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Multiple *cis*-elements in the 5′-flanking region of embryonic/larval fast-type of the myosin heavy chain gene of torafugu, $MYH_{M743\text{-}2}$, function in the transcriptional regulation of its expression

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ABSTRACT

The myosin heavy chain gene, $MYH_{M743\text{-}2}$, is highly expressed in fast muscle fibers of torafugu embryos and larvae, suggesting its functional roles for embryonic and larval muscle development. However, the transcriptional regulatory mechanism involved in its expression remained unknown. Here, we analyzed the 2075 bp 5′-flanking region of torafugu $MYH_{M743\text{-}2}$ to examine the spatial and temporal regulation by using transgenic and transient expression techniques in zebrafish embryos. Combining both transient and transgenic analyses, we demonstrated that the 2075 bp 5′-flanking sequences was sufficient for its expression in skeletal, craniofacial and pectoral fin muscles. The immunohistochemical observation revealed that the zebrafish larvae from the stable transgenic line consistently expressed enhanced green fluorescent protein (EGFP) in fast muscle fibers. Promoter deletion analyses demonstrated that the minimum 468 bp promoter region could direct $MYH_{M743\text{-}2}$ expression in zebrafish larvae. We discovered that the serum response factor (SRF)-like binding sites are required for promoting $MYH_{M743\text{-}2}$ expression and myoblast determining factor (MyoD) and myocyte enhancer factor-2 (MEF2) binding sites participate in the transcriptional control of $MYH_{M743\text{-}2}$ expression in fast skeletal muscles. We further discovered that MyoD binding sites, but not MEF2, participate in the transcriptional regulation of $MYH_{M743\text{-}2}$ expression in pectoral fin and craniofacial muscles. These results clearly demonstrated that multiple *cis*-elements in the 5′-flanking region of $MYH_{M743\text{-}2}$ function in the transcriptional control of its expression.

1. Introduction

A major structural protein which is expressed during differentiation of myoblast and myotube in vertebrates is sarcomeric myosin heavy chain (MYH) encoded by highly conserved multigene family. The MYH protein contains both the α-helical rod domain necessary for thick filament formation and the ATPase domain necessary for converting chemical energy into mechanical force (Weiss and Leinwand, 1996). It exists as a variety of isoforms adapted to function in individual muscles according to physiological requirements during development. These isoforms are evolved from primordial MYH genes (*MYH*s). During muscle fiber development, each *MYH* shows a complex expression pattern that continues throughout life cycle (Johnston et al., 1998; Mascarello et al., 1995). In mammals, at least 11 sarcomeric *MYH*s have been identified and their development-dependent and tissue-specific expressions contribute to the formation of various muscles with diverged muscle-fiber types such as embryonic, neonatal, fast, slow, and cardiac ones with different functional properties (Weiss et al., 1999). Meanwhile, fish are known to possess highly conserved MYH multigene family, although *MYH*s are much more than their higher vertebrate counterparts (Ikeda et al., 2007; Watabe and Ikeda, 2006). A higher number of *MYH*s expressed in fish is the result of many environmental factors such as water temperatures and physiological requirement in different developmental stages, resulting in changes of the composition of muscle-fiber type (Hirayama and Watabe, 1997; Liang et al., 2007; Watabe, 2002). Among *MYH*s family members in fish, specific *MYH*s are expressed

Abbreviations: ANOVA, analysis of variance; bp, base pair; CIP, calf intestinal phosphatase; DAPI, 4′-6′-diamidine-2-phenylindole dihydrochloride; dpf, days post fertilization; EGFP, Enhanced green fluorescent protein; *EGFP*, enhanced green fluorescent protein gene; kb, kilo base; MEF2, Myocyte enhancer factor-2; MRF, myogenic regulatory factor; MYH, myosin heavy chain; *MYH*, myosin heavy chain gene; MyoD, myoblast determining factor; NFAT, nuclear factor of activated T-cells; PCR, polymerase chain reaction; PFA, paraformaldehyde; Pax, paired box protein; RACE, rapid amplification of cDNA ends; RFP, red fluorescent protein; SPSS, statistical package for social science; SRF, serum response factor; TAP, tobacco acid pyrophosphatase; TBSTw, tris-buffered saline with 0.1% tween 20; TEEA, transient embryonic excision assay; TFsearch, transcriptional factor search.

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during embryonic and larval development. Such expression patterns of *MYHs* have been reported in some fish species such as rainbow trout *Oncorhynchus mykiss* (Rescan et al., 2001), common carp *Cyprinus carpio* (Ennion et al., 1999; Nihei et al., 2006), medaka *Oryzias latipes* (Ono et al., 2006, 2010), torafugu *Takifugu rubripes* etc. (Ikeda et al., 2007), providing some basic insight into embryonic and larval muscle development. These lines of information indicate that the expression of *MYHs* contributes to the formation of specific muscle-fiber types during development of fish in response to various internal and external stimuli. However, the molecular mechanisms of *MYHs* expression in fish remained mostly unknown.

Based on the primary structure, sarcomeric *MYHs* of fish are classified into fast and slow/cardiac types (Ikeda et al., 2004, 2007; McGuigan et al., 2004), and the fast type is further grouped into adult and embryonic types (Ikeda et al., 2010). Genomic structural analysis of torafugu revealed that fast-type *MYHs* in scaffold M743 (Fugu genome database Ver 3.0) form gene cluster C and are classified into an embryonic fast type (Ikeda et al., 2007) with its orthologous genes, all of which have also been reported to be expressed predominantly during embryonic and larval development in common carp (Nihei et al., 2006), medaka (Liang et al., 2007; Ono et al., 2006, 2010), and zebrafish *Danio rerio* (Berdougo et al., 2003; Bryson-Richardson et al., 2005; Wallace et al., 2005). The expression patterns of sarcomeric $MYH_{M743\text{-}2}$ in early developmental stages of torafugu were analyzed by our laboratory members. $MYH_{M743\text{-}2}$ appeared to be expressed at the somite formation stage (about 10–14 somites) which corresponds to 3 days post fertilization (dpf) and continued through successive embryonic and larval development but not in adult skeletal muscle (Yasmin et al., 2011; Unpublished observation). The whole mount *in situ* hybridization revealed that the transcripts of $MYH_{M743\text{-}2}$ were localized in fast muscle fibers of torafugu embryos and larvae (Ikeda et al., 2007). However, the transcriptional mechanisms in regulating the expression of $MYH_{M743\text{-}2}$ are not well defined. Transcriptional regulatory regions have been mapped for a small number of skeletal muscle-specific genes in fish, identifying both unique and shared *cis*-acting elements (Chen et al., 2007; Du et al., 2003; Kobiyama et al., 2006; Liang et al., 2008). Therefore it is important to identify *cis*-acting elements located at various distances upstream or downstream of the promoter responsible for the expression of torafugu $MYH_{M743\text{-}2}$.

The early steps in skeletal muscle development are controlled by combinatorial interactions between the members of myogenic regulatory factors (MRFs) (Bergstrom et al., 2002; Berkes and Tapscott, 2005), MADS (MCM1, Agamous, Deficiens, SRF) box transcription factors (Berkes and Tapscott, 2005; Jordan et al., 2004) and paired-box transcription factors (Pax3 and Pax7) (see reviews by Rawls and Olson, 1997; Buckingham, 2001). The MRF family of transcriptional activators includes MyoD, myogenin, myf-5 and MRF4 having a basic helix-loop-helix structure, and participates in the determination and differentiation of skeletal muscle fibers in higher vertebrates (Bergstrom et al., 2002; Berkes and Tapscott, 2005). Other important transcription factors in differentiation of skeletal muscle fibers are the myocyte enhancer factor-2 (MEF2) family members, which bind to an A/T-rich sequence existing in many muscle-specific promoters and enhancers (Berkes and Tapscott, 2005; Jordan et al., 2004). These are believed to underlie the expression of most, if not all, muscle-specific genes. Serum response factor (SRF), a MADS box transcription factor related to MEF2, also regulates skeletal, as well as cardiac and smooth muscle genes by binding a DNA sequence known as a CArG box (Davis et al., 2008; Miano, 2003; Norman et al., 1988). The previous studies revealed that MEF2 and SRF binding elements regulate fast skeletal *MYH* transcription *in vivo* (Allen et al., 2005). So far, the transcriptional activities of *MYHs* have been extensively investigated on the fast skeletal *MYHs* of mammals (Allen et al., 2005; Harrison et al., 2011; Lakich et al., 1998; Swoap, 1998) and fish (mainly carp and medaka) in a temperature dependent manner (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008). However, it is still far from comprehensive understanding of the transcription factors functioning in the regulation of embryonic and larval fast-type *MYHs* expression in fish.

The purpose of the present study was to identify the *cis*-regulatory regions within the upstream promoter that induce torafugu $MYH_{M743\text{-}2}$ promoter activity by transient analysis. The transient analyses by using expression vector containing various deletion and mutation forms of the 5′-flanking region of a particular gene are useful for identifying *cis*-acting elements responsible for its expression. Here, we used zebrafish to investigate the regulatory mechanisms involved in the expression of torafugu $MYH_{M743\text{-}2}$, as it is an economic animal model to generate transgenic lines and are easily accessible to transient reporter analysis for quantification. In addition, transgenic zebrafish that express EGFP under the control of muscle-specific promoters are useful for visualizing dynamic gene expression patterns, and dissecting regulatory transcription elements in live embryos (Jin et al., 2009; Park et al., 2009). We reported previously the isolation of the upstream promoter region of torafugu $MYH_{M743\text{-}2}$ and demonstrated that the 2.1 kb 5′-flanking region regulated developmental muscle-specific expression in zebrafish embryos (Yasmin et al., 2011).

In this study, we have characterized torafugu $MYH_{M743\text{-}2}$ expression using both transient and transgenic analyses in zebrafish embryos. Combining both transient and transgenic analyses, we demonstrated that the 2075 bp 5′-flanking sequences was critical for its muscle-specific expression in skeletal, craniofacial and pectoral fin muscles. A minimal promoter region containing SRF-like binding site was defined to direct $MYH_{M743\text{-}2}$ expression. We discovered that the SRF-like binding sites are required for promoting $MYH_{M743\text{-}2}$ expression in myotomal compartments and MEF2 and MyoD binding sites participate in the transcriptional regulation of $MYH_{M743\text{-}2}$ expression in myotomal compartments with other transcriptional factors. We further discovered that MyoD binding sites also participate in the transcriptional regulation of $MYH_{M743\text{-}2}$ expression in craniofacial and pectoral fin muscles.

