Studies on the mechanisms involved in the expression of myosin heavy chain genes in various muscle types of torafugu *Takifugu rubripes*

（種々のトラフグ筋タイプにおけるミオシン重鎖遺伝子の発現機構に関する研究）

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By

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DECLARATION

I hereby declare that the thesis entitled **" Studies on the mechanisms involved in the expression of myosin heavy chain genes in various muscle types of torafugu *Takifugu rubripes*"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

January 30, 2010

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DEDICATION

This thesis is dedicated to my lovable mother for her sacrifice, love and endless support

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CONTENTS

ABBREVIATIONS

ACD	:	Assembly competence domain
AP	:	Adapter primer
ATP	:	Adenosine 5'-triphosphate
BCF	:	Body caudal fin
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
BrdU	:	5-bromo-2'-deoxyuridine
cDNA	:	Complementary deoxyribonucleic acid
DIG	:	Digoxigenin
ED	:	Erectors and depressors
EF-1α	:	Elongation factor 1 alpha
LMM	:	Light meromyosin
LS	:	Lateralis superficialis
MPCs	:	Muscle precursor cells
MPF	:	Median paired fin
Myr	:	Million years
MYH	:	Myosin heavy chain
NADH	:	Nicotinamide adenine dinucleotide reduced
NBT	:	Nitro blue tetrazolium chloride
ORF	:	Open reading frame
PBS	:	Phosphate-buffered saline
PCNA	:	Proliferating cell nuclear antigen
PD	:	Paired box domain
PFA	:	Paraformaldehyde

PSM	:	Presomitic mesoderm
Pax	:	Paired box protein
RACE	:	Rapid amplification of cDNA ends
RT-PCR	:	Reverse transcription-polymerase chain reaction
S1	:	Subfragment-1
S2	:	Subfragment-2
SDH	:	Succinate dehydrogenase
SSC	:	Saline-sodium citrate
TGF-β	:	Transforming growth factor beta
UTR	:	Untranslated region
WGD	:	Whole genome duplication

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INTRODUCTION

1. General background

Morphologically muscles are mainly classified into striated and nonstriated smooth ones. Striated muscle is further divided into skeletal muscle, which control voluntary movements of the bone frame, and cardiac muscle that controls, in an involuntary way, the pumping of heart. Smooth muscle, which has no striations, is responsible for the majority of involuntary movements, such as the movement of alimentary canal. This muscle is found in stomach, intestine, uterus, artery walls, and secretary gland ducts. Skeletal muscle is by bulk the most extensive tissue in the body and consists of variable number of muscle fibers. One of the striking features of skeletal muscle is to perform a wide range of movements with respect to the functional demand. The attachment and location of muscle are arranged to provide the necessary mechanical advantage and muscle fibers are arranged within muscles to produce the appropriate power and degree of contraction. Fibers are highly heterogeneous because of different anatomical, physiological and biochemical features. The existence of such heterogeneous fiber types allows a division of labor, which enables each fiber to specialize in providing a particular type of contraction.

The striated appearance of skeletal muscle is due to the regular arrangement of contractile proteins. Myosin is the major component of thick filaments and consists of two myosin heavy chains (MYHs) and two pairs of light chains referred to as essential (or alkali) and regulatory (phosphorylatable) light chains, respectively (Harrington, 1984). The myosin molecule can be cleaved by limited proteolytic treatment into the N-terminal half of globular head called subfragment 1 (S1), and

the C-terminal half called rod, which has characteristic α-helical coiled coil structure with 7 residue and 28 residue repeats. S1 can be further cleaved into three fragments of 25, 50 and 20 kDa, while rod can be subdivided into the subfragment 2 (S2) and light meromyosin (LMM) fragments. Several MYHs have been found in mammals, bird and fish, which show strong amino acid sequence homology with each other.

The regions that vary in the amino acid sequence between MYHs are largely confined to two external loops associated with the ATP (loop 1) and actin binding sites (loop 2) in S1 (Weiss et al., 1999). S1 directly interacts with ATP and actin, resulting into the hydrolysis of ATP molecule. The energy released from ATP hydrolysis is directly used for muscle contraction. Thus, the speed of ATP hydrolysis correlates with contraction of muscle fibers (Larsson and Moss, 1993; Galler et al., 1994; Schiaffino and Reggiani, 1996). Due to the close correlation with fiber characteristics involving physiological, biochemical and metabolic ones, MYHs are most commonly used in the classification of muscle fiber types.

In mammals, most of skeletal muscles contain a mixture of various fiber types, enabling individual muscles in providing long-lasting, low-intensity contractions, together with fast burst of activity. On the basis of speed of contraction and predominant expression of a particular MYH, muscle fibers are classified into types I, IIa, IIx and IIb. Types I and IIa show oxidative metabolism, whereas types IIb and IIa are primarily glycolytic (Schiaffino and Reggiani, 1996; Pette and Staron, 2000, Wigmore and Evans, 2002). Type I fibers also termed as slow twitch fibers due to slow contraction owing to a slow ATPase activity associated with MYHI/β. Slow fibers rich in mitochondria have more capillaries surrounding each fiber, exhibit oxidative metabolism, low velocity of shortening and high resistance to fatigue. In

contrast, type II fibers termed as fast twitch fibers, exert quick contraction and fatigue rapidly.

In contrast with mammals, fiber types in fish are anatomically separated into distinct locations. Fast twitch fibers locate deeply in the myotomal muscle, whereas slow twitch fibers are present at lateral superficial to the myotome (Sanger and Stoiber, 2001). Fast twitch fibers occupy bulk of myotomal muscle, account for never less than 70% and have large fiber diameters up to 100 μm or even more, whereas slow twitch fibers, small in diameter (25-45 μm), usually account for less than 10% and never more than 30% of myotomal muscle (Greer-Walker and Pull, 1975; Sanger and Stoiber, 2001). In addition to the above major fiber types, minor ones such as intermediate or pink muscle fibers (Waterman, 1969; van Raamsdonk et al., 1982; Akster, 1983; Scapolo and Rowlerson, 1987; Gill et al., 1989), tonic fibers (Zowadowska and Kilarski, 1984; Sanger et al., 1997) and red rim fibers (van Raamsdonk et al., 1980) have been reported in fish. The properties of intermediate fibers are between fast and slow fibers in most regards including distribution, oxidative capacity, fiber diameter, fatigue resistance, contraction kinetics, etc. (Johnston, 1977; Akster, 1985; Akster et al., 1985; Sanger and Stoiber, 2001). The relative amount of intermediate muscle fibers differs both within fish species and developmental stage. Like slow twitch fibers, intermediate fibers have orientation parallel to the axis of body, lie between superficial slow and proximal fast twitch fibers and are to some extent intermingled with the most lateral fast twitch fibers (Scapolo and Rowlerson, 1987; Caughlin et al., 1996). The other fiber types such as red rim fibers and tonic fibers form a relatively small part of myotomal muscle and are considered to contribute less significantly to the swimming of fish.

Fiber types in fish skeletal muscles have been determined on the basis of histochemical demonstration of myofibrillar ATPase and immunocytochemitry using antibody raised against myosin of fish and higher vertebrates (van Raamsdonk et al., 1982; Akster, 1983; Mascarello et al., 1986; Scapolo and Rowlerson, 1987; Johnston and Horne, 1994). The ATPase of fast-twitch fibers is inactivated after preincubation at acidic pH, whereas species-specific differences have been reported with respect to alkaline incubation (Akster, 1983; Rowlerson et al., 1985). In contrast, slow twitch fibers show acid stable ATPase (Johnston and Bernard, 1982; Rowlerson et al., 1985). Slow-twitch fibers have also been identified using a histochemical stain for demonstrating the activity of enzymes involved in oxidative metabolic pathways such as succinic dehydrogenase (SDH), NADH diaphorase, etc. (Johnston and Lucking, 1978; Gill et al., 1989).

It is well established that the growth of trunk skeletal muscle in adult fish results from both increase in fiber number by the recruitment of new fibers (hyperplasia) as well as by the increase in fiber size (hypertrophy) (Stickland, 1983; Weatherley et al., 1988; Koumans et al., 1993; Stoiber and Sanger, 1996; Patruno et al., 1998). This phenomenon is in contrast with mammals, where hyperplasia ceases near the time of birth and further muscle growth results mainly from hypertrophy (Rowe and Goldspink, 1969; Watabe, 1999).

Fish skeletal muscle fibers grow by hypertrophy throughout post embryonic life until they reach a functional maximum diameter that is in the range of 100-300 μm for fast twitch fibers, although slow twitch fibers show small maximum diameters (Rowlerson et al., 1995; Rowlerson and Veggeti, 2001). Such hypertrophic growth in which fibers acquire additional nuclei (Johnston, 1993; Alami-Durante et al., 1997)

is considered to be similar with that described in mammalian muscle (Moss and Leblond, 1971; Cardasis and Cooper, 1975). The additional nuclei are supplied by a population of myogenic cells termed as muscle satellite cells to maintain the ratio of nuclei to the sarcoplasm volume in muscle fibers. Satellite cells also called myosatellite cells are mononuclear cells which occupy a sublaminar position in skeletal muscle. These cells, first discovered by Mauro in 1961, supply the postnatal source of DNA, contributing to the growth of muscle fibers. DNA accretion occurs through proliferation of satellite cells followed by differentiation and fusion with existing muscle fibers (Moss and Leblond, 1971). These cells have been studied in common carp *Cyprinus carpio* by Kouman and colleagues (Koumans et al., 1991; Komans et al., 1993; Koumans and Akster, 1995).

The hyperplastic growth is also termed as mosaic hyperplasia in adult fish which grow to a large size such as rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo solar*, seabream *Sparus aurata*, carp, etc. (Rowlerson and Veggeti, 2001). However, recently, such mosaic hyperplasia was also reported in adult zebrafish *Danio rerio* which grows to much smaller size, suggesting the use of this model fish in studying mechanisms involved in post embryonic muscle development (Patterson et al., 2008; Johnston et al., 2009). New small diameter fibers in growing and adult fish are thought either to originate from myosatellite cells or splitting of existing fiber (van Raamsdonk et al., 1978), although the mechanism underlying fiber splitting has not yet been confirmed. Immunohistological studies revealed that myosin in newly formed fibers differs from that in large diameter ones (Akster, 1983; Rowlerson et al., 1985). During the process of regeneration of mammalian skeletal muscles after injury or upon mechanical stress, hyperplastic growth occurs, thereby

generating new fibers (Goldspink 1972). Such new fibers have been reported to express embryonic- and neonatal-type MYH genes (*MYH*s) (Sartore et al., 1982; Matsuda et al., 1983; Butler-Browne and Whalen, 1984; Whalen et al., 1990). In this context, no direct evidence on the mechanisms involved in the expression of embryonic *MYH*s in new fibers generated by hyperplastic growth has been reported in fish.

Fish skeletal muscles are highly plastic and alter MYHs in response to activity levels like swimming as well as temperature fluctuations (Sanger and Stoiber, 2001). In eurythermal temperate fish, such as carp, acclimation temperature determines muscle contractile properties by altering the type of myosin cross bridge (Watabe et al., 1994; Watabe, 2002). The molecular mechanisms underlying muscle plasticity in terms of myosin expression in response to temperature acclimation have been extensively investigated by our group in common carp (Hwang et al., 1990; Guo et al., 1994; Watabe et al., 1994; Chaen et al., 1996; Hirayama and Watabe, 1997; Imai et al., 1997; Watabe, 2002). In fast muscles of evolutionarily tetraploid common carp, MYH_{F10}, MYH_{int} and MYH_{F30} have been reported to be expressed in a temperature-dependent manner (Hirayama and Watabe, 1997; Imai et al., 1997). Similar results have been obtained with temperate freshwater diploid fish, grass carp *Ctenopharyngodon idellus* (Tao et al., 2005), suggesting that such expression of multiple *MYH*s is not due to tetraploidization of fish, but common feature of temperate freshwater fish to adapt a wide range of temperature fluctuations occurring seasonally. Liang et al. (2007) also demonstrated that medaka *Orizias latipes* contains a pool of eight trascriptionally active fast-type *MYH*s and switch their expression depending on the fluctuation of surrounding water temperatures.

2. Objectives

Torafugu *Takifugu rubripes*, one of the most popular marine fish cultured in Japan, has a higher market price than that for yellowtail *Seriola quinqueradiata* and red seabream *Pagrus major*, which are two of the other important marine fish cultured in Japan (Kikuchi, 2006; Kikuchi et al., 2006). Apart from its commercial importance, torafugu has been introduced as the "genomic model" due to its compact genome size containing approximately the same number of genes as that of human (Brenner et al., 1993; Aparicio et al., 2002; Hedges and Kumar, 2002). Thus, the genome of torafugu is ideal for comparative genomics as well as for evolutionary biology research (Elgar et al., 1996; Venkatesh et al., 2000).

Fish under Tetraodontidae have a rigid body and median and paired fin (MPF; Webb, 1984) with a swimming style that classically has been considered to derive the thrust needed to propel themselves through the water from coordinated lateral oscillations of their dorsal and anal fins (Gordon and Kim, 1996). Thus the structure of muscle powering the MPF swimming in these fish deviates from that of fish having typical body caudal fin (BCF), as described by Winterbottom (1974). Dorsal and anal fin rays in the MPF swimming fish are generally controlled by paired erectors (erectors dorsalis/anales) and depressors (depressors dorsalis/anales) (called erectors and depressors, ED) that manipulate fin rays into an upright and depressed position, respectively. Erectors lie superficially to depressors and connect the front of pterygiophores, the bones between vertebral spines that support dorsal and anal fins (Winterbottom, 1974). In torafugu, the fast twitch fibers are located deep in the trunk myotomal muscle, whereas slow twitch fibers on the surface part of the myotome

called lateralis superficialis (LS) and at ED of median fins (dorsal and anal fins).

Recently, the whole genome analysis of *MYH*s family by *in silico* approach revealed that torafugu contains 20 sarcomeric *MYH*s (Ikeda et al., 2004; Watabe and Ikeda, 2006; Ikeda et al., 2007). The genomic structural analysis of torafugu *MYH*s together with phylogenetic analysis suggested a divergent evolutionary lineage of fish and tetrapod *MYH*s (Ikeda et al., 2007). Previously MYH multigene family has been reported for common carp *Cyprinus carpio* (Gerlach et al., 1990; Kikuchi et al., 1999). Kikuchi et al. (1999) showed 29 non-overlapping λ clones which encoded skeletal and cardiac-type *MYH*s. Furthermore, the expression patterns of several *MYH*s have been reported during development and temperature acclimation in medaka (Ono et al., 2006; Liang et al., 2007), common carp (Hirayama and Watabe, 1997; Imai et al., 1997; Nihei et al., 2006), silver carp *Hypopthalmichthys molitrix* (Fukushima et al., 2009) and grass carp *Ctenophyaringodon idella* (Tao et al., 2004). Although such a high number of *MYH*s have been evidenced in fish, much remains unknown regarding the following aspects. What would be the functional implication to have such a high number of sarcomeric *MYH*s in fish? Do the transcripts of each *MYH* can help to identify the fiber diversity in skeletal muscles and their implication in relation to the muscle growth? What are the mechanisms that control switching of *MYH*s expression in development-dependent and tissue-specific manners as well as in response to other environmental stimuli? The present study was carried in order to understand some of the above concerns in torafugu. The topics involved in the present study are as follow.

1. Characterization of fiber types in adult skeletal muscle of torafugu
2. Expression patterns of sarcomeric myosin heavy chain genes in adult torafugu

muscles

3. Expression patterns of myosin heavy chain genes in torafugu at embryos and larvae
4. Characterization of paired box protein genes as myogenic precursor cell markers in torafugu

3. Summary

This thesis consists of five Chapters. Chapter I deals with histochemical analysis of fiber types in adult skeletal muscles of torafugu. The expression patterns of sarcomeric *MYH*s in adult skeletal and cardiac muscles of torafugu forms a part of Chapter II. The regulation of *MYH*s expression in embryos and larvae were investigated in Chapter III. Paired box protein (Pax) genes belonging to *Pax3* and *Pax7* subfamily was characterized from embryos and adult skeletal muscles of torafugu and their potential roles in identification of myogenic precursor cells were discussed in Chapter IV. Chapter V was dedicated for general discussion based on the results obtained in previous chapters.

In Chapter I, myofibrillar ATPase was demonstrated in adult skeletal muscle of torafugu (body weight 290 g) by selective inhibition or activation of specific fiber groups after preincubation (2-3 min) either at acidic or alkaline pH. Fast muscle contained various fibers with different diameters. ATPase of fast fibers with large diameters was inactivated at pH 4.6, whereas that with small diameters was stable to this acidic pH. It was noted that the fibers with small diameters were more stable with those having smaller diameters. Such existence of fibers with small diameters in fast muscle suggests hyperplastic growth in adult fast muscle, since it has been reported that new fibers are formed by hyperplastic process in most adult fish which grow to a large final body size like torafugu. Meanwhile, most fibers in lateralis superficialis (LS) and erector and depressor (ED) slow muscles were resistant to pH 4.6, although some large-sized fibers were found to be slightly acid-labile. In contrast, ATPase of all fast fibers of juvenile torafugu was inactivated at pH 4.6, suggesting

that the existence of different fibers in fast muscle is a distinct feature of muscle growth in adult.

NADH-diaphorase staining was performed to identify oxidative fibers in skeletal muscles according to Novikoff et al. (1961). All fibers in LS and ED slow muscles were positive for NADH-diaphorase stain, suggesting that these muscles have oxidative metabolism. Importantly, fibers in LS slow muscle with large diameters adjacent to fast muscle showed lower NADH-diaphorase reaction compared with those in a superficial region with small diameters, demonstrating that the former fibers have an intermediate oxidative potential. In contrast, none of fibers in fast muscle was stained for NADH-diaphorase.

In Chapter II, cDNAs encoding sarcomeric MYHs were amplified by RT-PCR using *MYH*-specific degenerate primers. In total, seven sarcomeric *MYH*s were cloned from adult fast, slow and cardiac muscles of torafugu (body weight 1 kg). The nomenclature of torafugu *MYH*s found in the present study is described following Ikeda et al. (2007) who found 20 sarcomeric *MYH*s by *in silico* approach on the total genome database. Three *MYH*s, $MYH_{M86\text{-}1}$, MYH_{M8248} and MYH_{M880}, were cloned exclusively from fast, slow and cardiac muscles, respectively, whereas two *MYH*s, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, were cloned from both fast and slow muscles and another two *MYH*s, $MYH_{M2126\text{-}2}$ and MYH_{M5}, from both slow and cardiac muscles. Evolutionary relationships of torafugu MYHs with those reported from other fish were studied by phylogenetic analysis on the deduced amino acid sequences using the neighbor-joining method. $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034} belonged to fast type as they were placed in the same clade representing fast-type MYHs from other

fish on the phylogenetic tree. MYH_{M8248} and $MYH_{M2126\text{-}2}$ belonged to slow and cardiac types, respectively. MYH_{M5} and MYH_{M880} were found to have appeared in an early evolution of MYHs and thus regarded to belong to ancestral slow/cardiac type.

The frequencies of cDNA clones encoding above-mentioned *MYH*s in the cDNA clone libraries and relative mRNA levels determined by Northern blot analysis further revealed their tissue-specific expression in adult skeletal and cardiac muscles. The clones encoding fast-type $MYH_{M86\text{-}1}$ were most abundant in the cDNA clone library constructed from fast muscle. Both LS and ED slow muscles contained almost equally the clones of five *MYH*s including fast-type $MYH_{M2528\text{-}1}$ and MYH_{M1034}, slow-type MYH_{M8248}, cardiac-type $MYH_{M2126\text{-}2}$ and unique, slow/cardiac-type MYH_{M5}. Among three types of *MYH* clones from cardiac muscle, cardiac-type $MYH_{M2126\text{-}2}$ was most abundant.

In situ hybridization was performed to localize the transcripts of *MYH*s in skeletal muscles of adult torafugu (body weight 275 g). The transcripts of fast-type $MYH_{M86\text{-}1}$ were found in all fibers with different diameters in fast muscle. Fast fibers with smaller diameters tended to have transcripts of fast-type $MYH_{M2528\text{-}1}$ more abundantly. Given that such fast fibers with small diameters are generated by hyperplasia, the expression of $MYH_{M2528\text{-}1}$ is thought to be deeply correlated with generation of these fibers and those with the smallest diameter are considered to be most newly formed.

The fibers expressing slow-type MYH_{M8248} resided a superficial part of LS slow muscle with small diameters. Fibers expressing cardiac-type $MYH_{M2126\text{-}2}$ also occupied a superficial layer in LS slow muscle with small diameters. Interestingly, fast-type $MYH_{M2528\text{-}1}$ was expressed in fibers of LS and ED slow muscles with large

diameters which showed an intermediate oxidative potential as described above, implying their possible involvement in muscle generation by hyperplasia.

The expression levels of fast-type *MYH*s were also investigated in both wild and farm-cultured torafugu individuals (body weight 0.8 - 1 kg). Among three fast-type *MYH*s, the relative mRNA levels of $MYH_{M2528\text{-}1}$ were significantly higher in wild than farm-cultured fish.

In Chapter III, six sarcomeric *MYH*s were cloned from embryos and larvae of torafugu laboratory-reared at 18°C by using the same method as described for adult torafugu. These included four fast-type *MYH*s, MYH_{M743}, $MYH_{M86\text{-}2}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, cardiac-type $MYH_{M2126\text{-}1}$ and slow/cardiac-type MYH_{M5}. MYH_{M743} and $MYH_{M86\text{-}2}$ have been reported by Ikeda et al. (2007) using gene specific primers. Among all fast-type *MYH*s, the cDNA clone encoding MYH_{M743} was most abundant in all clone libraries from embryos and larvae, followed by those encoding $MYH_{M86\text{-}2}$ in embryos [5 and 7 days post fertilization (dpf)] and $MYH_{M2528\text{-}1}$ in larvae (10 and 16 dpf). The cDNA clone encoding MYH_{M1034} was marginally observed in clone libraries from larvae. While cDNA clones of slow/cardiac-type MYH_{M5} were found in all clone libraries from embryos and larvae, their abundance in larvae was found to be much lesser than in embryos.

RT-PCR using highly specific primers based on the 3' untranslated region nucleotide sequences of *MYH*s showed that the transcripts of MYH_{M743} appeared in embryos at 3 dpf, whereas those of $MYH_{M86\text{-}2}$ in embryos at 4 dpf. These two *MYH*s continued to be expressed during embryonic and larval development, suggesting their involvement in muscle development. The transcripts of fast-type $MYH_{M2528\text{-}1}$

appeared in embryos at 7 dpf and continued to be expressed at successive embryonic and larval stages, as well as in adult fast and slow skeletal muscles. The transcripts of slow/cardiac-type MYH_{M5} continued to be expressed from embryos at 3 dpf to larvae and as well in adult slow and cardiac muscles. Such expression patterns of $MYH_{M2528\text{-}1}$ and MYH_{M5} in adult muscles were consistent with those described in the previous section.

Whole mount *in situ* hybridization for embryos at 4 dpf with probes specific to fast-type $MYH_{M86\text{-}2}$ and slow/cardiac-type MYH_{M5} revealed that the former transcripts were localized in the whole embryonic myotome, whereas the latter transcripts were restricted to the superficial slow muscle as well as to the horizontal myoseptum. The transcripts of cardiac-type $MYH_{M2126\text{-}1}$ were localized adjacently to the notochord of embryos at 3 dpf.

Paired box protein (Pax) genes play pivotal roles in the formation of tissues and organs during development. This gene family encodes transcription factors characterized by the presence of paired box domain (PD), octapeptide motif and homeodomain. It has been reported that Pax3 and Pax7 regulate survival, proliferation and migration of myogenic precursor cells. In Chapter IV, *Pax3* and *Pax7* were cloned from torafugu embryos and adult fast skeletal muscle by using degenerate primers based on highly conserved amino acids in PD and homeodomain. Subsequent *in silico* analysis with the Fugu genome database (ver. 4.0) yielded two distinct genes each for *Pax3* (*Pax3a* and *Pax3b*) and *Pax7* (*Pax7a* and *Pax7b*). The 75th amino acid, glutamine (Glu75), from the N-terminus was replaced by proline in PD of Pax3b. Mammalian Pax3 and Pax7 both have alternatively spliced isoforms,

differing in the presence or absence of Glu75 (Q+/Q-) in PD which affects the DNA-binding specificity (Vogan et al., 1996). One single cDNA clone encoding Pax3a had deletion of Glu75 in PD, suggesting the presence of alternatively spliced variants (Q+/Q-) for torafugu Pax3a. This was further supported by identification of two adjacent alternative 3' splice acceptor sites for torafugu which produce Pax3a Q+ (aagCAGGGA) and Q- (aagcagGGA) variants. Interestingly, torafugu *Pax7b*, but not *Pax7a*, had an insert encoding five amino acid residues (GEASS) in a C-terminal region of PD in two out of three cDNA clones. Genomic analysis showed two alternate splice donor cites at exon 4 of *Pax7b* which is responsible for forming two alternately spliced variants.

RT-PCR revealed that the transcripts of *Pax3a*, *Pax3b*, *Pax7a* and *Pax7b* were found to appear in embryos at 3 dpf and later developmental stages, suggesting their key roles during development. Interestingly, the transcripts of *Pax7b* were observed in adult skeletal muscles. Thus, *Pax3* and *Pax7* can be used to monitor muscle precursor cells.

Finally, Chapter V is dedicated to general discussion based on the results obtained in the present study to discuss key findings and scope for future investigation.

The content of this thesis have been partly published as follows.

