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Isolation, Characterization, and Expression Analysis of Zebrafish Large Mafs[1]

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Large Maf proteins, which are members of the basic leucine zipper (b-Zip) superfamily, are involved in the determination and control of cellular differentiation. The expression patterns of various vertebrate large Maf mRNAs were described previously. Here, we report the cloning of a novel zebrafish large Maf cDNA, SMaf1 (Somite Maf1), and other zebrafish large Mafs, the N-terminus domains of which possess transactivational activity. We also analyzed the expression patterns of SMaf1 and SMaf2 (Somite Maf2)/Krml2 as well as MafB/Val and c-Maf during zebrafish embryogenesis. In particular, the robust expression of the novel SMaf1 mRNA, which overlapped that of MyoD, in somitic cells during somitogenesis was noteworthy. In addition, the expression patterns of SMaf2 and MafB in the blood-forming regions, and those of c-Maf and MafB in the lens cells showed spatial redundancy, although the temporal appearance of these genes at these sites differed. These data indicate that SMafs may play important roles in somitogenesis, and that Maf proteins may have overlapping and yet specific functions as to the determination and differentiation of cell lineages.

Key words: basic-leucine zipper (b-Zip), large Mafs, Maf recognition elements (MARE), somite, transcription factor.

The Maf protein family members are transcription factors that contain a basic-leucine zipper (b-Zip) motif, which is homologous to that of the v-Maf oncoprotein. The latter was isolated as the transforming component of the avian musculoaponeurotic fibrosarcoma virus, AS42 (*1*, *2*). Maf family proteins are divided into two subgroups, large Maf and small Maf proteins. The large Maf proteins, c-Maf (*3*), MafB (*4*), Nrl (*5*), and L-Maf (*6*), contain an acidic domain in the N-terminus of each factor that acts as the transcriptional activating moiety. By contrast, the small Maf proteins, MafK, MafF (*7*), and MafG (*8*), lack such an acidic domain.

Several experiments have demonstrated that the large Mafs are key factors involved in cellular differentiation. The c-Maf member activates the transcription of L7 gene expression in developing Purkinje cells (*9*), and controls the tissue-specific expression of interleukin-4 (IL-4) in $CD4^+$ T helper 2 cells (*10*). Nrl contributes to the maturation and establishment of neuronal cells (*11*), and regulates the expression of the rhodopsin gene in the retina (*12*). Kawauchi *et al.* (1999) reported that *c-maf* knock-out mice exhibit severe defects in the lens fiber cells (*13*). In the chicken, L-Maf takes part in the differentiation of the lens and induces the expression of crystallin in chick embryos (*6*). The MafB member has been identified as the affected gene in mice carrying the *kreisler* (*kr*) mutation (*14*), and the gene product is required for segmentation of the hindbrain because it controls Hoxb-3 expression (*15*). Furthermore, MafB down-regulates the expression of the transferrin receptor and inhibits erythroid differentiation in myelomonocytic cells (*16*). These results indicate that the large Maf proteins play indispensable regulatory roles in the cellular differentiation and morphogenesis of several distinct developmental lineages.

It has been reported that Mafs form either homodimers or heterodimers with other Maf, Jun, Fos (*17*, *18*), or other b-Zip–type transcription factors. The DNA target sites of Maf homodimers and heterodimers have been defined as a 13 bp palindromic sequence TRE (TPA-responsive element)-type Maf recognition element (T-MARE) (TGCTGACTCAGCA), or a 14 bp palindromic sequence CRE (cAMP-responsive element)-type MARE (C-MARE) (TGCTGACGTCAGCA) (*17*). The chicken L-Maf was shown to bind to the core site (TGCTGAC, −108 to −102) within the αCE2 sequence (CTCCGCATTTCTGCTGACCAC, −119 to −99), which was identified as the lens-specific enhancer element in the avian *αA-crystallin* promoter (*19*, *20*). The murine MafB recognizes *hoxb-3* r5 enhancer sequences (TGTCATC TAAGTCAGCAGTTAC and CCAAATTTGCA-

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Abbreviations: Hpf, hours post-fertilization, MARE, Maf recognition elements; SMaf, somite Maf.

GenBank accession numbers: SMaf1, AB006324; SMaf2, AB022286; MafB, AB006322; and c-Maf, AB006323.

GACACCTACATTCTTGGC) (*15*); the mouse *IL-4* promoter (CTCATTTTCCCTTCCTTTCAGCAACTTTAACTC) (*10*) is associated with c-Maf, and Nrl binds to the *rhodopsin* promoter (TGCTGATTCAGCA) (*21*). Hence, the large Maf factors are vital in various cell differentiation processes because they regulate target gene expression through MAREs.

Our goal is to understand the function of different members of the large Maf family during embryogenesis. Here, we describe the isolation of a novel large Maf transcription factor, in addition to the previously reported MafB/Val, SMaf2/Krml2, and c-Maf factors, from zebrafish (*22*, *23*). At the protein level, SMaf1 is related to the L-Maf-type of large Maf protein. Both SMaf1 and SMaf2 showed transcriptional activation activity, and the mRNAs were detected in the paraxial mesodermal cells after somite boundary formation. Analysis of the mRNA expression profiles of four zebrafish large Mafs showed spatial overlapping in the expression of each factor. Our investigation of zebrafish large Mafs indicated that SMafs may play important roles in somitogenesis, and that each Maf transcription factor may perform both overlapping and specific functions during embryogenesis in the vertebrate.