2. Materials and methods

2.1. Experimental fish

Artificial fertilized eggs of torafugu were collected from Oshima Fisheries Hatchery, Nagasaki, Japan, brought to The University of Tokyo and reared at 18–20 °C in a tank at the Department of Aquatic Bioscience. Dorsal fin muscle of adult torafugu (body mass about 1 kg) was used for the extraction of genomic DNA. Mature zebrafish were raised at the zebrafish rearing facility at the Department of Aquatic Bioscience, The University of Tokyo. Fish were maintained at a photoperiod of 14 h light and 10 h dark in small aquariums supplied with continuous freshwater in recirculatory system. Spawning of zebrafish was carried out by putting a pair of males with a pair of females. Embryos were staged and maintained at 28 °C as described (Westerfield, 1995).

2.2. Determination of transcription start site by 5′-RACE

GeneRacer™ kit (Invitrogen, Carlsbad, CA, USA) was used to define the transcriptional start site of $MYH_{M743\text{-}2}$ through RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-RACE). Total RNA from torafugu embryos at 4 dpf was extracted with ISOGEN solution (Nippon Gene, Tokyo, Japan) and treated with calf intestinal phosphtase (CIP). Dephosphorylated RNA was then decapped by tobacco acid pyrophosphatase (TAP) and ligated with GeneRacer RNA oligos. After reverse transcription, the 5′ cDNA end was amplified by PCR using a $MYH_{M743\text{-}2}$ specific reverse primer (5′-CCAGGACTTT-GACCGTGACCTTAGC) together with GeneRacer™ 5′ primer included in the kit. Amplified 5′-cDNA fragments were subcloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced with an ABI 3100 genetic analyzer (Applied Biosystems, CA, USA) after

labeling with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.3. Bioinformatics

Transcription factor-binding sites in the 5′-flanking region of $MYH_{M743\text{-}2}$ were predicted by MatInspector, V7.1 (http://www.genomatix.de) and TFSEARCH program, V1.3 (http://www.cbrc.jp /research/db/TFSEARCH.html). Genes orthologous to torafugu $MYH_{M743\text{-}2}$ were searched in zebrafish, medaka and green spotted pufferfish (*Tetraodon nigroviridis*) genomes using ensemble genome browser (http://www.ensembl.org). The homology search for the 5′-flanking region between torafugu $MYH_{M743\text{-}2}$ and green spotted pufferfish *MYH13* was carried out by rVISTA plot analysis through the Vista server (http://genome.lbl.gov/vista/index.shtml).

2.4. Generation of a non-deletion and a series of 5′-deletion constructs

To identify the minimal promoter, a series of 5′-deletion constructs within the 2075-bp fragment was generated by PCR using torafugu genomic DNA as a template. A total of 10 forward primers and one reverse primer (Table 1) were designed to amplify one non-deletion (2075 bp) and a series of 5′-distal deletion regions. All of these amplified PCR products were subcloned individually into the *Bam*HI-*Xho*I site of pT2AL200R150G vector (courtesy of Dr. Koichi Kawakami) by In-Fusion® Advantage PCR Cloning Kit (Clontech, CA, USA). The plasmid DNA for microinjection was isolated from each deletion construct using GenElute™ plasmid Mini-prep Kit (Sigma-Aldrich, Steinheim, USA). These constructs were P2075, P1482, P1006, P819, P600, P500, P468, P448, P425 and P340, in which numbers refer to the nucleotide positions upstream of the $MYH_{M743\text{-}2}$ translation start codon. P2075

Table 1
Nucleotide sequences of oligonucleotide primers used in various experiments.

Experiment	Primer name	Nucleotide sequence (5′————————3′)	Length (bp)
Generation of 5′-deletion constructs	P2075F	GGATCATAGCAGTAGGTTTA	20
	P1482F	CGTGAATCCAAAATGGCACTAC	22
	P1006F	CGGATGATTCCAAATACCCAG	21
	P819F	GCCTCATGAAATCAAAACTC	20
	P600F	CAAGAACAACTGGTATGAGGTG	22
	P500F	CACTACATATAACTTGGCTGTGTC	24
	P468F	CCTGTGAATATAAGGACCCCTTTG	24
	P448F	TTTGGAGACATCAGTGTGGAACA	23
	P425F	AGGAGACCCGTGAGTTGGTGGTAA	24
	P340F	CCAAATTCAACAAATCAAAATGTTCAC	27
	Reverse	ATGGTGGCGGCTTATGTCTG	20
Generation of SRF-like binding site deletion constructs	P2075ΔSRF1F	TTCGTAAACATATGGAGATAATTAG	25
	P2075ΔSRF1R	TTTAGAGACATTATTACGAGGGT	23
	P2075ΔSRF1-2 F	ACACTACATATAACTTGGCTGTGT	24
	P2075ΔSRF1-2R	GCTGACAACAAAAAAGGAGACAC	23
	P2075ΔSRF1-3 F	TTTGGAGACATCAGTGTGGAACA	23
	P2075ΔSRF1-3R	GGGCTTTGCTGACACAGCCAA	21
Generation of SRF-like binding site mutation constructs	P468M1-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M1-R	CAAAGGGGTCCTT**CC**ATTCACAG	23
	P468M2-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M2-R	CAAAGGGGTCCTTATA**CC**CACAG	23
	P468M3-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M3-R	CAAAGGGGT**GG**TTATATTCACAG	23
	P468M4-F	GAGACATCAGTGTGGAACAGGAG	23
	P468M4-R	C**GG**AGGGGTCCTTATATTCACAG	23
Generation of MyoD binding site deletion constructs	P2075ΔMyoD1F	GGGCAAAAACACTTAACCAAAGAA	24
	P2075ΔMyoD1R	ATGTTTAAAAAGAATCACATGGC	23
	P2075ΔMyoD1-2 F	AACATTTACCATGAAAAAGGTGT	23
	P2075ΔMyoD1-2R	GGATGGTTGTTGGGATTTTTTTG	23
	P2075ΔMyoD1-3 F	CATTTTACAATTTTAAGCCTCATG	24
	P2075ΔMyoD1-3R	TTCATAAAAATTACTGGGAGTTG	23
Generation of MEF2 binding site deletion constructs	P2075ΔMEF2_1F	AAACATATGGAGATAATTAGACAAGATGG	29
	P2075ΔMEF2_1R	GCAGTTTAGAGACATTATTTCGAGG	25
	P2075ΔMEF2_1-2 F	CAAACTAATATGTTACAAATAGATATCAAAC	31
	P2075ΔMEF2_1-2R	AATCACTTTTGCTCATATTTTGGC	24
	P2075ΔMEF2_1-3 F	AACAAAGCTCATCGTAGAAAAGT	23
	P2075ΔMEF2_1-3R	TTAGTTTGAGATCTATTTTCAGTGC	25
	P2075ΔMEF2_1-5 F	TCCAGGTGGCAAAAAGGTCGG	21
	P2075ΔMEF2_1-5R	TGAAAACAACCACATCAATGTTT	23
	P2075ΔMEF2_1-6 F	CCAGCCCAAAAAAATCCCAACAA	23
	P2075ΔMEF2_1-6R	TGTGGTTGTCTTGCATATTGTGT	23
	P2075ΔMEF2_1-7 F	ATCATTGATTATTGATCTGTTTAGG	25
	P2075ΔMEF2_1-7R	CACTCAACTTGATAATGTCCTAA	23
	P2075ΔMEF2_1-8 F	CACAAATTAGTCGAGTCATTATC	23
	P2075ΔMEF2_1-8R	ATCCAGCTTAATACTGTAATTTA	23
Conserved region deletion construct	P2075Δ664-364 F	CCACTTAAATATCTTTCCAAATTCAAC	27
	P2075Δ664-364R	GATAATGTCCTAAAAGGAATGTTTGAT	27
Real time PCR analysis	β-actin-F	TGCTGTTTTCCCCTCCATTG	20
	β-actin-R	TCTGTCCCATGCCAACCAT	18
	EGFP-F	AGCAAAGACCCCAACGAGAA	18
	EGFP-R	GCGGCGGTCACGAACTC	17

was generated as a non-deletion control and the rest deletion constructs contained a deleted $MYH_{M743\text{-}2}$ promoter region, *EGFP*, and SV40 polyA signal.

2.5. Generation of $MYH_{M743\text{-}2}$:EGFP transgenic line

For this purpose, RNA encoding functional Tol2 transposase enzyme was transcribed *in vitro* from pCS-TP vector (Kawakami et al., 2004) using the mMESSAGE mMACHINE SP6 Kit (Ambion, TX, USA). To generate the stable transgenic line, the Tol2-based construct (P2075) was co-injected with transposase mRNA into one- to two-cell staged embryos. After 8–10 h after the microinjection, embryos were subjected to transgenic embryonic excision assay (TEEA) to confirm whether the excision occurred properly. As TEEA showed successful results, *EGFP*-positive embryos were identified at 2 dpf and then transferred into the fish rearing unit at the Department of Aquatic Bioscience, The University of Tokyo, until sexual maturity. Individual founder fish were outcrossed with wild-type fish for examination of *EGFP*-positive expression in the offspring. *EGFP*-positive offspring of selected founder lines were raised to establish the F1 generation. The F2 generation was then established by incrossing F1 fish.

2.6. Generation of deletion and mutation constructs of various cis-elements

Inverse PCR-based site-directed mutagenesis technique was used to generate (1) the deletion and mutation constructs of SRF, MEF2 and MyoD binding sites, and (2) the deletion construct in the conserved region (−664 to −364 bp) between torafugu $MYH_{M743\text{-}2}$ and green spotted pufferfish *MYH13*. Specific forward and reverse primers were designed for the deletion of *cis*-elements (Table 1). Mutant constructs of SRF-like binding site (P468M1, P468M2, P468M3 and P468M4) were generated by using mutant reverse primers, each containing 2-bp mutant bases (Table 1, bold face). Inverse PCR was performed using KOD FX DNA polymerase (TOYOBO, Osaka, Japan) and the plasmid DNA of a specific construct as template with phosphorylated primers. Then the amplified inverse PCR products were digested by *Dpn*I at 37 °C for 1 h. The digested inverse PCR products were ligated with DNA Ligation Kit (Mighty Mix-Takara, Tokyo, Japan). The plasmid DNA for microinjection was isolated from the positive colony of each of deletion and mutation constructs using GenElute™ plasmid Mini-prep Kit (Sigma-Aldrich). Each construct was subjected to sequencing with an ABI 3100 genetic analyzer (Applied Biosystems) for confirming deletions and mutations in PCR-based mutagenesis technique.