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CHAPTER I

Characterization of fiber types in adult skeletal muscles of torafugu

Several unique features of fish skeletal muscles make them an excellent model for studies on muscle development. The most important feature is the anatomical separation of fiber types in contrast with higher vertebrates including mammals in which different types of muscle fibers form a mosaic within the same muscle. Fast-twitch fibers occupy a bulk of fish trunk myotomal muscle and are located deeply in the myotome, whereas slow-twitch fibers lie under the lateral line on the surface of myotome. Fast-twitch fibers contain low myoglobin and mitochondrial content in association with fast movement via glycolytic metabolic pathways, whereas slow-twitch fibers contain a high amount of myoglobin and mitochondria utilizing aerobic metabolism for the sustained swimming (Johnston et al., 1998, Goldspink et al., 2001; Sanger and Stoiber, 2001). Such fiber types are conventionally distinguished by histochemical staining for myofibrillar ATPase and each fiber type contains characteristic MYH which can be shown by immunostaining with MYH specific antibodies (Billeter et al., 1980; Rowlerson et al., 1985; Scapolo and Rowlerson, 1987). In addition, other qualitative and quantitative methods such as morphometry and electron microscopy have been used to characterize fiber types in fish such as carp (Akster, 1983), zebrafish (Van Raamsdonk et al., 1980; Van Raamsdonk et al., 1982), goldfish *Carassius auratus* (Johnston and Lucking, 1978), etc. Enzymes used for the histochemical analysis of fiber type include SDH and NADH-diaphorase to differentiate slow-oxidative fibers (Johnston and Lucking, 1978, Akster, 1983).

In the present chapter fiber types were analyzed by histochemical demonstration of ATPase and NADH-diaphorase in the skeletal muscle of adult torafugu. In order to understand fiber type variation during growth, fiber types were also identified by ATPase staining fast and slow muscles of juvenile torafugu.

Materials and Methods

Fish

A live specimen (sex unknown) of adult laboratory-reared torafugu (body weight 290 g) was used for histochemical demonstration of myofibrillar ATPase and NADH-diaphorase in fast and slow muscles. A juvenile specimen (sex unknown) of laboratory-reared torafugu (body weight 24.2 g) was used to perform histochemical analyses of ATPase.

Histochemical analysis

Fast, LS and ED slow muscles were dissected from of adult (body weight 290 g) and juvenile torafugu (body weight 24.2 g). Slow muscles were dissected from two locations that include LS beneath lateral surface of myotome and ED for dorsal fin (Fig. 1-1A, B). Muscles were snap-frozen by cooled isopentane and the sections prepared by a cryostat were air-dried and stained to determine acid and alkaline stabilities for myofibrillar ATPase by the method of Johnston et al. (1974) after a brief preincubation for 2-3 min at pH 4.3, 4.6, 9.4, 10.0 and 10.6.

NADH-diaphorase (NADH-tetrazolium reductase) staining was performed to determine glycolytic or oxidative metabolism by the method of Novikoff et al. (1961). Briefly, sections made from fast and slow muscles were incubated in 0.05 M Tris-HCl, pH 7.6 containing 2.25 mM NADH and 2.45 mM NBT for 30 min at 37°C and unbound NBT was removed by series of exchange in increasing concentrations of acetone and washed in deionized water before mounted in glycerol.

Results

ATPase was used to differentiate fiber types in fast and slow muscles of adult torafugu (Fig. 1-1). Fast muscle contained various fibers with different diameters (Fig. 1-1D). ATPase of fast fibers with large diameters was inactivated at pH 4.6, whereas those with small diameters were stable to this acidic pH treatment. It was noted that the stability of the fibers with small diameters was more apparent with fibers having smaller diameters. Meanwhile, most fibers in LS (Fig.1-1E) and ED (Fig. 1-1F) slow muscle were resistant to pH 4.6, although some large sized fibers were found to be slightly acid-labile. All fibers in fast muscle irrespective of their sizes were stained intensively for ATPase after incubation at pH 9.4, suggesting their stability at alkaline pH (Fig. 1-1F). On the other hand, all fibers in fast and LS and ED slow muscles were found to be inactivated after treatment at pH 4.3, 10.0 and 10.6 (Table 1-1).

In contrast with the occurrence of several fibers with differing ATPase in adult fast muscle, ATPase of all fibers in juvenile fast muscle was inactivated after preincubation in pH 4.6 (Fig. 1-2A). ATPase of fibers in juvenile LS slow muscle was resistant to preincubation at pH 4.6. In alkaline preincubation at pH 9.4, fast and LS slow twitch fibers maintained ATPase (Fig. 1-2C, D). Unfortunately, histochemical staining for ATPase of ED slow muscle of juvenile specimen was unsuccessful due to degradation of ATPase probably excessive storage of ED slow muscle sections at room temperature prior to staining.

NADH-diaphorase staining was performed to identify oxidative fibers in skeletal muscles. All fibers in LS and ED slow muscles were stained (Fig. 1-3). Importantly, fibers in LS slow muscle with large diameters adjacent to fast muscle showed lower

NADH-diaphorase reaction compared with those in a superficial region with small diameters, suggesting that the former fibers have an intermediate oxidative potential. However, none of fibers in fast muscle was stained.

Section 1. Discussion

Histochemical analysis using myofibrillar ATPase demonstrated that torafugu (290 g body weight) fast muscle contained various fibers with different pH stabilities and different diameters (Fig. 1-1). The mosaic appearance of fast muscle by the process of hyperplastic growth generally occurs in fish which grow to large size (Rowlerson and Veggetti, 2001). ATPase and immunohistochemical profile of the fibers with small diameter in fast muscle vary from those with large diameters. This has been demonstrated in fish such as mullet *Mugil saliens* (Carpene et al., 1982), sea bass *Sparus aurata*, common carp and eel *Anguilla anguilla* (Rowlerson et al., 1985; Scapollo and Rowlerson, 1987). The present results are almost consistent with those reported for torafugu having 154 g body weight (Fernandes et al., 2005). Fernandes et al. (2005) claimed that the threshold body size to have no small fibers with the cessation of recruitment in torafugu fast muscle is about 1.2 kg and 35 cm standard length. However fibers with different pH stabilities were not found in the fast muscle of juvenile torafugu (24.2 g body weight). This result indicates that fibers with different pH stabilities in the fast muscle of adult torafugu represents distinct growth phase (mosaic hyperplasia) and adult specimens used in present study are supposed to be at the stage to recruit fast fibers.

Slow muscles of fish are rich in mitochondria which exhibit oxidative metabolism and allow continuous muscle movement with less fatigue (Leary et al., 2003). In the present study, fibers in slow muscle were intensely stained for NADH-diaphorase, although some fibers with large diameter in LS slow present adjacently to fast muscle showed an intermediate oxidative potential (Fig. 1-3).

Such fibers had also showed distinct ATPase than the superficial slow-twitch fibers of LS slow muscle. In mammals, an intermediate fiber type IIx has been identified in adult skeletal muscle (Schiaffino and Reggiani, 1994), which has been found to have twitch attributes intermediate between slow-type IIa and fast-type IIb (Arany et al., 2007). Less is known about intermediate, fast/aerobic fibers in fish as they appear intermediate between those of slow and fast twitch fibers in most regards including distribution, oxidative capacity, fiber diameter, fatigue resistance, contraction kinetics, power output and optimal operating frequency (reviewed by Sanger and Stoiber, 2001). It will be an interesting to investigate above parameters for the fibers with large diameters in LS slow muscle for their functional importance.

In conclusion, distinct fiber types were examined in the fast and slow muscles of adult torafugu. Importantly, fibers with small diameter in fast muscle that were generated by hyperplasia contained distinct myosin than fibers with large diameters as revealed by myofibrillar ATPase. However, myosin heavy chain composition of such fibers is still unknown. In this regard it will be interesting to characterize myosin heavy chains from adult muscles of torafugu that were possibly expressed in a fiber type-specific manner. If such hypothesis holds true, it would be highly helpful to explain the role of different MYHs in adult skeletal muscle of torafugu that is known to have high number of *MYH*s.

CHAPTER II

Expression patterns of sarcomeric myosin heavy chain genes in adult torafugu muscles

Myosin is the most abundant protein in skeletal muscles make up the primary component of thick filaments and is involved in muscle contraction as well as phagocytosis, cell motility and vesicle transport in other organs of animals. Myosin superfamily consists of 18 classes of ATP-dependent motor proteins, although the best studied myosins belong to class II which include sarcomeric and smooth muscle and nonmuscle myosins (Berg et al., 2001; Foth et al., 2006). Class II myosin is a hexameric protein which consists of MYHs and four light chains. MYH possesses globular head at its N-terminal region called subfragment-1 (S1) and rod containing subfragment-2 (S2) and light meromyosin (LMM). S1 contains ATPase and actin-binding sites and functions as a molecular motor that transforms chemical energy generated from ATP hydrolysis into mechanical work. S2 links S1 at its N-terminal end and to LMM at its C-terminal end, and is believed to be loosely bound to thick filament surface (Harrignton and Rodgers, 1984). LMM is responsible for the thick filament formation through an assembly competence domain (ACD) under physiological conditions (Cohen and Parry, 1998).

The expression of *MYH*s in skeletal muscles has been extensively studied in mammals. Seven *MYH*s have been found to be predominantly expressed in the mammalian skeletal muscles that contain two developmental *MYH*s, *MYH-emb* (*MYH3*) and *MYH-peri* (*MYH8*), and five adult skeletal ones, *MYH-IIa* (*MYH2*), *MYH-IIb* (*MYH4*), *MYH-IIx/d* (*MYH1*) and *MYH-β* (*MYH 7*) (reviewed in Schiaffino

and Reggiani, 1996). *MYH-β* is known to be expressed in cardiac muscle along with another cardiac-type *MYH-α* (*MYH6*) (Lompre et al., 1984). *MYH-α* is expressed predominantly in atria and *MYH-β* in ventricles (Lyons et al., 1990). The *in situ* hybridization analysis of *MYH*s transcripts and immunohistochemical analysis identify distinct fiber types in mammalian skeletal muscles (Schiaffino and Reggiani, 1994; Ennion et al., 1995; Schiaffino and Reggiani, 1996). In addition to fibers containing a single type of *MYH* mRNA, fibers coexpressing two *MYH*s termed as hybrid fibers i.e., fibers containing both *MYH-β* and-*IIa*, *MYH-IIa* and *-IIx*, or *MYH-IIx* and *-IIb* have been found in higher vertebrates (DeNardi et al., 1993; Smerdu and Erzen, 2001).

Fish are known to possess highly conserved MYH multigene family, although MYHs are much more than their higher vertebrate counterparts (Gerlach et al., 1990; Kikuchi et al., 1999; Watabe and Ikeda, 2006; Ikeda et al., 2007). cDNAs of several *MYH*s have been cloned from adult skeletal muscles of other fishes such as medaka (Liang et al., 2007), grass carp (Tao et al., 2004), silver carp (Fukushima et al., 2009), rainbow trout (Gauvry and Fauconneau, 1996), Antartic rock cod *Notothenia coriiceps*, hawk fish *Paracirrhites forsteri* (Gauvry et al., 2000), chum salmon *Oncorhynchus keta* (Iwami et al., 2002), white croaker *Pennahia argentata* (Yoon et al., 2000), walleye pollack *Theragra chalcogramma* (Ojima et al., 1998), brushtooth lizardfish *Saurida undosquamis*, wanieso lizardfish *S. wanieso* (Hossain et al., 2008) and amberjack *Seriola dumerili* (Kawabata et al., 2000). However, less has been studied to localize their transcripts in order to characterize fiber types at the molecular level in above fishes. Identification of fiber types according to *MYH* expressed can facilitate greater understanding of the growth mechanism in adult fish

skeletal muscles.

In the present study, the transcripts of seven sarcomeric *MYH*s were identified in fast, slow and cardiac muscles of adult torafugu. While the expression of three *MYH*s were restricted to fast, slow or cardiac muscles, two *MYH*s were found to be expressed in both fast and slow fibers with different diameters. The functional significance of different *MYH*s is discussed.

Section 1. Molecular cloning and comparative expression patterns of *MYH*s

In vertebrates, *MYH*s are regulated developmentally as well as temporally according to the functional demand of muscles concerned. In adult skeletal muscle fibers of mammals, changes in expression occur in response to neuronal (Pette and Vrbova, 1985), hormonal (Izumo et al., 1988), mechanical (Loughna et al., 1990), electrical stimuli (Gorza et al., 1988; Termin et al., 1989, Ausoni et al., 1990), aging (Butler-Brown et al., 1984; Whalen et al., 1984; Larsson et al., 1991) and exercise (Andersen et al., 1994). In fish expressional regulations of *MYH*s has been studied in relation to development (Ennion et al., 1999; Yelon et al., 1999; Rescan et al., 2001; Weaver et al., 2001; Peng et al., 2002; Bryson-Richardson et al., 2005; Chauvigne et al., 2005; Nihei et al., 2006; Ono et al., 2006; Elworthy et al., 2008) and fluctuation of environmental temperature (Hirayama and Watabe, 1997; Imai et al., 1997; Watabe, 2002; Tao et al., 2004; Liang et al., 2007). Furthermore, cDNAs of *MYH*s have been cloned from adult fish (Gauvry and Fauconneau, 1996; Ojima et al., 1998; Kawabata et al., 2000; Yoon et al., 2000; Iwami et al., 2002; Hossain et al., 2008).

Fish are known to have more number of *MYH*s than their higher vertebrate counterparts; however, the former transcripts have not been observed in most studies probably due to inappropriate methods employed. Recently, the screening of *MYH*-specific cDNA clone libraries found the transcripts of multiple *MYH*s in medaka (Ono et al., 2006; Liang et al., 2007) and silver carp (Fukushima et al., 2008). Such approach is advantageous since multiple *MYH*s that are expressed at given tissues and under particular conditions can be cloned and predominant ones can be identified after analyzing the frequencies of cDNA clones.

In the present section, we screened *MYH* cDNA clone libraries from different adult trunk skeletal muscles of torafugu including cardiac one. This method enabled identification of seven *MYH*s and their frequencies suggested the predominant expression of *MYH*s in a tissue-specific manner. The accumulated mRNA levels of *MYH*s determined by Northern blot hybridization with highly specific probes further supported their tissue-specific expression patterns in adult torafugu muscles. The full length cDNA sequence was determined for fast-type $MYH_{M86\text{-}1}$, which was found to be predominantly and specifically expressed in adult fast muscle. In addition, the mRNA levels for fast-type *MYH*s were compared between wild and cultured torafugu specimens.

Materials and methods

Fish

A live male specimen of torafugu (body weight about 1 kg) grown in the University of Tokyo was humanly killed and subjected to the collection of fast, slow and cardiac muscles. The muscles collected were immediately frozen in liquid nitrogen (-196°C) and stored at -80°C until use for cDNA cloning, the construction of cDNA libraries and Northern blot analysis. LS and ED slow muscles were dissected as described in the previous section. Fast muscle from the dorsal trunk region of wild (n = 3) and farm-cultured (n = 3) torafugu females (body weight 0.8 - 1 kg) were used for Northern blot analysis.

Construction of cDNA clone library

Total RNAs were isolated from various muscles by using an ISOGEN solution (Nippon Gene, Tokyo, Japan). 5 μg of total RNAs were reverse-transcribed by using oligonucleotide (dT)-tailed primer (Table 2-1) and Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to the manufacture's instructions. PCR amplification of *MYH*s was performed by using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) with a forward primer (MYH-F) and a reverse primer (MYH-R) (Table 2-1) that were designed based on a highly conserved amino acid sequence from the LMM region of sarcomeric MYHs from teleost. Amplified DNA fragments were subcloned into the pGEM-T vector (Promega, Madison, WI, USA) and 50 cDNA clones each of various muscle types were randomly sequenced with an ABI 3100 genetic analyzer (Applied Biosystems) after labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems).

3' rapid amplification of cDNA ends (RACE)-PCR

3' RACE -PCR was performed to determine nucleotide sequences in the 3'untranslated region (UTR) from each cloned *MYH* by using a specific forward primer and an adapter primer (AP) (Table 2-1). PCR amplifications were carried out at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min with a final extension step for 5 min. A single PCR reaction of 20 μl contained 1 μl each of forward and reverse primers (10 μM), 1 μl of cDNAs template (dilution 1:100), 2 μl of 10x PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1 U *Taq* DNA polymerase and 13.8 μl of sterilized water. The amplified cDNA fragments were sequenced as described above.

Phylogenetic analysis

The deduced amino acid sequences of torafugu sarcomeric MYHs cloned in this study were aligned using multiple sequence alignment program CLUSTAL X (Thompson et al., 1997) along with corresponding amino acid sequences of MYHs from common carp, zebrafish and medaka. The phylogenetic tree was constructed by the neighbor-joining method in Mega4 (Tamura et al., 2007) with zebrafish smooth muscle MYH as an outgroup. Bootstrap sampling analysis from 1000 replicates was adopted to evaluate internal branches.

Determination of full-length cDNA encoding MYH_{M86-1}

The full-length cDNA encoding MYH_{M86-1} was amplified by using a degenerate forward primer (MYH-F1) together with a specific reverse primer (M86-1R) (Table 2-1) with PrimeSTAR™ Max DNA polymerase (Takara, Otsu Japan). PCR amplifications were carried out as described above. The entire sequence was determined by the primer walking method using designed internal forward (IFPs) and reverse primers (IRPs) (Table 2-1). To determine the translation start site and 5'UTR sequence for the full-length cDNA, 5'RACE was performed by using GeneRacer™ Kit (Invitrogen).

Dot blot and Northern blot analyses

For DNA dot blot analysis, digoxygenin (DIG)-labeled probes were prepared by PCR for each *MYH* in the highly variable 3'UTR region by using PCR-DIG Probe Synthesis Kit according to the manufacture's instructions (Roche Applied Science, Mannheim, Germany). The primers used for preparation of DIG-labeled probes for each *MYH* are listed in Table 2-1. Probes were hybridized separately with strip of positively charged nylon membranes (Biodyne®PLUS, Pall Corporation, USA) that were spotted with 1ng of plasmids encoding cloned *MYH*s at 42°C. The membrane was washed twice with low stringency buffer [2x SSC containing 0.1% SDS (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)] for 5 min each at room temperature followed by two wash in high stringency buffer (0.1x SSC containing 0.1% SDS) for 15 min each at 50°C. The membrane was incubated in blocking solution [1% blocking reagent (Roche Applied Science)] for one hrs at RT followed by incubation in anti-DIG Fab fragment (Roche Applied Science) at 1: 20000 dilution for 30 min followed by two washes in washing buffer (0.1 M maleic acid,

0.15 M NaCl, pH 7.5, containing 0.3% tween 20) for 20 min each. Hybridization signals were detected after incubating membranes with detection buffer (0.1 M Tris-HCL, 0.1 M NaCl, pH9.5) for 3 min followed by incubation with CDP-star substrate (Roche Applied Science) for five min. X-ray films were exposed over membranes under dark conditions for one hrs and X-rays films were developed successively.

For Northern blot analysis, 5 μg total RNAs were prepared from three different parts in each of fast, slow and cardiac muscles from the same specimen used for cDNA cloning or from fast muscles of wild (n = 3) and farm-cultured (n = 3) females. These RNAs were electrophoresed on 2% agarose gels containing 2% formaldehyde and transferred to nylon membranes (Biodyne®PLUS), and subjected to hybridization with DIG-labeled DNA probes described above. DIG-labeled DNA probes specific to the 18S rRNA (AB437876) and α-actin (U38958) genes were used as internal controls (Table 2-1).

The statistical analysis was carried out by using Student's *t*-test to compare mRNA levels of *MYH*s among fast, slow and cardiac muscles. The accumulated mRNA levels that showed a significant difference by Student's *t*-test were also confirmed by one-way ANOVA analysis.

Results

cDNA cloning of *MYH*s

Seven sarcomeric *MYH*s were cloned from adult torafugu fast, slow and cardiac muscles by RT-PCR (Table 2-2). Three *MYH*s, $MYH_{M86\text{-}1}$, MYM_{M8248} and MYH_{M880}, were cloned exclusively from fast, slow and cardiac muscles, respectively, dissected from a single laboratory-reared adult specimen, whereas two *MYH*s, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, were cloned from both fast and slow muscles and another two *MYH*s, $MYH_{M2126\text{-}2}$ and MYH_{M5}, from both slow and cardiac muscles. The nomenclature of torafugu *MYHs* found in the present study is described following Ikeda et al. (2007).

3'RACE-PCR determined nucleotide sequences at UTR and those encoding a C-terminal region of LMM domain for above seven MYHs (Fig. 2-1). These cDNA nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB465004 ($MYH_{M86\text{-}1}$), AB465006 ($MYH_{M2528\text{-}1}$), AB465005 (MYH_{M1034}), AB465007 (MYH_{M8248}), AB465009 ($MYH_{M2126\text{-}2}$), AB465008 (MYH_{M5}) and AB465010 (MYH_{M880}).

Phylogenetic analysis was carried out to compare torafugu *MYH*s with those from other fish such as common carp (Hirayama and Watabe, 1997; Imai et al., 1997; Nihei et al., 2006), zebrafish (Yelon et al., 1999; Bryson-Richardson et al., 2005) and medaka (Ono et al., 2006; Liang et al., 2007), whose expressions are well known. $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034} belonged to fast type as they were placed in the same clade representing fast *MYH*s (Nihei et al., 2006; Ikeda et al., 2007), whereas MYH_{M8248} belonged to slow type and $MYH_{M2126\text{-}2}$ to cardiac type as they were monophyletic with those of slow- and cardiac-type *MYH*s from other fish species,

respectively (Fig. 2-2). The phylogenetic tree showed that fast-type *MYH*s, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, were placed in the same clade with fast-type *MYHemb1* which is expressed in medaka from embryonic to adult stages (Ono et al., 2006), whereas $MYH_{M86\text{-}1}$ was placed in the same clade with fast-type *mMYH-11* which is expressed in adult medaka acclimated to 30°C (Liang et al., 2007). MYH_{M5} was a unique *MYH* as its phylogenetic position was independent of any other *MYH*s, whereas MYH_{M880} was monophyletic with zebrafish atrial *MYH* (Fig. 2-2). Such phylogenetic relationship of torafugu *MYHs* almost agreed with that described by Ikeda et al. (2007). MYH_{M5} and MYH_{M880} were found to have appeared in an early evolution of MYHs as far as the present phylogenetic tree was concerned, and these genes were regarded to belong to ancestral slow/cardiac type (Table 2-2).

Expression patterns of *MYH*s

We randomly sequenced 50 cDNA clones each from the cDNA clone libraries constructed from fast, slow and cardiac muscles of adult torafugu. The clones encoding fast-type $MYH_{M86\text{-}1}$ were most abundant in fast muscle (Table 2-2). Both LS and ED slow muscles gave almost equally the clones of five *MYH*s including fast-type $MYH_{M2528\text{-}1}$ and MYH_{M1034}, slow-type MYH_{M8248}, cardiac-type $MYH_{M2126\text{-}2}$ and unique, slow/cardiac-type MYH_{M5} (Table 2-2). Among three types of *MYH* clones from cardiac muscle, the type encoding cardiac-type $MYH_{M2126\text{-}2}$ was most abundant (Table 2-2).

Dot blot analysis revealed that all probes synthesized referring to fast, slow and cardiac-type *MYH*s were highly specific and hybridized only with corresponding *MYH*s (Fig. 2-3). Figure 2-4 shows hybridization patterns in Northern blot analysis

and mRNA levels relative to those of the 18S rRNA gene in three different parts each from fast, slow and cardiac muscles in the same specimen used for the construction of cDNA clone libraries. MYH_{M880} did not show any hybridization signal, suggesting its expression too low to be resolved by Northern blot analysis. Fast-type $MYH_{M86\text{-}1}$ was expressed only in fast muscle (Fig. 2-4B), whereas $MYH_{M2528\text{-}1}$ and MYH_{M1034} were expressed in fast as well as in LS and ED slow muscles. The relative mRNA levels of $MYH_{M2528\text{-}1}$ were significantly higher in fast than any of the two slow muscles (P<0.01) (Fig. 2-4C). However, no significant difference in the mRNA levels was observed with MYH_{M1034} in fast and two slow muscles (Fig. 2-4D).

The expression of slow-type MYH_{M8248} was observed specifically in slow muscles and its mRNA levels were significantly higher in ED than LS slow muscle (P<0.01) (Fig. 2-4E), whereas cardiac-type $MYH_{M2126\text{-}2}$ was found to be expressed in LS and ED slow, and cardiac muscles, with the relative mRNA levels being significantly higher in LS than ED slow and cardiac muscles (P<0.01) (Fig. 2-4F). The unique, slow/cardiac-type MYH_{M5} was expressed in the two slow muscles and mRNA levels in LS slow muscle were found to be significantly higher than those in ED slow muscle (P<0.05) (Fig. 2-4G). On the other hand, its signals were only marginally detected in cardiac muscle (Fig. 2-4A).

Three fast-type *MYH*s, $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, were found to be expressed both in wild (n = 3) and farm-cultured (n = 3) fish (Fig. 2-5). The relative mRNA levels of $MYH_{M2528\text{-}1}$ in wild fish were significantly higher than those in farm-cultured fish (P<0.05) (Fig. 2-5C). On the other hand, the mRNA levels of $MYH_{M86\text{-}1}$ and MYH_{M1034} did not differ significantly between the two groups (Fig. 2-5B, D).

The full-length cDNA sequence of MYH_{M86-1}

Since MYH_{M86-1} was found to be expressed predominantly and specifically in the fast skeletal muscle of adult torafugu, we determined the complete cDNA sequence of this gene, yielding a 5817 bp open reading frame (ORF) that encoded 1938 amino acid residues (Fig. 2-6). S1 encoded by MYH_{M86-1} consisted of 841 amino acid residues that possessed three each of ATP and actin-binding sites and a single site each for binding of essential light and regulatory light chains, along with 15 amino acid residues in the 25-50 kDa junction called loop 1 and 21 amino acid residues in the 50-25 kDa junction called loop 2 (Fig. 2-6). No proline residue was found in both loops 1 and 2, whereas five lysine residues were found in loop 2 with three lysine residues at a C-terminal site that are supposed to be necessary for interaction with actin (Joel et al., 2000).

