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Original Article

In vivo analysis of two striated muscle actin promoters reveals combinations of multiple regulatory modules required for skeletal and cardiac muscle-specific gene expression

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ABSTRACT We isolated two striated muscle actin genes from medaka *Oryzias latipes*. *OlMA1* is a skeletal muscle actin gene expressed in somitic muscle and head muscle and *OlMA2* is probably a cardiac muscle actin gene expressed in both somitic and cardiac muscle. The differential transcription mechanisms for these two genes were examined in embryos by introducing fusion genes in which the *OlMA1* or *OlMA2* upstream region was connected to the green fluorescent protein gene. Embryos were injected with these fusion genes at the 2-cell stage. A fusion gene containing the region up to -949 of *OlMA1* exhibited strong expression in somitic muscle. The coexistence of two regions, -949/-662 and -421/-201, is necessary for skeletal muscle-specific expression of *OlMA1*. Two E boxes and other unidentified sequences cooperatively function to achieve the full activity of the enhancer -949/-662. As for *OlMA2*, the region up to -520 is sufficient for strong muscle-specific expression. The region between -520 and -174 of *OlMA2* is necessary for specific expression in both skeletal and cardiac muscles. In addition to the CArG box located at -140, an E-box at -430 is important for the expression in cardiac muscle as well as skeletal muscle. When the enhancers for the two muscle actin genes were switched and combined with each other's promoter, they were able to upregulate tissue-specific expression according to their origin. These results suggest that distinct expression patterns of *OlMA1* and *OlMA2* are regulated by combination of regulatory modules, each of which contains multiple regulatory elements.

KEY WORDS: *muscle actins, muscle cell differentiation, green fluorescent protein, transcriptional regulation, cis-regulatory regions*

Introduction

Muscle is a popular tissue for studying cell differentiation because of the distinctive cell morphology specialized for the contractile system. Three types of muscle (skeletal, cardiac, and smooth) exist in different organs of vertebrates. Skeletal and cardiac muscles are bundles of huge elongated cells containing myofibrils in which actin thin filaments and myosin thick filaments are aligned in parallel. Actin filaments, anchored to the Z-discs, overlap with thick filaments to form a regular repetitive pattern of cross-striations. The two cell types in striated muscle are very different in their courses of differentiation (Buckingham, 1992). Skeletal myoblasts fuse with one another to form multinucleated skeletal muscle cells, whereas cardiac muscle cells have a single nucleus and they do not fuse into fibers. Different types of muscle cells express different sets of muscle-specific proteins, including muscle actins. In mammals and birds, skeletal α-actin is the main actin isoform expressed in skeletal muscle, and cardiac α-actin and smooth muscle actins (α and γ) are expressed in cardiac and smooth muscles, respectively. In early development of mice, skeletal and cardiac actins are coexpressed in developing somites, but the cardiac actin withdraws in later stages (Cox and Buckingham, 1992).

Different regulation mechanisms are thought to activate tissue-specific genes in skeletal and cardiac muscles. In skeletal muscle, myogenic determination factors of the bHLH that bind to E box (CANNTG) are the most famous muscle-specific transcription

Abbreviations used in this paper: GFP, green fluorescent protein; bHLH, basic helix-loop-helix; SRF, serum response factor; MEF2, myocyte enhancer factor-2; UTR, untranslated region; DIG, digoxigenin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline.

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A

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OlMA1  MCDDEETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRG
OlMA2  ....D...........................................................

OlMA1  ILTLKYPIEHGIITNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREKMTQIMFETFN
OlMA2  ...............................................................

OlMA1  VPAMYVAIQAVLSLYASGRTTGIVLDAGDGVTHNVPVYEGYALPHAIMRLDLAGRDLTDYLMKIL
OlMA2  ........................S.........I.............................

OlMA1  TERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEKSYELPDGQVITIGNERFRCP
OlMA2  ...............................................................

OlMA1  ETLFQPSFIGMESAGIHETAYNSIMKCDIDIRKDLYANNVLSGGTTMYPGIADRMQKEITALAPS
OlMA2  ...............................................................

OlMA1  TMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDEAGPSIVHRKCF
OlMA2  ...........................................
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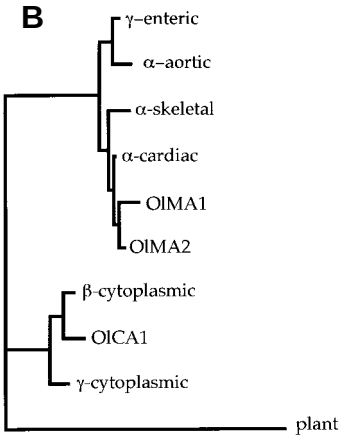

Fig. 1. Comparison of actin amino acid sequences. (A) *Comparison of the deduced amino acid sequences of OlMA1 and OlMA2. Dots indicate amino acid residues identical to those of OlMA1.* **(B)** *A phylogenetic tree constructed by the neighbor-joining method comparing the OlMA1, OlMA2, and OlCA1 amino acid sequences with six human actin isoforms. A plant actin (Arabidopsis thaliana AAc1) was included as an outgroup. Sources, references, and accession numbers for the actin sequences are described in Materials and Methods.*

factors (Lassar *et al.*, 1989; Weintraub *et al.*, 1991; reviewed by Yun and Wold, 1996). Multiple E boxes are found in upstream regions of *Xenopus* and mammalian muscle actin genes, and some of them have been confirmed to upregulate actin transcription when bound by myogenic determination factors such as MyoD (Sartorelli *et al.*, 1990; Taylor *et al.,* 1991; Biben *et al.*, 1994; Skerjanc and McBurney, 1994). Other important *cis*-regulatory sequences include CArG box [$CC(A/T)_6GG$] to which positive acting serum response factor (SRF) and negative acting YY1 factor competitively bind (Miwa and Kedes, 1987; Lee *et al.*, 1992; Moss *et al.*, 1994). CArG box and factors binding to it seem to function in both skeletal and cardiac muscles. In the chick cardiac actin gene, combinations of multiple SRF and E boxes have opposite effects in skeletal and cardiac muscles (Moss *et al.*, 1994). A dominant-negative mutant of SRF suppresses the activity of the skeletal actin promoter in chick myoblasts (Croissant *et al.*, 1996). In cardiac muscle, SRF recruits Nkx-2.5, a homeodomain transcription factor expressed in early cardiac progenitors, to provide strong transcriptional activation of the cardiac actin promoter (Chen and Schwartz, 1996). SRF was originally identified as a ubiquitous factor found in a variety of muscle and nonmuscle cells (Norman *et al.*, 1988). A recent study revealed that SRF is restricted in its expression to cardiac, skeletal, and smooth muscle lineages during avian and mammalian embryogenesis (Croissant *et al.*, 1996). SRF belongs to the family of MADS box transcription factors, which includes myocyte enhancer factor-2 (MEF2), another important component of regulatory mechanism of muscle development (reviewed by Black and Olson, 1998). Among cardiac muscle-specific regulators other than Nkx-2.5, nuclear factors GATA-4/5/6, expressed predominantly in the heart, have been reported to activate the cardiac actin gene in frog embryos (Jiang and Evans, 1996).

cis-Regulatory elements of actin genes have been investigated in various vertebrates, and the functional importance of short motifs has been investigated using cultured cells (Moss *et al.*, 1994; Croissant *et al.*, 1996). However, the sequences required for tissue-specific expression may not be identified using cell culture because a number of factors differ between the environment in cell culture and in the animal body. In a promoter analysis using the porcine skeletal actin gene, it has been shown that the same regulatory region acts differently in experiments using a transient transfection assay in cultured cells and by direct DNA injection into skeletal muscle (Reecy *et al.*, 1998). During embryogenesis, factors influencing gene expression would exhibit further dynamic change. Thus, in order to investigate the function of regulatory regions throughout development, an experimental system in which we can directly monitor the function of regulatory regions in living embryos would be required.

In this study, medaka *Oryzias latipes,* a small teleost with a short life and a relatively small sized genome, was chosen. The eggs of this species are transparent, which allows us to observe embryos through the chorion. We used the green fluorescent protein (GFP) gene as a reporter to which various upstream regions of muscle actin genes were linked, and the fusion genes were microinjected into fertilized medaka eggs. GFP fluorescence was directly monitored throughout development in living embryos. We characterized two striated muscle actin genes with different expression patterns in medaka. Relatively short upstream regions, less than 1 kb from the transcription start site (+1), were sufficient for the strong expression of both genes in muscle cells in early development. Succeedingly, we carried out a detailed analysis of the regulatory regions by introducing mutations and deletions into the fusion genes. The results provide us with a scheme of *cis*-regulatory mechanisms of striated muscle-specific gene expression by the combination of multiple regulatory sequences.