2.7. Microinjection

Each *EGFP* reporter constructs was diluted to 50 ng/mL with sterile distilled water containing 0.025% phenol red and introduced into fertilized eggs at one- to two-cell stages. Embryos were reared at 28 °C, and subjected to observation of EGFP expression patterns during development under a MVX10 macro-zoom microscope (Olympus, Tokyo, Japan) and a FV1000 confocal laser scanning microscope (Olympus). Older embryos were anesthetized with 0.6 μM tricaine methyl sulfonate (Sigma-Aldrich) to inhibit movement during observation.

2.8. Immunohistochemical analysis

For immunohistochemistry, embryos were fixed with 4% paraformaldehyde (PFA) in Tris-buffered saline (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% Tween 20 (TBSTw) overnight at 4 °C. Fixed embryos were washed with TBSTw, and blocking was performed using a 1.5% blocking reagent (Roche, Mannheim, Germany) in TBSTw. Transverse sections were prepared at a thickness of 16 μm with a cryostat Tissue-Tek Cryo3 (Sakura Finetech, Tokyo, Japan) at −20 °C before the first immunoreactions. The first antibodies used in this study were as follows. Living colors EGFP antibody (Clontech) was used at a dilution of 1:1000 in the blocking solution, and F310 and F59 supplied by Developmental Studies Hybridoma Bank at 1:20. Immunoreaction with the first antibody was performed overnight at 4 °C. After incubation, embryos were washed with TBSTw and labeled with the second antibodies, anti-mouse IgG Alexa Fluor 555 and anti-rabbit IgG Alexa Fluor 488 (Invitrogen) at a dilution of 1:250 for overnight at 4 °C. The signals in the cryosection samples were observed using an Olympus Fluo View1000 confocal laser scanning microscope (Olympus).

2.9. Real-time PCR analysis

For relative quantification, the reaction was performed in a total volume of 20 μL, containing 10 μL 2×SYBR premix *ExTaq*II kit (Takara), 10 μM each of primers, 1 μL diluted template cDNA (about 10 ng) and 0.4 μL ROX reference dye. Real-time quantitative PCR was performed in ABI Prism 7300 Sequence Detection System (Applied Biosystems). PCR consisted of pre-incubation at 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The samples were analyzed in triplicates. A housekeeping gene encoding β-actin (NM_131031) was selected as reference for the relative expression levels of target genes, which were calculated using the comparative C_T difference method (Schmittgen and Livak, 2008). Primers used in real-time PCR analysis are listed in Table 1.

2.10. Statistical analysis

To compare the percentages of embryos with EGFP expression in skeletal, craniofacial and pectoral fin muscles in various constructs and relative quantification of target gene, statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Tukey's test in Statistical Package for Social Science (SPSS) version 11.5 (SPSS Inc., IL, USA). Data were represented as the mean ± SD and the differences were considered significant at $P<0.05$.

3. Results

3.1. Characterization of 2075 bp 5′-flanking sequences of torafugu $MYH_{M743\text{-}2}$

To determine the regulatory region for the muscle-specific expression, we first characterized the 2075 bp 5′-flanking region of $MYH_{M743\text{-}2}$. The 2075 bp DNA fragment included an 1610-bp 5′-upstream region, three exons, and two introns. The transcription start site was determined by 5′-rapid amplification of cDNA ends (RACE) at 465 bp upstream of the ATG start codon in exon 3 (Fig. 1). Using the Genomatix Matinspector and TFsearch programs, we found that the 2075-bp 5′-flanking region contained transcription factor-binding sites for SRF, MEF2 and MyoD, all of which have been implicated in the expressional regulation of muscle-specific genes during development (Fig. 1). To analyze the transcriptional activity, the 2075 bp 5′-flanking region of $MYH_{M743\text{-}2}$ was fused to *EGFP* in pT2AL200R150G vector. The resultant gene construct (P2075) was microinjected into fertilized eggs of zebrafish for transient expression analysis. EGFP expression in injected embryos was monitored by direct observation under a fluorescent microscope. About 90% (n = 105) of the injected embryos showed a strong EGFP expression along skeletal muscle fibers. In zebrafish, the expression was detected at 1 dpf in the somite formation stage (Figs. 2A–B). After hatching, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 2C). In addition to the skeletal muscle expression, EGFP expression was also detected in pectoral fin and craniofacial muscles at 4 dpf (Figs. 2 D–F). These data confirmed that the 2075 bp 5′-flanking region of torafugu $MYH_{M743\text{-}2}$ contained the essential regulatory sequences for muscle-specific expression.

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GGATCATAGC AGTAGGTTTA CTACCCTACT TTAAATGCTG GAGTGGTTGC TTAAGTAGCC ATGTGATTCT TTTTAAACAT   -1996
GCAGCTGATT GGGCAAAAAC ACTTAACCAA AGAAAGCCAG TATACTAGGA CCCAGACACC TACGGGAATT CCTTCAAAAC   -1916
   MyoD
AATGAAAATT TAAATACAAA GTTTATCTTT AAGTCCTGTC TTGACCTTGC ACTTGACTGA CAATATGGTA TAACAAGGAC   -1836
CTGTTAGAAA AACTAATTTG CATATGAATT AACTCACAGT TCACTGGCTT TGACAATGGT TACACACATT AGCTTATGTT   -1756