Section 2. Fiber-type-specific expression of *MYH*s in skeletal muscles

Fiber types are distinguished by histochemical staining for myofibrillar ATPase and immunostaining with MYH-specific antibodies (Billeter et al., 1980; Rowlerson et al., 1985; Scapolo and Rowlerson, 1987). In addition, morphometry and electron microscopy analysis have been used to characterize fiber types in fishes such as carp (Akster, 1983), zebrafish (Van Raamsdonk et al., 1980; Van Raamsdonk et al., 1982), goldfish (Johnston and Lucking, 1978), etc. Histochemical analysis of enzymes involved in oxidative metabolism such as SDH and NADH-diaphorase has been used to differentiate slow-oxidative fibers (Johnston and Lucking, 1978, Akster, 1983).

The identification of fiber types by molecular methods involves localization of transcripts of *MYH*s. The multiple fibers that differ in fiber diameter and pH stabilities of myofibrillar ATPase were found for adult skeletal muscles of torafugu in Chapter I. Furthermore, the cDNAs of several *MYH*s were cloned and their tissue-specific expression was determined for adult skeletal and cardiac muscles of torafugu in section 1.

In the present section, the transcripts of *MYH*s were localized in the section of fast and slow skeletal muscles of adult torafugu by *in situ* hybridization with riboprobes. The transcripts of *MYH*s were localized in a fiber-type-specific manner and their implication with relation to the growth in adult muscles torafugu is discussed in the present section.

Materials and Methods

Fish

A laboratory-reared specimen (sex unknown) of adult torafugu (275 g) was treated as described in material and methods of section 1. Fast, LS and ED slow skeletal muscles of trunk were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and stored in absolute methanol at -20°C until their use for *in situ* hybridization.

In situ hybridization

The cDNA fragments of 250 to 300 bp which contain 3'UTR nucleotide sequences and those encoding a C-terminal part of MYH were cloned into pGEM-T vector (Promega) (Table 2-1). DIG-labeled antisense riboprobes were synthesized using *in vitro* translation by T7 and SP6 RNA polymerase according to the manufacture's instructions (Roche Applied Science). *In situ* hybridization was performed on transverse sections (12 μm thickness) made from adult fast and slow muscles by the method of Darby et al. (2006). Briefly, sections were rehydrated and digested with proteinase K (1 μg ml^{-1}) for 30 min at 37°C. Acetylation was performed with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min. After several rinses in 1x phosphate-buffered saline (PBS) and re-fixation in 4% PFA, sections were completely dehydrated before hybridization with riboprobes. Hybridization was performed in a humidified incubator at 45°C for at least 16 hrs followed by two high-stringency washes at 37°C for 30 min in 2x SSC and two rinses in 1x SSC, respectively. Unhybridized probes were removed after incubation with RNase A in solution containing 0.01 M Tris-HCl, 0.5 M NaCl, pH8.0, at 37°C for 30 min, followed by two washes in 0.1x SSC for 60 min at room temperature. The

sections were rinsed twice with buffer 1 (0.1 M Tris-HCl, 0.120 M NaCl, pH 7.5). Tissue sections were blocked with 2x blocking solution for 60 min at room temperature. Blocking buffer was replaced with anti-DIG Fab-alkaline phosphatase conjugate (1:500 in 2x blocking solution) for 2 hrs at room temperature. Unbound anti-digoxigenin primary antibodies were removed after two washes in buffer A for 15 min each. This was followed by incubation with buffer B (0.1 M NaCl, 0.1 M Tris-HCl, 0.05 M $MgCl_2$ pH 9.5) for 10 min at room temperature. Alkaline phosphatase activity was visualized by incubation with 5-bromo-4-chloro-3-indoyl -phosphate (BCIP) and nitroblue tetrazolium (NBT) in buffer B. The staining was carried out for 20 hrs at room temperature under dark conditions, rinsed with 1x PBS, and embedded in 80% glycerol.

Results

In situ hybridization was performed to localize the transcripts of predominantly expressed *MYH*s in skeletal muscles. The transcripts of fast-type $MYH_{M86\text{-}1}$ were found in all fibers with different diameters in fast muscle (Fig. 2-7A). On the other hand, the transcripts of fast-type $MYH_{M2528\text{-}1}$ were localized in fibers with small diameters in fast muscle (Fig. 2-7B), suggesting that these fibers express at least two *MYH*s, $MYH_{M86\text{-}1}$ and $MYH_{M2528\text{-}1}$. Interestingly, fast fibers with smaller diameters tended to have transcripts of $MYH_{M2528\text{-}1}$ more abundantly. The transcripts of $MYH_{M2528\text{-}1}$ were also expressed in fibers of LS (Fig. 2-7C) and ED (Fig. 2-7D) slow muscles with large diameters. The fibers expressing MYH_{M8248} resided a superficial part of LS and ED slow muscle with small diameters (Fig. 2-7E, F). Fibers expressing $MYH_{M2126\text{-}2}$ also occupied a superficial layer in LS slow muscle with small diameters. *In situ* hybridization for $MYH_{M2126\text{-}2}$ was not successful with fibers in ED slow muscle in the present study.

Section 3. Discussion

In the present study, seven sarcomeric *MYH*s were cloned from adult torafugu skeletal muscles. $MYH_{M86\text{-}1}$ was predominantly expressed in fast muscle (Table 2-2 and Fig. 2-4) and such predominant expression of $MYH_{M86\text{-}1}$ in fast muscle was also confirmed by N-terminal amino acid sequence analysis on purified myosin (data not shown). *In situ* hybridization analysis localized the transcripts of $MYH_{M86\text{-}1}$ to all fast fibers with different diameters (Fig. 2-7A). On the other hand, the transcripts of $MYH_{M2528\text{-}1}$ were found only in fast fibers with smaller diameters (Fig. 2-7B). Therefore, it seems that fast fibers with small diameters contain at least two fast-type *MYH*s, $MYH_{M86\text{-}1}$ and $MYH_{M2528\text{-}1}$. Interestingly, $MYH_{M2528\text{-}1}$ was also expressed in fibers of LS and ED slow muscles with large diameters (Fig. 2-7C, D). The transcripts of another fast-type *MYH*, MYH_{M1034}, were observed in fast muscle as revealed by cDNA clone library analysis (Table. 2-2). Unfortunately, *in situ* hybridization with a probe specific to MYH_{M1034} was not successful.

In contrast, MYH_{M8248} and $MYH_{M2126\text{-}2}$ were expressed only in fibers in a superficial part of LS slow muscle with small diameters, but not in fast fibers with any diameters and fibers in LS and ED slow muscles with large diameters (Fig. 2-7E, F, G). Earlier, we showed that fibers with large diameters in LS slow muscle had intermediate oxidative potential (Chapter I). Previously, the small-diameter fibers generated by hyperplasia in adult fast muscle were demonstrated in common carp which expressed a distinct *FG2MYH* (Ennion et al., 1995). *FG2MYH* showed the highest identity to $MYH_{M2528\text{-}1}$ in their deduced amino acid sequences among seven sarcomeric *MYH*s cloned from adult torafugu in the present study (data not shown). As described in the section of results, the transcripts of $MYH_{M2528\text{-}1}$ were expressed

more abundantly in fast fibers with smaller diameters. This relationship between fast fiber diameter and expression of $MYH_{M2528\text{-}1}$ agrees well with that between fast fiber diameter and ATPase staining intensity at pH 4.6. It is well known that fish muscles grow by hyperplasia even in adult (Stickland, 1983; Rowlerson et al., 1985; Weatherley and Gill, 1985; Rowlerson and Vegggetti, 2001) and this feature is in a marked contrast to mammals where adult muscles grow by hypertrophy (Rowe and Goldspink, 1969; Watabe, 1999). Given that such fast fibers with small diameters are generated by hyperplasia, the expression of $MYH_{M2528\text{-}1}$ is thought to be deeply correlated with this fiber generation and fibers with the smallest diameter are considered to be most newly formed. During growth of fast fibers from small to large diameters, the expression of $MYH_{M2528\text{-}1}$ would be gradually decreased. The expression of $MYH_{M2528\text{-}1}$ in slow fibers with large diameters also indicates their involvement in muscle generation by hyperplasia. However, the nature of such fibers responsible for the process of hyperplastic growth in fish is unknown. Furthermore, the expression switch of *MYH*s during maturation of fish muscle and the mechanisms underlying such switch are not clear. Thus it is interesting to disclose the regulatory mechanisms involved in the expression of $MYH_{M2528\text{-}1}$ in muscle fibers, because such investigation will possibly clarify how these fibers are recruited in fish with their indeterminate body size.

The expression levels of fast-type *MYH*s were also investigated in both wild and farm-cultured torafugu individuals. Among three fast-type *MYH*s, the relative mRNA levels of $MYH_{M2528\text{-}1}$ in wild fish were significantly higher than those in farm-cultured fish (Fig. 2-5). The number of muscle fibers recruited to reach a given body size of fish varies between species and different strains of the same species and

is also affected by diet, exercise training, and temperature (Johnston 1999). Therefore, it is again considered that the expression of $MYH_{M2528\text{-}1}$ participates with muscle generation.

CHAPTER III

Expression patterns of myosin heavy chain genes in torafugu embryos and larvae

The molecular, genetic and cellular bases for skeletal muscle growth have been documented in a number of vertebrate species. While all of the trunk skeletal muscles originate from presomitic mesoderm (PSM), a structure next to the notochord prior to somite formation, the limb/fin and cranial muscles appear distinctly from muscle progenitors other than PSM (Neyt et al., 2000; Shih et al., 2008). Somite arises by sequential segmentation of PSM on both sides of the neural tube in a rostro-caudal progression and each newly formed somites divides into ventral scelerotome which later forms axial skeleton, myotome which forms axial musculature, and dorsal dermomyotome that forms dermis and generate precursors for future myotomal growth (Stellabotte and Devoto, 2007). Such early developmental process which leads to the formation of primary myotome refereed as primary myogenesis and further growth of skeletal muscles are established by multiple wave of growth involving secondary, postnatal myoblast and adult satellite cells (Biressi et al., 2007).

Development of fish skeletal muscles differs considerably from higher vertebrates. In higher vertebrates, the primary muscle fibers are produced by the fusion of embryonic myoblasts independently of innervations, whereas the secondary fibers are also produced by the fusion of embryonic myoblasts forms on the surface of primary ones in smaller size (Evans et al., 1994). Furthermore, different fiber types develop in distinct proliferative zones in early embryogenesis of fish. For instance, in zebrafish, fast and slow muscle precursors develop in different cellular

environments (Devoto et al., 1996). Slow muscle fibers develop from adaxial cells, which are large cuboidal cells adjacent to notochord (Weinberg et al., 1996). Adaxial cells are the first ones to express myogenic transcription factor MyoD and differentiate into slow muscle fibers (Devoto et al., 1996). Adaxial cells then migrate superficially, whereas remaining non-adaxial precursors differentiate into lateral fast muscle fibers. Such process leading to the formation of early embryonic myotome within 24 hrs in zebrafish after fertilization marked as primary wave of myogenesis. Furthermore, in contrast to higher vertebrates, the post-embryonic growth in fish results from the recruitment of new fibers (hyperplasia) as well as by the increase in fiber size (hypertrophy) (Stickland, 1983; Rowlerson et al., 1985; Weatherley and Gill, 1985; Watabe, 1999; Rowelerson and Veggetti, 2001).

*MYH*s are expressed sequentially during development. This has been studied by immunohistochemistry using specific antibodies, *in situ* hybridization by specific probes, and gel electrophoresis. In higher vertebrates, the primary muscle fibers initially express embryonic *MYH-emb*, perinatal *MYH-peri* (Karsch-Mizrachi, et al., 1989; Weydert et al., 1987; Codon et al., 1990) and slow *MYH-β/I* (Narusawa et al., 1987; Barbet et al., 1991), whereas the secondary muscle fibers express embryonic, neonatal and fast *MYH*s but not slow *MYH-β/I* (Barbet et al., 1991). In fish such as zebrafish, adaxial cells are the earliest to express slow-type MYH, *smyhc1*, well before their migration towards the lateral surface of myotome (Devoto et al., 1996; Bryson-Richardson et al., 2005). Bryson-Richardson et al (2005) also showed that adaxial cells co-express fast-type MYH, *myhc4*, well before their radial migration. In rainbow trout, the expression of fast-type *MYH* was observed in adaxial cells before the expression of slow-type *MYH* (Rescan et al., 2001). The expression patterns of

multiple *MYH*s have been studied during embryonic development of other fish such as common carp, medaka, etc. (Ennion et al., 1999; Neihi et al., 2006; Ono et al., 2006), providing greater insights into embryonic muscle development.

Recently, the comprehensive analysis of the torafugu whole genome database by comparative genomics revealed over 20 sarcomeric *MYH*s (Ikeda et al., 2007). Furthermore, several *MYH*s were found to be expressed in fiber-type-specific manner in adult skeletal muscles (Chapter I). Interestingly, small diameter fibers that are known to appear due to hyperplastic growth in adult fast muscle of torafugu, expressed fast-type $MYH_{M2528\text{-}1}$. However, less is known regarding expression patterns of sarcomeric *MYH*s during development of torafugu.

The main objective of this Chapter was to clone cDNAs encoding sarcomeric *MYH*s from embryos and larvae of torafugu and to investigate their expression patterns during development.

Section 1. Molecular cloning of *MYH*s from embryos and larvae

In contrast to the formation of skeletal muscles in higher vertebrates where both fast and slow fiber types are intermingled, these fibers in fish are spatially separated during embryonic development (Devoto et al., 1996). In zebrafish, the embryonic slow muscle population is formed from adaxial cells, in response to the hedgehog signaling from notochord (Currie and Ingham, 1996; Blagden et al., 1997). Adaxial cells express a slow-type *MYH*, *smhyc1*, well before their migration to lateral surface of myotome. In contrast, a subpopulation of adaxial cells called muscle pioneer cells that remain in the medial location even after their differentiation also contain slow MYH (Bryson-Richardson et al., 2005). The rest of lateral myotome differentiates into fast muscle, and this differentiation begins after the migration of adaxially derived slow muscle cells. Such early muscle development in fish embryos has been elegantly shown by the transcripts of fast and slow-type *MYH*s in other fishes (Rescan et al., 2001; Nihei et al., 2006; Ono et al., 2006).

In the present section, cDNAs encoding MYHs were cloned from torafugu embryos and larvae by the method described in Chapter II. In addition, evolutionary relationship of MYHs from other fishes was also investigated by using phylogenetic analysis.

Materials and Methods

Fish

Artificially fertilized eggs of torafugu were collected from Oshima Fisheries Hatchery, Nagasaki, Japan, and brought to The University of Tokyo, where they were reared in 500 L tank in seawater at 18°C. About 100 embryos each from 1 to7 days post fertilization (dpf) and an equal number of larvae from 8, 10 and 16 dpf were collected and snap-frozen in liquid nitrogen (-196°C) and kept at -80°C for cDNA cloning of *MYH*s.

RT-PCR

Total RNAs were prepared from each developmental stage of torafugu by using ISOGEN (Nippon Gene) and 5 μg of each was reverse transcribed by using oligo-dT primer (Table 3-1) and superscript reverse transcriptase III™ (Invitrogen) according to the manufacture's instructions. *MYH*s were amplified by using *MYH*-specific degenerate primers as described in Chapter II. The amplified DNA fragments were subcloned into pGEM-T vector (Promega) and sequenced with an ABI 3100 genetic analyzer (Applied Biosystems) after labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3' RACE -PCR was performed to determine nucleotide sequences in the 3' UTR from each cloned *MYH* by using a specific forward primer (Table 3-1) and an adapter primer (AP) (Table 2-1). The amplified cDNA fragments were sequenced as described in Chapter II.

Phylogenetic analysis

The deduced amino acid sequences of *MYH*s cloned from torafugu embryos and larvae were aligned with the corresponding sequences reported for adult torafugu, common carp (Hirayama and Watabe, 1997; Imai et al., 1997; Neihi et al., 2006), medaka (Ono et al., 2006) and zebrafish (Yelon et al., 1997; Berdougo et al., 2003; Bryson-Richardson et al., 2004; Wallace et al., 2005) by multiple alignment program CLUSTAL X (Thompson et al., 1997). The phylogenetic tree was constructed by the neighbor-joining method by using phylogenetic software MEGA 4 (Tamura et al., 2007). The bootstrap values from 1000-replicates analysis are given at the nodes in percentages.

Results

cDNA fragments of 450 bp encoding partial amino acid sequences of LMM were amplified by RT-PCR with a pair of degenerate primer. 3'RACE-PCR was further performed to obtain nucleotide sequences that encode C-terminal portion of LMM and 3' UTR of each *MYH*s (Fig. 3-1). The deduced amino acid sequences of these cDNA fragments were grouped to six *MYH*s. They were identified as MYH_{M743}, $MYH_{M86\text{-}2}$, $MYH_{M2528\text{-}1}$, MYH_{M1034}, $MYH_{M2126\text{-}1}$ and MYH_{M5}, following Ikeda et al. (2007) (Table 3-2).

Phylogenetic analysis was carried out to study evolutionary relationship of torafugu MYHs with those reported for other fish such as common carp (Hirayama and Watabe, 1997; Imai et al., 1997; Neihi et al 2006), medaka (Ono et al., 2006; Liang et al., 2007) and zebrafish (Yelon et al., 1997; Berdougo et al., 2003; Bryson-Richardson et al., 2004; Wallace et al., 2005) (Fig. 3-2). Such phylogenetic analysis based on partial amino acids at the C-terminal end of LMM was almost

consistent with our pervious reports (Ikeda et al., 2007; Chapter II).

Fast-type $MYH_{M2528-1}$ and MYH_{M1034} were found to be expressed in larvae and adult fast and slow muscles (Table 3-2, Fig. 3-3 and Chapter II). $MYH_{M2528-1}$ and MYH_{M1034} belonged to a distinct subclade on phyogenetic tree along with embryonic fast-type $mMYH_{emb1}$ (Fig. 3-2) which is expressed in medaka from embryos to adult (Ono et al., 2006), suggesting that these MYHs perform wide functions in both embryos and in adult. In contrast, fast-type MYH_{M86-1}, slow-type MYH_{M8248} and slow/cardiac-type MYH_{M880} grouped in distinct clades were expressed in a tissue-specific manner in adult fast, slow and cardiac muscles, respectively (Fig. 3-3, Chapter II). MYH_{M743} and MYH_{M86-2} belonging to fast type and $MYH_{M2126-1}$ to cardiac type were expressed especially in embryos and larvae. (Fig. 3-3, Table 3-2, Chapter II). MYH_{M743} was monophyletic with embryonic fast-type MYHs such as mMYHL1, mMYHL2 and mMYHL3 which have been reported to be expressed in medaka embryos and larvae (Ono et al., 2006). Interestingly, MYH_{M86-2} was monophyletic with torafugu adult fast type MYH_{M86-1} which was found to be predominantly and specifically expressed in fast muscle of adult torafugu (Fig. 3-3 Chapter II), suggesting their distinct roles in embryos and larvae, and adult, respectively.

Section 2. Expression patterns of *MYH*s during development

The formation and growth of skeletal muscles in vertebrates proceed via successive phases of myogenesis that occur during embryonic and fetal development under the influence of various signals (Shi and Garry, 2006; Buckingham, 2007). However, spatial separation of fibers during embryonic development of fish ~~is~~ contrasts with mammals. In higher vertebrates, the first myotubes to form are known as primary fibers and these provide a framework along which remaining myotubes originate longitudinally and fuse to form the secondary fibers. A complex expression pattern of *MYH*s is observed during muscle fiber development. The primary muscle fibers initially express embryonic *MYH-emb*, perinatal *MYH-peri* (Weydert et al., 1987; Karsch-Mizrachi, et al., 1989), and cardiac *MYH-β* (Narusawa et al., 1987; Barbet et al., 1991), whereas the secondary fibers express embryonic, neonatal and fast *MYH*s, but not slow *MYH-β/I* (Barbet et al., 1991).

Fish are known to have more number of *MYH*s than their higher vertebrate counterparts (Gerlach et al., 1990; Kikuchi et al., 1999; Watabe and Ikeda, 2006; Ikeda et al., 2007). In this context, several *MYH*s have been cloned from torafugu embryos and larvae (Section 1). However, except for two *MYH*s, fast-type MYH_{M743} and slow-/cardiac-type MYH_{M5} (Ikeda et al., 2007), the expression of other torafugu *MYH*s during development remained unknown.

In the present section, cDNA libraries prepared from torafugu embryos and larvae were screened to determine frequencies of cDNA clones encoding each *MYH*. Furthermore, expression analysis of *MYH*s was carried out by RT-PCR and *in situ* hybridization.

Materials and methods

Fish

Artificially fertilized eggs of torafugu were collected from Oshima Fisheries Hatchery, Nagasaki, Japan, brought to The University of Tokyo, and treated as described in material and methods of section 1. About 100 embryos each from 1 to7 dpf and an equal number of larvae from 8, 10 and 16 dpf were collected and snap-frozen in liquid nitrogen (-196°C) and kept at -80°C for cDNA cloning of *MYH*s.

50 individuals each from embryos at 3, 4, 5 dpf were fixed in PFA overnight at 4°C and stored in absolute methanol at -20°C for whole mount *in situ* hybridization analyses.

A laboratory-reared specimen (sex unknown) of adult torafugu (275 g) was treated as described in material and methods of Chapter II. Fast, LS and ED slow skeletal muscles of the trunk were collected and snap-frozen in liquid nitrogen (-196°C) and kept at -80°C for RT-PCR.

Construction of cDNA clone libraries

*MYH*s were amplified by using MYH-specific degenerate primers as described in Section 1. The amplified DNA fragments were subcloned into pGEM-T vector (Promega) and sequenced with an ABI 3100 genetic analyzer (Applied Biosystems) after labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The frequencies of cDNA clones encoding *MYH*s were determined after randomly sequencing more than 40 cDNA clones from embryos at 3, 5 and 7 dpf, and larvae at 10, 16 dpf (Table 3-2).

RT-PCR

First strand cDNAs prepared using 5 μg of total RNAs from embryos at 1 to 7 dpf, larvae at 8, 10 and 16 dpf and fast, slow and cardiac muscles of adult torafugu (body weight 275 g) according to the procedure described in Section 1. RT-PCR was performed using gene specific primers (Table 3-1) that were designed based on the nucleotide sequences in the 3' UTR. PCR amplifications were carried out at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60-62°C for 30 s and extension at 72°C for 30 sec with a final extension step for 2 min. A single PCR reaction of 20 μl contained 1 μl each of forward and reverse primers (10 μmol 1^{-1}), 2 μl of cDNAs template (dilution 1:100), 2 μl of 10x PCR buffer (20 M Tris-HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1μl of 1 M dNTPs, 1 U *Ex Taq* DNA polymerase and 12.8 μl of sterilized water. The transcripts of β-actin (U38849) were used as the internal control for RT-PCR (Table 3-1).

Whole mount *in situ* hybridization

The cDNA fragments of about 250 bp which contain 3'UTR nucleotide sequences and those encoding a C-terminal part of MYH were subcloned into pGEM-T vector (Promega) (Table 3-1). DIG-labeled antisense riboprobes were synthesized using T7 and SP6 RNA polymerase (Roche Applied Science) according to method described in Section 2 of Chapter II. Whole mount single *in situ* hybridization was performed by the method of Westerfield (1993) using alkaline-phosphatase-conjugated anti-DIG antibody (1: 2000) (Roche Applied Science) and BCIP/NBT as a substrate (Roche Applied Science) or fast red

substrate (Roche applied science).

Whole mount double *in situ* hybridization was performed using DIG and fluoresceine labeled riboprobes and detection was performed by using AP conjugated anti-digoxigenin antibody (1: 2000) and anti-fluorescein antibody (1: 2000) by inactivating earlier antibody by low pH treatment according to the method of Jowett (2001). BCIP/NBT (Roche Applied Science) was used to obtain purple color, whereas fast red substrate (Roche Applied science) for red color.

Results

Frequencies of cDNA clones encoding sarcomeric *MYH*s were determined after random sequencing of at least 40 cDNA clones from libraries prepared from torafugu embryos (3, 5, 7 dpf) and larvae (10 and 16 dpf) (Table 3-2). Among all fast-type *MYH*s, cDNA clones encoding MYH_{M743} were most abundant in all libraries from embryos and larvae followed by those encoding for $MYH_{M86\text{-}2}$ in embryos at 5 and 7 dpf and $MYH_{M2528\text{-}1}$ in larvae at 10 and 16 dpf. Fewer clones encoding MYH_{M1034} were identified in the libraries from torafugu larvae (Table 3-2). While cDNA clones of slow/cardiac-type MYH_{M5} were found in every clone library from embryos and larvae, their abundance in larvae was found to be much less than in embryos (Table 3-2). Much less number of cDNA clones encoding cardiac-type $MYH_{M2126\text{-}1}$ was found in each of the cDNA clone libraries prepared from embryos and larvae.

RT-PCR using highly specific primers based on the 3'UTR sequences of *MYH*s revealed their expression in development-dependent and tissue-specific manners (Fig. 3-3). The expression of fast-type MYH_{M743} appeared in embryos at 3

dpf and continued to be expressed through successive embryonic and larval development. This result was almost consistent with those reported by Ikeda et al. (2007), who studied the expression of MYH_{M743} by *in situ* hybridization and RT-PCR analysis. The transcripts of fast-type $MYH_{M86\text{-}2}$ were found to appear in embryos at 4 dpf and continue to be expressed in later embryonic and larval development. No transcripts of MYH_{M743} and $MYH_{M86\text{-}2}$ were detected in adult fast, slow and cardiac muscles of torafugu (275 g body weight), suggesting their important role especially in embryonic and larval development of torafugu (Fig. 3-3). The transcripts of another fast-type $MYH_{M2528\text{-}1}$ appeared in embryos at 7 dpf stage and continued to be expressed in successive embryonic and larval stages as well as in adult fast and slow skeletal muscles. The transcripts of slow/cardiac-type MYH_{M5} were continued to be expressed from embryos at 3 dpf to larval and adult slow and cardiac muscles. Such expression patterns of $MYH_{M2528\text{-}1}$ and MYH_{M5} in adult skeletal muscles were consistent with our previous data (Chapter II).