METERIALS AND METHODS

Zebrafish Embryos—Breeding fish were maintained at 28.5°C with a 14 h light/10 h dark cycle. Embryos were collected by natural spawning and staged according to the method of Kimmel *et al.* (*24*).

cDNA Cloning and Sequencing of the Zebrafish Large Mafs—λgt10 and λZAP zebrafish cDNA libraries (kindly provided by Drs. H. Okamoto and B. Appel, respectively) were prepared from 18–23 hpf (hours post-fertilization) and 15–19 hpf zebrafish embryos. We screened approximately 4.8×10^6 and 3.2×10^7 plaques from the respective libraries under low stringency conditions (25% formamide, 42°C) with a probe containing the b-Zip domain of *Xenopus* MafB (provided by S. Ishibashi). A total of 180 clones was isolated on the first screening. After three further rounds of purification, the remaining 32 clones were subcloned into pBluescript SK+ (Stratagene) and sequenced. In an additional experiment, total RNA, which was prepared from 18 hpf zebrafish embryos using ISOGEN (Nippongene), was analyzed by RT-PCR (reverse transcriptase–polymerase chain reaction) using previously described degenerate sense and antisense oligonucleotide primers (*13*) on total RNA. The resultant PCR product of approximately 700 bp was cloned into pBluescript SK+ and sequenced. Full-length cDNA clones were isolated by screening the λZAP cDNA library using these PCR fragments as probes under high stringency conditions (50% formamide, 50°C). Overall, we isolated four different zebrafish large Maf cDNAs.

Luciferase Assay—cDNAs were subcloned into the pEFX eukaryotic expression plasmid (*6*). Constructs ΔSMaf1 and ΔSMaf2 contain 192 to 315 amino acids of SMaf1 and 200 to 318 amino acids of SMaf2, respectively. The reporter plasmid contains 6× cαCE2 (the core site of the αCE2 sequence) or 6× mtcαCE2 (the mutated cαCE2 sequence) in front of the *β-actin* basal promoter and Luciferase gene (Fig. 2A). Primary chick embryonic fibroblast (CEF) cells were prepared from 8-day-old chicken embryos previously (*19*). Transfection was carried out by the lipofection method using DMRIE-C Reagent (GIBCO). The CEF cells (2.2-mm dish) were co-transfected with test effector plasmid (30 ng), reporter plasmid (300 ng), and pEFX control plasmid (100 ng) containing a β-galactosidase gene for normalization of transfection efficiency. The cultured cells were harvested at 48 h post-transfection and then analyzed for luciferase activity.

Electrophoretic Gel Mobility Shift Assay (EMSA)—The intact Maf and truncated ΔMaf proteins were synthesized with a TNT coupled reticulocyte lysate or TNT coupled wheat germ extract *in vitro* transcription/translation system (Promega), respectively, using the pEFX-SMaf1, pEFX-SMaf2, pEFX-ΔSMaf1, and pEFX-ΔSMaf2 constructs. αCE2 (5′-GATCCCATTTCTGCTGACCACGTTGCCTTCA) (*20*), corresponding to the chicken *αA-crystallin* enhancer (−114 to −90), was labeled with [^{32}P-α]dATP using Klenow DNA-polymerase. Five microliters of *in vitro* translated proteins was mixed with 0.5 ng of a radiolabeled oligonucleotide in a reaction mixture containing 10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 0.1 mM EDTA, and 50 μg/ml of poly(dI-dC), and then analyzed on a 4% polyacrylamide gel in 0.25× TAE [10 mM Tris-HCl (pH 8.0), 10 mM acetate, 0.25 mM EDTA]. The gels were dried and exposed to X-ray films for 12 h. For competition assays, the EMSA reaction mixture was first incubated with a radiolabeled probe at 25°C for 10 min. For competition assays, unlabeled αCE2 or mutated αCE2 (mtαCE2, 5′-GATCCCATTTCTCAGGACCACGTTGCCTTCA) was added to the reaction mixture.

Whole Mount In Situ Hybridization—Digoxigenin-11-uridin-5′-triphosphate (DIG-11-UTP)–labeled RNA probes were synthesized using standard methods (Roche). For zebrafish embryos, the probes corresponding to the b-Zip coding region plus 3′UTR region [MafB (+709 to +1620 bp), c-Maf (+622 to +1015 bp), and SMaf2 (+718 to +1439 bp)], 5′UTR plus acidic region [SMaf1 (−263 to +410)], and full-length cDNA (MyoD) were used. For sectioned mouse embryos, the murine MafB probe, containing the ORF region (+52 to +755 bp), was used. Embryo fixation, pretreatment and whole-mount *in situ* hybridization for zebrafish were performed as described previously (*25*) except for slight modification of the hybridization mixture (5× SSC, 50 μg/ml of heparin, 5 mg/ml of yeast tRNA, 0.1% Tween-20, 50% formamide) and the blocking solution (1% DIG Blocking Reagent, 0.2% Tween20, 1× PBS). Post-stained zebrafish embryos were sectioned at a thickness of 50 or 10 μm. Whole mount *in situ* hybridization for e10.5 mouse embryos was carried out as described previously (*13*). Briefly, after hybridization with various riboprobes, embryos were incubated with alkaline phosphatase–conjugated anti-DIG antibodies (Roche). Hybridization signals were visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoryl phosphatase as a chromogen. After staining, the mouse embryos were sectioned at a thickness of 50 μm using a vibrating blade microtome.

Immunohistochemistry—Mice embryos were fixed in ice-cold 10% buffered formalin in PBS for 2 h, cryo-protected with 20% sucrose/PBS prior to embedding in O.C.T. compound (Tissue-Tek), and then sectioned at 8 μm thickness. The sections were then incubated with anti–β-galactosidase rabbit polyclonal antibodies (Cappel), and the signals were detected with DAB (dimethylaminoazobenzene) using an IHC (immunohistochemistry) system (Ventana).

RESULTS

Isolation of New Zebrafish Large Maf cDNAs—To isolate cDNAs encoding the large Maf family, we performed RT-PCR analysis using degenerate oligonucleotides, corresponding to the conserved region among the large Maf factors (v-Maf, Nrl), on total RNA isolated from whole zebrafish embryos at the 18-somite stage (18 hpf). The resultant RT-PCR products were subcloned and sequenced. One of the clones encoded part of the acidic and basic domains of zebrafish c-Maf. A full-length c-Maf cDNA was obtained by screening of a cDNA library, prepared from zebrafish embryos (at the stage of segmentation, 15–19 hpf), using the partial c-Maf clone as a probe.