Results

Characterization of medaka actin genes

We first isolated two medaka cDNA clones encoding different actin proteins that closely resemble human β-cytoplasmic actin (Nakajima-Iijima *et al.,* 1985) and human α-striated muscle actins (Hamada *et al.,* 1982; Taylor *et al.,* 1988), respectively. The former encodes a protein of 375 amino acids and was termed *OlCA1.* A comparison of 5' UTR sequences revealed that *OlCA1* cDNA

corresponds to the medaka cytoplasmic actin genomic DNA reported by Takagi *et al.* (1994). The latter cDNA clone, termed *OlMA1*, includes a 5' UTR with an open reading frame of 1134bp coding for a protein of 377 amino acids (Fig. 1A). Next, genomic DNA containing *OlMA1* or another muscle actin gene, *OlMA2*, was isolated from a genomic library using *OlMA1* cDNA probes. The *OlMA2* gene encodes a protein of 377 amino acids, out of which only three amino acid residues differ from OlMA1 (Fig. 1A). To locate introns in the 5' UTR of *OlMA2*, 5'-RACE was carried out. In contrast to the great similarity in the deduced amino acid sequences, no distinct similarity was found in the sequences of 5' and 3' UTRs between *OlMA1* and *OlMA2*.

A phylogenetic tree based on the amino acid sequences showed that both OlMA1 and OlMA2 resemble mammalian α-striated muscle actins (Fig. 1B). Cytoplasmic actin OlCA1 was clustered with mammalian cytoplasmic actins (Fig. 1B). We concluded that medaka actin isoforms are similar to those of mammals.

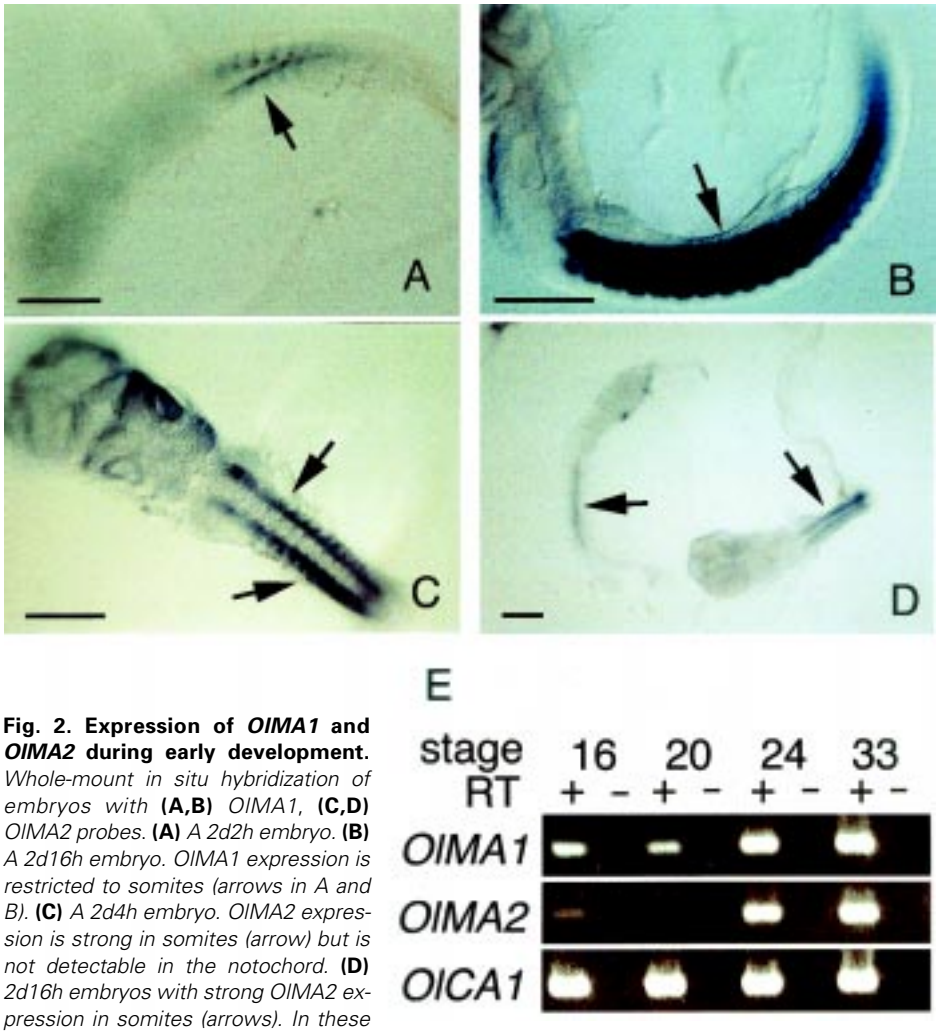

Fig. 2. Expression of *OlMA1* and *OlMA2* during early development. *Whole-mount in situ hybridization of embryos with* **(A,B)** *OlMA1,* **(C,D)** *OlMA2 probes.* **(A)** *A 2d2h embryo.* **(B)** *A 2d16h embryo. OlMA1 expression is restricted to somites (arrows in A and B).* **(C)** *A 2d4h embryo. OlMA2 expression is strong in somites (arrow) but is not detectable in the notochord.* **(D)** *2d16h embryos with strong OlMA2 expression in somites (arrows). In these embryos, the heart region was destroyed, and the signal in the cardiac muscle was not clear.* **(E)** *Stage-specific RT-PCR analysis. + and - represent amplification with and without reverse transcriptase (RT), respectively. Developmental stages were expressed as in Iwamatsu (1994). Bar, 200 μm in all photographs.*

Expression of medaka muscle actin genes during early development

The expression of *OlMA1* was analyzed by *in situ* hybridization with an RNA probe specific to *OlMA1* 3' UTR (Fig. 2A, B). In 1.5-day embryos (st. 20-22), no *OlMA1* expression was detected, although several segmental blocks of somites have formed by this stage. In 2-day embryos (st. 24-29), transcripts were detected exclusively in forming somites (Fig. 2A). Later in development, *OlMA1* expression was detected in striated muscle derived from somites (Fig. 2B). *OlMA1* is also expressed at the base of the pectoral fins and in the head, which also contains skeletal muscle (data not shown).

The expression of *OlMA2* was first detected at almost the same developmental stage as that of *OlMA1* (Fig. 2C,D). The hybridization signal was evident in forming somites and somitic muscle later in development. The signal was not clear in cardiac muscle, although we detected strong GFP expression in the heart driven by the *OlMA2* upstream region (see below). In contrast to the tissue-specific expression of *OlMA1* and *OlMA2*, *OlCA1* was expressed in the whole embryo (data not shown).

We also analyzed the expression of these medaka actin genes by RT-PCR (Fig. 2E). At stage 16 (late gastrula), when no somite has formed yet, weak expression of both *OlMA1* and *OlMA2* was detected. Transcripts accumulate markedly between st. 20 and st. 24, a period corresponding to an increase in somite number from 4 to 16 and formation of the primordial heart. This period also corresponds to the initial signal detection by *in situ* hybridization. These results show that *OlMA1* and *OlMA2* mRNAs, both of which are specific to striated muscle, appear prior to the formation of myotomes and accumulate rapidly during muscle development. These two genes seem to be expressed in adults with the same tissue-specificity as during embryogenesis because GFP fluorescence remains strong after hatching and in mature adults in promoter analysis with GFP fusion constructs (see below; data not shown). *OlCA1* was expressed abundantly in all stages examined.

Genomic organization of OlMA1 *and* OlMA2

Comparison of the sequences of *OlMA1* and *OlMA2* 5' upstream regions revealed that the first intron and a TATA box are located in similar positions (Figs. 3,6B,C), but the sequences of the introns are not conserved (data not shown). Each gene has the first intron at about ten nucleotides upstream from the start Met codon, and the transcription start site (+1) at about 65bp upstream from the 5' end of the first intron. A TATA box is located at about 30 nucleotides upstream of the start site. Upstream of the TATA box, a number of consensus binding sites of known transcription factors

such as E box (Lassar *et al.*, 1989), CArG box (Minty and Kedes, 1986), and Sp1-binding site (Gidoni *et al.*, 1984) were found in both genes. The locations of these binding motifs were compared between *OlMA1* and *OlMA2*, and with those of the muscle actin gene sequences of mammals (Taylor *et al.*, 1988; Sartorelli *et al.*, 1990) and amphibians (Mohun *et al.*, 1986), but no similarity in any two genes was found. However, when the *OlMA1* upstream region was compared with the skeletal actin gene α-*Sk1* of *Fugu rubripes* (Venkatesh *et al.,* 1996), sequences -418 to the start Met codon of α-*Sk1* and -420 to the start codon of *OlMA1* were found to be highly conserved (Fig. 6B). All three CArG boxes and one out of three E boxes in this region of *OlMA1* are perfectly conserved in *Fugu* α-*Sk1*. Moreover, there are long stretches of conserved sequences, including these known motifs and other AT- or GC-rich regions (Fig. 6B). The first introns are located at the identical site (8bp upstream from the start codon), but the sequences of the introns

TABLE 1

EXPRESSION OF THE REPORTER GENE IN MEDAKA EMBRYOS INJECTED WITH VARIOUS MEDAKA ACTIN-GFP CONSTRUCTS AT THE 2-CELL STAGE

A. The initial experiments to roughly estimate the 5' upstream regions required for muscle-specific expression

Construct	Concentration of DNA(ng/μl)	Expression in muscle	Negative	Number of embryos scored
MA1(-1430)intron(+)	50	133 (71.1 %)	54	187
MA1(-1430)intron(-)	50	118 (54.9 %)	97	215
MA2(-4096)	50	231 (83.4 %)	46	277

"Negative" includes those with only ectopic signal.