The transcripts of fast-type $MYH_{M86\text{-}2}$ and cardiac-type $MYH_{M2126\text{-}1}$ were localized by *in situ* hybridization analyses. The transcripts of $MYH_{M86\text{-}2}$ were almost undetectable in embryos at 3 dpf and became abundant in whole myotomal compartment of somites in embryos at 4 dpf (Fig. 3-4A-C). The transcripts of cardiac-type $MYH_{M2126\text{-}1}$ were localized apparently adjacent to the presumptive notochord of embryos at 3 dpf (Fig. 3-4D). However, no transcripts of $MYH_{M2126\text{-}1}$ were detected in developing cardiac precursor cells (data not shown).

Whole mount double *in situ* hybridization with probes for fast-type $MYH_{M86\text{-}2}$ and slow/cardiac-type MYH_{M5} revealed their spatio-temporal expression in embryos at 4 dpf (Fig. 3-5). The transcripts of MYH_{M5} were restricted to the

superficial slow cells as well as in the horizontal myoseptum, whereas the transcripts of $MYH_{M86\text{-}2}$ were localized in the epaxial and hypaxial domains of embryonic myotome (Fig. 3-5). However, cells that possibly expressed transcripts of both genes in the horizontal myoseptum as well as superficial slow muscle were not resolved by the current method.

In larvae at 10 dpf (hatching-stage), the transcripts of fast-type MYH_{M743} were localized to epaxial and hypaxial domains of the myotome, and eye and pectoral fin muscles, but not to slow muscle at the horizontal myoseptum (Fig.3-6 A-C, J). At the same stage, the transcripts of $MYH_{M86\text{-}2}$ were also found throughout the myotome and in pectoral fin muscles, but not in eye muscles (Fig.3-6 D-F, K), suggesting co-expression of two fast-type *MYH*s in the larval myotome and pectoral fin muscles. Interestingly, the transcripts of fast-type $MYH_{M2528\text{-}1}$ were specifically localized to fibers at the dorsal and ventral extremes in the larval myotome along with some craniofacial muscles in head (Fig.3-6, G-I).

Section 3. Discussion

In the present study, expression patterns of multiple sarcomeric *MYH*s were cloned in early developmental stages of torafugu (Table 3-2). While cDNAs encoding four *MYH*s cloned from embryos, six were cloned from in larvae. These included four fast-type *MYH*s, MYH_{M743}, $MYH_{M86\text{-}2}$, $MYH_{M2528\text{-}1}$ and MYH_{M1034}, cardiac-type $MYH_{M2126\text{-}1}$ and slow/cardiac-type MYH_{M5} (Fig. 3-1). Except for two MYHs, MYH_{M743} and MYH_{M5} whose expressions were investigated previously by Ikeda et al. (2007), the expressions of other genes was newly determined in the present study in embryonic and larval development.

Among fast-type *MYH*s, the expression of MYH_{M743} appeared at the somite formation stage (about 10-14 somites) which corresponds to 3 dpf, whereas the transcripts of fast-type $MYH_{M86\text{-}2}$ appeared later in developing embryos at 4 dpf (about 20-24 somites). MYH_{M743} was found to be predominantly expressed in embryos and larvae as revealed by cDNA library analysis (Table 3-2), whereas cDNA clones encoding $MYH_{M86\text{-}2}$ were less abundant in larvae than in embryos possibly due to the down regulation in larval development (Table 3-2, Fig. 3-3). Importantly, the transcripts of MYH_{M743} and $MYH_{M86\text{-}2}$ were restricted to embryos and larvae and not observed in adult skeletal muscles (Fig. 3-3), further suggesting their functional roles in early development of torafugu. Previously, the expression of embryonic *MYH*s such as *Eggs22* and *Eggs24* and their homologues, *MYHemb1* and *MYHemb2*, has been reported in embryonic fast fibers of common carp (Ennion et al., 1999; Nihei et al., 2006). The transcripts of *Egg22* and *Egg24* were detected in the developing myotomal muscle from 25 hpf and also in the developing pectoral fine muscle in post-hatching embryos (Ennion et al., 1999). Similarly, the expression

patterns of $mMYH_{L1}$ and $mMYH_{L2}$ have been demonstrated in the epaxial and hypaxial domains of medaka embryos at 4 dpf and found to be differentially expressed in cranial and pectoral fin muscles of hatching embryos (Ono et al., 2006). In this regard, it is interesting to investigate whether the transcripts of MYH_{M743} and $MYH_{M86\text{-}2}$ are also present in developing craniofacial and pectoral fin muscles of torafugu.

The transcripts of $MYH_{M86\text{-}2}$ were localized to the epaxial and hypaxial compartments throughout embryonic myotome; however, slow muscle precursors at prospective horizontal myoseptum also likely contain the transcripts $MYH_{M86\text{-}2}$ (Fig. 3-4F and Fig. 3-5F). Such high levels of transcripts of MYH_{M743} have been also noticed by Ikeda et al. (2007) in embryos at the same developmental stage, suggesting that either embryonic fibers are likely to have the transcripts of two *MYH*s. Such co-expression also evidenced in fast muscles of larval myotome and in the muscles at pectoral fins (Fig. 3-6). However, the functional importance of presence of two MYH-transcripts in single fiber remained to be understood.

The cDNA clones encoding the other fast-type *MYH*s, MYH_{M2528} and MYH_{M1034}, were found in the clone libraries from torafugu larvae (Table 3-2). Furthermore, the transcripts of $MYH_{M2528\text{-}1}$ were found to appear during late embryogenesis (7 dpf) as revealed by RT-PCR (Fig. 3-3). Unfortunately, RT-PCR failed to amplify the transcripts of MYH_{M1034} from cDNAs of torafugu embryos and larvae (data no shown). Previously, the new fibers generated by adult specific hyperplasia in trunk fast skeletal muscle of torafugu were found to express $MYH_{M2528\text{-}1}$ (Chapter II). In mammals, injury to skeletal muscle tissues, whether provoked experimentally or occurring as a part of a disease process, leads to the

degeneration and then the regeneration of muscle fibers (for review, see Schiaffino and Patridge, 2008). Several reports claim that the regenerating fibers contain embryonic and neonatal myosin isoforms (Sartore et al., 1982; Matsuda et al., 1983; Whalen et al., 1990). Earlier, Ennion et al. (1995) showed that new fibers generated by hyperplasia tend to express distinct *MYHFG2* in adult fast skeletal muscle of common carp. However, the transcripts of *MYHFG2* were undetected during development as well as in slow skeletal muscles of carp in a sharp contrast with the expression of torafugu $MYH_{M2528\text{-}1}$. Recently, Johnston et al. (2009) reported the up-regulation of fast-type *myhz1* in small diameter fibers of zebrafish skeletal muscle that had been still recruiting the myotubes. The present finding regarding the expression of $MYH_{M2528\text{-}1}$ in embryonic development has a high significance with regards to the establishment of hypothesis that the fibers generated during hyperplastic growth in fast skeletal muscle of fish initially tends to express embryonic fast-type *MYH*. Such finding in torafugu suggests the equivalent mechanism by which expression regulation is likely to work in fish, as a part of fiber generation.

Interestingly, the transcripts of $MYH_{M2528\text{-}1}$ were localized to the dorsal and ventral extremes in the larval myotome (Fig. 3-6). The dorsal and ventral extremes of the larval myotome are considered as the main growth engines for the recruitment of new fibers by stratified hyperplasia (Rowlerson and Veggetti, 2001; Stenibacher et al., 2007). During stratified hyperplasia, fibers are also recruited into layers between superficial slow and lateral fast muscles in fish larvae (Rowlerson and Veggetti, 2001; Stenibacher et al., 2007). However, the transcripts of $MYH_{M2528\text{-}1}$ were absent in these region, suggesting that new fibers between fast and slow muscles are yet to

be recruited at hatching stage. Earlier, we found that fibers located between lateralis supercficialis slow and fast muscles with an intermediate oxidative potential contained the transcripts of $MYH_{M2528\text{-}1}$ (Fig.1-3 and Fig. 2-7). Such fibers may appear either in late larval development (Scappolo et al., 1988) or at the onset of exogenous feeding where intermediate fibers appear (van Raamsdonk et al., 1982). It is interesting to examine the expression of $MYH_{M2528\text{-}1}$ in fibers which are generated by the stratified and mosaic hyperplasia in torafugu, where different genes are supposed to participate. Thus, availability of whole genome database, large final body size and complete *MYH* repertoire, torafugu could be considered as an ideal fish for studies on the mechanisms underlying post embryonic muscle growth.

Finally, several *MYH*s were cloned in the present study and their expression patterns were studied by RT-PCR and highly sensitive *in situ* hybridization that further suggested their developmental-specific and spatio-temporal expressions. This would greatly help to understand functional significance of a higher number of MYHs in fish and also to study the mechanisms involved in their expression switch with regards to diverse physiological stimuli.

CHAPTER IV

Characterization of paired box protein genes as myogenic precursor cell markers in torafugu

Skeletal muscle fibers are formed throughout the vertebrate life span, during either embryonic development or in regeneration; however, morphogenesis occurs during prenatal development in successive, distinct and overlapping steps involving different types of myoblast (Biressi et al., 2007; Messina and Cossu, 2009). With the exception of some craniofacial muscles, all skeletal muscles in vertebrate body are known to derive from progenitors present in somites (Christ and Ordahl, 1995). Somites are transient mesodermal units, which form in a rostro-caudal succession by segmentation of the paraxial mesoderm on both sides of neural tube. Each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome from which myogenic precursors originate. In mouse, at embryonic day E8.75, shortly after the onset of somitogenesis, some myogenic precursor cells give rise to the formation of terminally differentiated mononucleated muscle cells (myocytes) of the primary myotome. Primary myotome formation consists of a multistep process in which precursors also translocate from dermomyotome to a ventrally located domain where they elongate along the axis of embryos to span the entire somite length (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Denetclaw et al., 2001; Ordahl et al., 2001; Gros et al., 2005).

In higher vertebrates, distinct subpopulation of myoblasts is involved in different phases of myogenesis such as embryonic, fetal and adult skeletal myogenesis. While embryonic and fetal myoblasts contribute to the formation of the primary and

secondary fibers, satellite cells are involved in post natal growth and repair of skeletal muscle. Such myoblasts differ in morphology of myotubes they generate *in vitro*, and in the MYHs and muscle enzymes that they express (Bonner and Hauschka, 1974; Barbieri et al., 1990; Pin and Merrifield, 1993; Zappelli et al., 1996; Ferrari et al., 1997). They also differ with respect to media requirements (White et al., 1975), integrin–extracellular matrix interactions (Blanco-Bose and Blau, 2001), resistance to inhibitors of myogenesis such as phorbol esters (Cossu et al., 1988) and transforming growth factor beta (TGF-β) (Cusella-DeAngelis et al., 1994), and in the expression of number of genes that are involved in development and physiology of skeletal muscle (Birresi et al., 2007).

Myogenesis of fish has some unique features when compared to mammals, which includes spatial separation of fast and slow muscle precursors during somite formation, and generation of muscle fibers throughout development and growth (Devoto, 1996; Rowlerson and Veggetti, 2001). Fish are also controlled over their internal environment than mammals and in nature are subjected to marked seasonal fluctuations in food supply with conditions for growth varying throughout the year as well as in environmental temperatures which have profound consequences for the genetic mechanisms regulating myogenesis (Watabe, 2002; Johnston, 2006). At least three phases of myogenesis have been defined in fish which include embryonic, postembryonic stratified hyperplasia and mosaic hyperplasia (Rowlerson and Veggetti, 2001; Johnston, 2006). During stratified hyperplasia, new fibers are formed either mainly at the dorsal and ventral extreme of myotome, or in addition in a layer between the superficial muscle layer and the deeper fast fibers, whereas mosaic hyperplasia is distributed throughout myotome of adult skeletal muscle (Rowlerson

and Veggetti, 2001). Injury to the fish muscle also results in a new wave of myotube production to repair the damage even if muscle recruitment has stopped (Rowlerson et al., 1997). The distinction of embryonic, stratified and mosaic hyperplasia suggests that there must be distinct mechanisms underling development of muscle fibers and involvement of distinct muscle precursor cells.

The identification of muscle precursor cells is a key in determining the early events in the myogenesis and fiber generation in adult muscle. Traditionally, antibodies to the thymidine analogue, bromodeoxyuridine (BrdU) (McCormick and Schutz, 1992; Rowlerson et al., 1995) and proliferating nuclear cell antigen (PCNA) (Rowlerson and Veggetti, 2001) have been used to detect proliferating muscle precursor cells. Immunohistochemical and *in situ* hybridization analyses to detect muscle specific genes such as *MyoD* and *myogenin* have been used for the identification of muscle precursor cells since they are expressed during activation, differentiation and maturation of myotube (Fuchtbauer et al., 1992; Sasson, 1993). During myogenesis, desmin is expressed within undifferentiated muscle progenitor cells before myofibrillar proteins such as titin, skeletal muscle actin, MYHs and nebulin, and commonly used for the identification of progenitor cells (Hill et al., 1986). Desmin is found in the intermediate filaments of cytoskeleton of muscle cells (Lazarides and Hubbard, 1976), but its precise function in muscle progenitor cells is still unknown. A number of factors that have restricted expression in satellite cells serve as markers for their identification which include cell surface receptors and adhesion proteins such as c-met, m-cadherin (Cornelison and Wold, 1997) and cd34 (Lee et al., 2000), and transcription factors such as Foxk1 (Gary et al., 2000), Pax3/Pax7 (Relaix et al., 2005) and sox8/sox15 (Lee et al., 2004).

In order to identify muscle precursor population during development as well as in adult skeletal muscles, the present study was carried out to screen markers such as Pax3 and Pax7 during development of torafugu.

Section 1. Characterization of Pax3 and Pax7 genes and their expression patterns during torafugu development

Paired box protein genes play key roles in the formation of tissues and organs during development. This gene family encodes transcription factors that are characterized by the presence of paired box domain (PD), a octapeptide motif and homeodomain (Chi and Epstein, 2002; Lang et al., 2007). Based on some or all of the above features and sequence homologies between PD, four subfamilies of paralogous *Pax*s have been distinguished that include *Pax1/9*, *Pax2/5/8*, *Pax3/7* and *Pax4/6*, the last two subfamilies containing both PD and homeodomain (Gruss and Walther, 1992).

Pax3 and Pax7 have important roles in the formation of neural tube, neural crest formation (Mansouri and Gruss, 1998) and paraxial mesoderm formation (Goulding et al., 1991; Schubert et al., 2001) as well as myogenesis especially in regulating the survival, proliferation and migration of myogenic precursor cells and the formation of dermomyotomal lips (for details see Relaix, 2004; Buckingham and Relaix, 2007). During mouse embryonic development, *Pax3* is expressed in PSM as somites are formed and progressively restricted first to dermomyotome and later to dorsomedial and ventromedial lips, whereas *Pax7* expression is restricted to central region of the dermomyotome (Kassar- Duchossoy et al., 2005; Relaix et al., 2005). *Pax7* continues to be expressed in fetal and adult satellite cells, although few *Pax3* positive satellite cells also exist in adult skeletal muscles of mouse (Relaix, 2006). Recently, dermomyotome have been described in embryonic development of teleosts and indeed cells previously termed as “external cells” have the morphological and

molecular characteristics of dermomytome (Devoto et al., 2006; Stellabotte and Devoto, 2007). These cells are labeled for Pax3 and Pax7-specific antibodies in fish such as sturgeon *Acipenser ruthens* (Devoto et al., 2006), zebrafish (Devoto et al., 2006; Hollway et al., 2007; Stellabotte et al., 2007), cichlid *Astatotila burtoni* (Devoto et al., 2006), and common whitefish *Coregonus lavaretus* (Kacperczyk et al., 2009). Thus, Pax3 and Pax7 might play a role in the specification of muscle precursor cells in development of fish.

Pax3 is sufficient to induce MyoD and Myf5 *in vitro* (Maroto et al., 1997) and directly binds with enhancers of *Myf5* (Bajard et al., 2006) and *MyoD* (Hu et al., 2008). Pax7 directly regulate *Myf5* in the myoblast derived from adult satellite cells by the recruitment of a histone methyltransferase complex (McKinnell et al., 2008). Mammals and birds have a single gene each for Pax3 and Pax7, whereas in zebrafish, a single *Pax3* and at least four *Pax7* variants were reported, one of which encodes a protein with the sequence close to that of mammalian *Pax7* (Seo et al., 1998). Alternatively, spliced variants of *Pax3* and *Pax7* have been reported in mouse and human, although their exact functions remain poorly understood (Vogan et al., 1996; Ziman et al., 1997; Barber et al., 1999). Recently, cDNA clones encoding novel *Pax7* variants have been isolated from brain and skeletal muscle of tetraploid Atlantic salmon *Salmo salar* (Gotensparre et al., 2006).

In this regard, information regarding *Pax3* and *Pax7* in vertebrate genome model torafugu has remained unknown. In the present study, the partial cDNAs encoding *Pax3* and *Pax7* were cloned from embryos and fast skeletal muscle of torafugu and their expression patterns were investigated during different stages of development.

Materials and methods

Fish

Artificially fertilized eggs of torafugu were collected from Oshima Fisheries Hatchery, Nagasaki, Japan, and brought to The University of Tokyo, where they were reared in a 500 L tank in seawater at 18°C. About 100 individuals each from embryos at 1 to7 dpf and an equal number of larvae at 8, 10 and 16 dpf were collected, snap- frozen in liquid nitrogen and kept at -80°C for cDNA cloning and RT-PCR. Fast and slow skeletal muscles of the trunk was obtained from adult torafugu (275 g body weight) and used for cDNA cloning.

cDNA cloning of *Pax3* and *Pax7*

Total RNAs were prepared from each developmental stage of torafugu by using ISOGEN (Nippon Gene) and 5 μg of total RNAs each from embryos at 3 dpf and adult fast muscle were reverse-transcribed by using oligonucleotide (dT)-tailed primer (Table 4-1) and superscript reverse transcriptase III™ (Invitrogen) according to the manufacture's instructions. Degenerate forward primer (CATCCGGCA CAAGATCgtngaratggc) corresponding to the conserved amino acids (IRHKIVEMA) in paired box domain and reverse primer (GCCGGG CAGCAGGt grttraangc) to the amino acids (AFNHLLPG) at homeodomain of Pax proteins were used to amplify cDNAs of *Pax3* and *Pax7* from embryos at 3 dpf and adult fast muscle of torafugu. The amplified DNA fragments were subcloned into pGEM-T vector (Promega) and sequenced with an ABI 3100 genetic analyzer (Applied Biosystems) after labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

5’RACE-PCR was performed to determine nucleotide sequences in the 5’ UTR and those at the N-terminal end of Pax3 and Pax7 by using a gene-specific forward primer (Table 4-1) and AP (Table 2-1) as described previously. The amplified cDNA fragments were sequenced as described above.

Genomic analysis

The genomic organization of torafugu *Pax3* and *Pax7* were determined by comparison of cDNA and genomic sequences using ensemble genome browser (http://www.ensembl.org/Fugu_rubripes) and transcript predictions by GeneBuild. Syntenic relationships between torafugu *Pax3* and *Pax7* with corresponding genomic regions of green spotted puffer *Tetraodon nigroviridis*, medaka, zebrafish, mouse and human orthologues were analyzed with BioMart data mining tool (Ensembl).

RT-PCR

First strand cDNAs were prepared using 5 μg of total RNAs from embryos at 1 to 7 dpf, larvae at 8, 10 and 16 dpf and fast, slow and cardiac muscles of adult torafugu (body weight 275 g), according to the procedure described above. RT-PCR was performed using gene specific primers (Table 6) that were designed based on the nucleotide sequences in the 5’ UTR. PCR amplifications were carried out at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 sec with a final extension step for 2 min. A single PCR reaction of 20 μl contained 1 μl each of forward and reverse primers (10 μM), 2 μl of cDNAs template (dilution 1:100), 2 μl of 10x PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$), 1 μl of 1 M dNTPs, 1 U *Ex Taq* DNA polymerase

and 12.8 μl of sterilized water. Elongation factor one alpha (EF-1α) (AB193485) was used as the internal control in RT-PCR (Table 4-1).

Results

RT-PCR with the degenerate primers based on conserved amino acids at PD and homeodomain resulted in the amplification of DNA fragments about 600 bp from torafugu embryos at 3 dpf and adult fast skeletal muscle (data not shown). Blast search by using the partial cDNA sequences of *Pax3* and *Pax7* against the torafugu genome database (Ensembl release FUGU 4.0) led to the identification of two genomic sequences each for *Pax3* (*Pax3a*, scaffold_123; *Pax3b*, scaffold_73) and *Pax7* (*Pax7a*, scaffold_56; *Pax7b*, scaffold_382). The nucleotide sequences in the above scaffolds in the torafugu genome database encode putative full-length ORFs for all of the above genes except for *Pax7b* according to *in silico* predictions by genebuild (Ensembl) (Fig. 4-1).

5'RACE-PCR determined the nucleotide sequences encoding the N-terminal end of torafugu Pax3 and Pax7 proteins. The partial deduced amino acid sequences of Pax3 and Pax7 has all the characteristics of Pax proteins which include PD containing 128-134 amino acids, homeodomain containing 60 amino acid, and an octapeptide located in the region separating the two conserved DNA binding domains (Figs. 4-2 and 4-3). The deduced amino acid sequence of Pax3a showed 84.6% identity with that of Pax3b, whereas the sequence of Pax7a showed 89.6% identity with those of Pax7b (Table 4-3 and 4-4). Comparison in the amino acid sequences of torafugu Pax3 and Pax7 with those of humans, mice and zebrafish showed a high sequence similarity (Table 4-3 and Table 4-4). As in the case of mammalian and zebrafish Pax3 proteins, torafugu Pax3 PD domain was highly conserved (more than 90% identity) and torafugu homeodomain was identical with those of zebrafish, mouse and human Pax3 proteins (Fig. 4-2). The overall identity of deduced amino

acid sequences of both torafugu Pax3 and Pax7 were more than 80% with those of other vertebrates (Table 4-3).

Glutamine residue (Q+) was present at position 75 (Glu75) from the N-terminus of PD of Pax3a, Pax7a and Pax7b, but not in the sequence of Pax3b where glutamine was replaced by proline (Fig. 4-2 and Fig. 4-3). This region of PD is also termed as a linker region (amino acid residues from 60 to 82) (Vogan et al., 1996). Alternative splice variants that lack this Glu75 (Q-) of Pax3 and Pax7 proteins have been identified in mouse as well as in zebrafish, although cDNA clones of Q-variant are less abundant (Vogan et al., 1996; Seo et al., 1999). Alternative splice variants form by inclusion or removal of Glu75 through alternative use of the CAG glutamine codon as a 3' splice acceptor (Fig. 4-1) (Vogan et al., 1996). In the present study, among the three cDNA clones of *Pax3a*, one clone had deletion in Glu75 described above but not in clones of Pax3b (9 clones), Pax7a (2 clones) and Pax7b (3 clones) (Fig. 4-4). In addition, the deletion of five amino acid residues (SGEAS) was found in PD of *Pax7a* with of torafugu, zebrafish and salmon Pax7 proteins (Fig. 4-3). However, a single cDNA clone of *Pax7b* showed deletion in the above amino acids (data not shown). Such deletion of SGEAS in PD of Pax7b was due to alternative splicing of splice donor site at the start of exon 4 (Fig. 4-1).

The genomic neighborhood of *Pax3* and *Pax7* were examined by the synteny analysis, revealing high syntenic relationship of torafugu *Pax3a* and *Pax7a* with the corresponding regions of mammals and other teleosts (Fig.4-5 A and C). *Pax3b* showed syntenic relationship only with the corresponding regions in other teleost (Fig.4-5 B). However, no syntenic relationship of *Pax7b* was found within fish and mammals (data not shown).

The expression patterns of *Pax3* and *Pax7* were investigated by RT-PCR using gene-specific primers based on 5'UTR nucleotide sequences (Table 4-1). The transcripts of *Pax3a* and *Pax3b*, *Pax7a* and *Pax7b* were found to appear in embryos at 3 dpf and continue to be expressed in until hatching (Fig. 4-6). The transcripts of *Pax7a* and *Pax7b* were continued to be expressed in larvae and importantly those of *Pax7b* were found in adult skeletal muscles (Fig. 4-6).

Section 2. Discussion

In the present study, cDNAs encoding *Pax3* and *Pax7* were cloned from embryos and adult fast skeletal muscles torafugu. Two types of cDNA clones encoding Pax3 and Pax7 showed a high identity in their deduced amino acid sequences (Table 4-3 and Table 4-4) and subsequent *in silico* analysis with fugu genome database found two separate genes each for *Pax3* (*Pax3a* and *Pax3b*) and *Pax7* (*Pax7a* and *Pax7b*) (Fig. 4-1).