Three cDNA clones were isolated on the screening of two different cDNA libraries, using a probe encoding the b-Zip domain of *Xenopus* MafB, which were prepared from embryos at different segmentation stages (15–19 and 18–23 hpf). Sequence analysis allowed identification of these clones as zebrafish MafB/Val (*22*), a cDNA similar to Krml2 (*23*), and a novel large Maf cDNA. The latter two clones encode proteins containing acidic, basic and leucine zipper domain conserved amongst the large Maf subfamily, and their basic domain showed high homology (>80%) to that of other zebrafish large Maf members. These clones were named SMaf1 and SMaf2 to reflect the pronounced and specific expression in the somite (see below). SMaf1 cDNA exhibits 69% sequence identity with the chicken L-Maf, and its acidic, basic and leucine zipper moieties are 68, 93, and 86%, respectively, identical to those of L-Maf (Fig. 1B). By contrast, the amino acid sequence of SMaf2 does not exhibit a high homology to those of known large Mafs, except for the recently identified Krml2 (*23*). While 14 amino acid substitutions exist between SMaf2 and Krml2 (data not shown), the expression pattern of Krml2 resembles that of SMaf2. Although this creates ambiguity as to whether or not SMaf2 is actually the same gene as Krml2, we regarded SMaf2 as a paralog of Krml2.

A phylogenetic tree based on the entire amino acid sequence of each Maf protein (Fig. 1C) indicated that two of the isolated clones were indeed zebrafish homologues of c-Maf and MafB/Val (*22*), while the other two clones represent novel zebrafish large Mafs, SMaf1, and SMaf2/Krml2. Based on this sequence similarity, SMaf1 is possibly the homologue of chicken L-Maf (*6*) (Fig. 1, B and C), however, the expression pattern of SMaf1 is completely different from that of L-Maf (see below). SMaf2/Krml2 was not cate-

