanogaster*, M68897); Amphioxus *MyoD* (*Branchiostoma belcheri*, AY066009); *Xenopus MyoD* (*Xenopus laevis*, X16106); chicken *MyoD* (*Gallus gallus*, X16189); quail *MyoD* (*Coturnix coturnix*, L16686); sheep *MyoD* (*Ovis aries*, X62102); pig *MyoD* (*Sus scrofa*, U12574); mouse *MyoD* (*Mus musculus*, NM-010866); human *MyoD* (*Homo sapiens*, NM-002478).

paraformaldehyde in PBS (pH 7.4) and then stored in 100% methanol at −20°C. Embryos were dechorionated with fine forceps. Hatching stage embryos were treated with 10 μg/ml of Proteinase K for 10 min in phosphate-buffered saline (PBS) at room temperature. Next, the embryos were refixed with 4% paraformaldehyde for 30 min at room temperature. In situ hybridization was carried out as described by Du and Dienhart (2001). Embryos in glycerol were photographed under the microscope (DM LB2, Leica) with a Nikon 4500 digital camera.

Results and Discussion

Isolation and Characterization of the MyoD Gene from Flounder. The flounder *MyoD* gene was isolated by PCR from flounder Genome Walker libraries as described in Materials and Methods. During the first two rounds of PCR using the MRF consensus primers with the adaptor-specific primers, a 500-bp fragment was amplified. Sequence analysis revealed that this fragment was the homolog of *MyoD*. The complete genomic sequence of flounder MyoD was determined (GenBank accession no. DQ184914) and shown a size of approximately 4.1 kb. Sequence analysis predicted three exons and two introns with conserved consensus sequence GT...AG at the exon intron boundary. This structure was shared by all vertebrate MRFs and verified by RT-PCR. Flounder *MyoD* encodes a protein of 296 amino acids including a highly conserved bHLH domain.

The flounder *MyoD* is highly conserved compared with other fish *MyoD*s (Figure 1A). It shared 69%, 69%, 72%, 73%, 82%, and 86% identity with trout *MyoD1*, trout *MyoD2*, zebrafish *MyoD*, carp *MyoD*, takifugu *MyoD*, and tilapia *MyoD*, respectively. The bHLH domain of flounder *MyoD* exhibited more than 90% identity with that of other fish.

Protein alignments with all reported fish *MyoD*s revealed that the flounder *MyoD* shared

the highest identity with tilapia *MyoD* (86%) and seabream *MyoD1* (86%), and 72% with zebrafish *MyoD*. Phylogenetic analysis confirmed that flounder *MyoD* was more closely related to takifugu *MyoD*, seabream *MyoD1*, and tilapia *MyoD* (Figure 2). In addition, flounder *MyoD* contained a serine-rich insertion near the N-terminal region that also existed in tilapia *MyoD*, takifugu *MyoD*, and seabream MyoD1 (Tan and Du, 2002), but was missing in *MyoD* of other fish and vertebrates (Figure 1A). These data suggested that the flounder *MyoD* might be homolog of tilapia and takifugu *MyoD*, and sea bream *MyoD1*.

It had been reported that there were two *MyoD*s in trout (Delalande and Rescan, 1999) and sea bream (Tan and Du, 2002), which were specifically expressed in muscle. In sea bream embryos, *MyoD1* was expressed in both fast and slow muscles, while *MyoD2* expression was first detected in both fast and slow muscle precursors, and then decreased