Deletion in glutamine residue at the 75 amino acid from the N-terminus of PD was found for Pax3a (Fig. 4-1). However, no such deletion was observed in the amino acid sequences from the cDNA clones encoding *Pax7a* and *Pax7b*. PD of Pax3b had proline residue (Pro75) in this site. Mammalian Pax3 and Pax7 both have alternatively spliced isoforms, differing in the presence or absence of Glu75 (Q+/Q-) in the PD which influences the DNA-binding specificity (Vogan et al, 1996). Deletion of Glu75 (Q-) in the linker region of Pax3 and Pax7 PD unmasks DNA biding properties of the C-terminal subdomain of PD, whereas its addition (Q+) interferes with the DNA binding ability of C-terminal subdomain (Vogan et al., 1996). The occurrence of Q- relative to Q+ variant have been reported to be less in mouse (1:2) and zebrafish (2:3) Pax3 (Vogan et al., 1996; Seo et al., 1999). Further studies are required to investigate the presence of Pro75 in Pax3b PD on DNA binding properties.

Two adjacent alternative 3' splice acceptor sites were found for Q+ isoform (aagCAGGGA) and for Q- isoform (aagcagGGA) of torafugu Pax3a (Fig.4-1). Such a use of CAG codon for glutamine was consistent with the previous reports for mouse and zebrafish Pax3 (Vagan et al., 1996; Seo et al., 1999). Since the codon of

Glu75 for Pax7a and Pax7b is CAG, it is likely that torafugu Pax7a and Pax7b have Q- variant due to multiple splice sites. Interestingly, torafugu Pax7b variant had an insertion of five amino acid residues (GEASS) in the C-terminal part of PD in two out of three cDNA clones, but not for Pax7a (Fig. 4-3). Genomic analysis found two alternate splice donor sites at exon 4 of *Pax7b* which was responsible to form two alternately spliced variants (Fig. 4-1). Such insertion of amino acids at the same position has been reported for zebrafish and Atlantic salmon Pax7 variants (Seo et al., 1999; Gotensparreet al., 2006). An insertion of two amino acids, glycine and leucine, in the C-terminal part of PD has been reported for human Pax7 (Fig. 4-3) (Schafer et al., 1994). However, it was suggested in human that such insertion does not exhibit any differences in DNA binding (Schafer et al., 1994). In this context, it is interesting to investigate the functional role having an insertion of five amino acids in fish Pax7.

Mammals have single genes for *Pax3* and *Pax7*, whereas in protostomes, ascidians and amphioxus, a single ancestral gene is retained as cognates of vertebrate *Pax3* and *Pax7*. Separation of two genes is thought to appear in vertebrates by duplication of the ancestral gene; however, it is still unclear when such event occurred during vertebrate evolution (Kusakabe and Kuratani, 2005). Two distinct genes each for *Pax3* and *Pax7* in torafugu and in other fish possibly arose after additional round (3R) of whole genome duplication (WGD), which possibly occurred around 226-315 million years (Myr) ago (Hurley et al., 2007; Van de Peer et al., 2009). The genomic neighborhood of *Pax3* and *Pax7* were examined by the synteny analysis, revealing high syntenic relationship of torafugu *Pax3a* and *Pax7a* with corresponding genomic regions of mammals and other teleosts (Fig.4-5 A and C). *Pax3b* showed syntenic relationship with the corresponding region in other teleosts

(Fig.4-5B). However, no syntenic relationship of *Pax7b* was observed with other animals (data not shown), possibly due to divergent evolution.

Analysis of distribution patterns of *Pax3* and *Pax7* transcripts were investigated by RT-PCR during torafugu development (Fig. 4-6). The transcripts of *Pax3a*, *Pax3b*, *Pax7a* and *Pax7b* were found to appear in embryos at 3 dpf. Interestingly, the transcripts of *Pax7a* and *Pax7b* continued to be expressed in larvae, suggesting that they might regulate muscle precursor cells distinctly during different phases of muscle growth in torafugu. In zebrafish, *Pax3* transcripts are detected in embryos at neural plate stage and expression of *Pax7* starts later after neurulation is completed (Seo et al., 1999). External cell layers which are proposed to have dermomyotome-like function in fish also accumulate *Pax3* and *Pax7* transcripts (Devoto et al., 2006; Hollway et al., 2007; Stellabotte et al., 2007; Kacperczyk et al., 2009). Recently, Gotensparreet et al. (2006) reported that the transcripts of *Pax7* were present in the muscle precursor cells (MPCs) located at the periphery of juvenile/adult fast muscle of Atlantic salmon. In this regard, the transcripts of torafugu *Pax3* and *Pax7* could be used for further investigation as a marker for identification of muscle precursor cells during different phases of growth.

CHAPTER V

General discussion

The molecular, genetic and cellular bases for skeletal muscle growth and regeneration have been documented in a number of vertebrate species. Formation of skeletal muscle of fish differs in several aspects as compared with mammals. These include the spatial separation of fast and slow muscle precursor cells during somite formation in embryo (Deovoto et al., 1996) and the generation of muscle fibers during postembryonic growth phases by hyperplasia (Rowelerson and Vigeetti, 2001). Fish muscle growth occurs during distinct phases of myogenesis which include embryonic, post embryonic stratified hyperplasia and mosaic hyperplasia (Rowelerson and Vigeetti, 2001; Johnston et al., 2006). During stratified hyperplasia, new fibers are formed either mainly at dorsal and ventral extremes of myotome or in addition in a layer between the superficial muscle layer and deep fast fibers, whereas mosaic hyperplasia is distributed throughout myotome in adult skeletal muscle. This mosaic hyperplasia generally occurs in fish grows to large final size and is therefore extremely important for commercial aquaculture species.

Torafugu *Takifugu rubripes* is one of the most popular and high-valued marine fish in Japan (Kikuchi, 2006; Kikuchi et al., 2006). Torafugu is relatively a large marine fish that grows up to 70 cm in length. Recently, Fernandes et al. (2005) have reported that the number of fibers in fast muscle increased by hyperplasia until torafugu grew to 1.2 kg body weight and subsequent growth was accomplished by hypertrophy alone. New fibers and the age at the onset of mosaic hyperplasia vary widely between species (Rowelerson and Vigeetti, 2001). Less has been studied

especially the onset of mosaic hyperplasia in adult torafugu. In the present study, fiber types were investigated by histochemical methods in fast and slow muscles of adult torafugu (body weight 290 g). Fast-twitch fibers with small diameter that are known to be generated by hyperplasia showed higher stability towards acidic pH in their myofibrillar ATPase than those with large diameter. Such higher stability was possibly due to the presence of distinct MYH in fast-twitch fibers with small diameters.

Morphological, functional and metabolic characteristics of skeletal muscles are mainly related to the cellular expression of different MYHs and muscle fibers are characterized mainly based on MYHs they contain (Cobos et al., 2001). Meanwhile, fish are known to possess highly conserved MYH multigene family, although *MYH*s are much more than their higher vertebrate counterparts (Gerlach et al., 1990; Kikuchi et al., 1999; Watabe and Ikeda, 2006; Ikeda et al., 2007). However, functional implications for such a high number of *MYH*s have been less understood. In this context, the expression patterns of seven sarcomeric *MYH*s were determined in adult torafugu fast, slow and cardiac muscles. While three *MYH*s were specifically expressed in each of the muscles examined, the remainder showed a mixed expression pattern in fast, slow and/or cardiac muscle, suggesting their functional significance. Interestingly, the transcripts of *MYH*s were localized in a fiber-type specific manner in adult skeletal muscles. This is an important step ahead in understanding fiber type diversity and associated muscle growth in adult torafugu.

Muscle development in fish embryos has been elegantly shown by the transcripts of *MYH*s (Rescan et al., 2001; Bryson-Richardson et al., 2005; Nihei et al., 2006; Ono et al., 2006, Elworthy, et al., 2007). However, *MYH*s contributing to muscle

development in torafugu embryos and larvae have remained unknown. In this regard, multiple sarcomeric *MYH*s were cloned from torafugu embryos and larvae in the present study. Their expression patterns were studied by highly sensitive *in situ* hybridization. Expression patterns of some *MYH*s were restricted to embryos and larvae, whereas others were found to be tissue-specific in adult muscles. Interestingly, *MYH*s that showed mix expression patterns in adult skeletal muscles as well as in cardiac one, were found to be expressed in embryos and larvae. This would greatly help to understand evolutionary and functional significance of a higher number of *MYH*s in fish and also to study the mechanisms involved in their expression.

The distinction among embryonic, stratified and mosaic hyperplasia in fish suggests distinct mechanisms underling muscle development and involvement of distinct muscle precursor cells. Therefore, the identification of muscle precursor cells is a key factor in determining early events in myogenesis and fiber generation in adult muscle. Pax3 and Pax7 play an important role in regulating survival, proliferation, and migration of myogenic precursor cells (Relaix, 2004; Buckingham and Relaix, 2007). In this context, two genes each encoding Pax3 and Pax7 were cloned from embryonic and adult fast skeletal of muscle torafugu. In common with alternatively spliced variants of mammalian Pax3 that affect DNA-binding property, torafugu Pax3a has equivalent splice variants, suggesting their conserved function in torafugu. During mouse embryonic development, *Pax3* is expressed in PSM upon the formation of somites and progressively restricted first to dermomyotome and later to dorsomedial and ventromedial lips, whereas *Pax7* expression is restricted to central region of dermomyotome (Kassar- Duchossoy et al., 2005; Relaix et al., 2005). Although, the transcripts of torafugu *Pax3* and *Pax7*

appeared in embryos and larvae, only *Pax7b* was found to be expressed in adult skeletal muscles, suggesting that they have distinct functions in muscle precursors cells that might contribute to different phases of muscle growth.

Finally, the present study provides greater insight into complex regulation of *MYH*s in different muscle types of torafugu as well as in embryonic and larval development. The transcripts of these *MYH*s demonstrated the existence of distinct fibers types which is also evidenced by conventional histochemical methods. Less has been understood regarding distinct populations of muscle progenitor cells that might contribute differentially to muscle growth from embryo to adult. In this regard, it is interesting to investigate the existence of such muscle precursor populations in fish development. In particular, future investigation regarding roles of multiple *Pax* in fish can help to understand genetic requirements of fish muscle precursor cells.

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Table 1-1. Stabilities of myofibrillar ATPase in torafugu skeletal muscles in relation to preincubation at different pH.

Skeletal muscle	Preincubation for 2-3 min				
	pH 4.3	pH 4.6	pH 9.4	pH 10.0	pH 10.6
Fast	-	-,+,++	++	-	-
LS slow	-	+,++	++	-	-
ED slow	-	+,++	++	-	-

-, unstained; +, weakly stained; ++, intensely stained

Table 2-1. List of primers used in Chapter II

Experiment	Name	Nucleotide sequence (5'------ 3')*	Length (base)
cDNA clone library	MYH-F	GGH GAC CTS AAY GAR ATG GA	20
	MYH-R	TTC AGY TCC TCD GCC ATC ATG G	22
	Oligo (dT) primer	CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC$(T)_{17}$	52
3'RACE	M86-1 F	CAA TGG TAG AAC GTA GAA ATG GTC	24
	M1034 F	GCA CTC TCA GAA CAC CAG CCT T	22
	M2528-1F	AGC TGG AAG GTG ACC TTG TTC AGG	24
	M2126-2F	GCA CTC ACA GAA TAC AGC CTT GA	23
	M8248F	GCA CTC ACA GAA CAC TAG TTT ACT G	25
	M5F	GTT GCA CTC TCA GAA CAC ATC TT	23
	M880F	GCC AAG AAA GCC ATC AAT GAT GCT G	25
	AP	CCA GTG AGC AGA GTG ACG	18
Determination of full-length cDNA encoding MYH_{M86-1}	MYH-F1	GAY AAR ATT GAG GAC ATG GCV ATG	24
	M86-1R	AGG AGA CCC CAG TCA CCA TT	20
	IFP1	GTA AGA AAG CAG AGC AAG GC	20
	IRP1	GCT GTC CCT GGA CAT TTC TC	20
	IFP2	TGA TGC ATC ATG GTA CCA	18
	IRP2	TCA TCT TGA AGA GTT CAG	18
	IFP3	CCT GGA GCA AGA GGA GTA CAA	21
	IRP3	GTG TCT CTT AAG TTC CTC AAT	21
	IFP4	TGA TTC CCA ATG AGT CCA AGA	21
	IRP4	TTG GCC CGA GCA GCT CTC TCA	21
	IFP5	CTG GTC TGC TGG GTA CCC TGG A	22
	IRP5	CTT CTC CTT GGT TAA CTT GGC AAT	24
	M 86-1 F	GGG AAG TCT GAT TCT GCC GAG T	22
	M86-1R	TTA GAG ATA AAG TGA TGA TCT TT	24
	M1034 F	GTC AAG GTC AAG ACT ACA GAA TCA	24
	M1034 R	TGT TAC ATC AGG ACA CAA TAA A	22

Dot blot and Northern blot hybridization	M2528-1F	TCG GCA GGG GAA AGG ACG CTG AAG	24
	M2528-1R	TTG CGT ATA GAT TTA TTG CAC TT	23
	M2126-2F	GGA GCT TGC AGA AAT CCC TTT GCC	24
	M2126-2R	CTG GAG CAC TAC AGG TTG TGA A	22
	M8248 F	AGG CGA ATG AAG CAC GAA GAT GAA	24
	M8248 R	TTC TAC TGA ACA ACT GAC ATG C	22
	M5 F	GCG ACC AAG GCA GCA AGC TGG CT	23
	M5 R	CAG CTG TGC TAA ACT GTG AGA A	22
	M880 F	CTG AAG GCC AAA ACC CGG GAC GGT	24
	M880 R	TTA GAC TCA AAA TGT GAG GC	20
	18S rRNA F	GCA ATT ATT TCC CAT GAA CGA GGA A	25
	18S rRNA R	GAT CGT CTT CTC GGC GCT CCG CCA	24
	α-actin F	CGT CCA CAG GAA GTG CTT CTA AGC	24
	α-actin R	GGT AAA CAA AGA CAA GAA TTC GTG C	25
In situ hybridization	M86-1	CAC ATG TCC AGG CTG AGG AAG GTT	24
	M86-1	AGA GAT AAA GTG ATG ATC TTT ATT G	25
	M2528-1 F	TGA CCA GGT ACA GGA AGG TG	20
	M2528-1 R	TTG AAT TGC GTA TAG ATT TAT TGC	24
	M2126-2 F	TGA GGA GGA CCG CAA GAA TGT TGC	24
	M2126-2 R	TGC AGC ACA ATC ACC AGA ATT TAT TA	26
	M8248 F	TCG TAA AAA TCT TTC ACG TCT GCA A	25
	M8248 R	GAT ATG CAG ATT CTT TTT ATT CTA C	25

*Single-letter codes in primers: D, G+A+T; H, A+C+T; R, A+G; S, C+G; V, G+A+T; Y, C+T.

Table 2-2. List of *MYH*s cloned from fast, slow and cardiac muscles of adult torafugu

MYH type*		Muscle				Accession No.
		Fast	LS slow	ED slow	Cardiac	
Fast	$MYH_{M86\text{-}1}$	40				AB465004
	$MYH_{M2528\text{-}1}$	6	8	9		AB465006
	MYH_{M1034}	4	10	10		AB465005
Slow	MYH_{M8248}		10	8		AB465007
Cardiac	$MYH_{M2126\text{-}2}$		16	9	32	AB465009
Ancestral slow/cardiac	MYH_{M5}		6	14	17	AB465008
	MYH_{M880}				1	AB465010

* Based on Figure 2-2. MYH, myosin heavy chain; LS, lateralis superficialis; ED, erector and depressor.

Table 3-1. Nucleotide sequences of oligonucleotide primers used in Chapter III

Experiment	Primer	Nucleotide sequence (5'-----3')	Length (base)
3' RACE	M743	AGA AGC TCG AGT CTG ATC TGG TT	23
	M86-2	GGA AGC TGA CCT CGT CCA GGT T	22
	M2126-1	AGA AGC TTG AAG TCG ATG CGT CC	23
	β-actin F	CTG TAT GCC AAC ACT GTG CTG T	22
	β-actin R	TTA GAA ACA TTT GCG GTG GAC GAT	24
RT-PCR	M743 F	TGT TCA CTT GTC CAA GTG CCG GAA G	25
	M743 R	CAA GAG CAA AAA TGT TTA TTA TGG	24
	M86-2 F	CAT GTC CAG GCT GAG GAA GGT TCA	24
	M86-2 R	GCA GTA AAA CGG TTT ATT GAG AT	23
	M5 F	TAA GCT CCA GGT CAA AGT GAA GAG	24
	M5 R	TGA CGC ATT CTC ACA GTT TAG CAC	24
	M86-1 F	AAA CTG AGA CAA CAT CCC AGC ATC T	25
	M86-1 R	CTT TGC CAC CCT CTC TCT TGA TGA	24
	M2528-1	TCG GCA GGG GAA AGG ACG CTG AAG	24
	M2528-1	TTG CGT ATA GAT TTA TTG CAC TT	23
	M1034 F	GTC AAG GTC AAG ACT ACA GAA TCA	24
	M1034 R	TGT TAC ATC AGG ACA CAA TAA A	22
	M8248 F	TCG TAA AAA TCT TTC ACG TCT GCA A	25
	M8248 R	GAT ATG CAG ATT CTT TTT ATT CTA C	25
	M2126-2 F	TGA GGA GGA CCG CAA GAA TGT TGC	24
	M2126-2 R	TGC AGC ACA ATC ACC AGA ATT TAT TA	26
In situ hybridization	M86-2 F	AAA GTG AAA GCT TAC AAG AGA CAA G	25
	M86-2 R	GCA GTA AAA CGG TTT ATT GAG ATT	24
	M5 F	GAG TTA CAA AAG ACA GAC TGA GGA A	25
	M5 R	GAA GGT TGC AAG CAT TTT ATT G	22
	M2126-1 F	AAG TCA AAG CTT ACA AGA GAG CTG	24
	M2126-1 F	GAA TAA AAT AAA GAG CAT TTC AAT	24
	M2528-1F	TGA CCA GGT ACA GGA AGG TG	20
	M2528-1R	TTG AAT TGC GTA TAG ATT TAT TGC	24
	M743 F	TGT TCA CTT GTC CAA GTG CCG GAA G	25
	M743 R	CAA GAG CAA AAA TGT TTA TTA TGG	24

Table 3-2. Frequencies of cDNA clones encoding *MYH*s from the clone libraries prepared from torafugu embryos and larvae

MYH type*		Embryo			Larvae		Accession number
		3dpf	5dpf	7dpf	10dpf	16dpf	
Fast	MYH_{M743}	35	31	26	35	40	
	$MYH_{M86\text{-}2}$		7	6	2	2	
	$MYH_{M2528\text{-}1}$				4	6	AB465006
	MYH_{M1034}				1	3	AB465005
Slow/Cardiac	MYH_{M5}	7	5	5	2	1	AB465008
Cardiac	$MYH_{M2126\text{-}1}$	2	2	3	2	2	
Total		45	45	40	48	54	

*Based on Figure 3-2. MYH, myosin heavy chain.

Table 4-1. Nucleotide sequences of oligonucleotide primers used in Chapter IV

Experiment	Primer	Nucleotide sequence (5'-----3')	Length (Base)
RT-PCR	Pax3a F	ACT TTG GAC CGG TGT GAA CAA ACA A	25
	Pax3a R	ACC CTC CAC CAC TGC TGT GAT TCG G	25
	Pax3b F	TCG ATC CAA ATT CGG CGG AGT AG	23
	Pax3b R	TGA GTC CAC ATC GGA TGT GTC G	22
	Pax7a F	CCT CTG GGC CAG GGT AGG GTC AA	23
	Pax7a R	GGC TGA TGG AGC TCA CTG AAG GAA	24
	Pax7b F	TCT GAC CCT CCT GCT TCC TGG TTC T	25
	Pax7b R	CCT GGT ACC GAC AGA GGA TCT TGG	24
	EF1 α F	ACT GTT GCT GTC GGT GTC ATC AAG	24
	EF1 α R	TCA GCA TAA GCC AGT CCT TGA GG	23
5'RACE	Pax3a R	TTG TTC CCT GCT TCG GTT TGC TGC	24
	Pax7a R	CTT GAC CCA ACG GAG TAG AAA CTT	24
	Pax3b R	TTG GAG ACG CAG CCG TGG GAC ACC	24
	Pax7b R	CCT GGT ACC GAC AGA GGA TCT TGG	24

Table 4-2. Pax3 and Pax7 genes cloned from torafugu embryo and adult fast muscle

Pax genes	3 dpf	Fast muscle
Pax3a	+	-
Pax3b	+	-
Pax7a	+	-
Pax7b	-	+

Table 4-3. Amino acid sequence identities in percentages of torafugu Pax3 with those from other vertebrates

	Torafugu Pax3b	Zebrafish Pax3	Human Pax3	Mouse Pax3
Torafugu Pax3a	84.6	89.7	86.7	86.7
Torafugu Pax3b		89.0	81.6	81.6
Zebrafish Pax3			86.4	86.4
Human Pax3				100

Table 4-4. Amino acid sequence identities in percentages of torafugu Pax7 with those from other vertebrates

	Torafugu Pax7b	Zebrafish Pax7	Salmon Pax7	Human Pax7	Mouse Pax7
Torafugu Pax7a	89.6	95.2	91.2	92.4	92.7
Torafugu Pax7b		91.5	91.5	87.2	86.9
Zebrafish Pax7			94.4	93.5	93.1
Salmon Pax7				89.2	88.8
Human Pax7					96.5

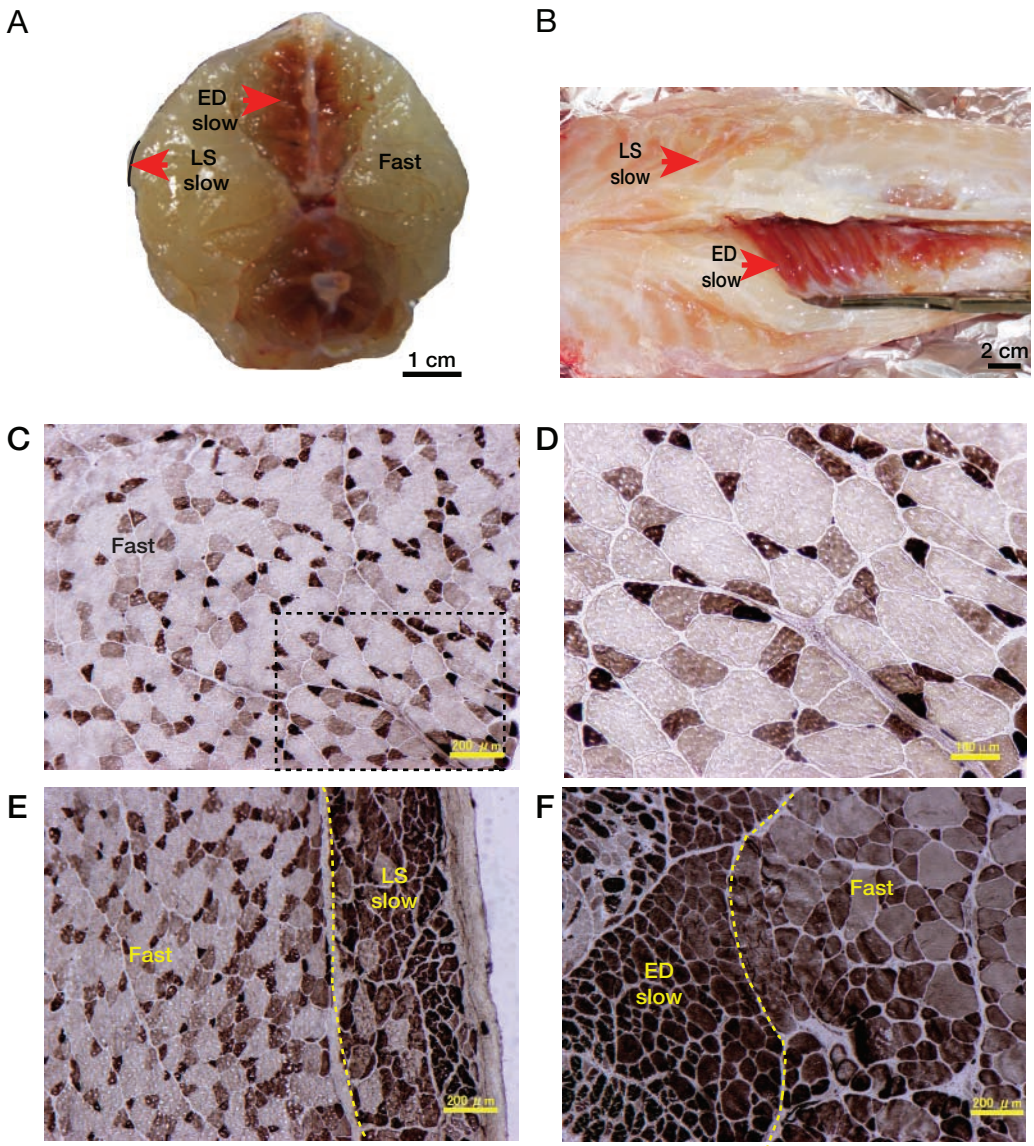

Fig. 1-1. The histochemical demonstration of myofibrillar ATPase in fast and slow muscles of adult torafugu (290 g body weight). A; the cross-sectional view of the whole trunk muscle at level of median fins of adult torafugu, showing anatomical locations of fast and lateralis superficialis (LS) and erector and depressor (ED) slow muscles. B; the dorso-lateral view of the whole trunk muscle,illustrating locations of LS and ED slow muscles. C; ATPase after preincubation at pH 4.6 in fast muscle. D; an magnified area marked in panel C. E; ATPase in LS slow muscle after preincubation at pH 4.6. F; ATPase in ED slow muscle after preincubation at pH 4.6. Scale bars indicate 1 cm for panel A; 2 cm for panel B; 200 μm for panels C, E, F, G, F; 100 μm for panel D.