ACCCTCGAAA TAATGTCTCT AAACTGCACG TATAAAGATA CTTTCGTAAA CATATGGAGA TAATTAGACA AGATGGTGGC   -1676
                      SRF                 MEF2
AGCGGCCAAA ATATGAGCAA AAGTGATTTT TCTGCACTGA AAATAGATCT CAAACTAATA TGTTACAAAT AGATATCAAA   -1596
                                  MEF2                 MEF2
CAAAGCTCAT CGTAGAAAAG TAAATTTGAA CACACCGGCA AAATAAAAGA TAGAAAACCA ATGCTCATTT AAGTTCAGTT   -1516
GCATATTAAA AAAACAGGTA TCTTGAGCTT TTTCGTGAAT CCAAAATGGC ACTACAAAGC CATCGAATGA CGTATTGTAT   -1436
TTATCTGATA AGTGTAATTC ACACTGATTC ACCCAAAACT GTAATTCGAG TTAGATTACA AAAGAATTCG AATGGGTGCT   -1356
TGTTTAAACG AAAACATTGA TGTGGTTGTT TTCAACCTGC TATACATAGT TCTATAGTTA GAATCCAGGT GGCAAAAAGG   -1276
                                  MEF2                 MEF2
TCGGGGGCTC CAGTTTCCAC CGCCATTCAT CTCAAACCGT TGCATTGACG GCACAGCTGA AGCTGCAGTG GTGTGTGTGT   -1196
GTGTGTGTGT GTGTCTTTGG ATTTAAGATC AGTAGATCTG CAAGACAGGG AGTGGAACAC ACAATATGCA AGACAACCAC   -1116
AGCTGCTATT TAAAACAAAA CCAGCCCAAA AAAATCCCAA CAACCATCCA TCATCTGTTT AACATTTACC ATGAAAAAGG   -1036
   MEF2                                              MyoD
TGTTTTATGT TGTGTGAGAG CAATTGTTCG GATGATTCCA AATACCCAGT AATCTCTACA TGGGGATAGT TGATGCATCC   -956
ATTTAATTTT TCTCCATCAA TACCTGAAAC CAGGATTAAA TTGTATTACA TATTTTAACT GATTATTCAG GAAGAGGAAT   -876
GGATTTACAA CTCCCAGTAA TTTTTATGAA GAGCACCTTT CATTTTACAT AATTAAGCCT CATGAAATCA AAACTCATTA   -796
                                  MyoD
AACTTAATTT CCAGTGAATA AGTGAATCAT TACACAAAAA AGTGTATTGT TGAATGAAAA ACTGTGGCAC AGTATAATGA   -716
TAAAACATGC AGCATTGGCA CAGAATCAAA CATTCCTTTT AGGACATTAT CAAGTTGAGT GATGCTTCAA AATAGCTTCC   -636
                                                                  MEF2
ATCATAGATT ATTGATCTGT TTAGGGTGCA TGTCCAAGAA CAACTGGTAT GAGGTGAAAC CTTAAACATC ACATGTATTG   -556
ACTTACAACG TGTGTCTCCT TTTTTGTTGT CAGCCTTTCC TTATTTGGCA CATACACTAC ATATAACTTG GCTGTGTCAG   -476
                                  SRF
CAAAGCCCCT GTGAATATAA GGACCCCTTT GGAGACATCA GTGTGGAACA GGAGACCCGT GAGTTGGTGG TAAATATTGT   -396
                SRF
TTAGTTGTTG TGTGTTTTTG TTTTTTCAAA TTAGTTAAAC CACTTAAATA TCTTTCCAAA TTCAACAAAT CAAAATGTTC   -316
ACATTTTTGA AATTTCTAAC TGTTGAATTT TCTTTTCTTA TCACATATAT TCTTTTAACA GCCACAGAGA ATACAATTCC   -236
CCATAGGAGC AGCAAGGTAA AGGCAACATA ACAATTAATT GATTGATCCT TAAATAACTA AAAATGTGTC AAATTATTCT   -156
GATATTCCCA GTTCAGCAGT AATATTATAC AGTATTAAGC TGGATCCCAT TGCTAAAAAA AACCACACAA ATTAGTCCAG   -76
                                                  MEF2
TCATTATCTT AATTAACATA ACTTAAGTCA TTCAAATCCT CTGTAATTAT CTAACAGACA TAAGCCGCCA CCATG        -1
```

Fig. 1. The DNA sequence of the 2075 bp 5′-flanking region of *MYH*$_{M743\text{-}2}$. The transcription initiation site determined by 5′-RACE is indicated by an arrow. Based on the transcription-factor binding site analysis by Genomatix and TFsearch program, MyoD, MEF2, and SRF-like binding motifs are indicated by boxes. Three exons are marked bold. The translation start codon located on the third exon is underlined.

3.2. Generation and expression pattern of MYH$_{M743\text{-}2}$*:EGFP transgenic zebrafish*

To confirm the expression patterns of P2075 during early muscle development more clearly, we attempted to establish stable transgenic lines. For this purpose, we used the Tol2 transposon vector system, which was originally identified in medaka (Koga et al., 1996) and later adapted as a vehicle to efficiently integrate ectopic DNA into the zebrafish genome (Kawakami et al., 2000, 2004). Transient injection of Tol2 based plasmids was previously shown to be a valuable method to analyze tissue-specific promoters in zebrafish (Fisher et al., 2006; Korzh, 2007). In this method, P2075 was co-injected with transposase mRNA, which was transcribed from pCS-TP vector. The injected embryos were subjected to the TEEA at 8–10 h after microinjection to confirm whether the excision occurred properly. The excision product was detected from all embryos injected with the transposase mRNA and a transposon-donor plasmid containing T2AL200R150G (data not shown). Muscle-specific EGFP expressing embryos were selected and raised to adulthood. After 3 months, the surviving founders were mated with wild-type zebrafish to find germline-transmitted transgenic

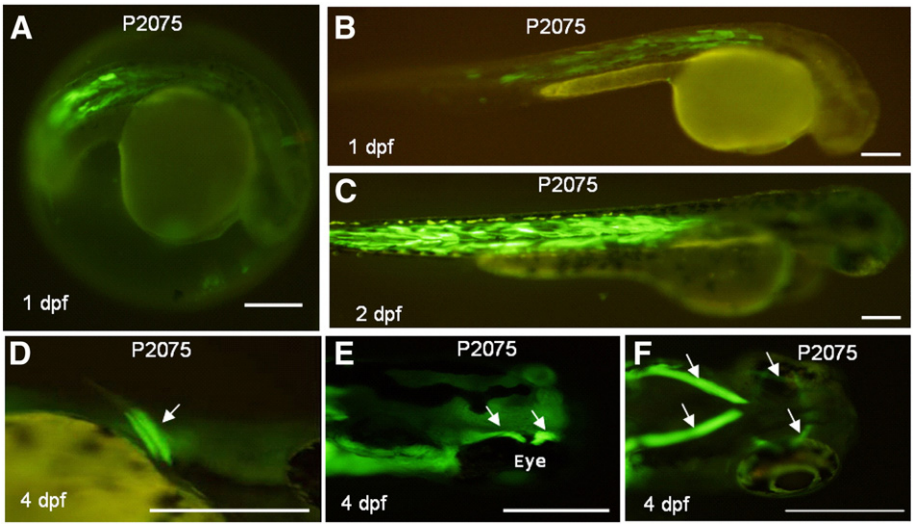

Fig. 2. The expression patterns of P2075 construct in embryos and larvae of zebrafish. A–C: EGFP expression patterns in whole myotomal region of zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C) injected with the reporter construct of P2075. D-F: EGFP expression patterns in pectoral fin (D) and craniofacial muscles (E, F) in zebrafish larvae at 4 dpf. Arrows indicate muscle fibers expressing EGFP. Scale bars: 100 μm.

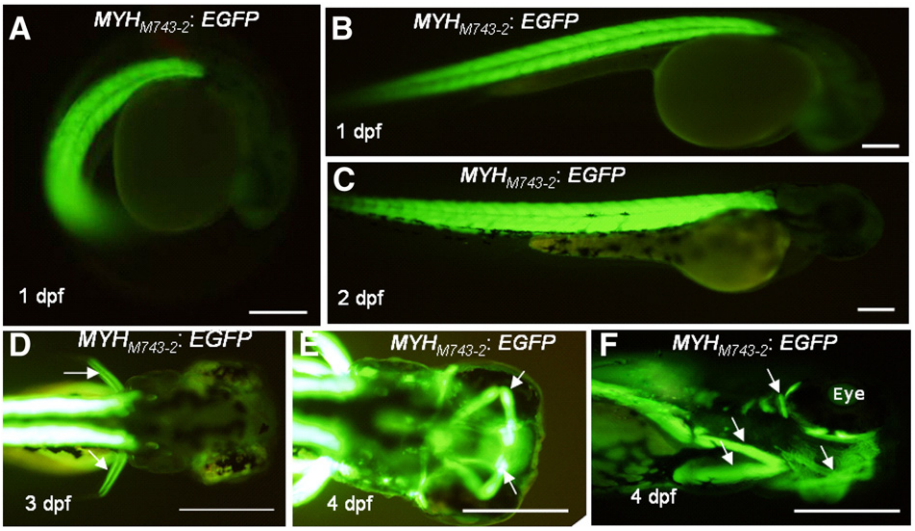

Fig. 3. The expression patterns of *MYH$_{M743-2}$:EGFP* in stable transgenic line embryos and larvae of zebrafish. A–C: EGFP expression patterns of *MYH$_{M743-2}$:EGFP* in the whole myotomal region of stable transgenic line zebrafish embryo (1 dpf, A, B) and larva (2 dpf, C). D–F: EGFP expression patterns in pectoral fin (D) at 3 dpf and craniofacial muscles (E, F) at 4 dpf in stable transgenic line zebrafish larvae. Arrows indicate muscle fibers expressing EGFP. Scale bars: 100 μm.

zebrafish. The 20% (4 out of 20 fish tested) of F0 injected zebrafish were found to be founders, which produced F1 embryos expressing EGFP in the myotomal compartments. About 50% of F2 offspring embryos carried muscle-specific EGFP expression by outcrossing F1 fish with wild-type fish, suggesting that the germline transmission rate of transgene complied with the Mendelian inheritance law. All of the F1 transgenic line fish were mated and produced normal offspring. The temporal and spatial EGFP expression was analyzed in these transgenic lines. Similarly with the transient assay, the embryos of stable zebrafish line also showed a strong expression of the reporter gene along muscle fibers. In the stable transgenic line, the expression was also detected at 1 dpf (Figs. 3 A–B). After hatching, EGFP continued to be expressed in the whole myotomal region of larva (Fig. 3C). The observation with a fluorescent microscope on 24 h intervals confirmed that zebrafish expressed EGFP in pectoral fin at 3 dpf and craniofacial muscles at 4 dpf (Figs. 3 D–F). The observed EGFP expression in the stable transgenic line was consistent with the expression pattern of P2075. Taken together, these data suggest that the 2075-bp 5′-flanking region contained the necessary regulatory elements for *MYH$_{M743-2}$* expression in skeletal, craniofacial and pectoral fin muscles.

3.3. Immunohistochemical observations

Immunohistological observations were conducted to clarify types of muscle fibers that express EGFP. EGFP-expressing embryos were stained with F310 (Crow and Stockdale, 1986) and F59 (Crow and Stockdale, 1986; Devoto et al., 1996) monoclonal antibodies specific to fast-type myosin light chain and slow-type MYHs, respectively. EGFP-expressing muscle fibers of P2075-injected larvae consisted of both fast and slow types reacted with F310 (Figs. 4 A–B) and F59 antibodies (Figs. 4C–D), respectively. *In situ* hybridization demonstrated that the transcripts of *MYH$_{M743-2}$* were restricted to fast muscle fibers in torafugu embryos (Ikeda et al., 2007). Transient analysis sometimes showed ectopic reporter gene expression. Therefore, immunohistochemical staining was also performed on the cryosection made from the *MYH$_{M743-2}$:EGFP* stable transgenic line zebrafish larvae at 3 dpf. Interestingly, EGFP-expressing muscle fibers in stable transgenic line larvae consisted of only fast types as reacted with F310 (Figs. 4 E–G) but not in the slow types as no EGFP muscle fibers reacted with F59 (Figs. 4 H–J). This study confirmed that *MYH$_{M743-2}$* expression is fast-muscle specific in agreement with the previous findings by Ikeda et al. (2007).