B. Experiments to analyze transcriptional activity in different regions of the *OlMA1* upstream sequence

Construct	Concentration of DNA (ng/μl)	Expression in muscle	Ectopic only	Negative	Number of embryos scored
MA1(-949)	50	26 (70.3 %)	8	3	37
	25	70 (90.9 %)	3	4	77
MA1(-802)	25	9 (20.0 %)	2	34	45
MA1(-746)	25	5 (13.9 %)	10	19	36
MA1(-662)	50	15 (23.4 %)	33	16	64
MA1(-557)	25	0	9	23	32
MA1(-421)	50	2 (3.8 %)	38	12	52
MA1(-201)	50	0	25	9	34
MA1(-54)	50	0	35	13	48
MA1(-421)+MA1e	25	34 (89.5 %)	0	4	38
MA1(-201)+MA1e	25	0	22	24	46
MA1(-54)+MA1e	25	0	2	35	37
MA1(-949)mE6	25	17 (70.8 %)	3	4	24
MA1(-949)mE7	25	22 (64.7 %)	2	10	34
MA1(-949)mOtx	25	54 (79.4 %)	3	11	68
MA1(-949)mE6E7	25	25 (41.7 %)	7	28	60
MA2(-174)+MA1e	25	[#]2 (9.1 %)	0	20	22

"Negative" excludes those with only ectopic signal. [#] There was 1 embryo with GFP signal in the notochord.

C. Experiments to analyze transcriptional activity in different regions of the *OlMA2* upstream sequence

		Region of Expression					
Construct	Concentration of DNA(ng/μl)	Somitic trunk muscle	Heart muscle	Trunk and heart muscle	Ectopic only	Negative	Number of embryos scored
MA2(-1570)	50	1 (3.3 %)	11 (36.7 %)	9 (30.0 %)	2	7	30
MA2(-520)	50	3 (7.5 %)	12 (30.0 %)	10 (25.0 %)	0	10	*40
	25	13 (23.6 %)	11 (20 %)	7 (12.7 %)	0	24	55
MA2(-174)	50	0	0	0	9	20	29
MA2(-68)	50	0	0	0	15	33	48
MA2(-1570)mCArG	50	2 (20.0 %)	1 (10.0 %)	0	0	7	10
	25	1 (2.7 %)	5 (13.9 %)	3 (8.3 %)	2	25	36
MA2(-1570)mE1	25	0	2 (22.2 %)	[$]4 (44.4 %)	0	3	9
MA2(-520)mE1	25	6 (21.4 %)	9 (32.1 %)	3 (10.7 %)	3	7	28
MA2(-520)mE2	25	0	6 (9.0 %)	1 (1.5 %)	0	60	67
MA1(-949)+MA2e	25	18 (60.0 %)	0	6 (20.0 %)	5	1	30

*There were 5 embryos with GFP signal in the notochord. Four of them also had the signal in the muscle. [$] There were 2 embryos with GFP signal in the notochord.

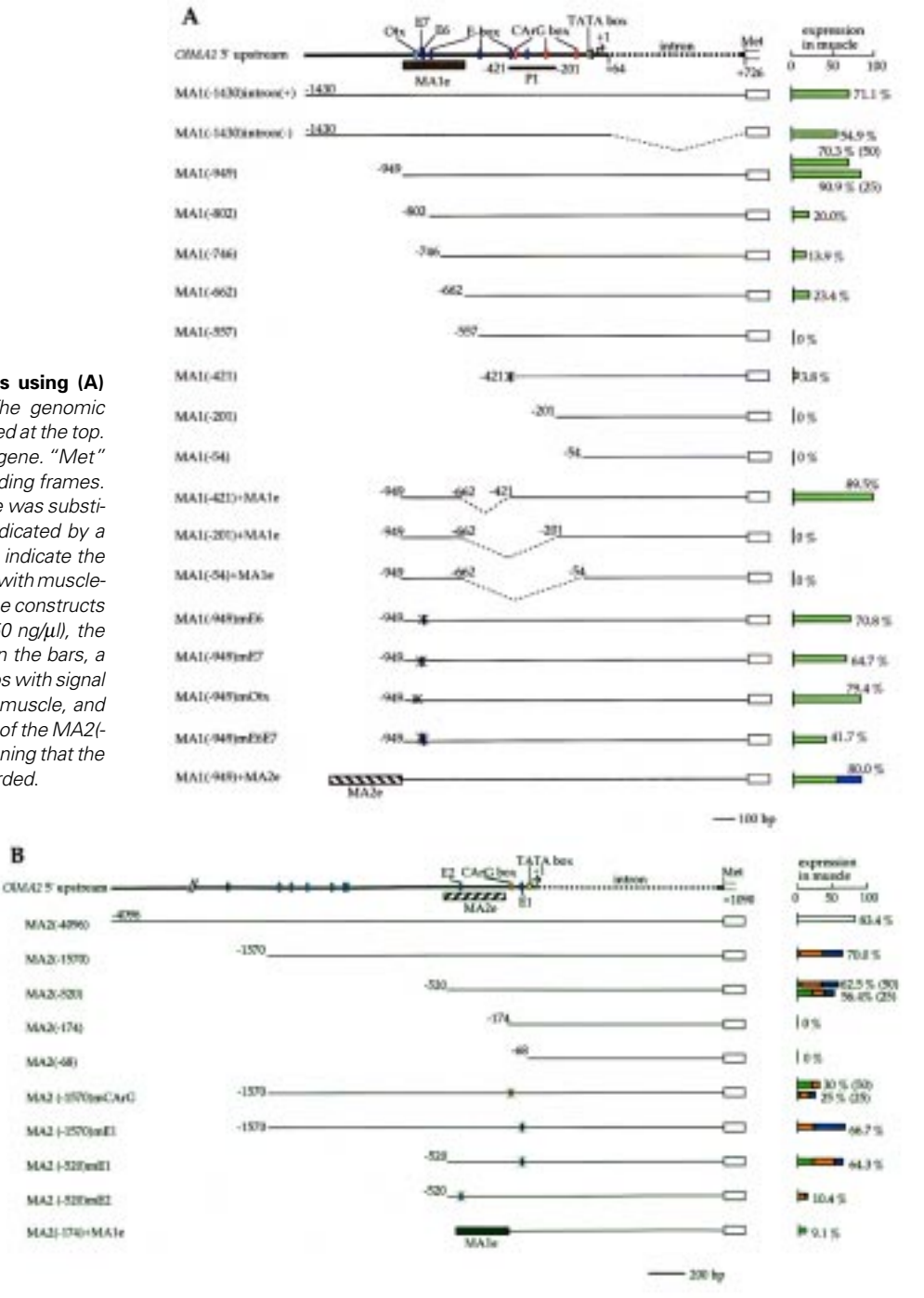

Fig. 3. Summaries of the 5′ region analysis using (A) *OlMA1*- and (B) *OlMA2*-GFP constructs. *The genomic structures of the endogenous genes are indicated at the top. Broken lines correspond to the first intron of the gene. "Met" indicates the start Met codon of actin open reading frames. In all fusion genes, the actin open reading frame was substituted with the GFP open reading frame, as indicated by a white box. At the right side of the figures, bars indicate the percentage of positive embryos, i.e., individuals with muscle-specific signals out of all embryos scored. For the constructs tested at two different concentrations (25 or 50 ng/μl), the concentrations are indicated in brackets. Within the bars, a green box stands for the ratio of positive embryos with signal only in skeletal muscle, red for only in cardiac muscle, and blue for both types of muscle. In* **(B),** *the results of the MA2(-4096) injection are indicated by a white bar, meaning that the ratio of different types of muscle was not recorded.*

are not conserved (not shown). Like *OlMA1*, α*-Sk1* is expressed predominantly in skeletal muscle and to a lesser extent in the heart (Venkatesh *et al.*, 1996). On the other hand, no similarity was found between the 5' upstream regions of *OlMA2* and any of the *Fugu* actin genes (Venkatesh *et al.*, 1996).

cis-Regulatory regions required for OlMA1 *expression*

In order to analyze the *cis*-regulatory sequences required for muscle-specific gene expression during development, we constructed fusion genes by inserting different parts of the 5' upstream regions into pEGFP-1 vector (Fig. 3). The plasmid DNA was microinjected into the cytoplasm of each blastomere of medaka 2-cell embryos. We first injected the fusion construct MA1(-1430)intron(+) containing the region from -1430 to the start Met codon of *OlMA1*, including the first intron (Fig. 3A). Most injected embryos developed normally and hatched. There have been reports of mosaic expression of the reporter gene in fish embryos developed from eggs injected with DNA constructs into cytoplasm (Takagi *et al.*, 1994; Higashijima *et al.*, 1997). Consistently, the GFP fluorescence was first detected in a subset of cells in the myotomes of day 3 embryos injected with MA1(-1430)intron(+) (Fig. 4A). Expression patterns varied from embryo to embryo. The initiation of GFP signal detection was delayed for 1 day compared to that of *OlMA1* mRNA by *in situ* hybridization, probably due to the accumulation of translated products to the detectable level. On day 4, embryos with normal morphology were scored, and ones with GFP signal in the trunk skeletal muscle were counted as positive (Table 1A). GFP signal was also ectopically detected in irregularly shaped epidermal cells on the yolk surface, as shown in Figure 4B. After hatching, many larvae grew to adulthood and the fluorescence was still visible in the trunk muscle, even with the naked eyes under a fluorescence lamp (data not shown). No GFP fluorescence was observed in the heart throughout development. Next, the fusion