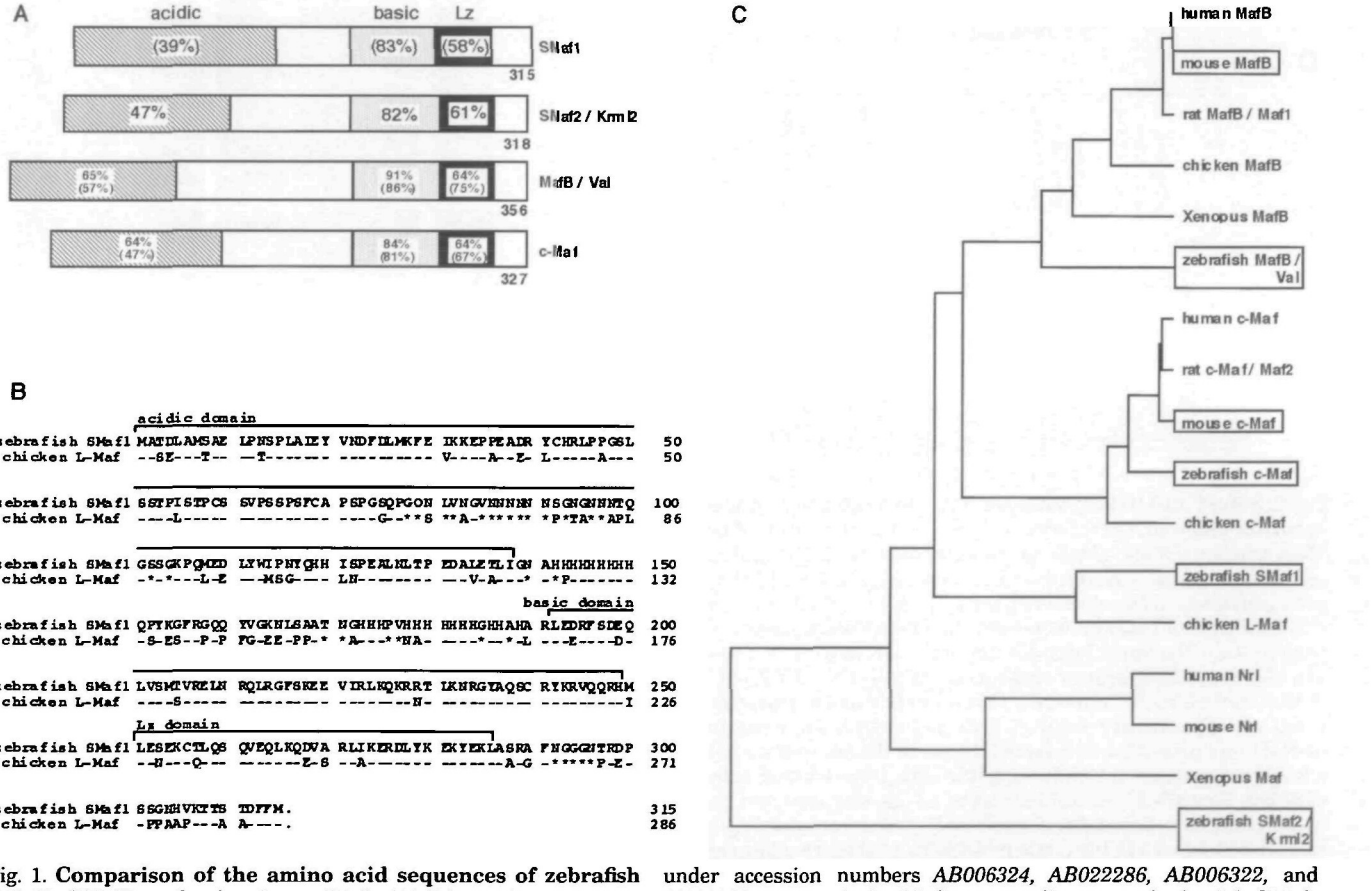

Fig. 1. **Comparison of the amino acid sequences of zebrafish SMaf1, SMaf2, and other large Mafs.** (A) Schematic representation of SMaf1, SMaf2, and other zebrafish large Mafs. Hatched boxes, acidic domains; grayboxes, basic domains; solid boxes, leucine zipper (Lz) domains. The percentages of the amino acid sequence identical to SMaf1 and SMaf2 are indicated in the domain boxes, those for SMaf2 being in parentheses. The nucleotide sequences of SMaf1, SMaf2, MafB, and c-Maf cDNA of zebrafish have been submitted to GenBank under accession numbers *AB006324, AB022286, AB006322,* and *AB006323,* respectively. (B) Sequence alignment of zebrafish SMaf1 and chicken L-Maf. Horizontal bars and asterisks represent identical amino acid residues to in SMaf1 and gaps, respectively. The dot denotes the stop codon. The acidic, basic, and Lz domains are indicated. (C) Phylogenetic tree of vertebrate large Mafs. The tree was generated by the UPGMA method using the whole amino acid sequences of the known large Mafs.

gorized as any of the known large Maf proteins (Fig. 1C).

Zebrafish SMaf1 and SMaf2 Contain Transactivation Domains—Since SMaf1 and SMaf2 each contained an acidic domain, the transactivational activity of both factors was examined by means of transient transfection assays using a reporter construct containing multiple copies of cαCE2, the core site of the chicken *αA-crystallin* enhancer (−108 to −102) (*20*) *cis*-linked to a luciferase gene under the control of the *β-actin* promoter (Fig. 2A). Chick embryonic fibroblast cells were co-transfected with the reporter plasmid and the expression plasmid of either SMaf1 or SMaf2. The zebrafish MafB expression plasmid was used as a positive control since chicken MafB has been shown to have transactivational activity (*4*). SMaf1 and SMaf2 exhibited 6- and 1.8-fold higher luciferase activity, respectively, than MafB (Fig. 2B). When transfected with a reporter plasmid containing a mutated cαCE2 sequence (mtcα-CE2), SMaf1 and SMaf2, like MafB, showed no activation. These data indicate that the transcriptional activation is modulated through MARE sites. To elucidate the functional importance of the acidic domain for transactivation, we constructed Mafs which lacked the acidic domain (ΔMafs, Fig. 2A) and measured their transactivational activity in a similar transfection paradigm. Neither ΔSMaf1 nor ΔSMaf2 activated the transcription of the reporter. To con-

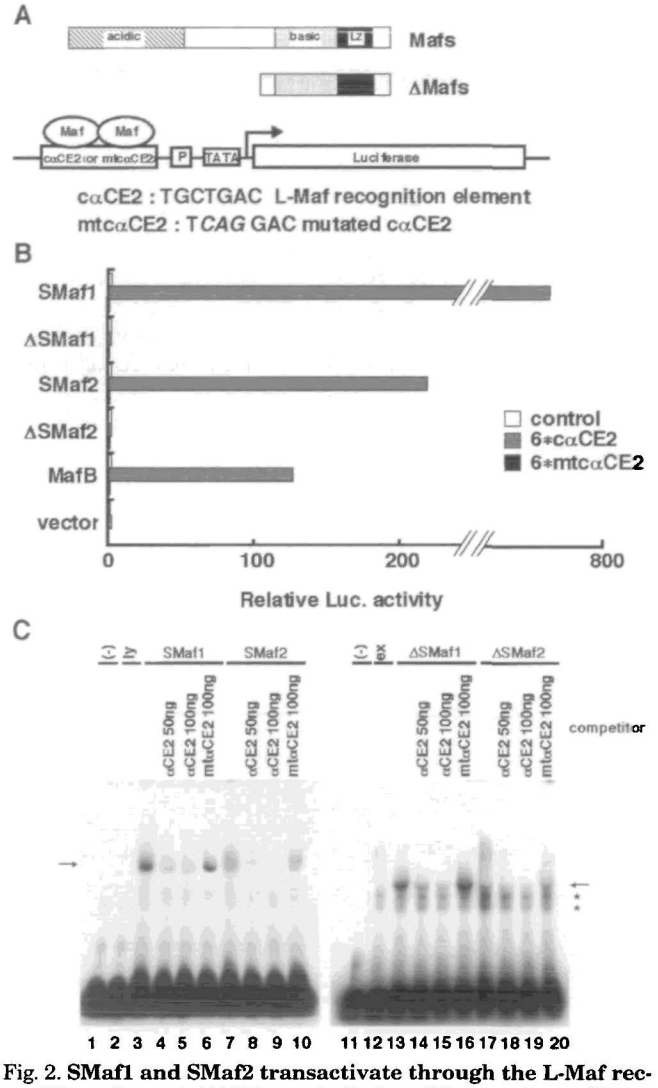

Fig. 2. **SMaf1 and SMaf2 transactivate through the L-Maf recognition element.** (A) Schematic display of the structures of large Mafs, truncated Mafs (ΔMaf), and reporter constructs. The luciferase reporter assay was performed with zebrafish large Maf and ΔMaf expression vectors using chick embryonic fibroblast cells. P and TATA indicates β-*actin* basal promoter. (B) Results of the transient transfection assay. The intact large Maf proteins activated the expression of a Maf-dependent reporter construct containing 6× cαCE2, but not of that containing 6× mtcαCE2. Control indicates the transfection result with the reporter construct without an Maf binding site. Both ΔSMaf1 and ΔSMaf2, which lacked the acidic domain, did not cause obvious activation. (C) Results of EMSA. The DNA binding activity of SMafs (lanes 3–10) or ΔSMafs (lanes 13–20) was analyzed using αCE2 as a probe. Lanes denoted as (−), ly and ex contained no proteins (lanes 1 and 11), the crude proteins in a rabbit reticulocyte lysate (lane 2), and those in a wheat germ extract (lane 12), respectively. The reaction mixture was incubated in the absence of unlabeled competitors (lanes 1–3, 7, 11–13, and 17), in the presence of a 100- (lanes 4, 8, 14, and 18) or 200- (lanes 5, 9, 15, and 19) fold excess of αCE2, or a 200-fold excess of mtαCE2 (lanes 6, 10, 16, and 20). Arrows indicate the positions of the DNA-Mafs complexes. Asterisks denote the non-specific bands, which are also observed in the control lane (lane 12).