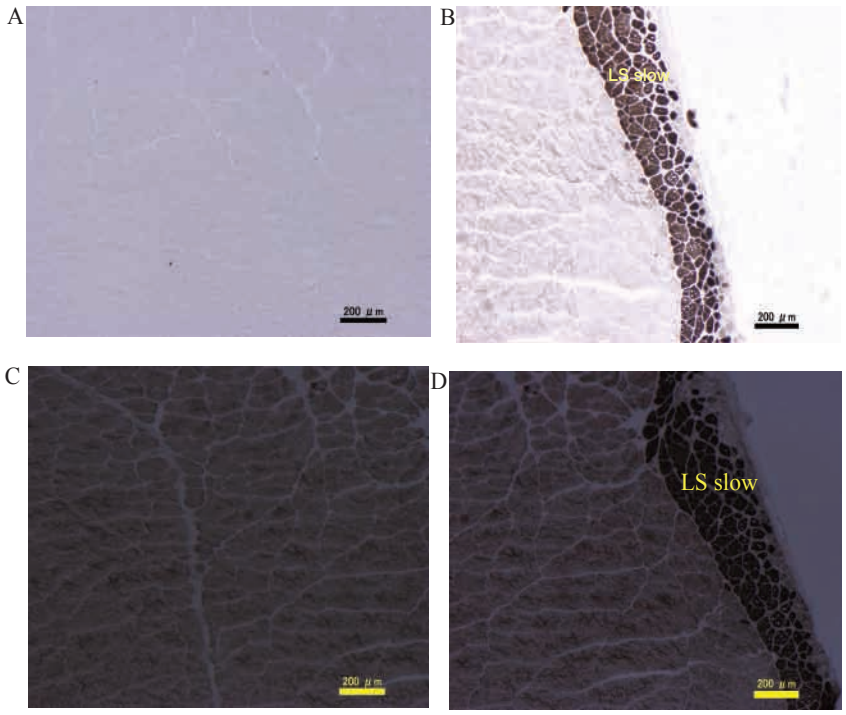

Fig. 1-2. The histochemical demonstration of myofibrillar ATPase in fast and slow muscles of juvenile torafugu (body weight 24.2 g). A; ATPase after preincubation at pH 4.6 in fast muscle. B; ATPase in LS slow muscle after preincubation at pH 4.6. C; ATPase in fast muscle after preincubation at pH 9.6. D; ATPase in LS slow muscle after preincubation at pH 9.6.Scale bars for panels A-D; 200 μm.

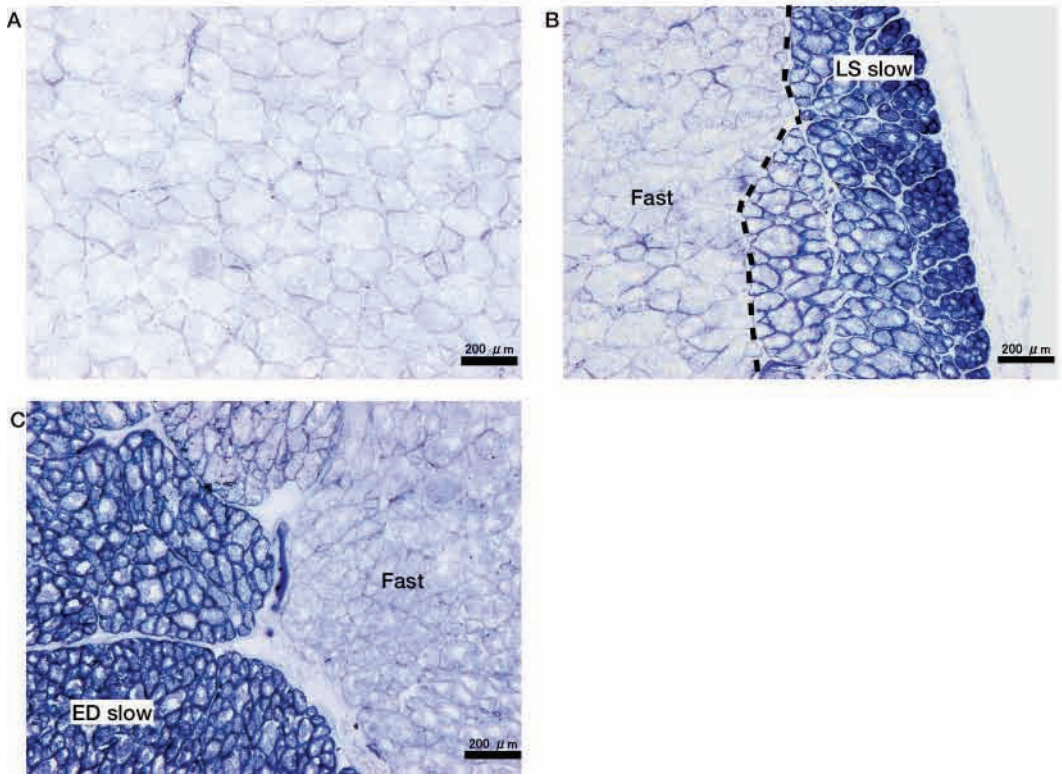

Fig. 1-3. The histochemical staining for NADH-diaphorase in skeletal muscles of adult torafugu. NADH-diaphorase was stained for fast muscle (A), lateralis superficialis (LS) (B) and erectors and depressors (ED) slow muscles (C). A dotted line indicates boundary between fast and slow muscles. Scale bars indicate 200 μm for panels A-C.

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M86-1    IQLSHANRQA AEAQKQLRNV QGQLKDAQLH LDDAVRGQED MKEQFAMVER RNGLMMAEIE  60
M2528-1  .......... .......... .......... ....I....E ....V..... ..N..L....  60
M1034    .......... .......... .......... ..E.I....E ..K.V..... ..N..L....  60
M8248    ....Q..... .......KS. HSH...S..Q ..ESL.VNDE I..NI.I... ..N.LQ..V.  60
M2126-2  ....Q..... .......KS. HAH...C.IQ ..ESM.ANDE ...NI.I... ..N.LQ..V.  60
M5       V..N...... ..S..L...L .V.I..I.ME ..ET.HQN.E L.D.VVVT.. ..N.LA..V.  60
M880     ....Q..... .D....IK.L .TFM..L.MQ ....HHAND. .R.NT.LL.. .HN.IQ..L.  60

M86-1    ELRAALEQTE RGRKVAEQEL VDASERVGLL HSQNTSLINT KKKLEADLVQ VQGEVDDSIQ  120
M2528-1  .......... .S........ .......... .....N.... .....G.... .....E....  120
M1034    .......... .S........ .......... .......... ........I. I....E....  120
M8248    ....S..... .S..L..... L.V....Q.. .......L.H .....V.TS. L.T..EEAV.  120
M2126-2  .......... .S..L..... L.V....Q.. .....A...Q .....V.AS. L.T..EEAV.  120
M5       ....L...ND .A..L..H.. LE.T...N.. ........SQ .....N..ST LSN....AV.  120
M880     .V...F.... .T..L..... T.IT..MQ.. .......L.Q ...H....L. L.T.TEEA..  120

M86-1    EARNAEEKAK KAITDAAMMA EELKKEQDTS AHLERMKKNL EVTVKDLQHR LDEAESLAMK  180
M2528-1  .......... .......... .......... .......... .......... .....N....  180
M1034    .......... .......... .......... .......... .......... .....N....  180
M8248    .C........ .......... .......... .........M .Q.I...... .....QI...  180
M2126-2  .C....D... .......... .......... .........M .Q.I...... .....QI...  180
M5       .C....D... .......... .......... .........M .Q......M. .....QI.L.  180
M880     .N........ ...N...... ...NE..... .........M .Q.I...... .....QV...  180

M86-1    GGKKQLQKLE SRVRELEAEV EAEQRRGVDA VKGVRKYERR VKELTYQTEE DKKNVSRLQD  240
M2528-1  .......... A......S.. .......SE. I......... ....A..... .....A....  240
M1034    .......... A......G.. ....K..A.. I......... .......... ....LV....  240
M8248    .....I..M. A.I....I.L ......SSES ...I...... I......... .R..L.....  240
M2126-2  .....V.... A......N.. .S..KKSTE. A..I...... I......... .R...A....  240
M5       .....V.... A..K...N.L .S..KKSQEF Q......... I...S..A.. ....LI...E  240
M880     .......... T.IK...S.L .....K.IES I..I...... L......... .R..MA...E  240
                                ACD domain
                 + +   ++  -- - -     +  +  ++
M86-1    LVDKLQLKVK AYKRQAEEAE EQANTHMSRL RKVQHEMEEA QERADIAESQ VNKLRVKSRD  300
M2528-1  .......... .....S.... ......LT.Y .......... .......... .....A....  300
M1034    .........M S......D.. ....S.LT.Y ......L... .......... .........E  300
M8248    .A........ S..KAT.... ....SNLTKF ..I...LD.. E......... .....A....  300
M2126-2  .........I S...V..... ....V.LGKF ..L...LD.. E......... .....I....  300
M5       .I....V... S....T.... ....CNL.KY ..L...LND. E....M..T. ......RT..  300
M880     .......... S...A..... .AG.AN.AK. ..L...L... E......... ....KA.T..  300

M86-1    MGKSDSAE-- - 308
M2528-1  I.RGKGR--- - 307
M1034    IV.VKTT.SE E 311
M8248    V.SKKGF.GE - 310
M2126-2  T.SKKGFDEE - 310
M5       Q.SKLAE--- - 307
M880     GSTKKGLDE- - 309
```

Fig. 2-1. Comparison of the deduced amino acid sequences of cDNA clones encoding adult torafugu MYHs. Amino acids identical to those of torafugu MYH_{M86-1} are shown by dots and gaps to maximize alignment are represented by dashed lines. A 29-residue assembly competence domain (ACD) region is shaded, whereas one motif of five amino acids residues is boxed. Positively and negatively charged amino acid residues are shown as + and -, respectively.

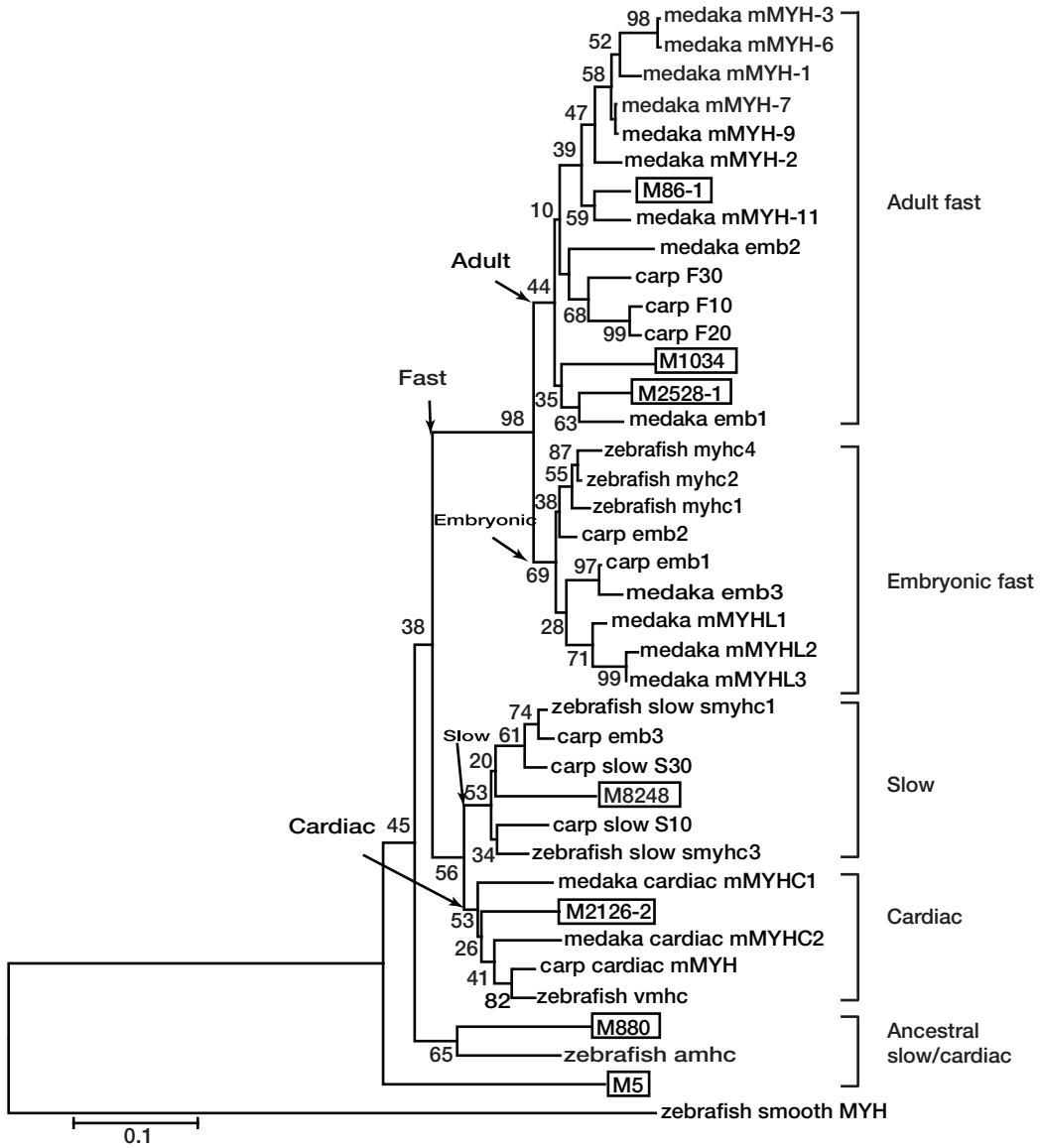

Fig. 2-2. Phylogenetic tree based on partial deduced amino acid sequences in the C-terminal region of adult torafugu MYHs. Sequences were aligned with the corresponding sequences of common carp, medaka and zebrafish MYHs by multiple alignment program CLUSTAL X (Thompson et al., 1997) and the phylogenetic tree was constructed by the neighbor-joining method by using phylogenetic software MEGA 4 (Tamura et al., 2007). The bootstrap values from 1000-replicates analysis are given at the nodes in percentages. Sequences sited were from common carp *Cyprinus carpio*: carp F10, D89990; carp F20, D89991; carp F30, D89992; carp S10, AB104625; carp S30, AB104626; carp emb1, AB104622; carp emb2, AB104623; carp emb3, AB104624; carp cardiac MYH, AB104627. Zebrafish *Danio rerio*: zebrafish myhc1, AF180893; zebrafish myhc2 , BC071279; zebrafish myhc4, AY333450; zebrafish smyhc1, AY921649; zebrafish smyhc3, EU218876; zebrafish vmhc, AF114427; zebrafish amhc, AY138982; zebrafish smooth, DQ022376. Medaka *Oryzias latipes*: medaka MYHemb1, AB256928; medaka emb2, AB256929; medaka emb3, AB256930; medaka mMYHL1, AB256931; medaka mMYHL2, AB256932; medaka mMYHL3, AB256933; medaka cardiac mMYHC1, AB256934; medaka cardiac mMYHC2, AB256935; medaka mMYH-1, AB258222; medaka mMYH-2, AB258223; medaka mMYH-3, AB258224; medaka mMYH-6, AB258220; medaka mMYH-7, AB258225; medaka mMYH-9, AB258225; medaka mMYH-11, AB258227.

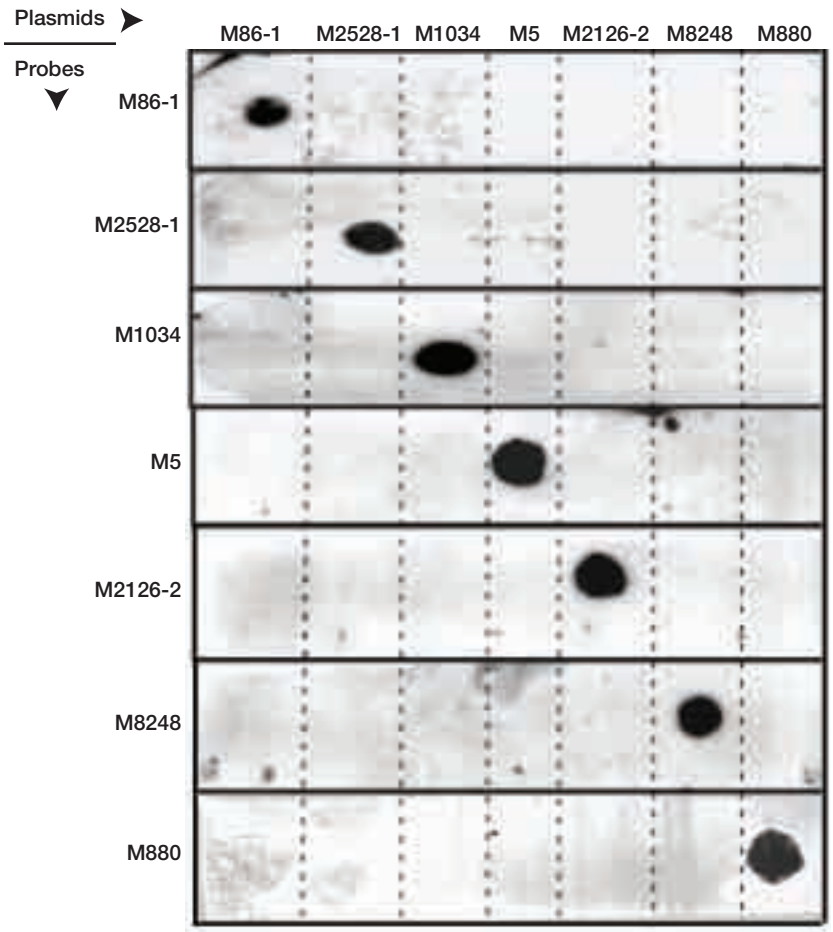

Fig. 2-3. Specificity of DIG-labelled DNA probes for Northern blot hybridization of seven cDNA clones encoding adult torafugu *MYH*s. Nylon membranes spotted with 1 ng of plasmids containing $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$, MYH_{M1034}, MYH_{M5}, $MYH_{M2126\text{-}2}$, MYH_{M8248} and MYH_{M880} were hybridized with DIG-labelled DNA probes (see Table 2-1).

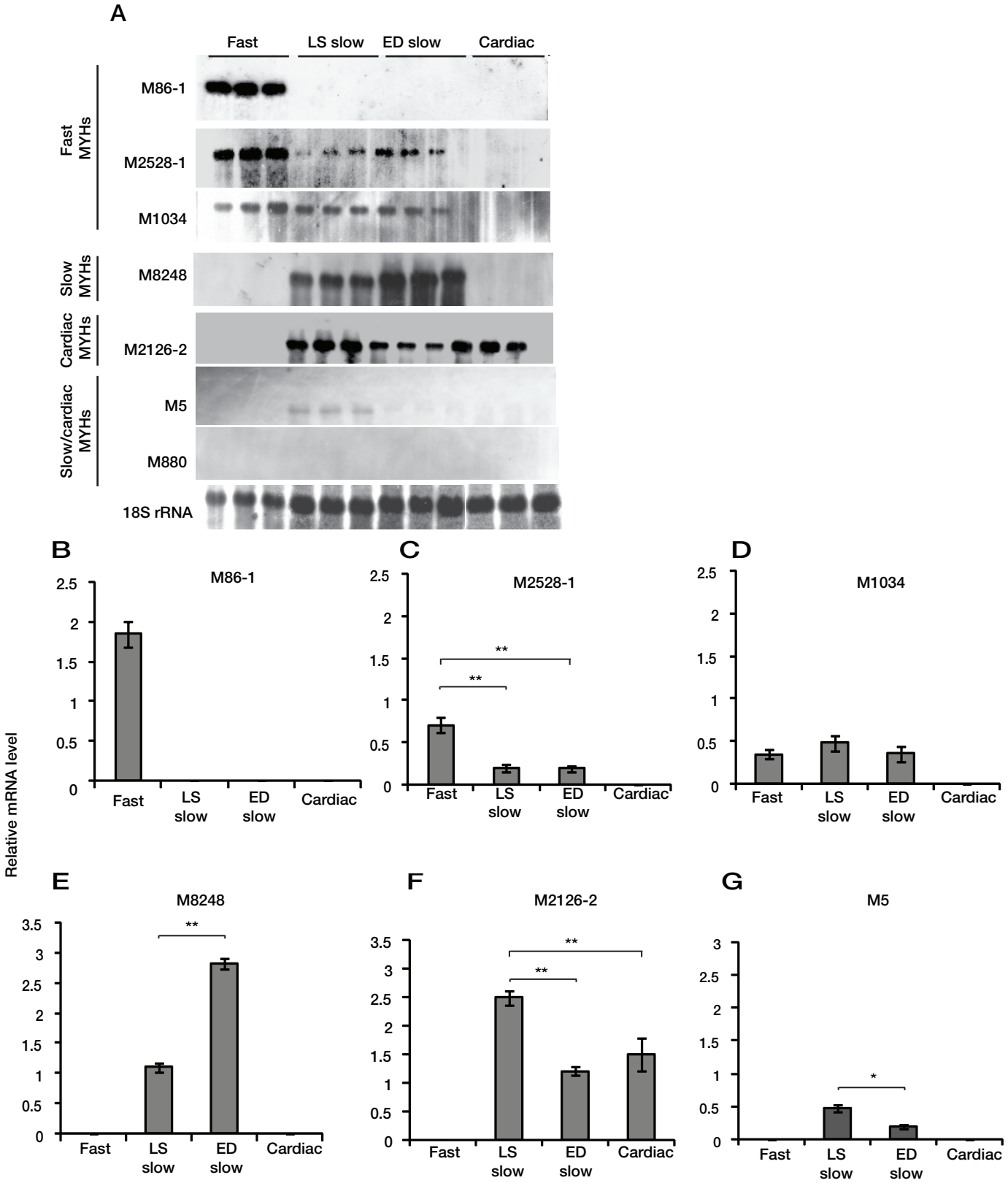

Fig. 2-4. The accumulated mRNA levels of torafugu MYHs in adult fast, lateralis superficialis (LS) and erector and depressor (ED) slow, and cardiac muscles. A; total RNAs (5 μg) prepared from three different parts each of fast, slow and cardiac muscles of torafugu used in Table 2-2 were electrophoresed in a 2% agarose gel containing 2% formaldehyde and transferred to nylon membranes followed by hybridization with the DIG-labelled probe. First three lanes contain RNAs form fast muscle, middle six lanes contain RNAs from LS and ED slow muscles, and last three lanes contain RNAs from cardiac muscle. The 18S rRNA probe was used as the internal standard (AB437876). Panels B, C, D, E, F and G indicate mRNA levels of $MYH_{M86\text{-}1}$, $MYH_{M2528\text{-}1}$, MYH_{M1034}, MYH_{M8248}, $MYH_{M2126\text{-}2}$ and MYH_{M5}, respectively. Each data point represents the mean value (n = 3) for *MYH* relative to that of the 18S rRNA gene calculated from densitometric analysis on Northern blots with significant differences at $P<0.05$ (*) and $P<0.01$ (**)

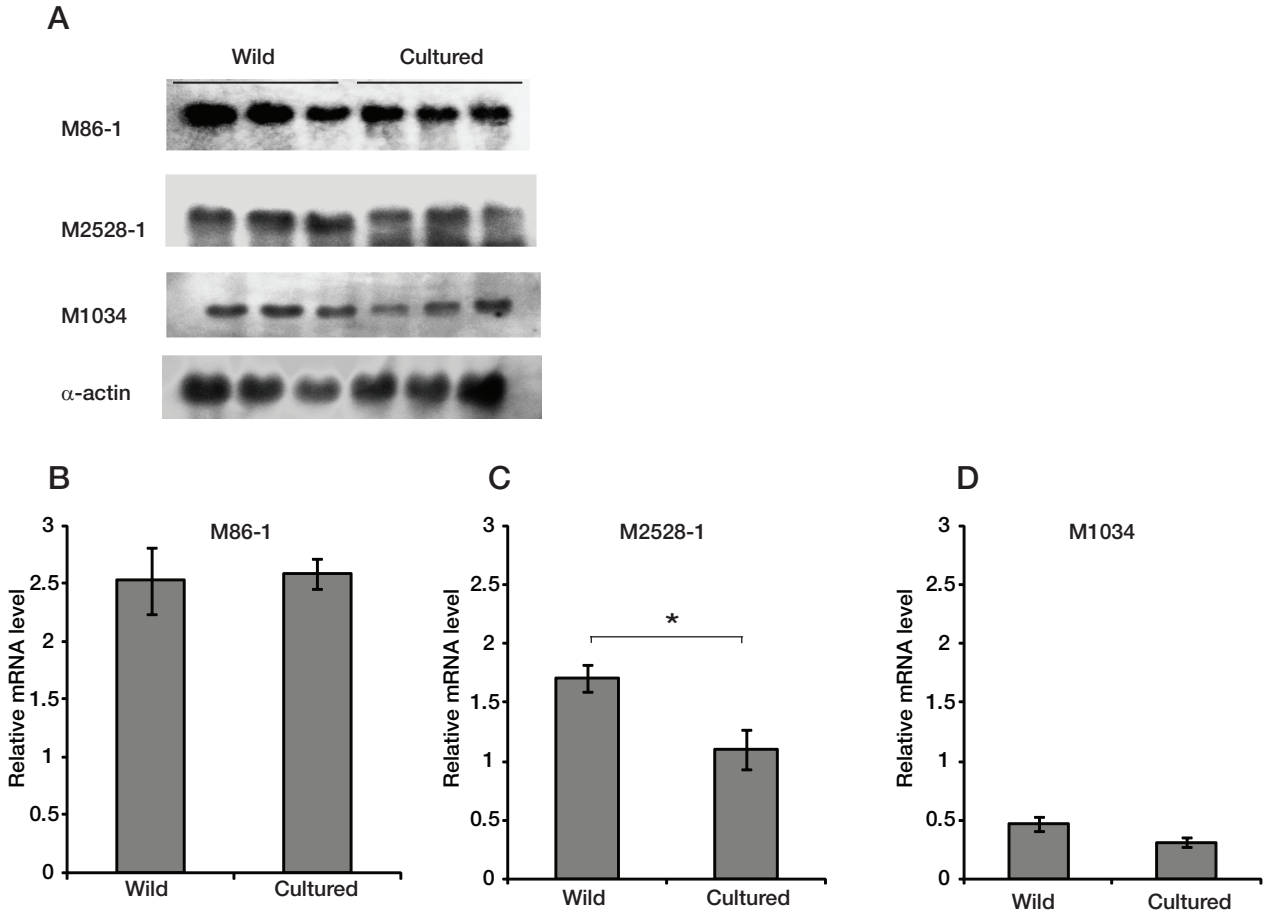

Fig. 2-5. Expression patterns of fast-type *MYH*s in fast muscle from wild and farm-cultured torafugu. A; Northern blot hybridization for three individuals each from wild and farm-cultured torafugu. The mRNA levels of the α-actin gene (U38958) were used as the internal control. B, C, D; mRNA levels of torafugu fast-type *MYH*s in fast muscle from wild and farm-cultured fish. Refer to the legend of Fig. 2-5 for further details.

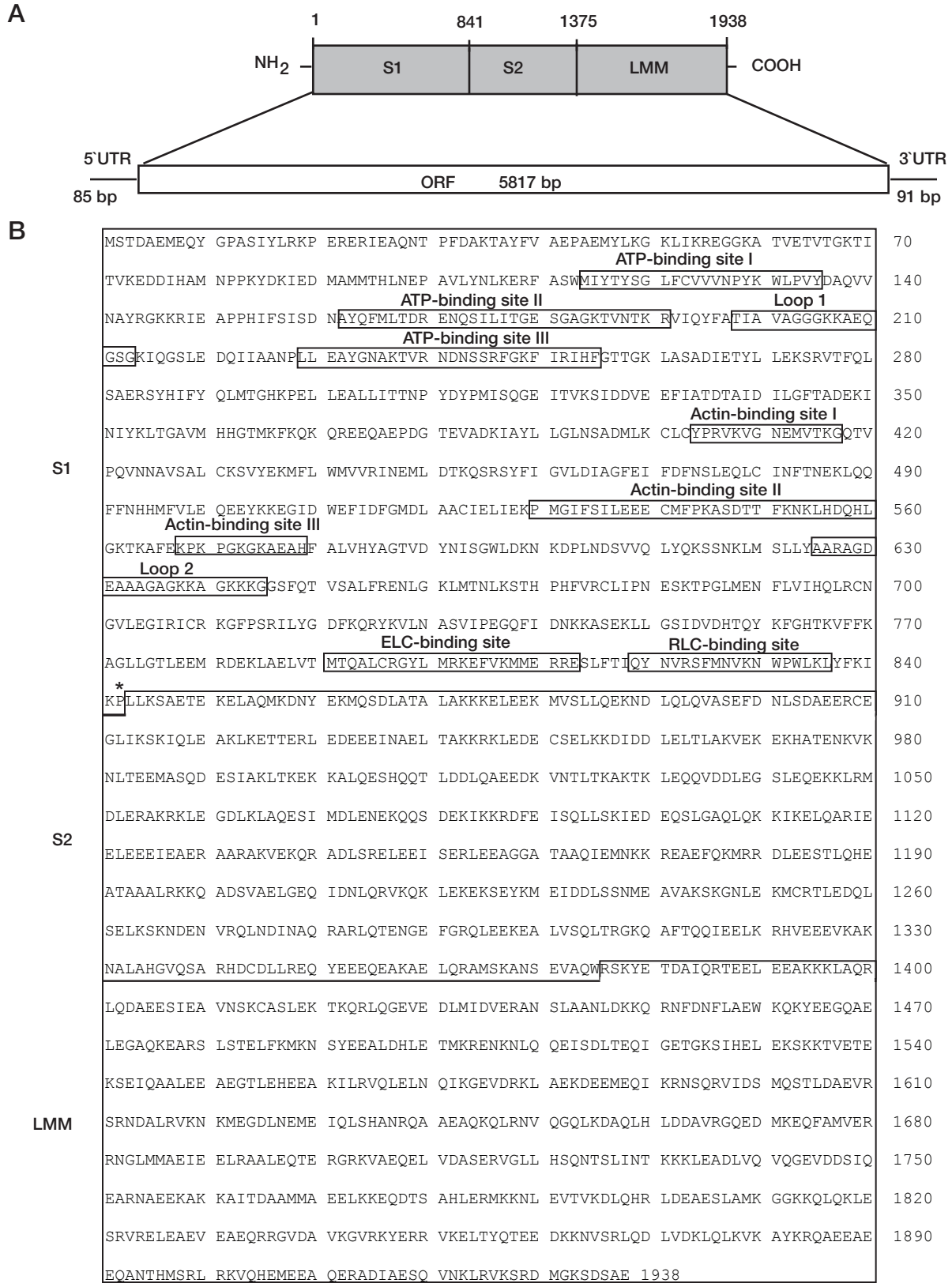

Fig. 2-6. The full-length cDNA and deduced amino acid sequences of MYH_{M86-1}. A; schematic representation of deduced amino acid sequences of myosin subfragment-1(S1), myosin subfragment-2 (S2) and light meromyosin (LMM) domains (top) encoded by MYH_{M86-1} (bottom). Open reading frame (ORF) of MYH_{M86-1} contains 5817 bp that encode 1938 amino acids. B; the deduced amino acid sequence of MYH_{M86-1} containing important putative sites in S1 that includes ATP (I, II, III)-, actin (I, II, III)-, essential light chain (ELC)- and regulatory light chain (RLC)-binding sites. Amino acids of 1-197, 214-624 and 645-841 encompass 25 kDa, 50 kDa and 20 kDa domains of S1. 25 kDa-50 kDa junction called loop-1 and 50 kDa-20 kDa junction called loop-2 have 14 and 21 amino acids, respectively. Proline residue in the boundary between S1 and S2 is marked by an asterisk (*).

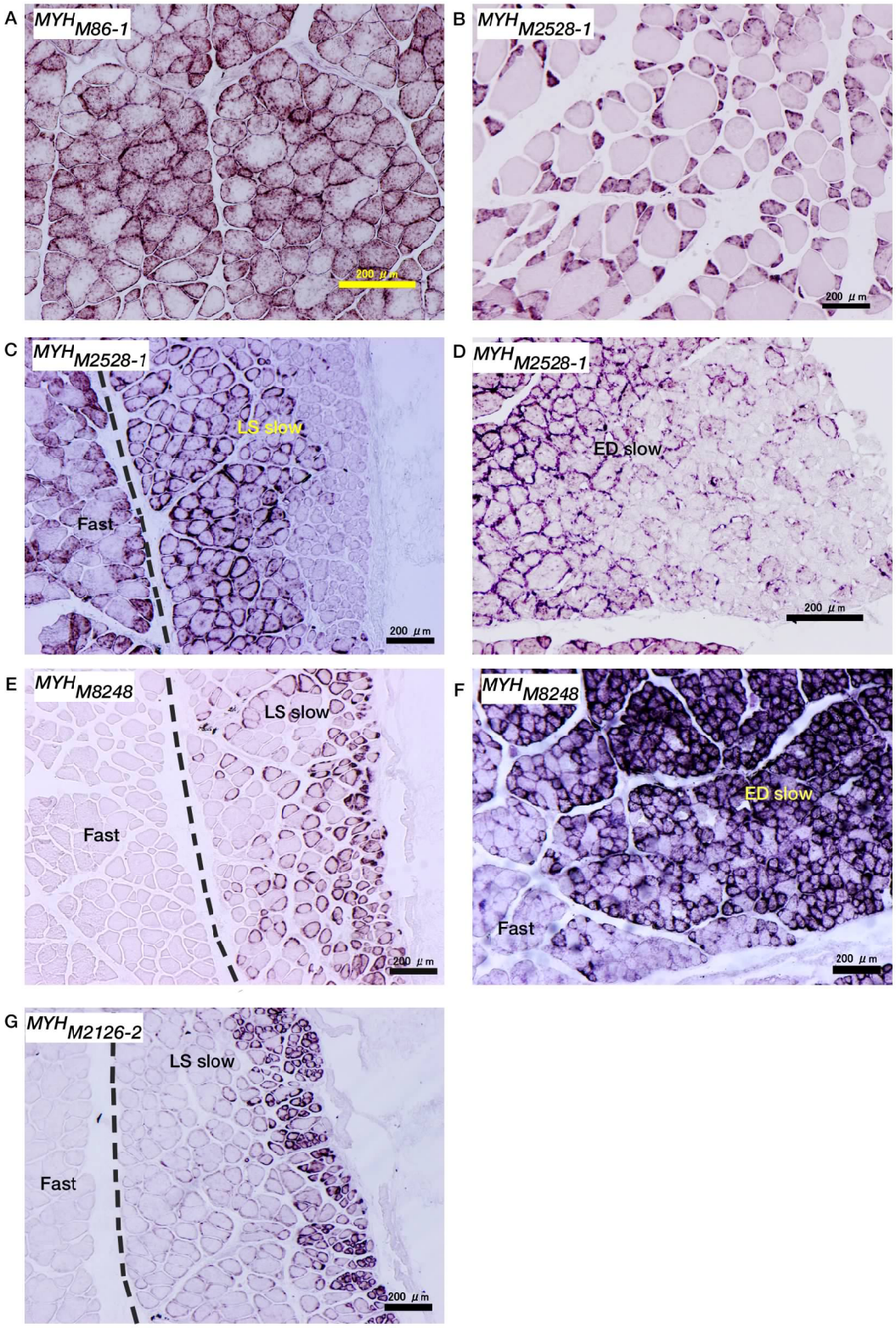

Fig. 2-7. *In situ* hybridization of adult torafugu *MYH*s in fast (A, B) and slow muscles (C-G). The transcripts are localized for fast-type $MYH_{M86\text{-}1}$ in fast muscle (A), for fast-type $MYH_{M2528\text{-}1}$ in fast muscle (B) and lateralis superficialis (LS) (C) and erectors and depressors (ED) (D) slow muscles, for slow-type MYH_{M8248} in LS (E) and ED slow muscle (F), and for cardiac-type $MYH_{M2126\text{-}2}$ in LS slow muscle (G). Dotted line in panels C, E and G mark boundary between fast and slow muscles. Scale bars indicate 200 μm for panels A-G.

