Abstract Members of the myogenic regulatory gene family, including \textit{MyoD}, \textit{Myf5}, \textit{Myogenin} and MRF4, are specifically expressed in myoblast and skeletal muscle cells and play important roles in regulating skeletal muscle development and growth. They are capable of converting a variety of non-muscle cells into myoblasts and myotubes. To better understand their roles in the development of fish muscles, we have isolated the \textit{MyoD} genomic genes from gilthead seabream (\textit{Sparus aurata}), analyzed the genomic structures, patterns of expression and the regulation of muscle-specific expression. We have demonstrated that seabream contain two distinct non-allelic \textit{MyoD} genes, \textit{MyoD1} and \textit{MyoD2}. Sequence analysis revealed that these two \textit{MyoD} genes shared a similar gene structure. Expression studies demonstrated that they exhibited overlapping but distinct patterns of expression in seabream embryos and adult slow and fast muscles. \textit{MyoD1} was expressed in adaxial cells that give rise to slow muscles, and lateral somitic cells that give rise to fast muscles. Similarly, \textit{MyoD2} was initially expressed in both slow and fast muscle precursors. However, \textit{MyoD2} expression gradually disappeared in the adaxial cells of 10- to 15-somite-stage embryos, whereas its expression in fast muscle precursor cells was maintained. In adult skeletal muscles, \textit{MyoD1} was expressed in both slow and fast muscles, whereas \textit{MyoD2} was specifically expressed in fast muscles. Treating seabream embryos with forskolin, a protein kinase A activator, inhibited \textit{MyoD1} expression in adaxial cells, while expression in fast muscle precursors was not affected. Promoter analysis demonstrated that both \textit{MyoD1} and \textit{MyoD2} promoters could drive green fluorescence protein expression in muscle cells of zebrafish embryos. Together, these data suggest that the two non-allelic \textit{MyoD} genes are functional in seabream and their expression is regulated differently in fast and slow muscles. Hedgehog signaling is required for induction of \textit{MyoD} expression in adaxial cells.

Keywords Seabream · \textit{MyoD1} · \textit{MyoD2} · Muscle · Zebrafish

Introduction

\textit{MyoD} is a key basic helix-loop-helix transcription factor that plays essential roles in specification and differentiation of skeletal muscle (Weintraub 1993). Together with \textit{Myf5}, \textit{Myogenin} and MRF4, they form the myogenic regulatory factor (MRF) family. Members of the MRF family are expressed in highly regulated spatial and temporal patterns during myogenesis. \textit{MyoD} and \textit{Myf5}, expressed in developing somites, are essential for initiating the skeletal muscle program in the embryo, whereas \textit{Myogenin} and MRF4, expressed relatively later than \textit{MyoD} and \textit{Myf5} during myogenesis, are involved in final differentiation/maturation of myofibers. Knockout mice lacking a functional \textit{MyoD} gene have no significant skeletal muscle defect (Rudnicki et al. 1992). In a similar fashion, targeted inactivation of the \textit{Myf5} gene generates mice with normal skeletal muscle but which die from severe rib abnormalities (Braun et al. 1992, 1994). However, \textit{MyoD} and \textit{Myf5} double knockout mice fail to develop skeletal muscles suggesting that \textit{MyoD} and \textit{Myf5} play a redundant role in regulating muscle formation (Rudnicki et al. 1993). In contrast to \textit{MyoD} and \textit{Myf5} mutants, newborn \textit{Myogenin}-deficient mice have normal numbers of \textit{MyoD}-expressing myoblasts, however, the myoblast cells fail to differentiate into myofibers (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995).

Vertebrate skeletal muscle fibers can be divided into multiple fiber types based on contraction speed, innervation, metabolism, morphology and the expression of distinct isoforms of myosin heavy chain (MyHC). Unlike other vertebrates, in which fast and slow muscles are
intermingled with each other, fish skeletal muscles have a clear separation of slow and fast muscles. Therefore, fish have been used as models to facilitate many kinds of studies that were difficult or impractical in other vertebrates. Recent studies in zebrafish show that fast and slow muscle fibers are derived from distinct populations of myoblast precursors in the embryos. Slow muscles develop from adaxial cells, which are located adjacent to the notochord, whereas fast muscle fibers develop from cells that are lateral to adaxial cells in segmental plate and somites (Devoto et al. 1996). Signaling molecules like Hedgehog secreted by the notochord and floor plate play an important role in slow muscle induction (Currie and Ingham 1996; Blagden et al. 1997; Du et al. 1997).

Mutants with disrupted notochord were found to have a developmental defect in slow muscle formation which could be rescued by ectopic expression of sonic hedgehog (Shh) mRNA (Halpern et al. 1993; Blagden et al. 1997). Also, mutation in Shh, its receptor or its downstream gene Gli2 was found to cause a developmental defect in slow muscles in zebrafish embryos (Lewis et al. 1999; Barresi et al. 2000; Du and Diernhart 2000). In contrast, the development of fast muscle appears to be independent of notochord signal (Blagden et al. 1997; Lewis et al. 1999; Du and Diernhart 2000).