3.4. Deletion analysis of torafugu MYH$_{M743-2}$ promoter in zebrafish embryos

To identify the minimal promoter that could drive *MYH$_{M743-2}$* expression in the myotomal compartment, a series of constructs containing progressive deletions from the 5′-end of the 2075-bp fragment were generated. The resulting six deletions constructs (P1482, P1006, P819, P600, P500 and P340) contained fragments of 1,482, 1,006, 819, 600, 500 and 340 bp, respectively, which were fused to *EGFP* and SV40 polyA signal (Fig. 5A). P2075 was generated as a non-deletion control. When P2075 was microinjected into one to two-cell embryos, approximately 90% of the injected embryos had EGFP expression in the myotomal compartments (Fig. 5B). Embryos microinjected with P1482, P1006, P819, P600 and P500 displayed a gradual reduction in percentages of EGFP expression in myotomal compartments (Fig. 5B). Notably, EGFP expression was not detected in any embryos microinjected P340. Fluorescent optics also showed that EGFP expression was reduced in embryos microinjected with these deletion constructs and such EGFP fluorescent reduction typically correlated with a smaller *MYH$_{M743-2}$* promoter region (Figs. 5 C–G). To precisely map the minimal active promoter, three additional deletion constructs, P468, P448 and P425, were generated and microinjected into one to two-cell embryos. Only embryos microinjected with P468 had EGFP expression in the myotomal compartments, and embryos injected with P448 and P425 did not show any EGFP expression in myotomal compartments (Fig. 5B). Thus, the 468-bp regulatory sequence contained the minimum basal promoter that could drive EGFP expression in the myotomal compartment. These results also suggest that 20 nucleotides spanning the region from −468 to −449 bp contain positive *cis*-elements required for *MYH$_{M743-2}$* expression (Fig. 5H).

3.5. SRF-like binding elements are necessary for directing MYH$_{M743-2}$ expression

Deletion analysis suggested that the 20 nucleotides spanning the region from −468 to −449 bp (CCTGTGAATATAAGGACCCC) may contain positive *cis*-elements required for *MYH$_{M743-2}$* expression. Genomatix Matinspector program predicted a transcription factor known as SRF-like binding site within this 20 bp sequence of *MYH$_{M743-2}$*. SRF is a member of the MADS box superfamily of transcription factors (Shore and Sharrocks, 1995) and regulates skeletal as well as cardiac and smooth muscle genes by binding to a DNA sequence known as the CArG box (Camoretti-Mercado et al., 2003; Miano, 2003). Therefore, we

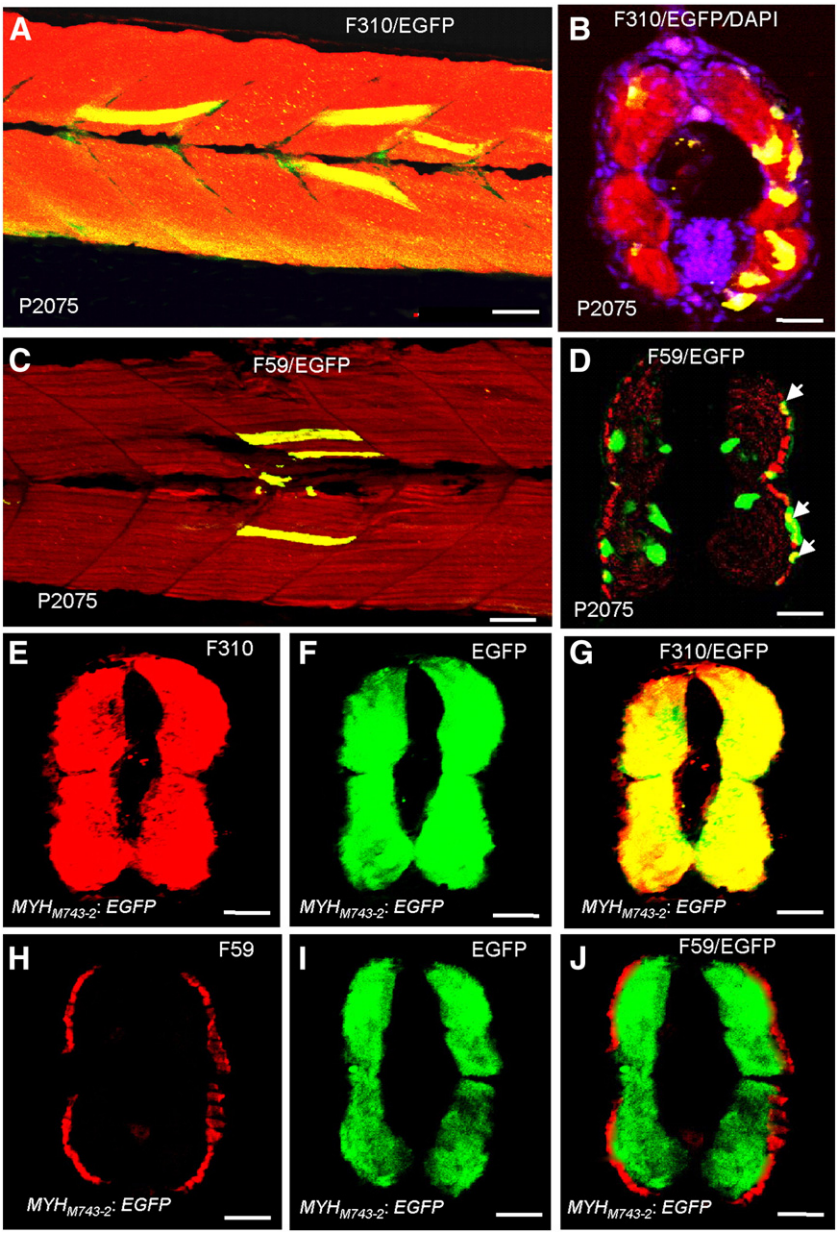

Fig. 4. EGFP expression was localized to both fast and slow muscle fibers in P2075-injected larvae and only fast muscle fibers in stable transgenic line (*$MYH_{M743\text{-}2}$:EGFP*) larvae. A–B: Fast muscle fibers expressing EGFP as reacted with F310 antibody in a P2075-injected larva at 3 dpf (A, lateral view; B, transverse section). C-D, Slow muscle fibers also expressing EGFP as reacted with F59 antibody (C, lateral view; D, transverse section) in a P2075-injected larva at 3 dpf. Some EGFP expressing muscle fibers were stained with F59 antibody (arrows in D). E-J: *$MYH_{M743\text{-}2}$:EGFP* transgene in a stable transgenic line larva showed EGFP expression in fast muscle fibers as reacted with F310 antibody (E, F, G; transverse sections) but not in slow muscle fibers as no *EGFP* expressing muscle fiber reacted with F59 antibody (H, I, J; transverse sections). Scale bars: 20 μm.

hypothesized that this SRF-like binding site may be necessary to mediate *$MYH_{M743\text{-}2}$* expression in the myotomal compartments. To examine this, four primers, each having two altered bases within and outside of SRF-like binding site, were used to generate mutant forms of P468 (Table 2). Embryos microinjected with P468M1, containing two base substitutions in the central core sequence, showed the complete disappearance of EGFP expression in the myotomal compartments (Table 2). Mutation of two bases adjacent to the core sequence (P468M2 and P468M3) caused a marked reduction of EGFP expression in the myotomal compartments (Table 2). In contrast, dinucleotide replacement (P468M4), which was apart from the SRF-like binding site, resulted in relatively higher levels of EGFP expression compared with P468M2 and P468M3 (Table 2).

Genomatix Matinspector program identified three SRF-like binding sites within the 2075-bp regulatory region of *$MYH_{M743\text{-}2}$*. To examine functional redundancy of these SRF-like binding sites, we deleted these sites in P2075 and established the P2075ΔSRF1, P2075ΔSRF1-2 and P2075ΔSRF1-3 with deletion of one, two, and three SRF-like binding sites, respectively (Fig. 6A). Embryos microinjected with P2075ΔSRF1 did not significantly reduce EGFP expression percentages in myotomal compartments compared to P2075-injected embryos (Fig. 6A). P2075ΔSRF1-2 injection exhibited a marked reduction in EGFP expression, and embryos microinjected with P2075ΔSRF1-3 displayed the largest reduction in EGFP expression percentage (Fig. 6A). Fluorescent optics showed that EGFP expression pattern in myotomal compartments was variable from embryo to embryo. Therefore, EGFP expressing embryos were grouped into three categories (high, medium and low), according to the intensity of fluorescence by visual observation under a fluorescent microscope (Figs. 6 B–D). The purpose of this grouping was to investigate whether

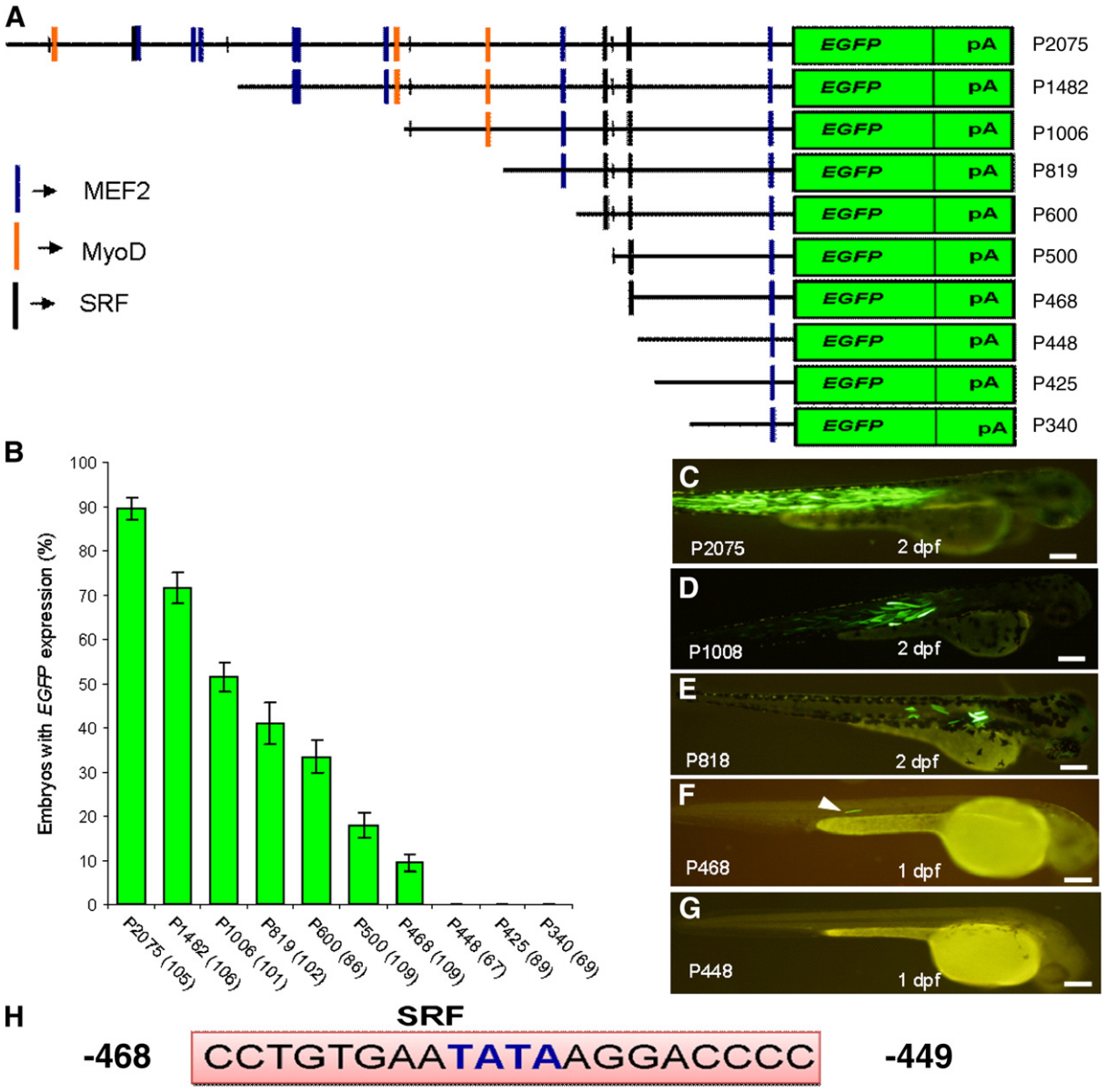

Fig. 5. Effects of $MYH_{M743\text{-}2}$ promoter deletions on EGFP expression in the myotomal compartments of zebrafish embryos and larvae. A: Schematic representations of consensus-binding sites within 2075-bp upstream region of torafugu $MYH_{M743\text{-}2}$, as well as a deletion series in the torafugu $MYH_{M743\text{-}2}$ promoter region. Vertical lines represent consensus-binding sites for transcriptional factors of MEF2, MyoD and SRF. All promoter deletions (black lines) are linked to *EGFP* and SV40-polyA sequence in pT2AL200R150G vector. B: Bar graph showing percentages of embryos that express EGFP in the myotomal compartments in microinjection of each deletion construct. The total number of embryos injected with each construct is shown in parentheses. C–G: Embryos injected with a series of distal promoter deletion constructs at one- or two-cell stages were examined for transient EGFP expression at 1–2 dpf using fluorescent microscopy. Reduced EGFP fluorescence typically correlates with a smaller $MYH_{M743\text{-}2}$ promoter (C–F) and no EGFP expression was observed in P448 (G). An arrowhead indicates a single fiber expressing EGFP in P468 (F). Scale bars: 100 μm. H: 20 nucleotides spanning the region from −468 to −449 contain positive *cis*-elements required for $MYH_{M743\text{-}2}$ expression. Blue color with bold face in the SRF sequence indicates a central core sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there was any correlation between the levels of transient expression and the deletion of SRF-like binding sites. Deleting all three SRF-like binding sites resulted in a marked reduction of high-EGFP expressing embryos and an increase of medium- to low-EGFP expressing embryos (Fig. 6A). In order to confirm this, we validated the reduction of EGFP expression in P2075ΔSRF1-2 and P2075ΔSRF1-3 using real-time PCR analysis (Fig. 6E). The deletion of all SRF-like binding sites reduced EGFP expression in P2075, although it failed to eliminate $MYH_{M743\text{-}2}$ expression completely. Collectively, these data suggest that SRF-like binding sites, together with other *cis*-elements, are necessary for directing $MYH_{M743\text{-}2}$ expression.