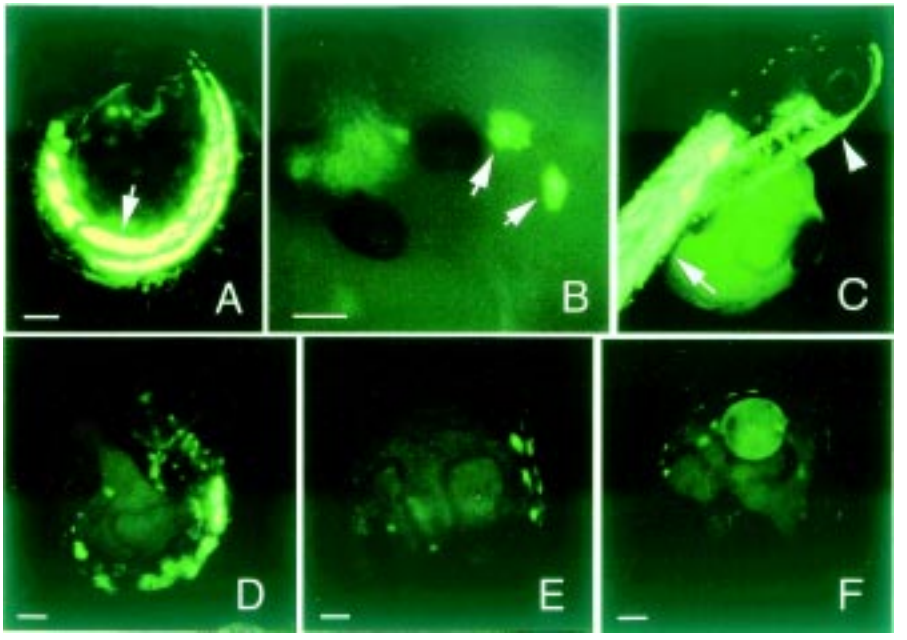

Fig. 4. **Expression of GFP in embryos injected with *OlMA1*-GFP constructs. (A)** *A 5-day embryo injected with MA1(-1430)intron(+). Strong signal is seen in somites (arrow).* **(B)** *A MA1(-54)-injected embryo. Irregularly shaped epidermal cells (arrows) had signal, but no muscle-specific signal was detected.* **(C)** *A hatched MA1(-1430)intron(-)-injected embryo. The signal was strong in somitic (arrow) and head (arrowhead) muscle.* **(D,E,F)** *Examples of* **(D)** *+++,* **(E)** *++, and* **(F)** *+ embryos injected with MA1(-949). Bar, 200 μm in all photographs.*

construct MA1(-1430)intron(-) containing the region from -1430 to +45 but not the first intron (Fig. 3A) was injected. The GFP signal was observed with the same tissue specificity as that of MA1(-1430)intron(+) (Table 1A). In addition to skeletal muscle in the trunk region, both constructs gave GFP signal in the head skeletal muscle, as shown in Figure 4C. These results suggest that the *OlMA1* upstream region between -1430 and +45 is sufficient to drive gene expression predominantly in skeletal muscle cells. The fluorescence was relatively weak in most of the MA1(-1430)intron(-)-injected embryos and the proportion of positive embryos decreased (54.9%), compared to MA1(-1430)intron(+)-injected embryos (71.1% positive). Therefore, the first intron of *OlMA1* seems to have enhancer activity. It has been suggested that introns are important components of the more consistent expression of transgenes in zebrafish (Amsterdam *et al.*, 1995) and in mice (Brinster *et al.*, 1988).

A series of deletion constructs, MA1(-949), MA1(-802), MA1(-746), MA1(-662), MA1(-557), MA1(-421), MA1(-201), and MA1(-54), was prepared from MA1(-1430)intron(+) by deleting the 5' end of the upstream region down to -949, -802, -746, -662, -557, -421, -201, and -54, respectively, and these constructs were microinjected into 2-cell embryos (Fig. 3A). The injected embryos were scored as described above, and positive embryos were further classified into three ranks, +++ (strong), ++ (moderate), and + (weak), according to the fluorescence intensity in a positive cell of the trunk skeletal muscle (Fig. 4D,E,F), with +++ being the highest intensity seen in MA1(-1430)intron(+) (Tables 1B and 2). MA1(-949) gave a strong (+++) GFP signal in muscle cells in nearly 40% of the scored embryos (Table 2). The ratio of each intensity rank was not influenced by the concentration of the DNA solution (50 or 25 ng/μl, Table 2). In contrast, embryos injected with MA1(-557) or shorter constructs gave no or very low GFP fluorescence in muscle cells (Table 1B). These results suggest that there exist skeletal muscle-specific enhancer activities between -949 and -557. When MA1(-802), MA1(-746) or MA1(-662) was injected, the GFP signal became weaker than that from MA1(-949) (Table 2), and the proportion of positive embryos decreased to 20% (Table 1B). Therefore, within the region -949/-557, there appear to be two critically important subregions for consistent expression; one is in -949/-802, which reduced the positive embryos to 20% when deleted, and the other in -662/-557, which reduced the positive embryos to 0% when deleted.

To investigate the roles of different parts of the *OlMA1* upstream region in muscle-specific gene expression, we designated the region -949/-662 as MA1e (*OlMA1* enhancer) and connected this region to the 5' end of MA1(-421), MA1(-201), or MA1(-54), each of which by itself has very low or apparently no transcrip-

TABLE 2

LEVELS OF GFP EXPRESSION IN EMBRYOS INJECTED WITH REPRESENTATIVE ACTIN-GFP CONSTRUCTS

Fluorescence level	Concentration	Strong	⟷	Weak
Construct	of DNA (ng/μl)	+++	++	+
MA1(-949)	50	10 (38.5 %)	9 (34.6 %)	7 (26.9 %)
	25	27 (38.6 %)	20 (28.6 %)	23 (32.9 %)
MA1(-802)	25	0	4 (44.4 %)	5 (55.6 %)
MA1(-746)	25	0	3 (60.0 %)	2 (40.0 %)
MA1(-421)+MA1e	25	18 (52.9 %)	9 (26.5 %)	7 (20.6 %)
MA1(-949)mE6	25	3 (17.6 %)	10 (58.8 %)	4 (23.5 %)
MA1(-949)mE7	25	6 (27.3 %)	11 (50.0 %)	5 (22.7 %)
MA1(-949)mOtx	25	28 (51.9 %)	17 (31.5 %)	9 (16.7 %)
MA1(-949)mE6E7	25	2 (8.0 %)	14 (56.0 %)	9 (36.0 %)

The total number of embryos shown here equals the number for "expression in muscle" in Table 1B. The percentage indicates the ratio of each levels in relation to those with expression in muscle.

tional activity in muscle cells [MA1(-421)+MA1e, MA1(-201)+MA1e, and MA1(-54)+MA1e, Fig. 3A]. The injection of MA1(-421)+MA1e produced a high frequency of positive embryos (89.5%) with strong expression in muscle cells (Tables 1B and 2). On the other hand, MA1(-201)+MA1e and MA1(-54)+MA1e gave no GFP fluorescence in muscle cells (Table 1B). These results suggest that MA1e has muscle-specific enhancer activity and that it requires other *cis*-regulatory elements that reside between -421 and -201; this region was designated as P1 (Fig. 3A). MA1e and P1 seem to function cooperatively to achieve full activation of transgenes.

Ectopic GFP signal in the epidermal cells of the yolk was constantly observed in embryos injected with the DNA constructs described above, including MA1(-557) and shorter constructs that exhibited no GFP signal in muscle cells. In control embryos with no transgene injected, epidermal fluorescence was never detected. Thus the ectopic signal in the epidermal cells may derive from the minimum promoter region which has basal transcriptional activity.

Two E boxes cooperatively contribute enhancer activity in the OlMA1 *upstream region*

The MA1e region contains an Otx-binding consensus motif TAATCC (Hanes and Brent, 1989, 1991; Treisman *et al.*, 1989) and three E boxes (Figs. 3A, 6A). We predicted that the Otx site and two upstream E boxes (E6 and E7) might be important for MA1e enhancer activity because MA1(-802), which contains none of these sites but the most downstream E box (E5), gave significantly reduced transcriptional activity in muscle cells. To test if any of the three upstream motifs plays an important role in muscle-specific gene regulation, a mutation was introduced in each of the motifs, resulting in the constructs MA1(-949)mE6, MA1(-949)mE7, and MA1(-949)mOtx (Fig. 3A). These constructs gave almost the same strength and frequency of GFP fluorescence in the skeletal muscle as the intact MA1(-949). We also created the construct MA1(-949)mE6E7 in which both E6 and E7 and the intervening four bases were deleted (Fig. 3A). MA1(-949)mE6E7 gave moderately reduced transcriptional activity in terms of both frequency and signal intensity; the proportion of positive embryos (41.7%) was about half that of those injected with intact MA1(-949) (70-90%, Table 1B), and among these embryos very few (8.0%, +++) gave strong signal in the muscle cells (Table 2). We therefore concluded that the two upstream E boxes E6 and E7 cooperatively contribute to muscle-specific enhancer activity in MA1e *in vivo*, although neither of them significantly affects the transcriptional activity when mutated by itself. However, since MA1(-949)mE6E7 still retains some muscle-specific enhancer activity, other unidentified *cis*-regulatory elements probably also contribute to the MA1e enhancer activity.