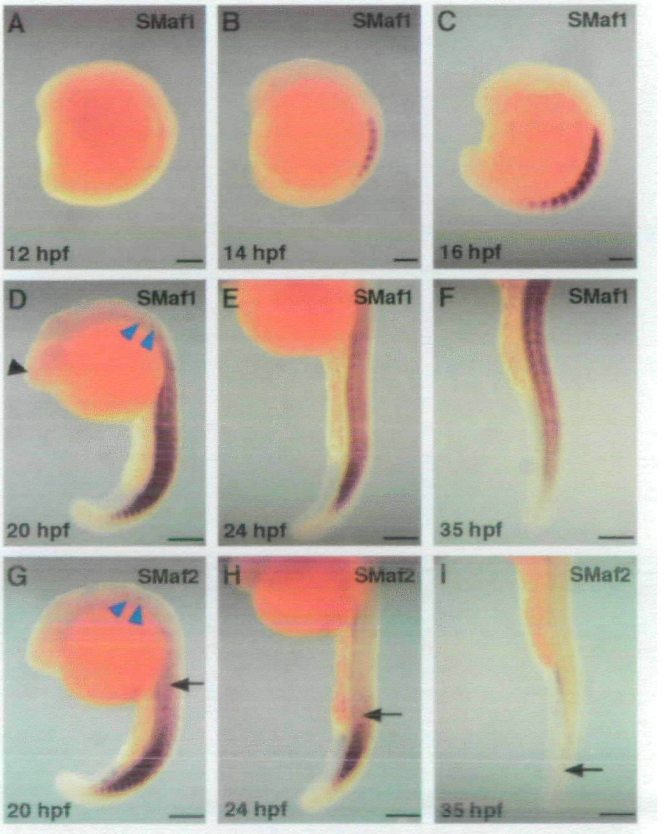

Fig. 3. **Expression of SMaf1 and SMaf2 mRNAs in the developing somite.** (A–F) SMaf1 or (G–I) SMaf2 mRNA expression in a lateral view. Anterior to the top (A–I). (A) 12 hpf, (B) 14 hpf, (C) 16 hpf, (D,G) 20 hpf, (E,H) 24 hpf, and (F,I) 35 hpf embryos. In the head of zebrafish embryos, SMaf1 and SMaf2 mRNAs (blue arrowheads) exist in cells in the hindbrain and SMaf1 mRNA is expressed in the olfactory cells (black arrowheads, D). Note that the SMaf1 expression is retained in all the somites at the later stage (F). On the other hand, a reduction of SMaf2 mRNA in the somite is observed in the 20 hpf embryo (G, arrow), and this reduction proceeds to posterior somites (H, arrow). Finally, the SMaf2 transcript has disappeared in the most posterior somite (I, arrow). Scale bars, 50 μm.

firm that a reduction in transactivational activity on deletion of the acidic domain is not due to a loss of DNA-binding, we carried out EMSA using αCE2 as a probe. Figure 2C shows that all of SMaf1 (lane 3), SMaf2 (lane 7), ΔSMaf1 (lane 13), and ΔSMaf2 (lane 17) could bind to αCE2. To examine the specificity of the DNA binding, unlabeled αCE2 or mtαCE2 was added to a binding reaction as a competitor. The addition of unlabeled αCE2 but not mtαCE2 reduced the shifted complexes containing SMafs proteins in all cases. We concluded that SMaf1 and SMaf2 possess transactivational ability which requires the acidic domain. These data further confirm that the two newly identified Mafs are members of the large Maf protein family.

SMaf1 and SMaf2 were Expressed Prominently in the Somites during Embryogenesis—In order to analyze the spatial-temporal expression profiles of SMaf1 and SMaf2 during zebrafish embryogenesis, we performed whole mount *in situ* hybridization using DIG-labeled probes. SMaf1 mRNA was first detected in each somite at the 7-somite stage (12 hpf) (Fig. 3A). In vertebrates, somitogenesis includes two steps: segmentation and maturation. Segmentation is the separation of cells from the mesoderm into smaller units (somites) by epithelial cells, which is called the "boundary," and it proceeds in an anterior-posterior direction. Expression of SMaf1 mRNA increased as somitogenesis proceeded (Fig. 3, B and C). By the 24 hpf stage, SMaf1 mRNA was detected in each newly formed somite (Fig. 3, D and E). A low level of SMaf1 transcripts continued to be detected in the cells of each maturing somite until after the 35 hpf stage of embryonic development (Fig. 3F).

On the other hand, SMaf2 mRNA was first detected in the somitic cells at an earlier stage than SMaf1. The expression pattern of SMaf2 in the somite was similar to that of SMaf1 in the 16 hpf embryo (data not shown), however, expression of SMaf2 in anterior somites, the maturing somites, decreased by the 20 hpf stage (Fig. 3G, arrow). In the 24 hpf embryo, the expression of the SMaf2 was further decreased in posterior somites (Fig. 3H, arrow), and was completely undetectable in the 35 hpf embryonic somite cells (Fig. 3I, arrow), whereas SMaf1 expression was still prominent in all somites. SMaf1 mRNA was also expressed in cells in the hindbrain (Fig. 3D, blue arrowheads) and in olfactory cells (Fig. 3D, black arrowhead, and Fig. 6G, red arrowheads). SMaf2 mRNA also existed in cells in the hindbrain (Fig. 3G, blue arrowheads) as described previously (*23*). We concluded that the expression patterns of SMaf1 and SMaf2 in the somitic cells were similar spatially, but not temporally, during zebrafish embryogenesis.