3.6. MyoD and MEF2 binding sites are involved in the transcriptional control of $MYH_{M743\text{-}2}$ expression in skeletal muscle

Deletion analysis confirmed that EGFP expression in myotomal compartments typically correlated with a smaller $MYH_{M743\text{-}2}$ promoter region. These results indicate that the key *cis*-acting element(s) that regulates the transcription of $MYH_{M743\text{-}2}$ is located within the deleted promoter region. Analysis of the 2075 bp 5′-flanking region by Genomatix Matinspector and TFsearch programs identified three putative MyoD and eight putative MEF2 binding sites (Fig. 1). These binding sites have been reported to be involved in the transcriptional

Table 2
Mutations in the SRF-like binding site in the P468 construct and their effects on EGFP expression in the myotomal compartments.

Constructs	Sequence and mutation	EGFP expression (%)
P468	5′- CCTGTGAA**TATA**AGGACCCCTTTGGAGA	9.17 (n = 109)
P468M1	5′- CCTGTGAA**TGGA**AGGACCCCTTTGGAGA	0 (n = 89)
P468M2	5′- CCTGTGGG**TATA**AGGACCCCTTTGGAGA	2.67 (n = 75)
P468M3	5′- CCTGTGAA**TATA**ACCACCCCTTTGGAGA	3.84 (n = 78)
P468M4	5′- CCTGTGAA**TATA**AGGACCCCTCCGGAGA	5.81 (n = 8 6)

Underlined, SRF-like binding site; bold, central core elements of the SRF-like binding site. n, total number of microinjections for each construct. Blue font color indicates mutation within and outside the SRF-like binding site.

regulation of many muscle-specific genes during development in fish (Du et al., 2006; Kobiyama et al., 2006; Liang et al., 2008). To determine the role of these MyoD and MEF2 binding sites, we deleted these sites individually or in various combinations (Fig. 7A). All deletion mutant constructs were microinjected into zebrafish embryos for transient expression analysis. Deleting any one of these three MyoD or eight MEF2 binding sites alone had little or no effect on the $MYH_{M743\text{-}2}$ promoter activity and EGFP expression from these single MyoD or MEF2 binding site deletion constructs was almost comparable with the wild-type construct (P2075) (data not shown). Therefore, we decided to generate the multiple MyoD and/or MEF2 binding site-deleted mutant constructs by deleting these sites one by one (Fig. 7A). Deleting multiple MyoD and/or MEF2 binding sites significantly reduced EGFP expression percentage compared with the non-deletion construct (P2075) (Fig. 7A). However, fluorescent optics also showed that EGFP expression levels in myotomal compartments were variable from embryo to embryo. Therefore, similarly with the SRF-like binding site deletion analysis, EGFP-expressing embryos were also grouped into high, medium and low categories. Deleting all three MyoD or eight MEF2 binding sites resulted in a significant reduction of high-EGFP expressing embryos (Fig. 7A). The reduction of such EGFP expression in MyoD and MEF2 binding sites deletion constructs was also validated by using real-time PCR analysis (Fig. 7B). These findings suggest that the deletion of MyoD and MEF2 binding sites reduced EGFP expression in myotomal compartments. However, it should be noted that EGFP expression was not completely inhibited in skeletal muscles in all MyoD and/or all MEF2 binding sites deletion constructs. Collectively, these data suggest that multiple transcriptional factors including MyoD and MEF2 participate in the transcriptional regulation of $MYH_{M743\text{-}2}$ expression.

3.7. MyoD binding sites participates in the transcriptional control of $MYH_{M743\text{-}2}$ expression in craniofacial and pectoral fin muscles

At larval stage (10 dpf) of torafugu, the indigenous transcripts of fast-type $MYH_{M743\text{-}2}$ were localized to epaxial and hypaxial domains of myotome, and eye and pectoral fin muscles (Unpublished observation). Similarly with the indigenous expression, EGFP expression was

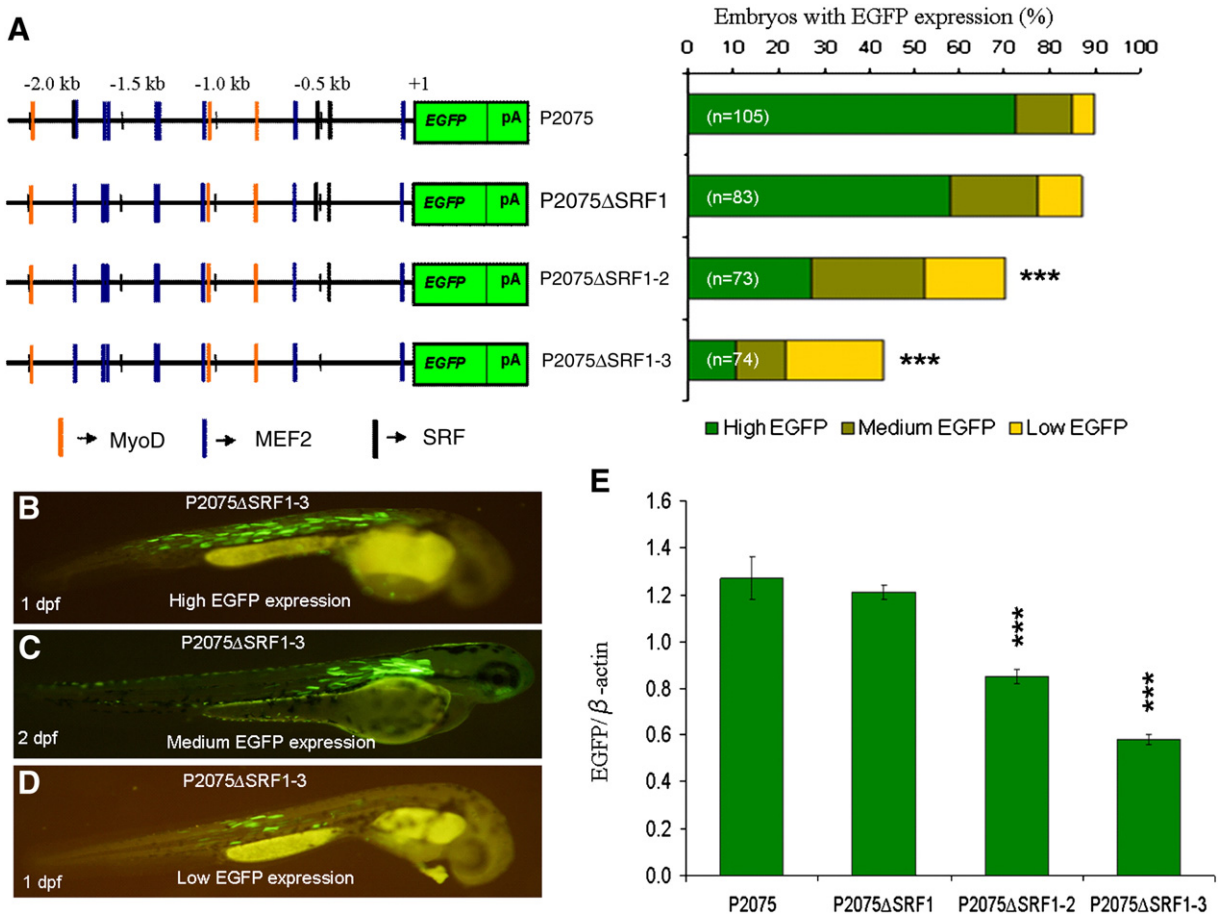

Fig. 6. Effects of SRF-like binding sites deletions on EGFP expression in the myotomal compartments. A: Schematic representation showing SRF-like binding sites deleted one by one and the percentages of EGFP-expressing embryos (high, medium and low) in each SRF-deletion constructs. The total number of embryos injected with each construct is shown in parentheses. B-D: Lateral view showing high (B), medium (C) and low (D) EGFP expression in myotomal compartments in embryos injected with the P2075ΔSRF1-3 construct. E: Bar graph showing reduced relative EGFP expression in the P2075ΔSRF1-2 and P2075ΔSRF1-3 constructs using real-time PCR analysis. Differences are significant in ANOVA followed by Tukey test at $^{***}P<0.001$.

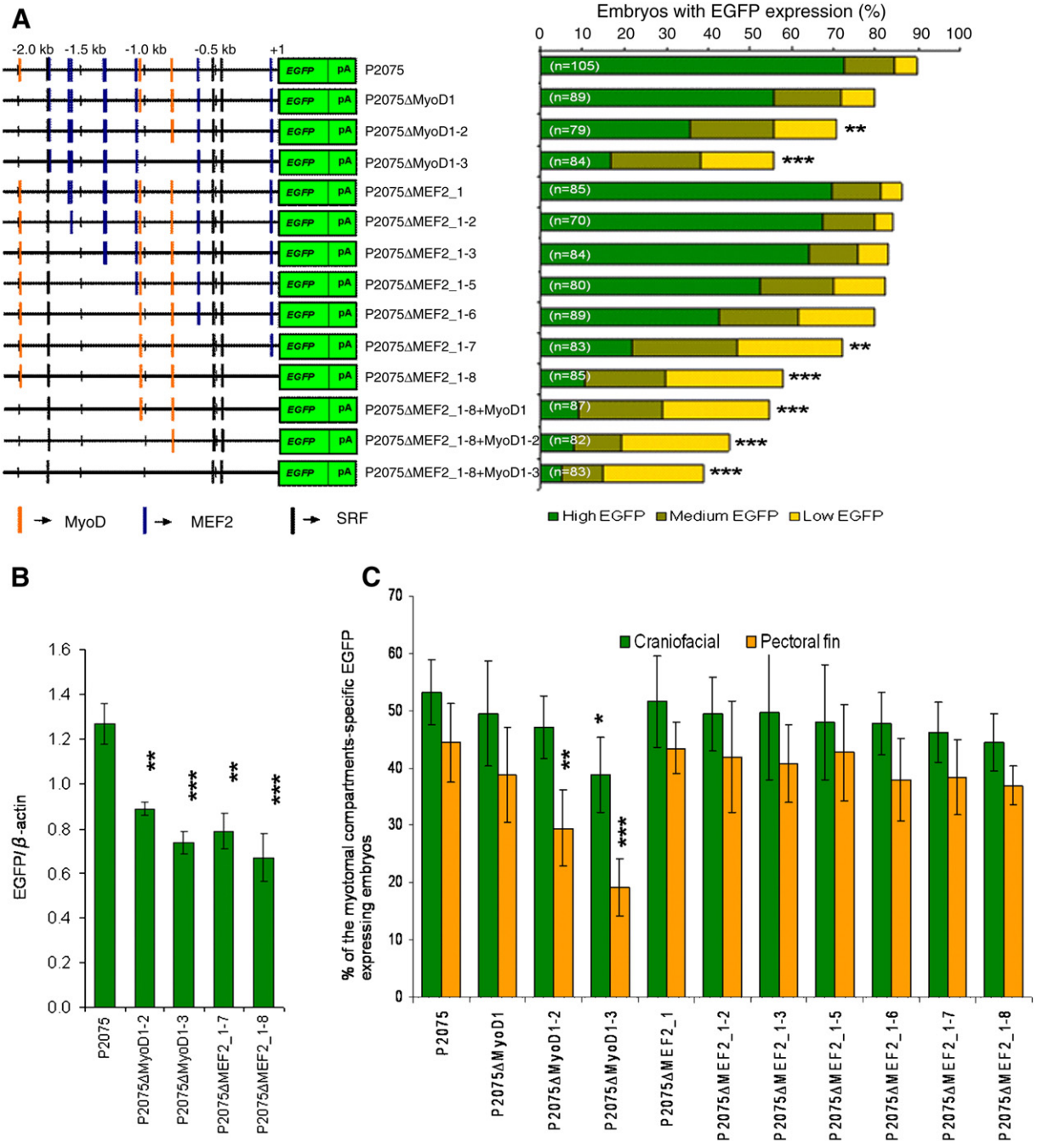

Fig. 7. Effects of MyoD and/or MEF2 binding sites deletions on transgene EGFP expression in the myotomal compartments, pectoral fin and craniofacial muscles. A: Schematic representation of different constructs in which MyoD and/or MEF2 binding elements were deleted one by one and the percentages of EGFP-expressing embryos (high, medium and low) in each MyoD and/or MEF2 binding site deletion constructs. The total number of embryos injected with each construct is shown in parentheses. B: Bar graph showing reduced relative EGFP expression in MyoD and MEF2 binding sites deletion constructs using real-time PCR analysis. C: Bar graph showing percentages of embryos showing EGFP expression in craniofacial and pectoral fin muscles to those expressing EGFP in myotomal compartments. Differences are significant in ANOVA followed by Tukey test at $^{*}P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$.

also observed in craniofacial and pectoral fin muscles in both P2075-injected and stable transgenic line larvae. Because $MYH_{M743\text{-}2}$ was expressed in several types of muscle, it would be interesting to determine if the same or distinct regulatory elements are responsible for its transcriptional regulation in skeletal, craniofacial and pectoral fin muscles using transient expression and transgenic approach. Previous studies reported that MRFs such as MyoD and Myf5 had a significant role in driving pectoral fin and head myogenesis (Hinits et al., 2009; Lin et al., 2006). We therefore selected MyoD and MEF2 (in our interest) binding sites to investigate their roles in the transcriptional control of $MYH_{M743\text{-}2}$ expression in craniofacial and pectoral fin muscles. During expression analyses, we observed that all skeletal muscle-specific EGFP-expressing embryos did not show EGFP expression necessarily in the above two muscles and that there were no embryos which exhibited EGFP expression only in these two muscles. Therefore, we calculated EGFP expression in these two muscles in terms of percentage in myotomal compartments-specific EGFP-expressing embryos. In non-deletion construct (P2075), about 53.2% and 44.2% of the muscle-specific EGFP-expressing embryos showed EGFP expression in craniofacial and pectoral fin muscles, respectively, at 4 dpf (Fig. 7C). In the case of craniofacial muscle, only embryos microinjected the construct deleting all MyoD binding sites could reduce EGFP expression (Fig. 7C). For pectoral fin

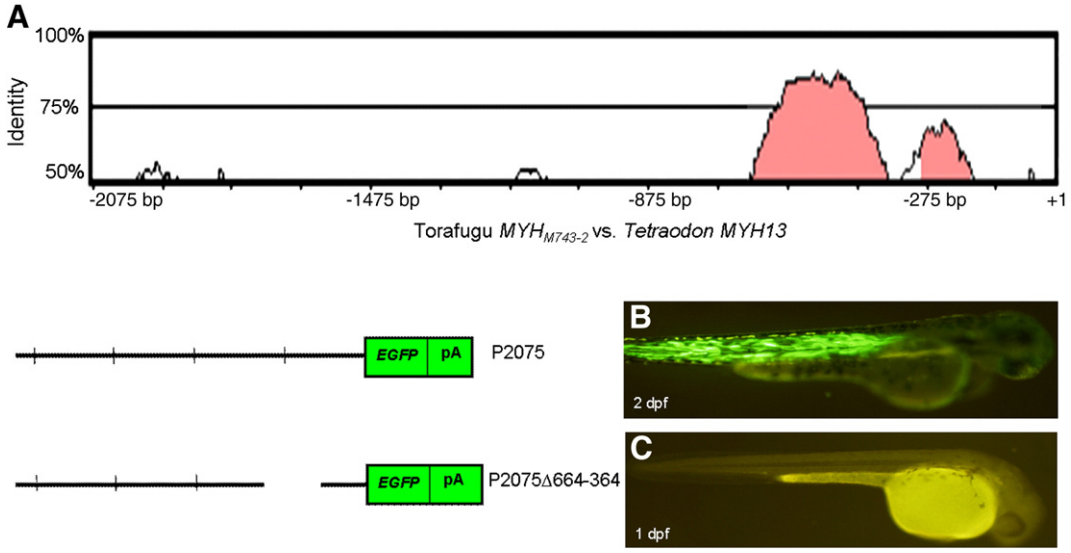