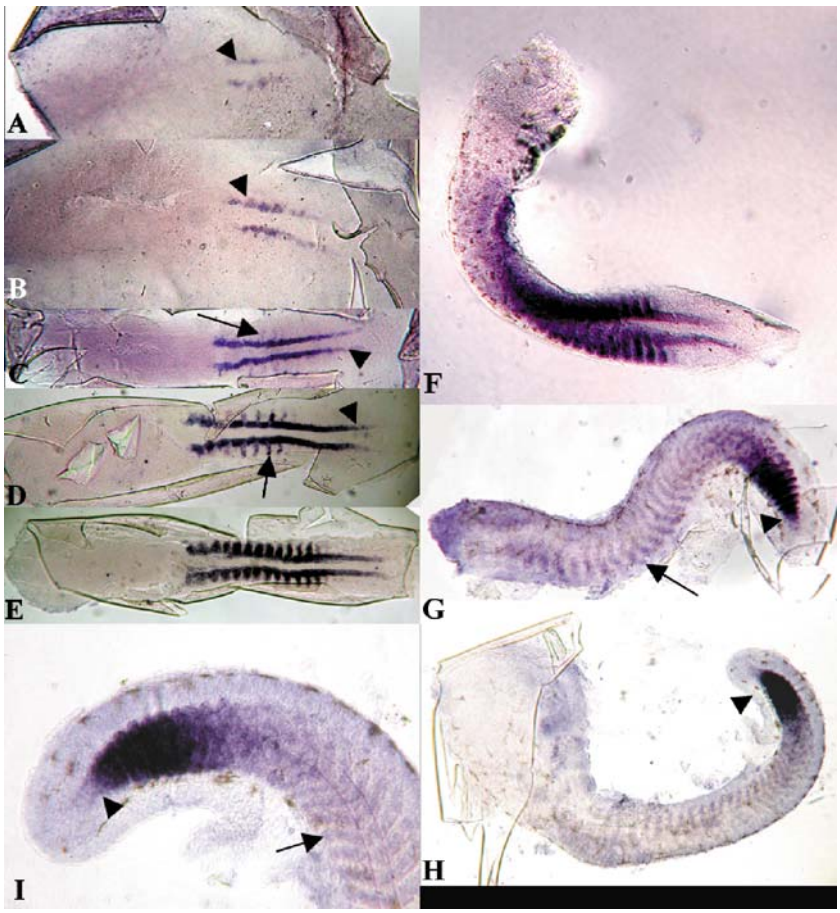

Fig. 3. Temporal and spatial expression of *MyoD* in flounder embryos. (**A**–**E**) Anterior is to the left and dorsal view. (A) Stage 1 embryo (completion of epiboly, before somite formation). *MyoD* labeling was present in two cords of cells (arrowhead) adjacent to the prospective notochord. (**B**) Stage 2 embryo (3 somites). *MyoD* expression was detected in the somitic medial cells and the presomitic cells in the segmental plate. (**C**) Stage 3 embryo (5 somites). *MyoD* expression remained to the adaxial cells of the somites (arrowhead) and weak expression was also detected in the lateral somitic cells (arrow). (**D**) Stage 4 (7 somites): lateral expansion of *MyoD* labeling was observed within the somites (arrow). (**E**) Stage 5 (15 somites). *MyoD* transcripts were detected within the somites. (**F**) Stage 6 (about 25 somites). Lateral view. Anterior to the top: *MyoD* expression was detected in the myotome. (**G**) Stage 7 (30 somites). Anterior to the left. (**H**) Stage 10 (about 40 somites). Lateral view. Anterior to the top. *MyoD* expression decreased in older somites (arrow) while it was still strong in the neoformed somites (tail region) (arrowhead). (**I**) Magnification of positive signals in tail region of H. *MyoD* expression decreased in anterior somites (arrow) while it was still strong in the posterior somites (tail region) (arrowhead). (**J**) Hatching stage embryo. Lateral view, anterior to the left. (**K**) Ventral view. Magnification of MyoD expression in the adductor mandibulae (arrow) and adductor operculi (arrowhead). yolk removed. (**L**) Side view. Magnification of *MyoD* expression in extraocular muscle (arrow). (**M**) Side view. Magnification of *MyoD* expression in dorsal anterior myotome muscle cells (arrowhead). (**N**) Side view. Magnification of *MyoD* expression in dorsal/ventral (weak) posterior myotome muscle cells (arrowhead) and caudal somites (arrow). **Continues.**