Expression of SMaf1, SMaf2, and MyoD during the Segmentation Period—To examine potential involvement of SMaf1 and SMaf2 in myogenesis, we next compared the expression of SMaf1 with that of SMaf2 and MyoD. Myogenesis is a part of somitogenesis, and proceeds in an anterior-posterior direction as in somitogenesis. Figure 4A to F show the expression patterns of SMaf1, SMaf2, and MyoD at the 14-somite stage (16 hpf). The SMaf1 mRNA was distributed in the area where SMaf2 and MyoD were expressed. These three transcripts were observed at the boundary formed by the paraxial mesoderm (Fig. 4, A–C); the expression patterns in the paraxial mesoderm were similar, but not identical. An obvious difference was that SMaf1 and SMaf2 mRNAs were not expressed in adaxial cells while MyoD expression was distinctly prominent (Fig. 4C, arrows). Adaxial cells are on either side of the notochord (*26*) and can be distinguished from lateral MyoD-expressing myotome cells. They differentiate earlier than lateral MyoD-expressing myotome cells and develop into specific cells, such as the muscle pioneers and so on. The most posterior expression of SMaf1, SMaf2, and MyoD in paraxial mesoderm cells was not the same (Fig. 4, A–C, red

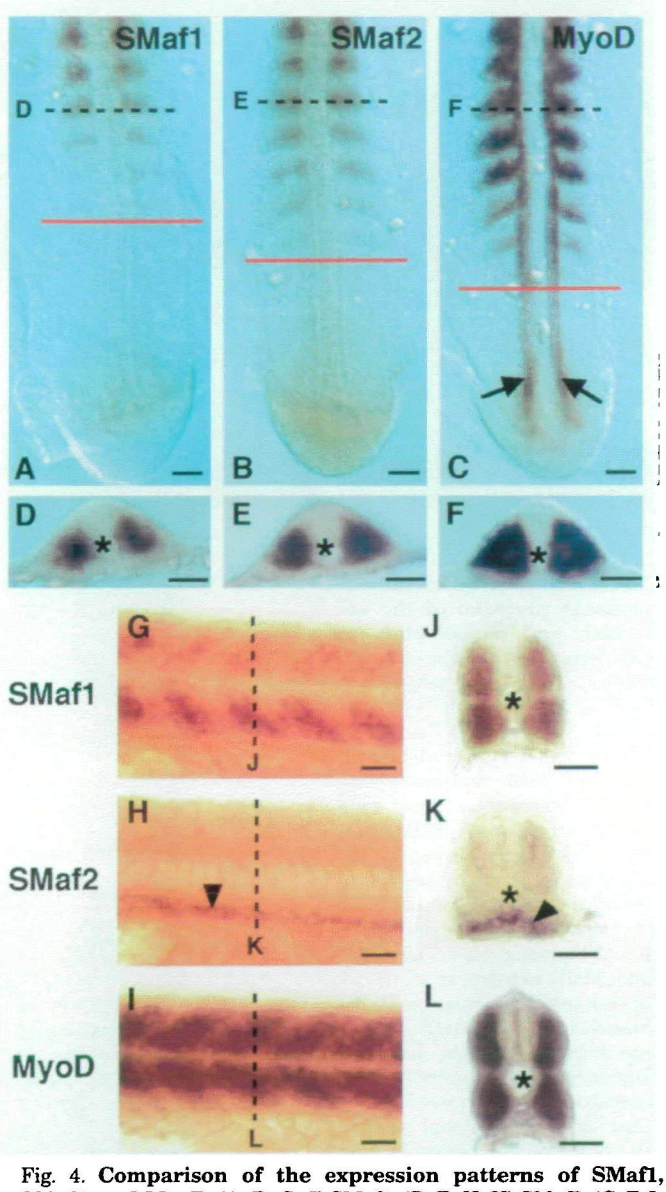

Fig. 4. **Comparison of the expression patterns of SMaf1, SMaf2, and MyoD.** (A, D, G, J) SMaf1, (B, E, H, K) SMaf2, (C, F, I, L) MyoD mRNA. (A–F) 16 hpf and (G–L) 24 hpf embryos. The planes of sections (D–F) and (J–L) are indicated by dotted lines in (A–C) and (G–I), respectively. (A–C) Dorsal views, anterior to the top, (G–H) lateral views, anterior to the left. (A–F) SMaf1, SMaf2, and MyoD mRNAs are detected in the paraxial mesoderm. Note that the most posterior expression of each mRNA is different (red lines). (G–L) SMaf1 and MyoD transcripts are retained in the myotome cells. SMaf2 mRNA is not observed in the myotome cells, but in ventral cells below the notochord. Arrows and arrowheads indicate adaxial cells and ventral cells derived from the lateral mesoderm, respectively. Asterisks indicate the notochord (D–F, J–L). Scale bars, 50 μm.

lines). During somite maturation, the cells first expressed MyoD, followed by SMaf2, and finally SMaf1. Thus, MyoD is expressed in the forming somite, while SMaf1 and SMaf2 are only expressed in the developmentally more mature somite. Cross sections of these embryos showed that the intensities of the SMaf1 and SMaf2 mRNA signals were higher in the medial myotome cells than in the lateral myotome cells (Fig. 4, D–F). At the 30-somite stage, the expression of SMaf1 and MyoD persisted in the developing myotome cells (Fig. 4, G, I, J and L). In contrast, as the somite matures, SMaf2 expression decreased in cells originating from the paraxial mesoderm (Fig. 4, H and K). However, the SMaf2 transcript was still present in the ventral cells, which were situated below the notochord and were derived from the lateral mesoderm (Fig. 4, H and K). Thus, in MyoD-expressing myotome cells of a newly formed somite, SMaf2 mRNA appears before SMaf1 mRNA, whereas in maturating myotome cells with sustained expression of MyoD, the SMaf2 expression subsides before SMaf1 transcription is silenced.