We carried out a database search for transcription factor binding sites within MA1e. An AT-rich region that contains the Otx-binding consensus was identified as resembling the binding sites of several homeobox transcription factors such as Bcd, Dfd, CdxA, S8, and Oct-1 (“a” in Fig. 6A; score = 85-100). There are three other sites with similarity to binding sites of homeobox transcription factors such as Dfd, S8, CdxA, and Pbx-1 (“b”, “c”, and “d” in Fig. 6A; score= 90-100). The two upstream E boxes E6 and E7, although fitting with the consensus sequence (CANNTG), did not appear to be a binding site of any particular bHLH transcriptional factor. E box E5 has a score of 86.8 for c-Myc which has both bHLH and leu-

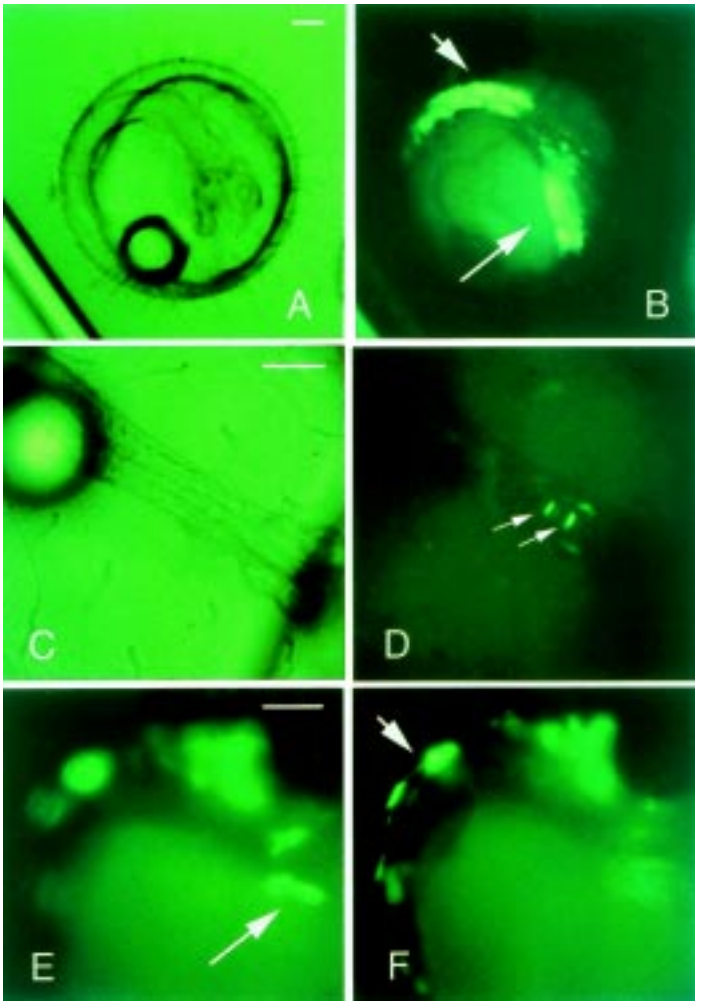

Fig. 5. Expression of GFP in embryos injected with *OlMA2-GFP* constructs. (A) *An MA2(-4096)-injected embryo, in light view.* **(B)** *The same embryo as in A under a fluorescence microscope. GFP expression is strong in both somitic (short arrow) and cardiac (long arrow) muscles.* **(C,D)** *A MA2(-520) embryo with distinctive signal in notochordal cells (arrows). The muscle cells beside the notochord also have specific signal.* **(E)** *An MA1(-949)+MA2e embryo focused at the heart region. GFP signal is visible in the beating heart (long arrow).* **(F)** *The same embryo as in* **(E)**, *focused at the somites. GFP signal is also visible in the somites (short arrow). Bar, 200 μm in all photographs.*

zipper domains. Close to the 3’ end of MA1e, there is a motif similar to the binding sequence of Dorsal, a *Drosophila* transcription factor with a Rel homology domain (“e” in Fig. 6A; score= 98.9).

Transcriptional regulatory sequences required for OlMA2 *expression*

To analyze the function of the 5’ upstream region of *OlMA2*, we first constructed a fusion gene containing the sequence from -4096 to the first Met codon of *OlMA2* linked to the GFP gene [Fig. 3B, MA2(-4096)]. In embryos injected with MA2(-4096), GFP fluorescence became visible in the somites and in the beating heart (Fig. 5A,B; Table 1A) on day 3, and this expression pattern persisted throughout development, suggesting that *OlMA2* is a cardiac

muscle actin gene. Like *OlMA1*-fusion constructs, MA2(-4096) was also ectopically expressed in epidermal cells. Deletions were introduced to the 5' end of MA2(-4096), resulting in MA2(-1570), MA2(-520), MA2(-174), and MA2(-68) (Fig. 3B). Among these constructs, MA2(-520) was the shortest fusion gene with strong expression in skeletal and cardiac muscle cells (about 60% of embryos, Table 1C). Constructs shorter than MA2(-174) gave no GFP signal in muscle, although ectopic expression in the yolk epidermal cells was observed. We therefore concluded that the region from -520 to -174 is necessary for expression in both skeletal and cardiac muscles, and this region was designated as MA2e (MA2 enhancer).

The region from -520 to the transcriptional start site (+1) of *OlMA2* contains two E boxes (E1 and E2) and a CArG box (Figs. 3B, 6C). To examine whether either of the two E boxes plays a regulatory role in *OlMA2* expression, we introduced mutation in E1 or E2 of the *OlMA2* fusion constructs [Fig. 3B; MA2(-1570)mE1, MA2(-520)mE1, and MA2(-520)mE2]. Mutated E1 did not affect transcriptional activity, while mutated E2 greatly reduced the frequency and intensity of the GFP signal in both skeletal and cardiac muscle (Table 1C). These results suggest that the E box E2 but not E1 is required for gene expression in both skeletal and cardiac muscles. Mutation in the CArG box at -140 also reduced the proportion of positive embryos to 25-30% [MA2(-1570)mCArG; Fig. 3B, Table 1C]. Thus this CArG box was also confirmed to be important for both the skeletal and cardiac muscle expression of *OlMA2*.

In addition to the muscle-specific signal, we observed GFP fluorescence in a subset of cells in the notochord of some MA2(-520)- and MA2(-1570)mE1- injected embryos (Fig. 5C,D). This implies the possibility that tissue-specific expression of *OlMA2* might be achieved in part by the suppression of transcription in notochordal cells and that overlapping sets of transcription factors might be involved in the somite and notochord development (see Discussion).

A database search identified several AT-rich regions within MA2e as being similar to the binding sites of homeobox transcription factors such as CdxA, Pbx-1, and S8 ("a", "b", "e", and "f" in Fig. 6C; scores= 85-100). The "c" sequence in Figure 6C is similar to the binding sites for several Zn-finger proteins such as MZF1 and MIG1 (scores =89-95), and "d" is a possible Su(H)-binding consensus (score= 87.2). There is a GATA motif similar to the GATA-1 and -2 binding consensus (Fig. 6C; scores=85-90). However, no motif similar to the binding sites of heart-specific transcription factor, such as GATA-4/5/6 (Evans, 1997) or Nkx-2.5 (Harvey, 1996), was identified through this database search.

