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Cloning and expression analysis of *myogenin* from flounder (*Paralichthys olivaceus*) and promoter analysis of muscle-specific expression

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Abstract

Myogenin is a bHLH transcription factor of the *MyoD* family. It plays a crucial role in myoblast differentiation and maturation. We report here the isolation of flounder *myogenin* gene and the characterization of its expression patterns. Sequence analysis indicated that flounder *myogenin* shared a similar structure and the conserved bHLH domain with other vertebrate *myogenin* genes. Flounder *myogenin* gene contains 3 exons and 2 introns. Sequence alignment and phylogenetic showed that flounder myogenin was more homologous with halibut (*Hippoglossus hippoglossus*) myogenin and striped bass (*Morone saxatilis*) myogenin. Whole-mount embryo in situ hybridization revealed that flounder *myogenin* was first detected in the medial region of somites that give rise to slow muscles, and expanded later to the lateral region of the somite that become fast muscles. The levels of *myogenin* transcripts dropped significantly in matured somites at the trunk region. Its expression could only be detected in the caudal somites, which was consistent with the timing of somite maturation. Transient expression analysis showed that the 546 bp flounder *myogenin* promoter was sufficient to direct muscle-specific GFP expression in zebrafish embryos.
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Keywords: Flounder; Myogenin; Myogenesis; Somites; *Myogenin* promoter; Zebrafish

1. Introduction

Muscle regulatory factors (MRFs), including *MyoD* (Davis et al., 1987), *Myf-5* (Braun et al., 1989), *myogenin* (Wright et al., 1989), and *MRF4* (Rhodes and Konieczny, 1989; Braun et al., 1990), are specially expressed in skeletal muscle and play key roles during myogenesis (Sassoon, 1993). In vitro studies have demonstrated that members of the MRF family are capable of converting non-muscle cell lines to a muscle phenotype (Choi et al., 1990; Schäfer et al., 1990; Edmondson and Olson, 1993). The four MRFs share a highly conserved basic helix–loop–helix (bHLH) domain (Murre et al., 1989). They can dimerize with ubiquitous bHLH proteins (E12 and E47) (Lassar et al., 1991) to form complexes that bind to a DNA consensus sequence E-box (CANNTG), and to activate muscle-specific transcription (Edmondson and Olson, 1993). Studies on the structure of MRFs showed that they also contain several other functional domains responsible for transcriptional activation, chromatin remodeling, nuclear localization and heterodimerization (Tapscott et al., 1988; Weintraub et al., 1991; Vandromme et al., 1995; Gerber et al., 1997; Schwarz et al., 1992).

Various knock-out experiments in mice have shown that *MyoD* and *Myf-5* are required for muscle determination (Rudnicki et al., 1993), whereas myogenin acts as crucial differentiation factors to control the onset of myoblast fusion and maturation of myofibers (Hasty et al., 1993; Nabeshima et al., 1993), and MRF4 partly serves both roles (Zhu and Miller, 1997; Sumariwalla and Klein, 2001; Kassar-Duchossoy et al., 2004). These studies also demonstrated a regulatory network of the MRFs in myogenesis. *MyoD* and *Myf-5* play overlapping roles in myoblast specification (Kablar et al., 1998; Tajbakhsh and Buckingham, 2000); while myogenin functions in a genetic pathway downstream of *MyoD* and Myf-5 (Rawls et al., 1995). It appears that myogenin does not have overlapping functions as *MyoD* and MRF4 because myogenin plays a

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central role in muscle differentiation and this function could not be replaced by other MRFs (Wang et al., 1996; Wang and Jaenisch, 1997; Myer et al., 2001; Valdez et al., 2000).

Generally, during embryogenesis, *MyoD* and *Myf-5* are expressed earlier than *myogenin* and *MRF4* which are observed when cells start differentiated. The temporal and spatial expression patterns of these four MRF factors vary among different species (Chanoine et al., 2004). In mouse embryos, *Myf-5* is the first myogenic genes being expressed in the epithelial somites followed by *myogenin*, *MRF4*, and later by *MyoD1* which are expressed consecutively in myotome (Sassoon et al., 1989; Ott et al., 1991; Bober et al., 1991). However during quail embryogenesis, *qmf1* (*MyoD1*), *qmf3* (*Myf-5*) and *qmf2* (*myogenin*) are expressed sequentially during somite formation (Pownall and Emerson, 1992). Studies on their expression patterns of different fishes have also shown that these three genes (*MyoD*, *Myf-5* and *myogenin*) express in the same order: *Myf-5*, *MyoD* first, and *myogenin* late, although there are nuances among these fish species (Weinberg et al., 1996; Delalande and Rescan, 1999; Temple et al., 2001; Tan and Du, 2002; Cole et al., 2004). In the herring (*Clupea harengus*) *myogenin* shows a more transient expression pattern than that in zebrafish (Weinberg et al., 1996), trout (Delalande and Rescan, 1999) and carp (Cole et al., 2004), disappearing from the somites before the downregulation of *MyoD* (Temple et al., 2001).