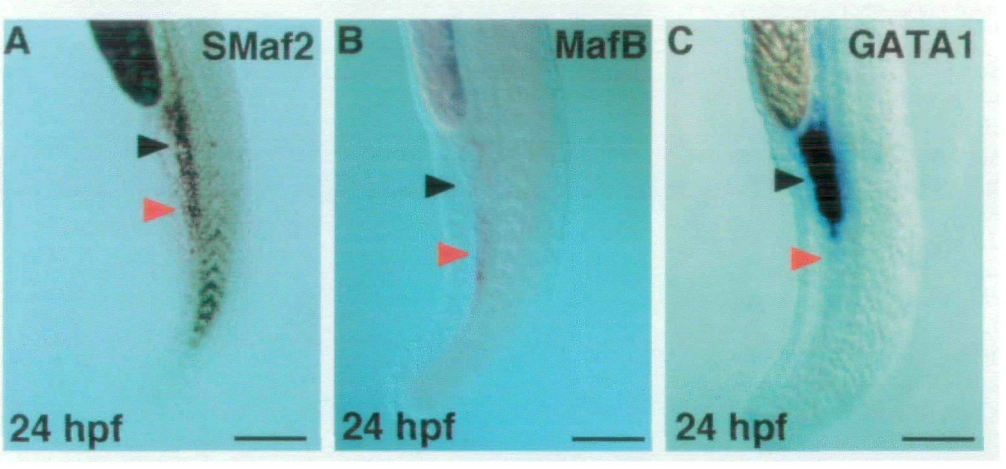

Fig. 5. **Expression of the zebrafish large Maf mRNAs in the blood-forming region.** (A) SMaf2 mRNA, (B) MafB mRNA, (C) GATA1 mRNA. (A, B, C) 24 hpf embryo, lateral views, anterior to the top. SMaf2 and MafB transcripts were detected in both anterior (black arrowheads) and posterior (red arrowheads) portions of the blood-forming region. Scale bars, 50 μm.

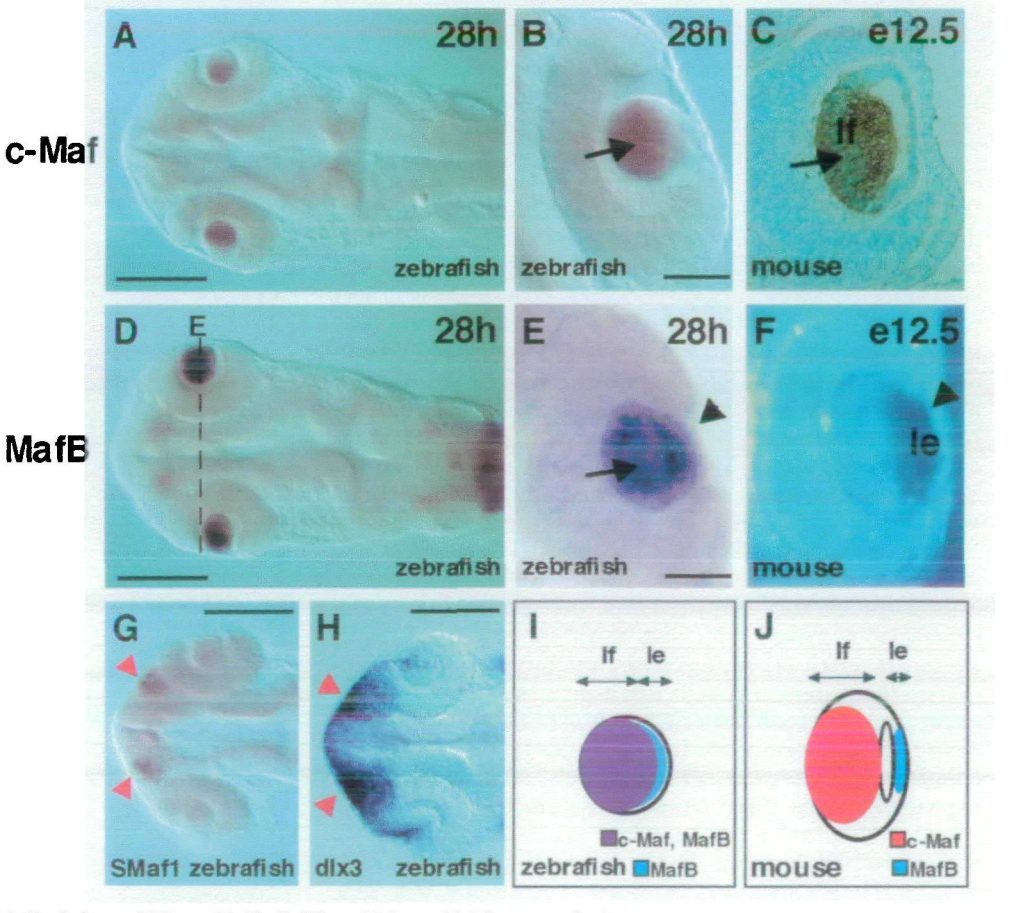

Fig. 6. **Comparison of the expression patterns of the zebrafish and mouse Mafs in the lens.** Results of *in situ* hybridization for (A, B) zebrafish c-Maf, (D, E) zebrafish MafB, (F) mouse MafB, (G) zebrafish SMaf1, and (H) zebrafish dlx3, and of (C) LacZ immunostaining in the heterozygous *c-maf* gene knock-out mouse. (A, B, D, E) zebrafish 28 hpf embryos, (C, F) e12.5 mouse embryos, (G, H) zebrafish 24hpf embryos. (A, D, G, H) Dorsal views, anterior to the left. Schematic views of c-Maf and MafB expression in (I) zebrafish and (J) mouse. (A, D) Zebrafish c-Maf and MafB mRNAs are expressed in the lens. (B) Higher magnification of (A). Zebrafish c-Maf is expressed in lens fiber cells. (E) Coronal section, the plane of the section is indicated in (D). Zebrafish MafB mRNA is observed in the lens fiber cells and lens epithelial cells. (C) LacZ protein, whose expression pattern was thought to be identical to that of endogenous c-Maf, is detected in lens fiber cells. (F) Mouse MafB mRNA is detected in the lens epithelial cells. Black arrows and arrowheads indicated the lens fiber (lf) and lens epithelial cells (le), respectively. (G) SMaf1 transcripts exist in olfactory cells (red arrowheads). Scale bars, 100 μm (A, D, G, H) and 20 μm (B, E), respectively.