It appears that MyoD is differentially expressed in fast and slow muscle precursor cells. The slow muscle precursor cells, also known as adaxial cells, are the first to express MyoD in zebrafish embryos (Weinberg et al. 1996) and the first to differentiate into muscle fibers (Van-Raamsdonk et al. 1978; Devoto et al. 1996). In rainbow trout, two MyoD genes have been identified and they exhibit different expression patterns in fast and slow muscles in adult fish as well as in embryos (Delalande and Rescan 1999; Rescan et al. 1999). At present, little is known about the genomic structure of MyoD genes in fish and regulation of their muscle-specific expression (see reviews by Watabe 1999, 2001). To date, only a few MRF genomic genes have been isolated and characterized (Chen et al. 2001; Couteell et al. 2001; Tan et al., in press), although several members of the MRF cDNAs have been cloned in several fish species and found to be expressed specifically in developing somites and skeletal muscle (Rescan et al. 1994, 1995; Weinberg et al. 1996; Kobiyaama et al. 1998; Chen et al. 2000, 2001; Couteel et al. 2001).

In this study, we have isolated and characterized two MyoD genes from seabream. Sequence analysis revealed that these two MyoD genes share a similar gene structure. Both of them contain three exons and two introns, and a highly conserved basic helix-loop-helix domain. Promoter analysis demonstrated that both MyoD1 and MyoD2 promoters could direct green fluorescence protein (GFP) expression in muscle cells of zebrafish embryos. Expression analysis showed that these two genes were expressed in overlapping but distinct patterns in seabream. MyoD1 was expressed in both slow and fast muscle precursors, whereas MyoD2 was initially expressed in both slow and fast muscle precursors, but gradually disappeared in slow muscle precursors. This type of expression pattern appeared to be maintained in adult seabream skeletal muscles because MyoD1 was expressed in both fast and slow muscles, whereas MyoD2 was specifically expressed in fast muscles. The expression of MyoD1 in slow muscle was inhibited by treating embryos with forskolin, a protein kinase A (PKA) activator but expression in fast muscle precursors was not affected. These data indicate that seabream contain two functional MyoD genes that are differentially expressed in seabream and adult muscle precursors.

Materials and methods

Isolation of sea bream MyoD1 and MyoD2 genomic genes

Seabream MyoD genomic genes were isolated as DNA fragments by PCR using DNA from the seabream GenomeWalker libraries. Specifically, the promoter sequence and part of the first exon I of the seabream MyoD1 gene were isolated by two rounds of PCR using MRF consensus primers (MRF-p1 and MRF-p2) and adapter primers (Ap1 and Ap2). The promoter sequence and part of the first exon I of the seabream MyoD2 gene were isolated by two rounds of PCR using gene-specific primers (sbmd2-p1, sbmd2-p2, sbmd2-p3 and sbmd2-p4) and adapter primers (Ap1 and Ap2). The remaining parts of the MyoD1 and MyoD2 genomic sequencess were cloned by several rounds of PCR using MyoD1 or MyoD2 consensus or specific primers together with the Ap1 and Ap2 adapter primers. The consensus primers for cloning part of the first exon and intron were sbmd1-1 and sbmd1-2. The MyoD1 gene-specific primers for cloning the rest of the gene were sbmd1-1, sbmd1-2, sbmd1-3 and sbmd1-4, and sbmd1-5 and sbmd1-6, whereas the MyoD2 specific primers were sbmd2-1 and sbmd2-2. All PCR fragments were cloned into pGEM-T easy cloning vector (Promega) and used for sequence analysis.

- **Ap1**: 5'-GTAATACGACTCACTATAGGCCG-3'
- **Ap2**: 5'-ACTATAGGGCGCCGGTGTG-3'
- **MRF-p1**: 5'-TT(T/C)(C/G)AGGATCCCGATCTGAGC-3'
- **MRF-p2**: 5'-CCACCTTGGG(C/A)AG(T/C/A)(T/G)CGTGTGAGC-3'
- **MRF-p3**: 5'-CGCCCGCTCAACAAATGCAAGCAGCCGCT-3'
- **Sbmd1-1**: 5'-TAAATACGACTCACTATAGGCCG-3'
- **Sbmd1-2**: 5'-TTAAATACGACTCACTATAGGCCG-3'
- **Sbmd1-3**: 5'-GTCTCCAGTCTGGACTGTCTGTC-3'
- **Sbmd1-4**: 5'-AAATGGTACAGCCTGCTTGTCTG-3'
- **Sbmd1-5**: 5'-GATCTCCGACACCGACACAGGCG-3'
- **Sbmd1-6**: 5'-GATCTCCGACACCGACACAGGCG-3'
- **Sbmd2-1**: 5'-TACTGGTTTTCAGCGGCTT-3'
- **Sbmd2-2**: 5'-ATGGTACAGCCTGCTTGTCTG-3'
- **Sbmd2-3**: 5'-ATGGTACAGCCTGCTTGTCTG-3'
- **Sbmd2-4**: 5'-CTCACGTCGCTTGTCTG-3'
- **Sbmd2-5**: 5'-CTCACGTCGCTTGTCTG-3'
- **Sbmd2-6**: 5'-CTCACGTCGCTTGTCTG-3'

RT-PCR

To determine if the MyoD1 and MyoD2 exhibited the same or a distinct pattern of expression in seabream embryos, and fast and slow muscles of adult fish, total RNAs were extracted from seabream embryos 20 h post fertilization as well as fast and slow muscles from adult seabream of 17, 20 and 24 months old. cDNA was synthesized using the first strand cDNA synthesis kit (Life Sciences). The expression of MyoD1 and MyoD2 was analyzed by RT-PCR using specific primers for MyoD1 (sbmd1-c) and MyoD2 (sbmd2-c) together with a consensus primer (sbmd1-c: 5'-CGCCCGCTCAACAAATGCAAGCAGCCGCT-3', sbmd2-c: 5'-TACTGGTTTTCAGCGGCTT-3').