Flounder (*Paralichthys olivaceus*) is one of the most important economic cultured marine fish living in north-east Asian seacoast. Though studies on its genetics and development have been performed recently, there is little information about its muscle development and growth. Understanding the molecular regulation of muscle growth in flounder will be useful for aquaculture. Herein, we reported the isolation and characterization of flounder *myogenin*, its expression pattern during embryogenesis, the muscle-specific activity of its promoter, and the expression order of *MyoD*, *Myf-5* and *myogenin* during embryogenesis.

2. Material and methods

2.1. Fish and embryo 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 with commercial particle food twice a day. The fertilized eggs were obtained by mixing sperm and eggs after they were collected from matured males and females by gently stripping. The embryos were cultured at 15± 1 °C in 1 m^3 tank under natural or controlled conditions.

2.2. Isolation of flounder myogenin gene

The myogenin genomic gene was isolated from a flounder GenomeWalker library by a Nest-PCR method (Clontech, USA). Specifically, a 1 kb DNA fragment containing the 5′-flanking region and part of the first exon was generated during the first two rounds of PCR using MRF consensus primers (MRF1 and MRF2) (Table 1) and the adapter primers (AP1 and AP2) (Table 1). The remaining part of myogenin genomic sequences was cloned by another two rounds of PCR using myogenin-specific primers (MG1 and MG2) together with the adapter primers (Table. 1). All of the fragments were cloned into pUCm-T vector (Sangon, Shanghai, China) and sequenced.

2.3. RT-PCR

To determine the intron–exon junctions in myogenin genomic sequence, myogenin cDNA was isolated by RT-PCR. Flounder embryos of different developmental stages were pooled and flash-frozen in liquid nitrogen. Total RNA was extracted from flounder embryos using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. cDNA was synthesized using the first strand cDNA synthesis kit (Promega, USA). PCR was performed with *pfu* DNA polymerase (Promega, USA) using myogenin-specific primers (FLMGc1 and FLMGc2) (Table 1). The PCR fragments were cloned into pBluescript II SK (Stratagene, USA) *Sma* I site and sequenced.

To analyze *myogenin* expression in different tissues of adult flounder, RT-PCR with specific primers (FLMGc1 and FLMGc2) was performed from cDNA libraries of different tissues (described in Zhang et al., 2006). *Actin* was used as a control. The specific primers for flounder *actin* were Act-5 and Act-3 (Table 1).

To determine the expression of flounder *MyoD*, *Myf-5* and *myogenin*, RT-PCR was carried out using cDNA libraries of different flounder developmental stages with specific primers FLMDc2 and MyoD1, FLMFc and MyF, FLMGc1 and FLMGc2 (Table 1), respectively. *actin* was used as the internal control. All of the PCR was run as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C 1 min, and then 72 °C 10 min for elongation.

2.4. Protein sequence alignment and phylogenetic analysis

Protein sequence alignment and phylogenetic analysis were performed using the Clustal W. Program (http://www.ebi.ac.uk/

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
MG1	AAAGCCTCTCCATCCAGCCTGTCAC
MG2	AAGAAAGACAGTGACCATGGACCGTC
FLMGc1	CATCCAGTAATGGAGCTTTTCGAG
FLMGc2	TGGATCTCTGTGCATTTATGGGTC
Act-5	AGAGCAAGAGAGGCATCCTGAC
Act-3	CGATGGGTGATGACCTGTCC
MyoD1	CTGCAAGGCCTGCAAGTGGAAG
FLMDc2	TATGAGTGGACTGTGTGAGAGG
MyF	GGAGGCTGAAGAAGGTCAACCATG
FLMFc	GTCTGTTTTGGTGAAGGCACACAG
FLMGp	GTCGACTACTGGATGGCACTGTGGTCTGG

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