Fig. 8. The −664 to −364 bp region in the 5′-flanking region of MYH_{M743-2} is crucial for its promoter activity. A:. rVISTA plot showing homology (−664 to −364 bp region) in pairwise sequence alignments between −2075 bp of torafugu MYH_{M743-2} and the corresponding region of the *Tetraodon* orthologue. Peaks are shown relative to their positions in MYH_{M743-2} and their percent identities (50%–100%) are indicated on the vertical axis. B: EGFP expression was observed in the whole myotomal region in larvae microinjected with the P2075 construct. C: No EGFP expression was observed in embryos injected with the P2075Δ664-364 construct.

muscle, deleting MyoD binding sites gradually reduced EGFP expression (Fig. 7C). However, the deletion of all MEF2 binding site did not significantly reduce EGFP expression irrespective of pectoral fin and craniofacial muscles. These data suggest that the MyoD binding sites, but not the MEF2 binding sites, are crucial for MYH_{M743-2} promoter activity in craniofacial and pectoral fin muscles. It should be noted that the deletion of all MyoD binding elements could not completely abolish EGFP expression in these two muscles. Therefore, additional regulatory sequences other than MyoD binding sites, but not MEF2 binding site, might also be involved in the transcriptional control of MYH_{M743-2} expression in these muscles.

3.8. The sequence spanning −664 to −364 bp is a key regulatory region in the MYH_{M743-2} promoter

Initially, we aligned −2,075 bp 5′-flanking region of torafugu MYH_{M743-2} with the corresponding region of orthologues from zebrafish. The sequence analysis showed that there was no homology in the 5′-flanking region of torafugu MYH_{M743-2} with those of zebrafish (data not shown). However, torafugu MYH_{M743-2} showed homology over 55% in the 5′ flanking region from −664 to −364 bp with the corresponding region of green spotted pufferfish orthologous gene, *MYH13* (Fig. 8A), suggesting a similar regulatory element function for these two genes. Therefore, to examine the functional roles of this conserved region, we deleted the −664 to −364 bp region from P2075 by an inverse PCR technique and established the P2075Δ664-364 construct. When P2075 was microinjected into one to two-cell embryos, approximately 90% of them showed EGFP expression in the myotomal compartments (Fig. 8B). However, the embryos microinjected with P2075Δ664-364 showed no fluorescence of EGFP in the whole myotomal region (Fig. 8C). These results indicate that the key *cis*-acting element(s) that regulates muscle-specific expression of MYH_{M743-2} is located in this region.

4. Discussion

In embryonic and larval stages of torafugu, multiple sarcomeric *MYHs* are expressed in different muscles in a sequential fashion. A greater understanding of factors regulating the expression of these embryonic- and larval-type *MYHs* in different muscles would provide insights into how these muscles are established and maintained. Although a considerable progress has been made in elucidating the molecular genetics underlying the muscle-specific expression of *MYHs* in carp and medaka mostly in a temperature dependent manner (Gauvry et al., 1996; Kobiyama et al., 2006; Liang et al., 2008), little is currently known regarding the molecular mechanisms governing gene expression even within the fast subfamily at embryonic and larval stages of torafugu. We recently reported that the 2.1 kb 5′-flanking region of torafugu MYH_{M743-2} regulated developmental muscle-specific expression (Yasmin et al., 2011). This study identified several regulatory elements involved in the expression of MYH_{M743-2} in skeletal, craniofacial and pectoral fin muscles by transient and transgenic analyses.

4.1. The 2075 bp 5′-flanking region of MYH_{M743-2} is sufficient for muscle-specific expression

Combining both transient and transgenic techniques, we demonstrated here that a 2075-bp fragment upstream of torafugu MYH_{M743-2} could recapitulate muscle-specific gene expression. In transient injection assay, the 2075 bp fragment of torafugu MYH_{M743-2} was cloned into a Tol2-based vector to dramatically reduce mosaicism, and significantly increase sensitivity. The expression patterns of the 2075 bp construct (P2075) from the transient assay were confirmed by generating stable transgenic fish lines for P2075. In both transient and transgenic analyses, strong EGFP expression was observed along the whole myotomal regions in zebrafish embryos. This observation was in agreement with an earlier report by Ikeda et al. (2007), where the transcripts of MYH_{M743-2} were localized in the whole myotome of torafugu embryos.

Embryonic *MYHs* have been also reported to be expressed distinctly in craniofacial and pectoral fin muscles in common carp (Ikeda et al., 2010). Similarly, we observed zebrafish larvae expressed *EGFP* in pectoral fin and craniofacial muscles. These results were also in agreement with the expression of embryonic MYH orthologues in zebrafish (Xu et al. 2000) and medaka (Ono et al., 2006, 2010). The endogenous expression of MYH_{M743-2} by *in situ* hybridization was also found to be expressed in pectoral fin and eye muscles of torafugu by our laboratory member. EGFP expression analysis suggests the functional requirement of MYH_{M743-2} during larval pectoral fin muscle development which entirely consist of fast fibers (Patterson et al., 2008; Thorsen and Hale, 2005). It also suggests that MYH_{M743-2} functions in craniofacial muscles

development which consists of both fast and slow fibers at larval stage (Hernandez et al., 2005).

The whole mount *in situ* hybridization for torafugu embryos and larvae showed the $MYH_{M743\text{-}2}$ expression specific to fast-type muscle (Ikeda et al., 2007). The orthologous genes of $MYH_{M743\text{-}2}$ have been also reported to be expressed only in fast muscle of common carp (Nihei et al., 2006), zebrafish (Xu et al., 2000) and medaka embryos (Ono et al., 2006). While the injected P2075 showed EGFP expression in both fast and slow muscle fibers of larvae as revealed by immunohistochemical analysis, zebrafish larvae from the stable transgenic line expressed EGFP in fast muscle fibers only. Finally, we can say that expressions directed by the 2,075 bp 5′-flanking region of torafugu $MYH_{M743\text{-}2}$ in skeletal, craniofacial and pectoral fin muscles in zebrafish is consistent with its endogenous expression patterns in torafugu. Taken together, these data suggest that this region of torafugu $MYH_{M743\text{-}2}$ is sufficient for muscle-specific expression in zebrafish.

4.2. SRF-like binding sites as a critical element of $MYH_{M743\text{-}2}$ promoter activity