TABLE 3

PRIMERS USED TO CONSTRUCT FUSION GENES

Primer	Fusion genes constructed with the primer	Primer sequence
MA1-pro3	MA1(-1430)intron(+)	cctgtcgacTCCTCGTCGTCACACATGA
MA1-pro4	MA1(-1430)intron(-)	cgggtcgaCGGCTCGGAGAGAACGACC
MA1-pro5	MA1(-662)	GATGGaaTTCTCATAGACTGCAGTT
MA1-pro6	MA1(-421)	AAATgaaTTCAGAcaAGGGAAGCG
MA1-pro7	MA1(-201)	ggcgaattcATGGAAGCTGATGGACG
MA1-pro8	MA1(-54)	TGCTGAattCAATGAATGGTGGAAGT
MA1-pro5EVR	MA1(-421)+MA1e	tcttgatatcAACTGCAGTCTATGAGA
MA1-490F	MA1(-949)	CCCgAATTCTCTCTCACACGC
MA1-pro6EVF	MA1(-421)+MA1e	TGTTgAtATcAGGGAAGCGTTTTCCCA
MA1-mOtxF	MA1(-949)mOtx	tagcactagTCCATCGCTCTGTGAC
MA1-mOtxR	MA1(-949)mOtx	gtacactagTacATTATAACTCTTATGCA
MA1-mE7SacIF	MA1(-949)mE7	tagcgagctCAatTATGGTTTCAGCTGCTGG
MA1-mE7SacIR	MA1(-949)mE7, MA1(-949)mE6E7	gtacgagctCAGGGGGTGCTCTGCCC
MA1-mE6SacIF	MA1(-949)mE6, MA1(-949)mE6E7	agcgagctCTGCTGGCAGTACAAAC
MA1-mE6SacIR	MA1(-949)mE6	gtacgagctCatAAACCATATGTGCAGGG
MA1-490XhoIF	MA1(-201)+MA1e, MA1(-54)+MA1e	ACACCtcgagCAATTCTCTCTCACACGC
MA1-pro5EIR	MA1(-201)+MA1e, MA1(-54)+MA1e	CTTTGAAttcAACTGCAGTCTATGAGA
MA1-490SacIF	MA2(-174)+MA1e	CCACgagCTCCCCAATTCTCTCTC
MA1-pro5KIR	MA2(-174)+MA1e	CTTTGgtaccAACTGCAGTCTATGAGA
MA2-pro1	MA2(-4096)	tctggatcCTCATCGTCGTCGCACATGT
MA2-pro11KpnI	MA2(-1570)	tagcggtaccATTGGGGGTATGTACTT
MA2-pro12KpnI	MA2(-174)	ataggtaCCTCTCCAAACACGCGGTGA
MA2-pro13	MA2(-68)	tcgaagcttAAAAGGGAAGGAATCCG
MA2-dCArGR	MA2(-1570)mCArG	ccgatatcttTCGCGCGCGGGGGTCAC
MA2-dCArGF	MA2(-1570)mCArG	ccgatatcttAGGAAGGGCGGCTGAAT
MA2-pro14	MA2(-520)	CATGTCAAAAGCTTCTGCATTCCC
MA2-mE1F	MA2(-1570)mE1, MA2(-520)mE1	gatcgggccCTGTCTGCAGCGAAGGAA
MA2-mE1R	MA2(-1570)mE1, MA2(-520)mE1	acatgggccCATTCAGGAATGTCCGAAGA
MA2-mE2F	MA2(-520)mE2	TTCAATggtaccTTTGTCGATCAACAAAA
MA2-mE2R	MA2(-520)mE2	GACAAAggtaccATTGAATTTTAAAATGT
MA2-pro14XhoI	MA1(-949)+MA2e	ATGTCtcgAGCTTCTGCATTCCCAC
MA2-pro12EIR	MA1(-949)+MA2e	GGGGGaattCGCGTGTTTGGAGAGGGA

In the column of primer sequences, small letters indicate bases that do not match the template. Underlines indicate restriction enzyme recognition sites.

The muscle actin enhancers tissue-specifically activate heterologous promoters

To test if MA1e serves as a tissue-specific enhancer to drive a heterologous promoter, MA1e was connected to the 5' end of MA2(-174), which has no transcriptional activity in muscle cells [MA2(-174)+MA1e] (Fig. 3B). Two out of 22 embryos had GFP signal in skeletal muscle but no signal in the heart (Table 1B). Thus MA1e can activate transcription in skeletal muscle when linked to a basal promoter of another muscle actin gene.

MA2e was also analyzed for its cardiac muscle-specific enhancer activity. This region was connected to the 5' end of MA1(-949), which by itself has strong transcriptional activity in the skeletal muscle but not in cardiac muscle [MA1(-949)+MA2e] (Fig. 3B). Eighteen out of 30 embryos injected with MA1(-949)+MA2e had GFP signal only in skeletal muscle (Table 1C). Notably, six additional embryos had fluorescence in both skeletal and cardiac muscle (Fig. 5E,F, Table 1C). Thus MA2e, when combined with a skeletal muscle actin promoter, appears to be capable of promoting expression in cardiac muscle.

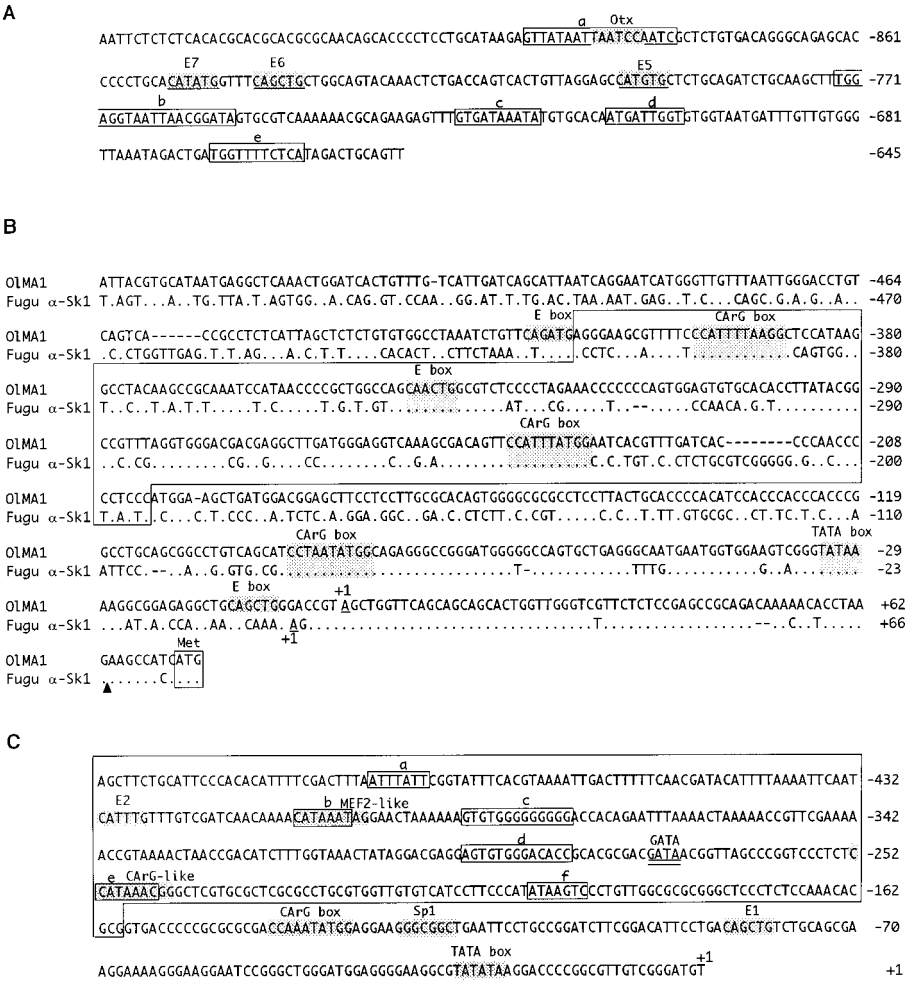

Fig. 6. Nucleotide sequences of the 5' upstream regions of *OlMA1* and *OlMA2*. *The nucleotide positions are indicated relative to the start site of transcription (+1). E box, CArG box, Sp1-binding motif, Otx-binding motif, MEF2-like, and TATA box sequences are shaded.* **(A)** *MA1e sequence of the OlMA1 gene. The predicted binding sites of the transcription factors are boxed and labeled a to e. E boxes are shaded and underlined.* **(B)** *Comparison of nucleotide sequences of the 5' upstream regions between OlMA1 and Fugu α-Sk1. Identities to OlMA1 are indicated by dots. Dashes indicate gaps introduced in the sequences to optimize the alignment. The black triangle indicates the conserved position of the first introns. The sequences corresponding to the P1 region are framed.* **(C)** *The 5' upstream sequence of OlMA2. Predicted binding sites of transcription factors are boxed and labeled a to f. A GATA consensus motif is doubly underlined. MA2e region is framed.*

Discussion

Actin genes in medaka and other animals

Actins fall into two categories of isoforms, cytoplasmic or muscle, depending on their physiological roles within cells. Cytoplasmic actin is a major component of the cytoskeleton and is seen in all kinds of cells. Muscle actin plays an important role in the muscle contractile system and therefore exists only in muscle cells. In vertebrates and invertebrate chordates, the amino acid sequences of cytoplasmic and muscle actin are similar; they do, however, differ at a number of amino acid positions, which serves to distinguish the two types of actin (Vandekerckhove and Weber, 1984; Kovilur *et al.*, 1993; Bovenschulte and Weber, 1997; Kusakabe *et al.*, 1997a,b). In contrast, in nonchordate invertebrates, actins that are more similar to vertebrate cytoplasmic actins than to vertebrate muscle actins function as contractile proteins in the muscle (Vandekerckhove and Weber, 1984). Previous analysis of phylogenetic trees including various chordate actins (Kusakabe *et al.*, 1997a) has shown that four isoforms of vertebrate muscle actin (α-skeletal, α-cardiac, α-vascular, and γ-enteric) appeared after the ancestral vertebrate muscle actin had diverged from amphioxus and ascidian muscle actins. A large number of actin pseudogenes (Moos and Gallwitz, 1983; Ng *et al.*, 1985) and only single genes for each six actin isoforms (β-cytoplasmic, γ-cytoplasmic, plus four muscle actins described above) have been mapped in the human genome (Miwa *et al.*, 1991).

The deduced amino acid sequences of medaka actins reported here clearly fall into categories of corresponding actin isoforms of other vertebrates. In the phylogenetic analysis, both OlMA1 and OlMA2 were clustered together with mammalian α-actin isoforms, and both are most similar to mammalian α-cardiac actin. OlMA1 and OlMA2 seem to have diverged from the ancestral α-striated actin (skeletal and cardiac) after it diverged from smooth muscle actins (α-vascular and γ-enteric) within the vertebrate muscle actin lineage. Actin genes have been isolated in several species of fish (Liu *et al.*, 1989; Takagi *et al.*, 1994; Venkatesh *et al.*, 1996; Higashijima *et al.*, 1997). *Fugu rubripes* has nine actin genes, including two skeletal and three cardiac actin genes (Venkatesh *et al.*, 1996). Notably, no actin pseudogene has been found in *Fugu.* It is possible that yet unidentified muscle actin genes correspond-

ing to multiple skeletal and cardiac actin genes of *Fugu* exist in medaka.