```
M743      IQLSHANRQA SESQKQLRNV QAQLKDAQLH LDDAVRAQED LKEQAAMVDR RNGLMIAEIE  60
M86-2     .......... A.A....... .G........ ......G... M...V...E. .....M....  60
M2528-1   .......... A.A....... .G........ ....I.G..E M...V...E. ..N..L....  60
M1034     .......... A.A....... .G........ ..E.I.G..E M.K.V...E. ..N..L....  60
M2126-1   ....Q..... A.A....KS. H.H...C.IQ ..E.M..NDE M..NI.I.E. ..N.LQ..V.  60
M5        V..N...... A....L...L .V.I..I.ME ..ET.HQN.E ..D.VVVTE. ..N.LA..V.  60

M743      ELRAALEQTE RSRKVAEQEL VDASERVGLL HSQNTSLMNT KKKLESDLVQ IQSEVDDTVQ  120
M86-2     .......... .G........ .......... .......L.. .....A.... V.G....S..  120
M2528-1   .......... .......... .......... .....N.I.. .....G.... V.G..E.SI.  120
M1034     .......... .......... .......... .......I.. .....A..I. ..G..E.SI.  120
M2126-1   .......... ....L..... L.V....Q.. .....G.I.Q .....V.AS. L.T..EEA..  120
M5        ....L...ND .A..L..H.. LE.T...N.. .......ISQ .....N..ST LSN....A..  120

M743      EARNAEEKAK KAITDAAMMA EELKKEQDTS AHLERMKKNL EVAVKDLQHR LDEAENLAMK  180
M86-2     .......... .......... .......... .......... ..T....... ..........  180
M2528-1   .......... .......... .......... .......... ..T....... ..........  180
M1034     .......... .......... .......... .......... ..T....... ..........  180
M2126-1   .C....D... .......... .......... .........M .QTI...... .....QI...  180
M5        .C....D... .......... .......... .........M .QT.....M. .....QI.L.  180

M743      GGKKQLQKLE SRVRELETEV ETEQRRGADA VKGVRKYERR VKELTYQTEE DKKNVTRLQD  240
M86-2     .......... .......A.. .A.....V.. .......... .......... .....N....  240
M2528-1   .......... A......S.. .A.....SE. I......... ....A..... .....A....  240
M1034     .......... A......G.. .A..K..... I......... .......... ....LV....  240
M2126-1   .....V.... A...D..... .M..KKSSE. L..I...... I.....R... .R...V....  240
M5        .....V.... A..K...N.L .S..KKSQEF Q......... I...S..A.. ....LI...E  240
                                ACD domain
                   + +   ++  - - - -    +  +  ++
M743      LVDKLQLKVK AYKRHSEEAE EQANVHLSKC RKLQHELEEA EERADIAESQ VNKLRAKSRD  300
M86-2     .......... ....QA.... ....T.M.RL ..V...M... Q......... ..........  300
M2528-1   .......... ....Q..... ....T..TRY ..V...M... Q......... ..........  300
M1034     .........M. S...QA.D.. ....S..TRY ..V....... Q......... .....V...E  300
M2126-1   ......M... ....AA.... ....T..G.F ........D.. .......... ..........  300
M5        .I....V... S...QT.... ....CN...Y ........ND. .....M..T. .....VRT..  300

M743      SGKGKDAAE     310
M86-2     V...N.S..     310
M2528-1   I.R..GR       308
M1034     IV.V.TTESE E  312
M2126-1   V.SK.GLD.E    310
M5        Q.SKLAE       308
```

Fig. 3-1. Comparison of the deduced amino acid sequences of *MYH*s from torafugu embryos and larvae. Amino acids identical to those of torafugu MYH_{M743-2} are shown by dots and gaps to maximize alignment are represented by dashed lines. A 29-residue assembly competence domain (ACD) region is shaded, whereas one motif of five amino acids residues is boxed. Positively and negatively charged amino acid residues are shown as + and -, respectively.

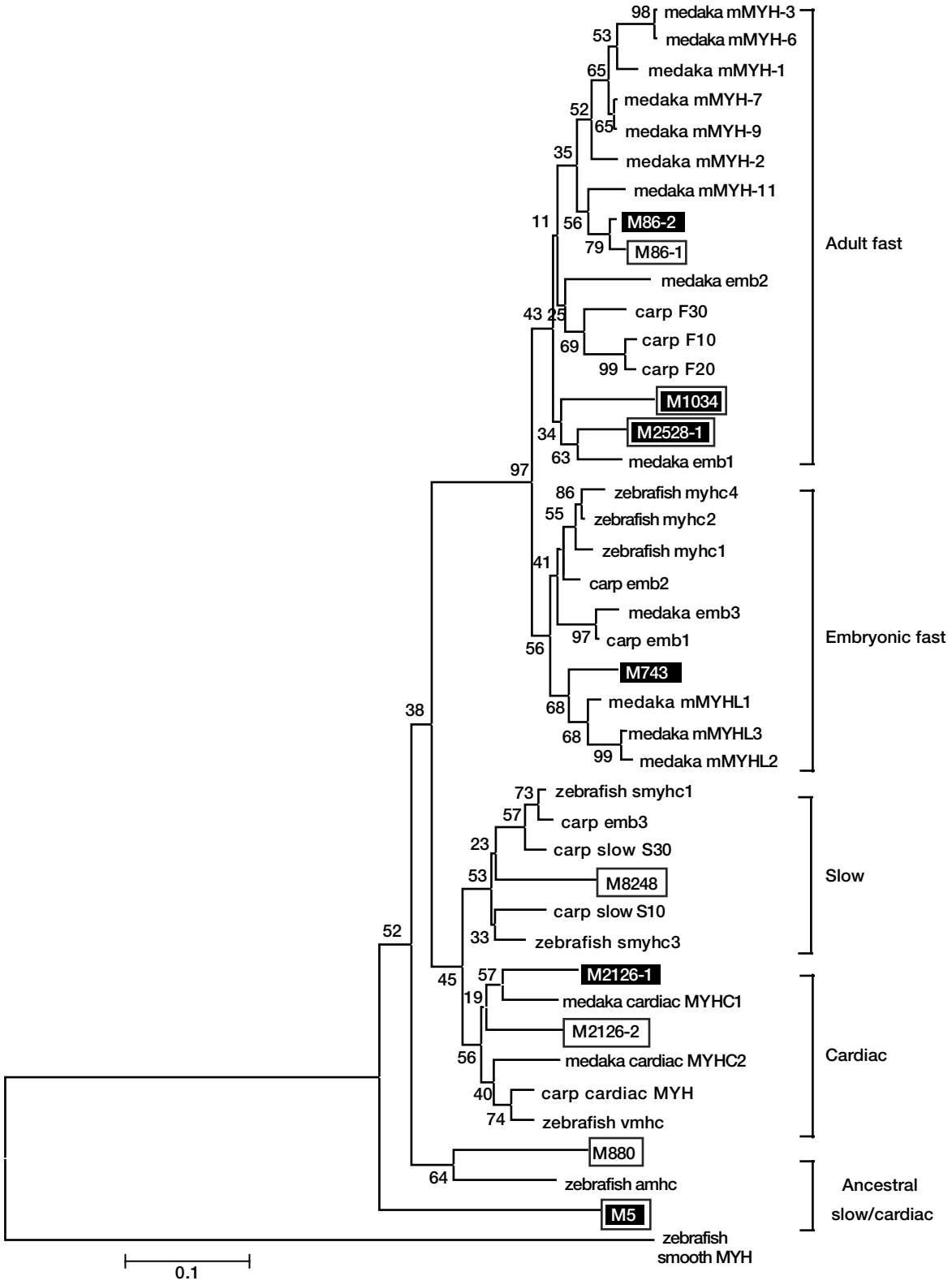

Fig. 3-2. Phylogenetic tree based on partial deduced amino acid sequences at the C-terminal end of MYHs. The phylogenetic tree was constructed using neighbor joining (NJ) method. The bootstrap values from 1000-replicates analysis are given at the nodes in percentages. MYHs of common carp *Cyprinus carpio* (Hirayama and Watabe, 1997; Imai et al., 1997; Neihi et al 2006), medaka *Oryzias latipes* (Ono et al., 2006; Liang et al., 2007) and zebrafish *Danio rerio* (Yelon et al., 1997; Berdougo et al., 2003; Bryson-Richardson et al., 2004; Wallace et al., 2005) were used in the analysis. *MYH*s cloned from torafugu embryos and larvae are shown in shaded boxes, whereas those cloned from adult muscles are boxed and that from adult and embryos/larvae indicated by both.

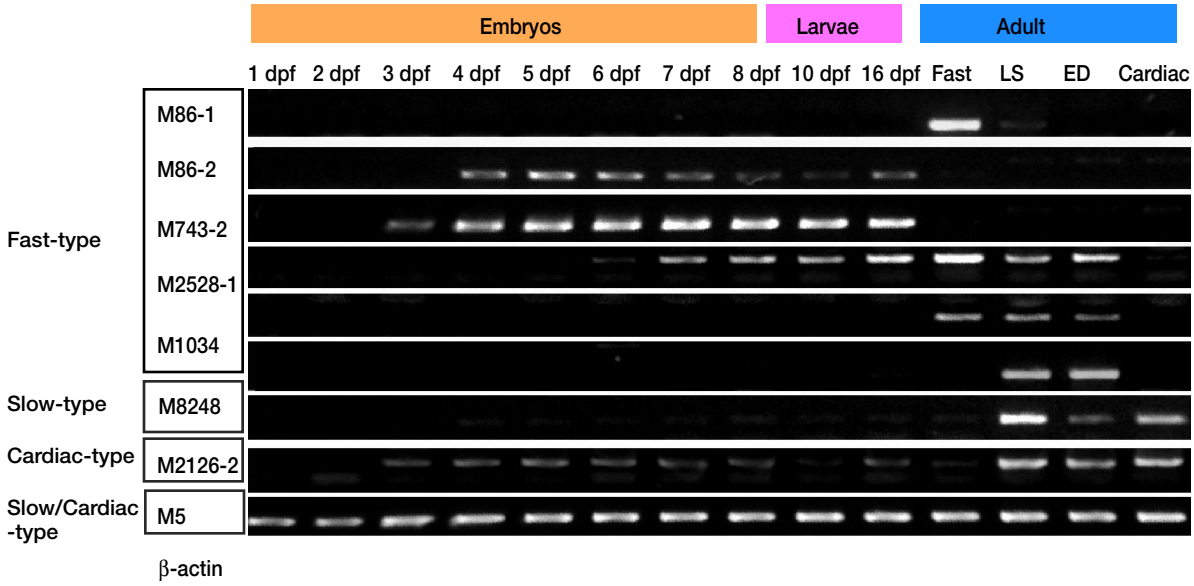

Fig. 3-3. Expression analysis of *MYH*s in during development of torafugu by RT-PCR. Embryos at 1-8 dpf, larvae at10 and 16 dpf and adult skeletal muscles including cardiac one were used in RT-PCR analysis. The transcripts of β-actin (U38849) were used as the internal control. LS, lateralis superficialis; ED, erectors and depressors.

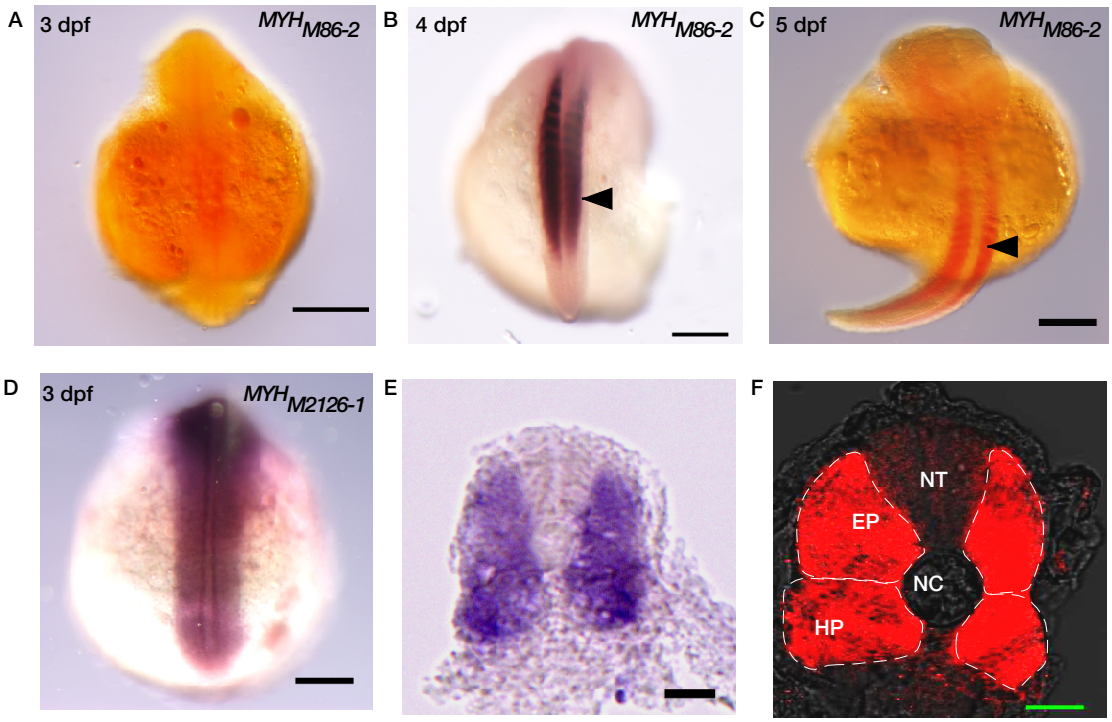

Fig. 3-4. The expression of fast-type MYH_{M86-2} and cardiac-type $MYH_{M2126-1}$ in torafugu embryos at 3 to 5 dpf. A, B and C, expression of MYH_{M86-2} in embryos at 3, 4 and 5 dpf, respectively, and D, expression of $MYH_{M2126-1}$ in embryos at 3dpf. Views in panel A, B, C, D are dorsal. Panel E and F are across sectional views marked by an arrowhead in panel B and C, respectively. Dotted lines indicate epaxial (EP) anpd hypaxial (HP) domain of embryonic myotome in panel F. Scales for panel A- C are 200 µm and 20 µm for panel D. NT, neural tube; NC, notochord.

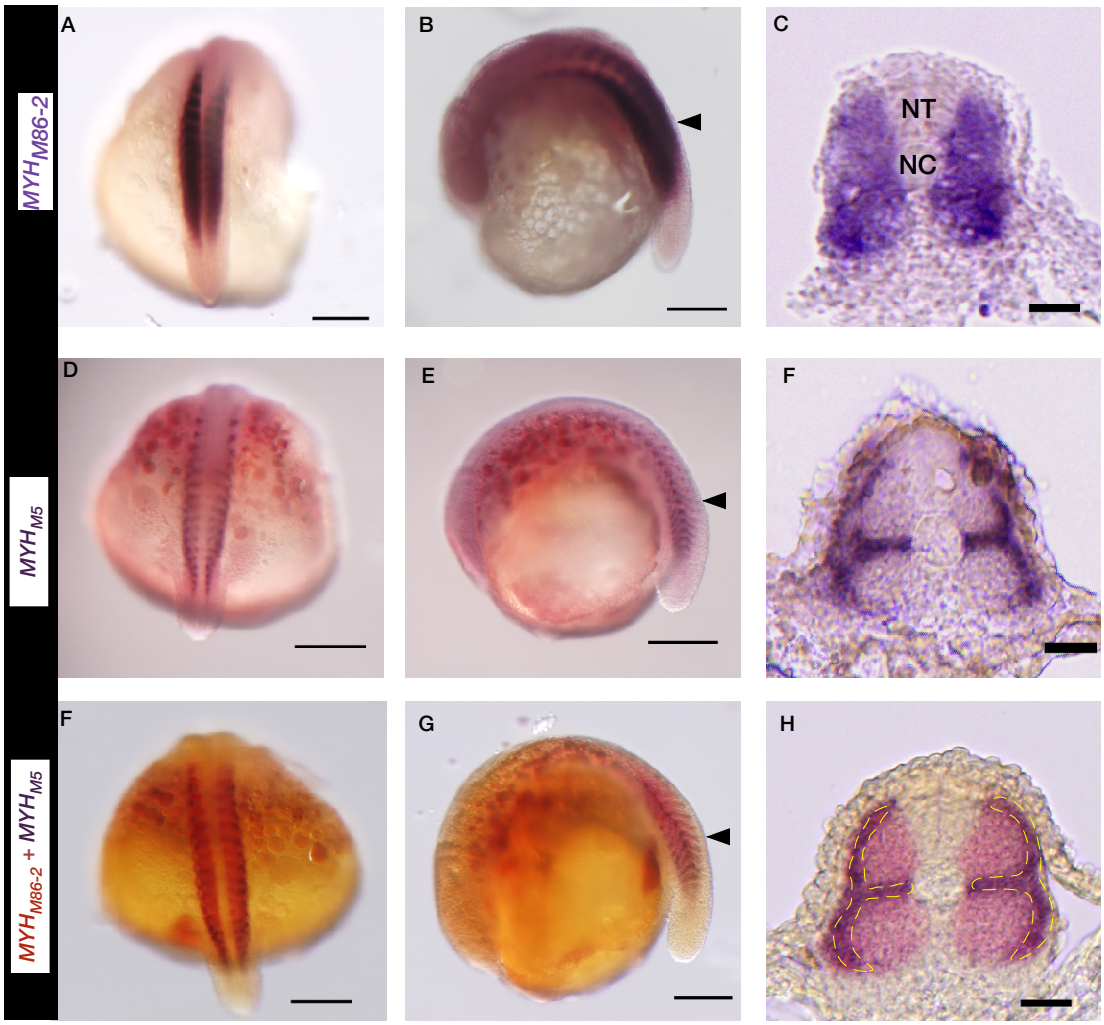

Fig. 3-5. Expression of fast-type MYH_{M86-2} and slow/cardiac-type MYH_{M5} in torafugu embryos at 4 dpf. A-C, the expression of MYH_{M86-2}; D-F, the expression of MYH_{M5}; E-H, merged with MYH_{M86-2} and MYH_{M5}. Views in panels A, D and F are dorsal; B, E and G are anterior at left; Panels C, F and H are across sectional view marked by arrowhead in panels B, E and G, respectively. Dotted line in panel H indicates layers of slow fibres at horizontal myoseptum and at proximity to myotome. Scales for panels A, B, D, E are 200 µm and 20 µm for panels C and F. NT, neural tube; NC, notochord.

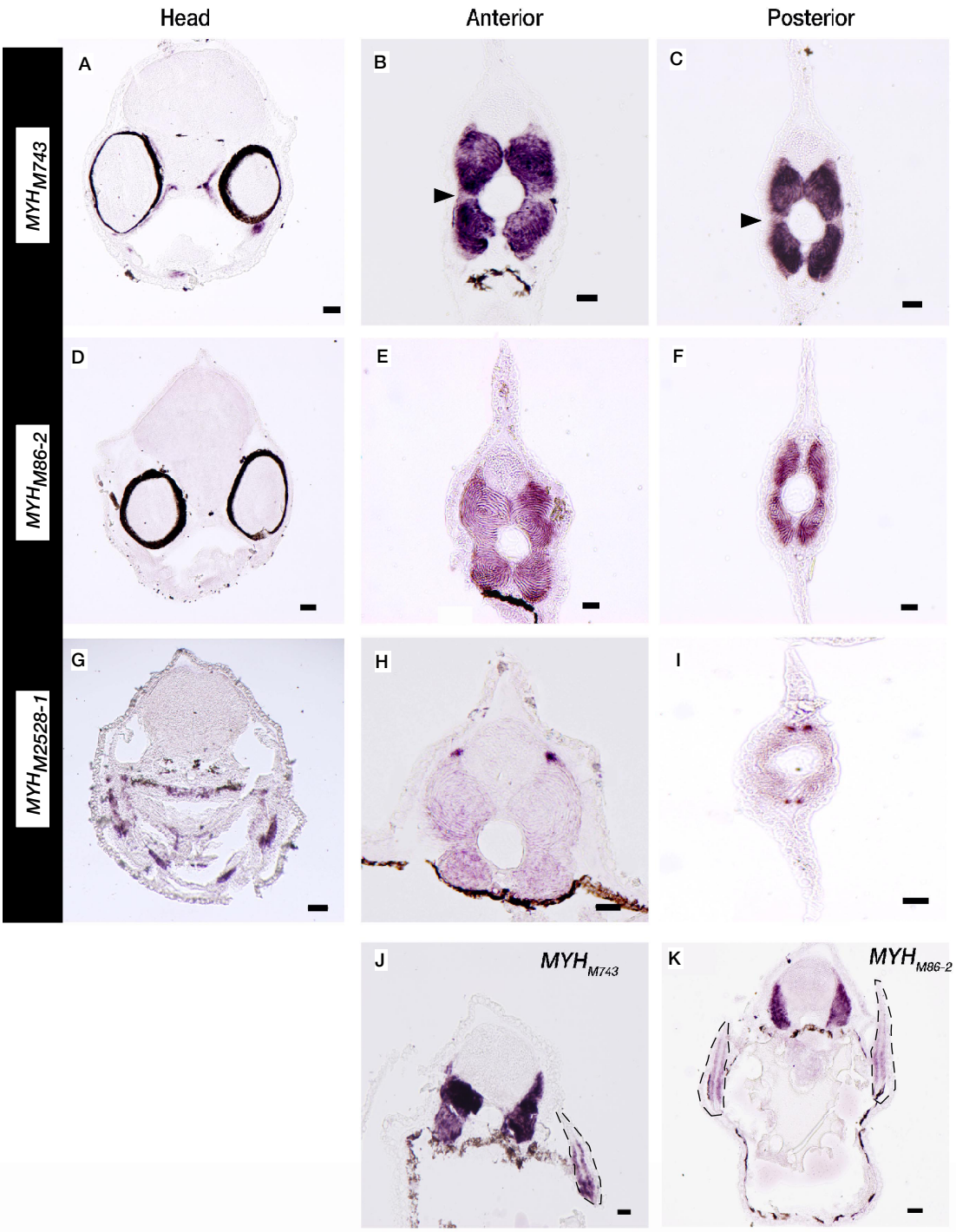

Fig. 3-6. The expression of fast-type *MYH*s in torafugu larvae at 10 dpf (hatching-stage). A-C, sections showing the expression of MYH_{M743}; D-F, the expression of $MYH_{M86\text{-}2}$; G, H, I, the expression of $MYH_{M2528\text{-}1}$. Panels J and K show sections made from pectoral fin that expresses the transcripts of MYH_{M743} and $MYH_{M86\text{-}2}$, respectively. Scales are 25 µm for panels A-I and 50 µm for panels J and K. An arrowhead in panels B and C indicates slow muscle at the horizontal myoseptum. Pectoral fins in panels J and K are shown by a dotted line.

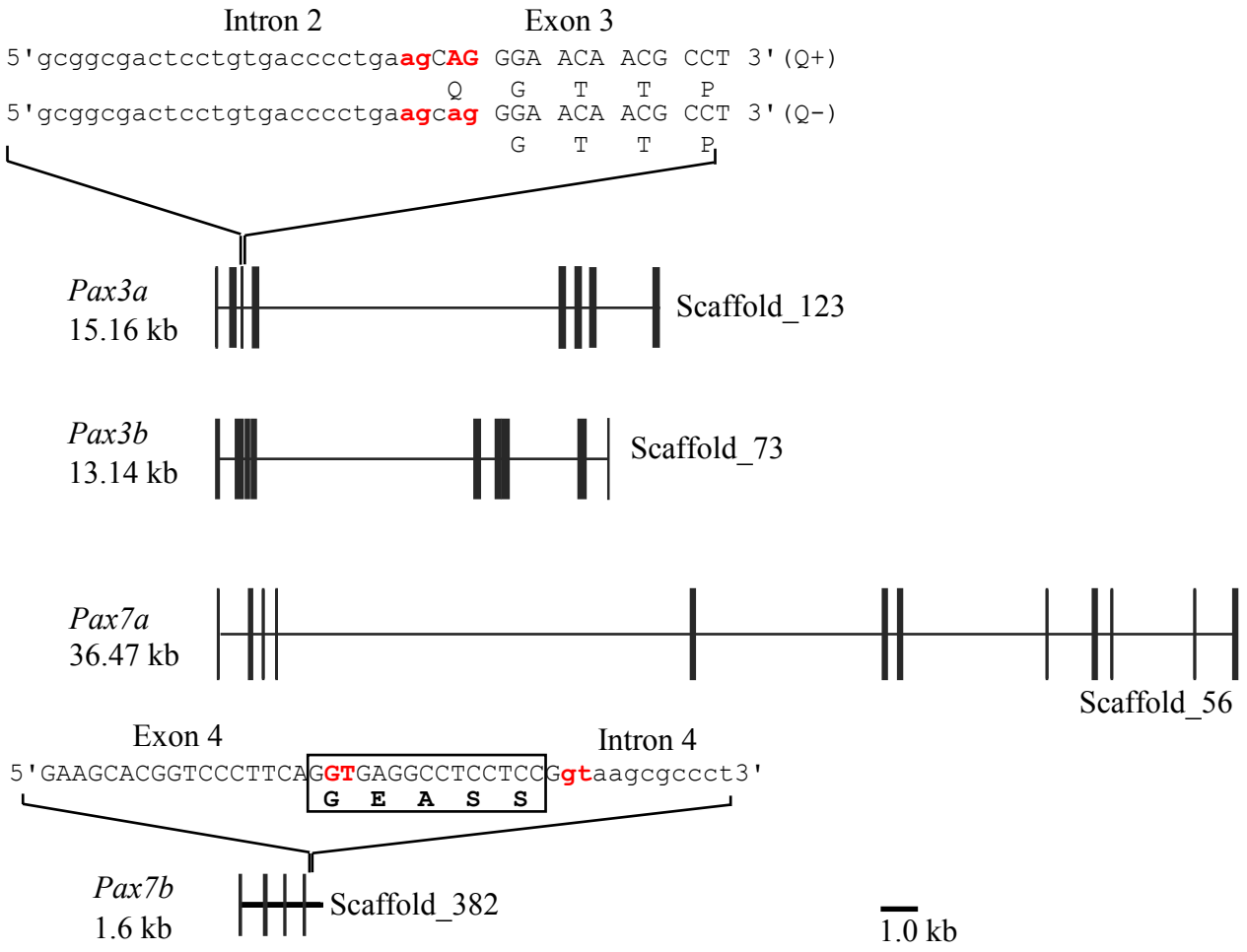

Fig. 4-1. Genomic organization of torafugu *Pax3* and *Pax7*. A Scale bar indicates 1.0 kb. The nucleotide sequences at the end of intron 2 and the beginning of exon 3 of *Pax3a* are shown above the gene structure of *Pax3a* and those at the end of exon 4 and beginning of intron 4 of *Pax7b* are shown above the gene structure of *Pax7b*. Splice acceptor and splice donor cites are marked by red color.

