Original Article

Characterization of Muscle-Regulatory Gene, \textit{MyoD}, from Flounder (\textit{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 \textit{MyoD}, \textit{Myf5}, \textit{myogenin}, and \textit{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 \textit{MyoD} gene from flounder (\textit{Paralichthys olivaceus}) and analyzed its structure and patterns of expression. Sequence analysis showed that flounder \textit{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 \textit{MyoD} was highly conserved with other fish \textit{MyoD} genes. Sequence alignment and phylogenetic analysis indicated that flounder \textit{MyoD}, seabream (\textit{Sparus aurata}) \textit{MyoD1}, takifugu (\textit{Takifugu rubripes}) \textit{MyoD}, and tilapia (\textit{Oreochromis aureus}) \textit{MyoD} were more likely to be homologous genes. Flounder \textit{MyoD} expression was first detected as two rows of presomitic cells in the segmental plate. From somitogenesis, \textit{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, \textit{MyoD} expression decreased in the somites except the caudal somites, coincident with somite maturation. In the hatching stage, \textit{MyoD} was expressed in other muscle cells and caudal somites. It was detected only in muscle in the growing fish.

Keywords: flounder — muscle — \textit{MyoD} — myogenesis — somites

Introduction

Members of the myogenic regulating factors (MRFs) family are basic helix–loop–helix (bHLH) transcription factors, and include \textit{MyoD}, \textit{Myf5}, \textit{myogenin}, and \textit{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 ([C]ANNTG), present in the regulatory regions of many skeletal-muscle-specific genes (Lassar et al., 1989; 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 ([C]ANNTG), present in the regulatory regions of many skeletal-muscle-specific genes (Lassar et al., 1989; 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 ([C]ANNTG), present in the regulatory regions of many skeletal-muscle-specific genes (Lassar et al., 1989; Murre et al., 1989; Sun and Baltimore, 1991; Lin and Konieczny, 1992; Langlands et al., 1997).

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 \textit{MyoD} and \textit{Myf5} were required for myogenic determination, whereas \textit{myogenin} and \textit{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). \textit{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 myogenesis 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 1m³ 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 (DraI, EcoRV, HpaI, PvuII, ScaI, SmaI, and StuI) 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)ICT(C/G)AGGATCTCCACCTTGGG(C/A)AG |
| MRF2         | CCACCTTGGG(C/A)AG(T/C/A)C(T/G)CTG(A/G)(T/C)TGGGGTT |
| Ap1          | GATATACGACTCATACTAGGGCC |
| Ap2          | ACTATAGGCGACGCCTGGT |
| MyoD1        | CTGCAAGGCCCTGCAAGTGAAGG |
| MyoD2        | CTGCAAGTGGGAAGACACCAAC |
| MyoD3        | GTGATAGGGGAATCACAAGTTCG |
| MyoD4        | CACATCACACAGTCTAAAGTAC |
| MyoD5        | TCTTCAGGTGTCAGAAAGTAC |
| MyoD6        | AAAGTGGACGCACTGGGTC |
| MyoD1R       | GGAAAGACATATCCGCAACTCC |
| MyoD3R       | GAAACTCATAATGTCAGTCSTGG |
| FLMDC-1      | AGACTCCCTGTGGTTCCTCACTG |
| FLMDC-2      | TATGAGTGGACTGTTGAGG |
| Act-5        | AGAGCAAGAGAGGCACTCC |
| Act-3        | CGATGGGATGACCTGTCC |
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.

### 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], AF270970), 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.
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 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%
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 10-2 g/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 MyoDs [Figure 1A]. It shared 69%, 69%, 72%, 73%, 82%, and 86% identity with trout MyoD1, trout MyoD2, zebrafish MyoD, carp MyoD, takifugu 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 MyoDs 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 MyoDs 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

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**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 extracutaneous 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.
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;
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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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.
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