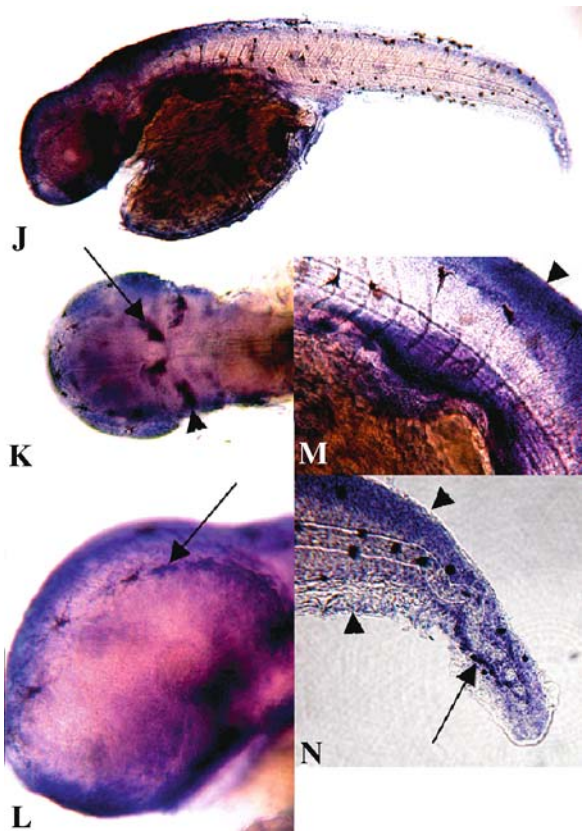

Fig. 3. Continued.

gradually in slow muscle precursors. In adult, seabream fast muscles expressed both *MyoD1* and *MyoD2*, while slow muscles predominantly expressed *MyoD1* (Tan and Du, 2002). Trout *MyoD1* was similarly expressed as seabream *MyoD1* but the expression pattern of trout *MyoD2* was quite different. Trout *MyoD2* was first detected in late-stage embryos and expressed only in slow muscles of adult trout (Delalande and Rescan, 1999). Both *MyoD1* were expressed similarly to the flounder *MyoD*. It is possible that the differential expression of two *MyoD* genes in the fast and slow muscles of late embryos and adult reflect the difference of these two types of muscle fibers in different fish species.

Analysis of the promoter sequences identified five putative E-box sites (CAnnTG), one MEF-2, NF-Y, SP1, USF, and some other transcriptional factor binding sites in the *MyoD* promoter (Figure 1B). A putative TATA box (TATAA) and a polyadenylation site (AATAAA) were found in the 5′- and 3′-flanking regions of the *MyoD* gene. In the four homologs, only the promoters of sea bream *MyoD1* and flounder *MyoD* are long enough to be compared. Comparison of their promoter sequences revealed that there was a highly conserved region of 187 bp in the sea bream *MyoD1* and flounder *MyoD* promoters (Figure 1C). Interestingly, within this conserved region, there were one putative NF-Y and one putative SP1 binding sites separated by 25 nucleotides, followed by USF, TATA-box, and E-box in the relative same sites.

The Temporal and Spatial Expression of MyoD in Flounder Embryos. The temporal and spatial expression of *MyoD* was examined in flounder embryos by whole-mount in situ hybridization. Before somitogenesis, *MyoD* was expressed as two parallel rows of cell on the elongating embryonic shield (Figure 3A). As embryos develop, these cells broadened to include more lateral paraxial cells. At the beginning of somitogenesis (stage 2), *MyoD* was expressed in the medial somitic cells and as two single rows of presomitic cell adjacent to the notochord in the segmental plate (Figure 3B). These cells correspond to the adaxial cells that have been described in other fish (Thisse et al., 1993; Devoto et al., 1996; Delalande and Rescan, 1999; Rescan et al., 1999; Tan and Du, 2002). As the somite formed from anterior to posterior, *MyoD* expression was detected both in the medial and the lateral regions of the somites (Figure 3C–F). At stage 3 (Figure 3C), *MyoD* expression was observed in the adaxial cells of the somites and the presomitic cells. At this time, weak expression of *MyoD* appeared in the lateral region of the somites. At stages 4, 5, and 6, *MyoD* transcripts were present in the medial and lateral somitic cells (Figure 3D–F). After stage 7 (30 somites), the expression of *MyoD* decreased in the anterior somites where somitic cells differentiated but the expression in the posterior somites was still strong where new somites formed (Figure 3G–J). At stage 7, strong *MyoD* signals were detected in the caudal somites and weak signals were present in the anterior somites (Figure 3G). At hatching stage, the *MyoD* transcripts were present not only in the caudal somites (Figure 3J, N) but also in other muscles such as adductor mandibulae (Figure 3J, K), adductor operculi (Figure 3J, K), extraocular muscle (Figure 3J, L), pectoral fin muscle (Figure 3J, M), dorsal anterior myotome muscle cells (Figure 3J, M), and dorsal/ventral posterior myotome muscle cells (Figure 3J, N).