```
torafugu Pax3a     MTALAGSIPR MMRPALAQNY PRSGFPLEVS TPLGQGRVNQ LGGVFINGRP  50
torafugu Pax3b     ......GL.. ....SPH... ..G.YS.... .......... ..........  50
zebrafish Pax3a    ......G.S. ....TPP... ..G.YS.... .......... ..........  50
Human Pax3a        ..T...AV.. ....GPG... .......... .......... ..........  50
Mouse Pax3a        ..T...AV.. ....GPG... .......... .......... ..........  50

torafugu Pax3a     LPNHIRHKIV EMAHHGIRPC VISRQLRVSH GCVSKILCRY QETGSIRPGA 100
torafugu Pax3b     .......... .......... .......... .......... .......... 100
zebrafish Pax3a    .......... .......... .......... .......... .......... 100
Human Pax3a        .......... .......... .......... .......... .......... 100
Mouse Pax3a        .......... .......... .......... .......... .......... 100
                          *                PD
torafugu Pax3a     IGGSKPKQGT TPDVEKRIEE YKRENPGMFS WGIRDKLLKD GICDRNNVPS 150
torafugu Pax3b     .......PTA S.ELD.K... .......... .E........ ...H......  150
zebrafish Pax3a    ........S. ......K... .......... .E........ ..........  150
Human Pax3a        ......Q..V. ......K... .......... .E........ AV....T...  150
Mouse Pax3a        ......Q..V. ......K... .......... .E........ AV....T...  150
                                                                Octapeptide
torafugu Pax3a     VSFISRIMRG KFGVKCDDEE DEDEIEKKEQ EDNERRAKHS IEGILGDRSS 200
torafugu Pax3b     ..A...VI.S ...GVG.... .DG.V-.R.M DES.P.T... .D........  199
zebrafish Pax3a    ..S...ML.C ...GNG.ED. .D..V..R.I .E........ .D........  200
Human Pax3a        ..S....L.S ...---KG.. E.ADL.R..A .ES.KK.... .D...SE.A.  197
Mouse Pax3a        ..S....L.S ...---KG.. E.ADL.R..A .ES.KK.... .D...SE.A.  197

torafugu Pax3a     --HSDEGSDI ESEPDLPLKR KQRRSRTTFT AEQLEELERA FERTHYPDIY 248
torafugu Pax3b     --...DT..V D...G..... .......... .......... ..........  247
zebrafish Pax3a    --.......V D...G..... .......... .......... ..........  248
Human Pax3a        APQ....... D......... .......... .......... ..........  247
Mouse Pax3a        APQ....... D......... .......... .......... ..........  247
                                          Homeodomain
torafugu Pax3a     TREELAQRAK LTEARVQVWF SNRRARWRKQ AGANQLMAFN HLLPG 293
torafugu Pax3b     ....V..... .......... .......... ...S...... ....A 292
zebrafish Pax3a    .......... .......... .......... .......... ..I.. 293
Human Pax3a        .......... .......... .......... .......... ..I.. 292
Mouse Pax3a        .......... .......... .......... .......... ..I.. 292
```

Fig. 4-2. Amino acid sequences alignment of torafugu Pax3 with those reported for zebrafish (Seo et al., 1999), human (Tsukamoto et al., 1994) and mouse (Goulding et al., 1991). Paired box domain (PD), an octapeptide motif and homeodomain are indicated in box. Identical amino acid residues are indicated by dots. Glutamine residue at 75th position (Glu75) from N-terminus of PD is indicated by an asterisk. Q- indicates an alternative splice variant reported for mammalian Pax3.

```
torafugu Pax7a    MATLPGTVPR MVRPAPGQNY PRTGFPLE-- ---------- -VSTPLGQGR  37
torafugu Pax7b    .SS....I.. .M........ ........AH TLGPQSQHLN I.........  50
zebrafish Pax7    .......... .M........ ........-- ---------- -.........  37
Salmon Pax7       .......... .M........ ........GQ YAGPEYVYCG T.........  50
Human Pax7        ..A....... .M........ ........-- ---------- -.........  37
Mouse Pax7        ..A...A... .M..G..... ........-- ---------- -.........  37

torafugu Pax7a    VNQLGGVFIN GRPLPNHIRH KIVEMAHHGI RPCVISRQLR VSHGCVSKIL  87
torafugu Pax7b    .......... .......... .......... .......... ..........  100
zebrafish Pax7    .......... .......... .......... .......... ..........  87
Salmon Pax7       .......... .......... .......... .......... ..........  100
Human Pax7        .......... .......... .......... .......... ..........  87
Mouse Pax7        .......... .......... .......... .......... ..........  87
                                       *PD
torafugu Pax7a    CRYQETGSIR PGAIGGSKPR QVATPDVEKR IEEYKRENPG MFSWEIRDKL  137
torafugu Pax7b    .......... .......... .......... ......D... ..........  150
zebrafish Pax7    .......... .......... .......... .......... ..........  137
Salmon Pax7       .......... .......... .......... .......... ..........  150
Human Pax7        .......... ..........Q-........K .......... ........R.  137
Mouse Pax7        .......... ..........Q-........K .......... ........R.  137

torafugu Pax7a    LKDGVCDRST VP-----SVS SISRVLRARF GKKDDEDDCD KKDEDGEKKT  182
torafugu Pax7b    .......... ..SGEAS... .......... ......EE.. ..........  200
zebrafish Pax7    ........G. ..SGEAS... .......... .....D.E.. ..........  187
Salmon Pax7       .......... ..SGEAS... .......... .....D..S. ..........  200
Human Pax7        ....H..... ..SG---L.. .......IK. ...EE..EA. ..ED.....A  184
Mouse Pax7        ....H..... ..-----... .......IK. ...E.DEEG. ..E......A  182
                  Octapeptide
torafugu Pax7a    KHSIDGILGD KSSGNRIDDG SDVDSEPDLP LKRKQRRSRT TFTAEQLEEL  232
torafugu Pax7b    .......... .--.S...EV .......... .......... ..........  248
zebrafish Pax7    .......... .--...T.E. ...E...... .......... ..........  235
Salmon Pax7       .......... .--...T.E. ...E...... .......... ..........  248
Human Pax7        .......... .--...L.E. ...E...... .......... ..........  232
Mouse Pax7        .......... .--...L.E. ...E...... .......... ..........  230
                                                   Homeodomain
torafugu Pax7a    EKAFERTHYP DIYTREELAQ RTKLTEARVQ VWFSNRRARW RKQAGANQLA  282
torafugu Pax7b    .......... .......... .......... .......... ..H......T  298
zebrafish Pax7    .......... .......... .......... .......... ..........  285
Salmon Pax7       .......... .......... .......... ........K. ..........  298
Human Pax7        .......... .......... .......... .......... ..........  282
Mouse Pax7        .......... .......... .......... .......... ..........  280

torafugu Pax7a    AFNHLLPG 290
torafugu Pax7b    ........ 306
zebrafish Pax7    ........ 293
Salmon Pax7       ........ 306
Human Pax7        ........ 290
Mouse Pax7        ........ 288
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Fig. 4-3. Amino acid sequences alignment of torafugu Pax7 with those reported for zebrafish (Seo et al., 1999), human (Schafer et al., 1994) and mouse (Seale et al., 1991). Paired box domain (PD), an octapeptide motif and homeodomain are indicated in box. Identical amino acid residues are indicated by dots. Glutamine residue at 75th position (Glu75) from N-terminus of PD is indicated by an asterisk, Q- indicates an alternative splice variant reported for mammalian Pax7.

```
                                        *
Pax3a   Clone 1   60  TGSIRPGAIG GSKPK-GTTP DV 82
        Clone 3   60  .......... .....Q.... .. 82
        Clone 5   60  .......... .....Q.... .. 82

        Clone 6   60  TGSIRPGAIG GSKPKPTASP EL 82
        Clone 8   60  .......... .......... .. 82
        Clone 9   60  .......... .......... .. 82
        Clone 10  60  .......... .......... .. 82
Pax3b   clone 11  60  .......... .......... .. 82
        Clone 12  60  .......... .......... .. 82
        Clone 13  60  .......... .......... .. 82
        Clone 14  60  .......... .......... .. 82
        Clone 15  60  .......... .......... .. 82
                                        *
Pax7a   Clone 2   60  TGSIRPGAIG GSKPRQVATP DV 82
        Clone 16  60  .......... .......... .. 82
                                        *
        Clone 17  60  TGSIRPGAIG GSKPRQVATP DV 82
Pax7b   Clone 19  60  .......... .......... .. 82
        clone 20  60  .......... .......... .. 82
```

Fig. 4-4. Deduced amino acid sequences of cDNA clones encoding torafugu Pax3 and Pax7 at a linker region of PD (residues 60-82). Identical amino acid residues are shown by an dots. Glu75 in Pax3a, Pax7a and Pax7b are indicated by an asterisk.

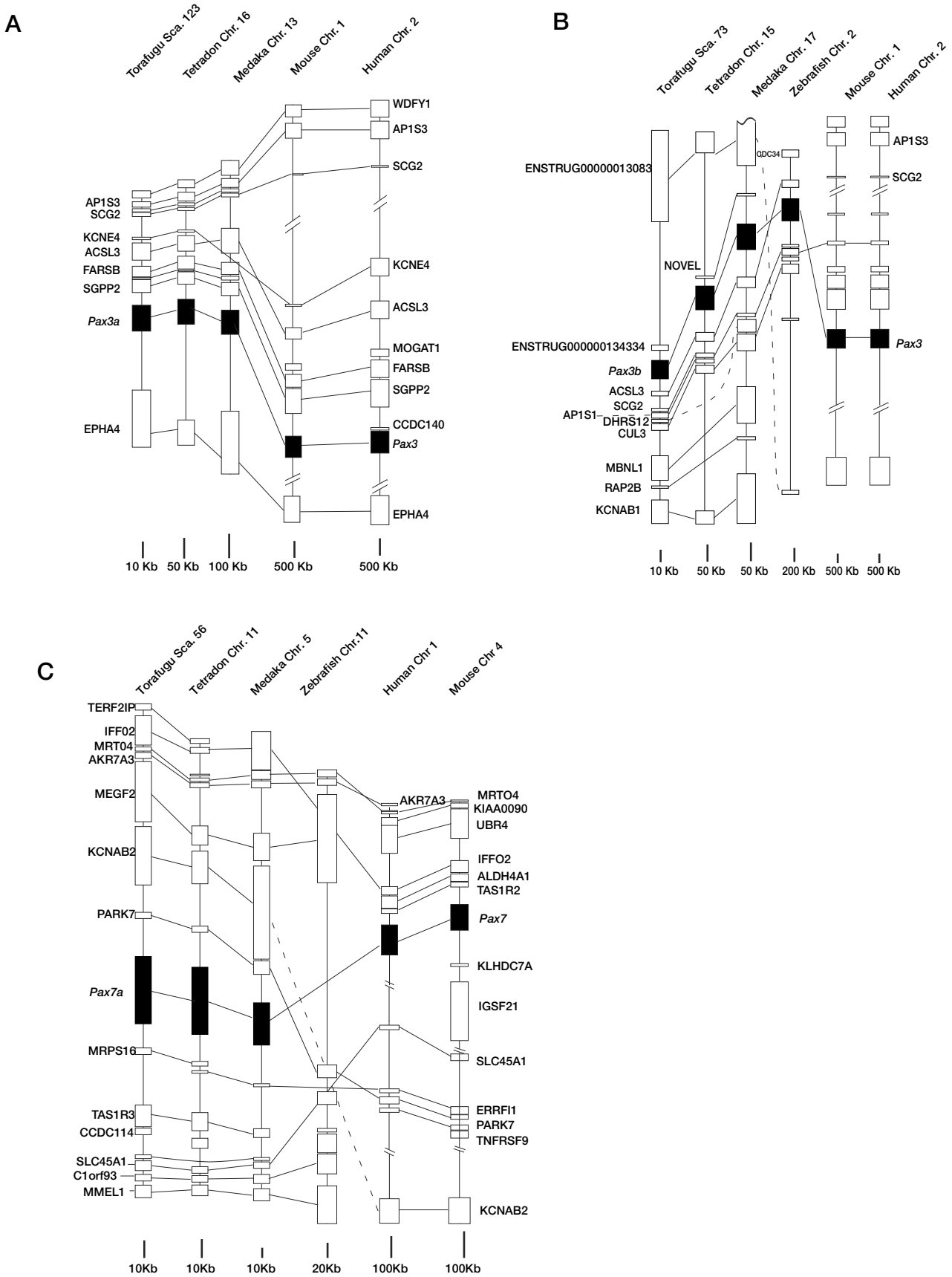

Fig. 4-5. Syntenic relationship of *Pax3* and *Pax7* genomic neighborhood in fish and mammals. Torafugu *Pax3a* and *Pax7a* are located on scaffold_123 and scaffold_56, respectively, which shows high syntenic relationship with *Pax3* and *Pax7* bearing regions on mammal and fish genome (A, C). Torafugu *Pax3b* is located on scaffold_73 that shows syntenic relationship only with additional copy of *Pax3* in fish but less with *Pax3* containing region with mammals (B).

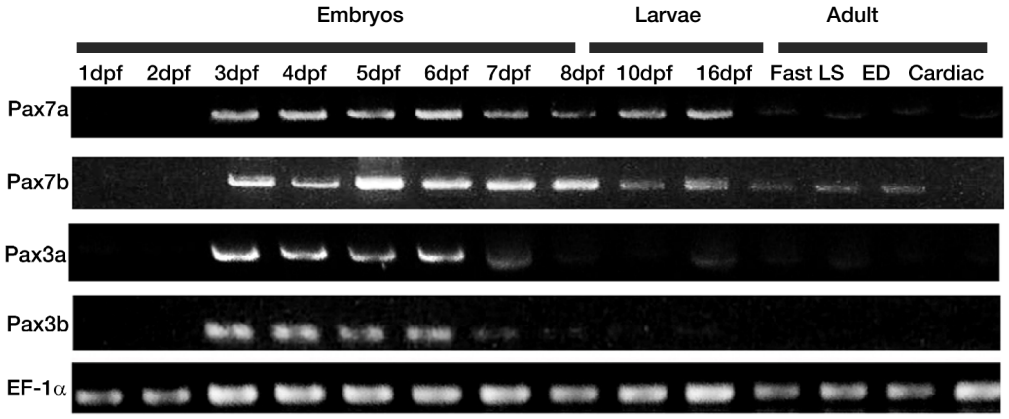

Fig. 4-6. Expression patterns of torafugu *Pax7* and *Pax3* during development and adult by RT-PCR. LS, lateralis superficials slow; ED, errectors and depressor slow.