In higher vertebrates, somitic muscle first expresses the cardiac actin isoform, but its place is taken by the skeletal actin isoform later in development (Cox and Buckingham, 1992). In medaka, strong cardiac actin expression in somitic muscle persists throughout development, and probably after hatching. This phenomenon might also be true in *Fugu*, in which Northern blot analysis has allowed for the detection of abundant cardiac actin mRNA in adult skeletal muscle (Venkatesh *et al.*, 1996).

Transient and stable expression of actin-GFP fusion genes in medaka embryos

Higashijima *et al.* (1997) have established transgenic zebrafish lines that stably express GFP driven by zebrafish actin promoters. They raised the injected embryos to sexual maturity and mated them with wild-type adults. Twenty to 50% of the resulting F1 progenies expressed the GFP either in whole muscles when muscle actin-GFP construct was used, or the whole body when cytoplasmic actin-GFP was used, indicating a transmission of reporter genes to germlines. In our analysis, the activity of various actin-GFP constructs was monitored in embryos undergoing development after microinjection. We predicted that some of the injected DNA molecules would be incorporated into the medaka genome in the early stages of development and would then be stably expressed. The earlier the incorporation would occur, the more cells would express GFP. The expression could also be influenced by the site into which transgenes settled in the genome. Although GFP expression in muscle would be highly mosaic, it would reflect the activity of the upstream regions included in the fusion constructs, and the variety in the expression patterns within muscle would be normalized by observing as many embryos as possible. This procedure enabled us to test many fusion constructs containing deletions or mutations in sites of interest, in a much shorter time compared to the establishment of transgenic lines.

In fact, this type of analysis has been carried out in many marine invertebrates such as sea urchins (Kirchhamer and Davidson, 1996) and ascidians (Kusakabe, 1997). In these cases, actin promoters have been very popular for use in detailed characterizations of functional elements. For example, using β-galactosidase gene as a reporter, it has been demonstrated that -103 to +1 of an ascidian muscle actin gene *HrMA4* is sufficient for muscle-specific expression (Hikosaka *et al.*, 1994), and that within this region there are two very short sequences (9 and 13bp) essential for the muscle-specificity (Satou and Satoh, 1996). Interestingly, the ectopic expression of reporter genes in cells of epidermal lineage has been reported in these cases, a phenomenon also observed in the injected medaka embryos in this study and in a study of medaka soluble guanylyl cyclase genes (Mikami *et al.*, 1999). In this study and previous studies (Hikosaka *et al.*, 1994; Satou and Satoh, 1996), even constructs with no muscle-specific activity has been expressed in the epidermis. Thus embryonic epidermal cells may serve as an environment in which the 5' upstream region of transgenes with minimum transcriptional elements could be activated regardless of additional associated enhancer elements.

There was another body region in which the ectopic expression of muscle actin-GFP constructs was observed - the notochord. The notochord is a supportive organ characteristic of chordates, and it has some muscle-like characteristics. In amphioxus, both somites and the notochord form as evaginations from the gut wall that pinch off (Flood, 1975). The notochord contains some myofilaments, and its major protein is paramyosin, most similar to the paramyosin in molluscan catch muscle (Flood, 1975). However, the amphioxus muscle actin gene *BfMA1* is not expressed in the notochord (Kusakabe *et al.*, 1997a). Recent studies in zebrafish and ascidians have revealed that relatively few transcription factors establish the boundary between muscle and the notochord within the mesoderm, and that some muscle- and notochord-specific transcription factors interact with each other (Corbo *et al.*, 1997; Amacher and Kimmel, 1998; Fujiwara *et al.*, 1998). In the present study, some of the constructs containing *OlMA2* upstream regions gave GFP signal in a subset of notochordal cells, although endogenous *OlMA2* expression was not detected in the notochord. There may be a positive-acting element capable of driving expression of the muscle actin gene in the notochord, and this activity could be down-regulated *in vivo*. Within the *OlMA2* upstream region, this notochord-specific element could be located in -174 to +1, since this region is shared by all fusion genes that gave notochord-specific signal.

Insights into differential transcriptional control of muscle genes

We found that the muscle-specific expression of both *OlMA1* and *OlMA2* is regulated by relatively short regions directly upstream from +1. As for skeletal actin *OlMA1*, a sequence beginning only 949bp upstream from +1 is sufficient for strong expression in skeletal muscle. The most upstream end of this region, from -949 to -662, which we named MA1e, seems to act as a skeletal muscle-specific enhancer, and it cooperates with the region between -421 and -201, which we named the P1 region. Constructs with deleted MA1e gave significantly reduced muscle-specific signal, even if they contain P1 region. MA1e was capable of upregulating the skeletal muscle-specific expression of the heterologous gene, which by itself is not expressed in muscle. Interestingly, the P1 region is contained in a highly conserved region between *OlMA1* and *Fugu* α*-Sk1* (Fig. 6B, Venkatesh *et al.*, 1996), suggesting that a mechanism for skeletal muscle-specific expression of skeletal actin gene is conserved in *Fugu* and medaka.

Biben *et al.* (1996) have succeeded in roughly locating separate but partly overlapping enhancer regions for skeletal and cardiac muscle-specific expression of the murine cardiac actin gene during embryogenesis. The regions consist of two distal enhancer elements (0.8 and 1.9 kb long) and a proximal promoter (0.7 kb). As for medaka cardiac actin *OlMA2*, a sequence beginning only 520bp from +1 is sufficient to activate both skeletal and cardiac muscle-specific expression. When the region MA2e (-520 to -174) was deleted, no specific expression of the reporter gene was observed either in skeletal or cardiac muscle. Further analysis may enable us to divide MA2e, which is as short as 350bp, into subregions with enhancer activity exclusively for skeletal or cardiac muscle. MA2e requires a CArG box located downstream of MA2e to achieve strong activation of the *OlMA2* promoter in both skeletal and cardiac muscles. MA2e was capable of expanding gene expression to cardiac muscle when coupled with MA1(-949), which by itself is not expressed in cardiac muscle. In this case, MA2e might interact with either or both of the two CArG boxes in the P1 region of MA1(-949). In addition to the CArG box at -140, *OlMA2* also contains a CArG-like region (CCATAAACGG) that does not perfectly fit the consensus sequence [CC(A/T)$_6$GG],

and a region similar to the MEF2-binding consensus sequence [YTA(A/T)$_4$TAR; Black and Olson, 1998] (Fig. 6C). These motifs might play regulatory roles either independently or in cooperation with other *cis*-elements.

Binding of bHLH factors to an E box has been accepted as the main component of transcriptional regulation in skeletal muscle. We consistently found that multiple E boxes in the MA1e region of *OlMA1* function cooperatively for activation in skeletal muscle. On the other hand, there has been a long debate on the necessity of E boxes for muscle actin gene expression in cardiac muscle. No bHLH myogenic factors such as MyoD and myf5 have been reported to be expressed in cardiac muscle, and any other putative *trans*-acting factors that would bind to E box in cardiac muscle have not been characterized. Sartorelli *et al.* (1992) showed that an E box in human cardiac actin promoter activates transcription by interacting with bHLH proteins in cardiomyocytes. In contrast, Skerjanc and McBurney (1994) reported that an E box is not essential for the activity of the cardiac actin promoter in cardiac muscle, an insight based on experiments using embryonal carcinoma cells. Moss *et al.* (1994) showed that avian cardiac actin promoter is regulated through a pair of complex elements composed of E boxes and SREs in chick cardiomyocytes, and that the multiple E boxes act differentially in two striated muscle cell types. In these cases, gene expression was monitored in cell cultures, which might not exhibit precise gene regulatory mechanisms in skeletal and cardiac muscle tissue differentiation in early development. Our experimental system enabled us to directly observe if a mutated E box in cardiac actin genes affects the expression in heart muscle. The result was remarkable; mutating the second E box upstream of +1 reduced both the frequency and intensity of both cardiac and skeletal muscle-specific signals. This is the first evidence that an E box functions in cardiac muscle to promote muscle actin expression in early development.

Materials and Methods

Animals and embryos

Mature adults of the orange-red variety of medaka *Oryzias latipes* were purchased from a dealer. The adults and embryos were maintained as described in Seimiya *et al.* (1997). The definitions of developmental stages are according to Iwamatsu (1994). The developmental stage was also expressed in days, and the day of fertilization was referred as day 0. Hatching usually occurs at day 10.

Isolation of actin clones from cDNA and genomic libraries

A cDNA library of hatched larva of medaka (Mikami *et al.*, 1998) was screened to obtain clones corresponding to actin genes. The templates for the DNA probes were actin cDNA fragments amplified directly from the *O. latipes* cDNA library by the polymerase chain reaction (PCR) with oligonucleotide primers ACT-F2 (5'-AATTGGGATGATATGGAGAA-3') and ACT-R2 (5'-ATCCACATTTGTTGGAAKGT-3'; K= G or T) (Kusakabe *et al.*, 1997a). DNA probes were labeled with [^{32}P]dCTP using the Random Primer DNA labeling kit ver.2 (Takara Shuzou Co., Ltd., Otsu, Japan). Library screening and DNA sequencing were carried out as described in Kusakabe *et al.* (1997a), except that the hybridization solution was 50% formamide, 5xDenhardt's solution, 6xSSC (1xSSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 250 μg/ml sheared and denatured salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS). Two different cDNA clones encoding a muscle actin OlMA1 and a cytoplasmic actin OlCA1 were obtained.