Like *MyoD* genes of other fish, flounder *MyoD* is also initially expressed in medial (adaxial) cells close to the notochord that will migrate through the somites and form superficial cells later. This type of cell will differentiate into slow muscle fibers while the fast muscle fibers arising from the differentiation of lateral somitic cells (Devoto et al., 1996;

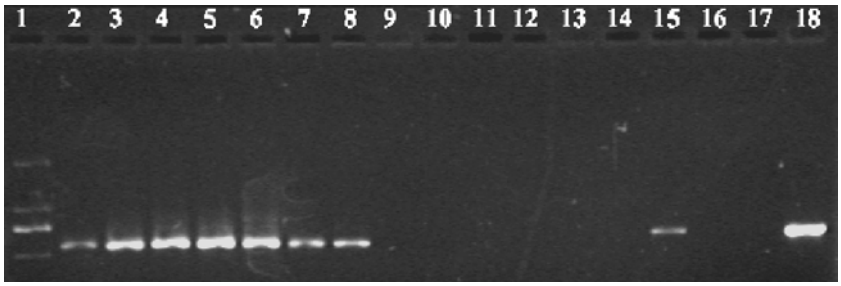

Fig. 4. RT-PCR analysis of *MyoD* expression in different tissues of post-hatching flounder fish. 1, DNA marker; 2–9, actin RT-PCR; 10–17, *MyoD* RT-PCR; 2, 10, liver; 3, 11, spleen; 4, 12, head kidneys; 6, 14, heart; 7, 15, muscle; 8, 16, intestine; 9, 17, negative control; 18, positive control, *MyoD* cDNA plasmid as the PCR template.

Stoiber et al., 1998). Before 5 somites, *MyoD* expression was restricted to the adaxial and presomitic cells. From 5 somites to 30 somites, the signals were expanded to the lateral somitic cells. After 30 somites, *MyoD* transcripts were decreased in the anterior and older somites, while still strong in the caudal and newly formed somites. Skeletal muscle cells were not the only muscle cells that can express *MyoD*.

The Distribution of MyoD in Different Tissues of Growing Fish. In situ hybridization analysis showed that *MyoD* played a role only in the skeletal muscle in the embryonic stage. To determine whether the *MyoD* plays role only in muscle of post-hatching fish, total RNAs were extracted from different tissues of post-hatching flounder. The expression of *MyoD* was analyzed by RT-PCR using *MyoD* specific primers (Figure 4). In post-hatching fish, *MyoD* expressed only in the muscle. These data showed that *MyoD* played a role in muscle growth in the growing fish.

Fish are usually the important commercial products of seafood. Fish skeletal muscles are the most abundant tissue in fish. Our RT-PCR results showed that *MyoD* was expressed only in the muscle in growing fish. In a group of cultured fish of the same age, there exist fast-growing and slow-growing fish. Their muscle growth rates are different. However, we do not know whether their expression levels of *MyoD* are different. A recent study showed that different growth rates were related to the expression level of *MyoD*, which af-fect muscle growth rates in turkey (Liu et al., 2005). In rainbow trout, the *TMyoD2* expression level related to muscle fiber numbers (Johansen and Overturf, 2005), which is muscle hyperplastic growth. Therefore, it will be more interesting to study the relationship between the expression level of *MyoD* and the growth rate of flounder. This information can be used to help to select strains of fast growing fish that are profitable for products.

In summary, we isolated and characterized the flounder *MyoD* gene, and analyzed its expression pattern during embryogenesis. Sequence analysis revealed that the flounder *MyoD* gene contained the same structure as that of other vertebrates MRF genes. Phylogenetic analysis showed that it was more likely to be the homolog of tilapia and takifugu *MyoD* and seabream *MyoD1*. The flounder *MyoD* gene was expressed in the somitic cells that would give rise to skeletal muscle. In the hatching stage, *MyoD* was also expressed in other muscle cells. In the growing fish, *MyoD* was expressed only in the muscle.

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