SRF is a transcription factor, which binds to a serum response element associated with a variety of genes including muscle-specific genes such as those encoding actin and MYH. By regulating the expression of these muscle-specific genes, SRF controls muscle development and function (Camoretti-Mercado et al., 2003; Miano, 2003). It binds to the 10 bp sequence CArG box [$CC(A/T)_6GG$], a regulatory element identified in many muscle-specific genes (Miano, 2003). In this study, we revealed that the minimum 468-bp basal promoter drove EGFP expression in the myotomal compartment and suggested that the 20 nucleotides spanning from 468 to 449 bp and containing a SRF-like binding site may be required for $MYH_{M743\text{-}2}$ expression. The predicted SRF-like binding site within this 20 bp basal promoter contained a CArG-like box that differed from the consensus sequence by two nucleotides (CCTGT**GAATATAAGG**ACCCC, with bold face for CArG-like box and differing nucleotides underlined). Mutations in the central core sequence (TATA→TGGA) of this SRF-like binding site completely eliminated $MYH_{M743\text{-}2}$ expression in the myotomal compartment, suggesting that this SRF-like binding site was necessary for mediating the minimum basal expression of $MYH_{M743\text{-}2}$. In general, no insertions or deletions are allowed in the central AT core as this would disrupt the planar topology of the G base and alter the inherent bending of the CArG element that appears critical for SRF binding (Pellegrini et al., 1995). Substitutions with C or G are best tolerated at the first and last positions of the AT core whereas similar substitution at the middle severely impaired SRF binding (Hautmann et al., 1998; Leung and Miyamoto, 1989; Miano et al., 2000). A very recent study reported that a single bp difference (at 8th base pair position) in CArG motif of human *MyHC-IIb* sequence greatly reduced or abolished SRF binding (Harrison et al., 2011). Therefore, further studies are needed to confirm whether the difference at 1st and 2nd base pair position (TG in stead of CC) in the CArG motif of 486-bp promoter alters the SRF binding.

A considerable progress has been made in elucidating the role of SRF underlying the transcriptional regulation of muscle-specific expression of *MYH* in mammals. A previous study showed that a CArG-like box (SRF binding site) residing in the −120 to −80 bp promoter region act as an enhancers of *MyHC-IIb* transcriptional activity predominantly in fast muscle of adult mice (Allen et al., 2005). *In silico* analysis combined with deletion and site-directed mutagenic analyses revealed that the low transcriptional activity of human *MyHC-IIb* is largely the result of reduced SRF binding within the proximal promoter region (Harrison et al., 2011). Deletion studies using transgenic mice demonstrated that multiple SRF binding sites were involved in the expressional regulation of the smooth muscle-α actin gene (Mack and Owens, 1999). Functional analyses also indicated a major role of SRF in skeletal muscle fiber growth and maturation in mice (Arsenian et al., 1998; Li et al., 2005). Consistent with all of these studies, we revealed that deleting all three SRF-like binding sites together significantly reduced EGFP expression in myotomal compartments. This data argue that SRF-like binding sites participate in the transcriptional activity of $MYH_{M743\text{-}2}$ expression. Although the role of SRF in transcriptional control of *MYH* expression has been reported in mammals (Allen et al., 2005; Harrison et al., 2011), the combined deletion and mutation analyses in this study provided the new insight that SRF binding sites within the upstream promoter regulate the transcriptional activity of *MYH* in fish too.

4.3. MyoD and MEF2 binding sites participate in $MYH_{M743\text{-}2}$ transcriptional activity in skeletal muscle

Members of MRF (MyoD, myogenin, myf5, and MRF4) and MEF2 have all been shown to regulate the transcription of numerous muscle-specific genes. The transcriptional regulation of these genes is often correlated with the presence of DNA consensus sequences in the regulatory region like E box (CANNTG) and AT rich sequence [$(C/T)TA(T/A)_4TA(A/G)$]. In this study, deleting multiple MyoD and/or MEF2 binding sites together significantly reduced EGFP expression in myotomal compartments of fish. These findings argue that E box to bind MyoD family and AT rich sequences to bind MEF2 may regulate $MYH_{M743\text{-}2}$ expression in skeletal muscles. The 5′-flanking region in three adult fast skeletal *MYHs* of mouse was found to have *cis*-elements such as MEF2 binding site, E-box and NFAT binding site (Lakich et al., 1998; Swoap, 1998). Members of the MRF and MEF2 families of myogenic transcription factors appear to be necessary for high level, muscle-specific expression of *MYH-IIb,* whereas NFAT preferentially activates the *MYH-IIa* promoter. It was also shown that the expression of common carp fast skeletal *MYH*, *FG2*, required the sequence in the 5′-flanking region between −901 to −824 bp, which contained both E box and MEF2 binding site (Gauvry et al., 1996). Previous studies also reported that MEF2 binding sites are crucial for a temperature-dependent expression of *MYHs* in medaka and common carp (Kobiyama et al., 2006; Liang et al., 2008). Functional analysis also indicated a major role of MyoD and Myf5 for fast muscle myogenesis in individual somites in zebrafish (Hinits et al., 2009). All of these studies indicate that MyoD and MEF2 binding sites are involved in the transcriptional regulation of *MYHs* expression in fish and mammals. It should be noted that deleting all MyoD and/or MEF2 binding sites in the torafugu $MYH_{M743\text{-}2}$ regulatory region did not completely abolish its activity in directing EGFP expression in skeletal muscles, suggesting that other regulatory element(s) might be involved in its expression. Regulation of muscle specific *MYHs* expression by multiple regulatory sequences appears to be a common mechanism for the control of muscle-specific gene expression. Supporting this idea, because the deletion of −664 to −364 bp completely abolished the $MYH_{M743\text{-}2}$ expression, it remains to be determined which *cis*-elements within this region regulate its expression in fast skeletal, craniofacial and pectoral fin muscles.

4.4. MyoD also participates in the transcriptional regulation of $MYH_{M743\text{-}2}$ expression in craniofacial and pectoral fin muscles

MyoD has an important role in abaxial pectoral fin and craniofacial muscle formation in zebrafish. Inactivation of Myf5 and/or MyoD leading to malformation of different populations of craniofacial muscles indicates distinct functions of Myf5 and MyoD during craniofacial myogenesis (Lin et al., 2006). Unlike most craniofacial muscles, pectoral fin muscle is somite derived and composed exclusively of fast fibers (Patterson et al., 2008). Hinits et al. (2009) showed that MyoD knockdown prevented myosin accumulation in pectoral fin buds. Therefore, MyoD is also the major MRF required for fast pectoral myogenesis. Because $MYH_{M743\text{-}2}$ was also predominantly expressed in both craniofacial and pectoral fin muscles, we hypothesize that MyoD binding site may regulate $MYH_{M743\text{-}2}$ expression in these muscles. In our observation, deleting multiple binding sites of MyoD

reduced EGFP expression in craniofacial and pectoral fin muscles. This data suggest that E-box binding to MyoD participate in $MYH_{M743\text{-}2}$ expression in the two muscles. However, the deletion of all MyoD binding sites did not completely inhibit EGFP expression in these muscles, suggesting that other regulatory elements might be involved in the transcription regulation of $MYH_{M743\text{-}2}$ expression, as in the case of skeletal muscle. Lin et al. (2006) showed that MyoD knockdown completely abolished red fluorescent protein (RFP) signal in some of the extraocular (superior rectus, medial rectus, inferior rectus, lateral rectus) and ventral pharyngeal muscles in the Tg (α-actin:RFP) fish, but other extraocular and dorsal pharyngeal muscles retained RFP with slightly reduced signals. Because of a certain level of mosaic problem with the transient expression assay, we did not characterize which craniofacial muscles are actually affected EGFP expressions by the deletion of MyoD binding sites. Further studies of MyoD knockdown by using $MYH_{M743\text{-}2}$:*EGFP* transgenic fish are required for this purpose. This ambiguity is the next target of our research.

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