A genomic library of medaka (white strain) constructed in the lambda FIXII vector (Stratagene) was screened using actin cDNA probes. The same RNA probe as synthesized for *in situ* hybridization analysis of *OlMA1* (see below), which corresponds to the 3' untranslated region (UTR) of *OlMA1*, was used to isolate genomic clones containing the *OlMA1* gene. The whole *OlMA1* cDNA clone was used as a probe to isolate a different medaka muscle actin gene, *OlMA2*. Probes were labeled with digoxigenin (DIG)-dUTP using the DIG-High Prime (Boehringer, Mannheim). The hybridization was performed in 50% formamide, 5xSSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer, Mannheim) at 42°C. Washes were in 2xSSC, 0.1% SDS, 2 times for 15 min at 50°C. Detection was carried out with the DIG Nucleic Acid Detection Kit (Boehringer, Mannheim) according to the manufacturer's instructions. Positive clones were digested with *Bam*HI, *Eco*RI, and *Hin*dIII, and analyzed by Southern hybridization using the same probes used for the screening. The digested fragments containing actin genes were subcloned into pBluescriptII KS(-) (Stratagene) and sequenced as described in Kusakabe *et al.* (1997a).

The nucleotide sequences reported in this paper have been submitted to the GenBank with accession numbers D89627 (*OlCA1* cDNA), D87740 (*OlMA1* cDNA), AB015886 (*OlMA1* genomic DNA), and AB016259 (*OlMA2* genomic DNA).

Molecular phylogenetic analysis

The deduced amino acid sequences were aligned with those of other animals and a plant using the SeqPup sequence editor (D. Gilbert, Indiana University). The gaps at the N-terminus were inserted manually to optimize the sequence alignment. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using PROTODIST and NEIGHBOR programs in the PHYLIP package (version 3.572; Felsenstein, 1989). The following is a list of the accession numbers for previously published sequences obtained from GenBank/EMBL/DDBJ; M20543, human α-skeletal muscle (Taylor *et al.*, 1988); J00073, human α-cardiac muscle (Hamada *et al.*, 1982); X13839, human α-aortic smooth muscle (Kamada and Kakunaga, 1989); X16940, human γ-enteric smooth muscle (Miwa and Kamada, 1990); M10277, human β-cytoplasmic (Nakajima-Iijima *et al.*, 1985); M19283, human γ-cytoplasmic (Erba *et al.*, 1988); M20016, *Arabidopsis thaliana* AAc1 (Nairn *et al.*, 1988).

5' Rapid amplification of cDNA ends (5'-RACE)

To determine the sequence of the 5' end of *OlMA2* mRNA, a 5' portion of the cDNA was amplified by the 5'-RACE method (Frohman *et al.*, 1988) using the 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Life technologies, Inc.). One μg of total RNA was reverse-transcribed with a gene-specific antisense oligonucleotide primer GSP1 (5'-ATCGTCGTCGCACATGTCT-3'). The cDNA was tailed with dCTP using terminal deoxynucleoticyl transferase, and amplified by PCR with the Abridged Anchor Primer (Life Technologies, Inc.) and another gene-specific antisense oligonucleotide primer GSP2 (5'-GTCGCACA-TGTCTGCAGA-3'). The PCR product served as a template of the second PCR with GSP2 and the Abridged Universal Amplification Primer (Life Technologies, Inc.). The final PCR product was cloned into pBluescript II KS(-) and sequenced.

Database search

Transcription factor binding sites were searched within the enhancer elements using the TFSEARCH program of GenomeNet (Heinemeyer *et al.*, 1998; http://www.rwcp.or.jp/papia/).

Primer extension

In order to locate the transcription start sites, primer extension analysis of RNA was carried out essentially as described by Kusakabe *et al.* (1996). A mixture of an end-labeled oligonucleotide primer (5'-TAGGTGTTTTTGTCTGCG-3') and 35 μg of total RNA was heated at 65°C for 90 min, then allowed to anneal at room temperature for 90 min. Extension reactions were carried out with Superscript II Reverse Transcriptase (Life Technologies, Inc.) at 42°C for 1 h. The extension products were analyzed on a 6% polyacrylamide gel.

In situ *hybridization*

Fixation of larvae and whole-mount *in situ* hybridization were carried out by the methods in Westerfield (1995). The antisense RNA probes were synthesized from 3' UTR of *OlMA1* and *OlMA2*. Since an *OlMA2* cDNA clone has not been obtained, the 3' end of the *OlMA2* 3' UTR was estimated from the genomic sequence with respect to the putative poly(A) signal. *Bam*HI-digested 3' fragments of *OlMA1* cDNA (nt position 1240-1518) and a PCR-amplified fragment of *OlMA2* (nt position 6864-7103) were used as templates. After *in situ* hybridization, the whole mounts were observed in a 1:2 mixture of benzyl alcohol and benzyl benzoate.

RT-PCR

Total RNA (1μg) was used as the template to synthesize the first-strand cDNA using an oligo(dT) primer according to the manufacturer's protocol (SuperScript Preamplification System for First Strand cDNA synthesis, Life Technologies, Inc.). cDNA fragments containing 3' UTRs of *OlMA1*, *OlMA2*, and *OlCA1* were amplified by PCR from the first-strand cDNA. The primer sets used were as follows: *OlMA1*, 5'-TAAATCCTCCACCTTCTC-3' and 5'-GGTAGATGCATGGTTCAG-3'; *OlMA2*, 5'-TAACCGTCAGAACTGCAG-3' and 5'-GCGCATCAGTTCATACTT-3'; *OlCA1*, 5'-CAGACACGTA-TTTGCCTCTG-3' and 5'-CAAGTCGGAACACATGTGCA-3'.

The PCR was performed for 30 cycles (94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min) using 10% of the cDNA reaction mixture as a template, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase. To eliminate the possibility of PCR amplification derived from contaminated genomic DNA, the same reaction was carried out without reverse transcriptase as a control experiment. Twenty percent of the PCR products were separated on 1.2% NuSieve/0.4% SeaKem GTG agarose (FMC corp.) gel. The gel was soaked with ethidium bromide solution (10 μg/ml) and photographed under UV irradiation.

Construction of muscle actin - GFP plasmids

Figure 3 is a diagrammatic representation of the fusion gene constructs used in this study. To generate MA1(-1430)intron(+), MA1(-1430)intron(-), MA1(-949), MA1(-662), MA1(-421), MA1(-201), MA1(-54), MA2(-4096), MA2(-1570), MA2(-520), MA2(-174), and MA2(-68), corresponding to the upstream regions of *OlMA1* and *OlMA2* genes, were amplified by PCR using a heat-stable DNA polymerase with proofreading activity (LA *Taq*; Takara Shuzou) and inserted into the pEGFP-1 Promoter Reporter Vector (Clontech). These constructs succeedingly served as templates for the production of mutated constructs. Mutagenesis on MA1(-949)mE6, MA1(-949)mE7, MA1(-949)mOtx, MA1(-949)mE6E7, MA2(-1570)mCArG, MA2(-1570)mE1, MA2(-520)mE1, and MA2(-520)mE2 was performed by PCR using LA *Taq* and primers containing mismatches against desired sites in the wild-type template. The whole plasmid constructs were amplified and the products were trimmed at both ends with restriction enzymes before self-ligation. MA1(-421)+MA1e was generated similarly using primers with no mismatch and MA1(-949) as template. MA1(-802), MA1(-746), and MA1(-557) were generated by deleting 5' region of MA1(-949) using Exonuclease III (Takara Shuzou). The addition of enhancer regions to pre-existing constructs generated MA1(-201)+MA1e, MA1(-54)+MA1e, MA1(-949)+MA2e, and MA2(-174)+MA1e. Enhancer regions were amplified by PCR using LA *Taq* and inserted into the 5' end of MA1(-201), MA1(-54), MA1(-949), or MA1(-174) . The primers used in PCR are listed in Table 3. Nucleotide sequences of actin upstream regions in the fusion constructs were verified by sequencing with oligonucleotide primers EGFP-F (5'-GCTCACATGTTCTTTCCTGCGT-3') and EGFP-N (5'-AGAACTTGTGG-CCGTTTACGTC-3') using an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Microinjection

To prevent the egg chorion from hardening after fertilization, the eggs were incubated in 20 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)-NaOH (pH 10.5) containing 2 mM glutathione at 4°C for several hours (Iwamatsu, 1983). Micropipets were made using a horizontal puller (PN-3, Narishige) from a siliconized and sterilized 1x90 mm fiber-filled glass capillary tube (GD-1, Narishige). The circular plasmid DNA was dissolved in PBS containing 0.1% phenol red in the concentrations described in Table 1, and loaded into the micropipets. Under a dissection microscope, embryos at the 2-cell stage were stabilized by suction using a glass holding pipet and injected with DNA solution into each blastomere through the chorion. The injected embryos were washed with distilled water and incubated at 26°C. GFP fluorescence was detected and photographed under a fluorescence microscope (IX70, Olympus).

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