Redundant Expression of Large Maf mRNAs in the Blood-Forming Region—In addition to its expression in myotome cells, SMaf2 was also detected as thin lines on both sides of the lateral mesoderm cells at the 7-somite stage (12 hpf) (data not shown). In the 24 hpf embryo, SMaf2 mRNA was observed in the blood-forming region (Fig. 5, arrowheads). The expression pattern was similar to that of MafB (*22*) but not to that of GATA1 (*27*), whose expression was confined to the anterior portion (Fig. 5, black arrowheads).

Expression Patterns of Large Maf mRNAs in Lens Cells—Like the expression of SMaf1 and SMaf2 in myotome cells, c-Maf and MafB mRNAs were both detected in lens cells during zebrafish embryogenesis. The first appearance of the c-Maf transcript in the lens placode coincided with that of MafB (data not shown). The expression of MafB in the lens resembled that of c-Maf (Fig. 6, A and D) during lens formation in the zebrafish embryo. In the mouse, both c-Maf and MafB transcripts were also detected in lens cells. In common with zebrafish c-Maf, the expression of mouse c-Maf was observed in lens fiber cells (Fig. 6C, arrow). Although zebrafish MafB expression was detected in all the lens cells (Fig. 6E), mouse MafB was only expressed in lens epithelial cells (Fig. 6F, arrowhead). The expression of c-Maf and MafB in the lens in zebrafish and mouse is summarized in Fig. 6, I and J, respectively. Although SMaf1 was shown to be a chicken L-Maf homologue based on the amino acid sequence (Fig. 1, B and C), it was not expressed in lens cells (Fig. 6G). Instead, SMaf1 mRNA was expressed in olfactory cells, where dlx3 is expressed (*28*) (Fig. 6, G and H, red arrows).

DISCUSSION

In this paper, we described the cloning of a novel large Maf family member, SMaf1, and other zebrafish large Mafs. Using a MARE-responsive reporter gene for a co-transfection assay and EMSA, we showed that these proteins have transactivational activity, which resides within the acidic domain. Additionally, data obtained on whole mount *in situ* hybridization indicated that SMaf1 and SMaf2 were co-expressed in myotome cells and that their expression overlapped spatially. Furthermore, the embryonic expression patterns of four zebrafish large Maf mRNAs were found to overlap. Considering the facts that these proteins are transactivation factors and that their expression profiles overlap each other, it is anticipated that the large Maf proteins might have indispensable functions in cellular differentiation and specification.

As we reported in this paper, SMafs are mainly expressed in somites. The differentiation of somites is a major event in vertebrate mesodermal development; first, the paraxial mesoderm separates into blocks and forms "somites"; next, somitic maturation results in two differentiated compartments being formed, the ventral sclerotome and a dorsal structure, the myotome. In fish, the sclerotome is small (*29*), and the somite mainly gives rise to muscle, which is probably the primary fate of the paraxial mesoderm during early chordate evolution (*30*). The myotome is the origin of the skeletal muscles of the trunk, appendicular skeleton and muscles of appendages. Understanding of the molecular events leading to the differentiation of myotome cells has been advanced by the discovery of myogenic regulatory factors (MRFs), such as MyoD (*31–33*). Both SMaf1 and SMaf2 transcripts were detected in the MyoD-positive myotome cells (Fig. 4) (*34*), so SMafs may be among the factors which orchestrate the specification of somite cells.

Prominent expression of SMaf1 and SMaf2 was detected in somite cells, although the timing of their first appearance was different. The co-expression of two large Maf genes was observed at other sites, such as in blood-forming regions (Fig. 5) and in the lens (Fig. 6). These large Mafs are transcriptional activators, as shown on *in vitro* transfection assaying (Fig. 2B), and they can recognize the same target sequence, αCE2, which contains half site of "MARE" (Fig. 2C). It is, therefore, plausible that they might have similar functions. The zebrafish *valentino* (*mafB*) mutant does not exhibit a lens defect, although this mutation exhibits defective hindbrain patterning (*35*). In the lens, it is likely that the deficiency of MafB may be compensated for by c-Maf. On the other hand, we previously reported that c-Maf-deficient mice exhibited a developmental lesion in lens fiber cells (*13*). This phenotypic incongruity in the two species can be explained by the difference in the expression profiles of both Mafs in the mouse, since c-Maf expression is restricted to lens fiber cells, while MafB is only detected in lens epithelial cells (Fig. 6, C and F). MafB may be unable to compensate for the c-Maf function in lens fiber cells where it is not expressed. The *MafB* mutant mouse (*kreisler*) mutant shows normal lens development (Kawauchi, S., personal communication) like the zebrafish mutant. However, it must be noted that the *kreisler* is not a null-mutant (*36*), and the mRNA of MafB can be detected in the lens (our unpublished observation). Therefore the *kreisler* mutant is not equivalent to c-Maf deficient mice. More thorough analysis is needed for a more comprehensive understanding of the function of MafB during lens development.

In this study, four zebrafish large Maf genes were identified (SMaf1, SMaf2, c-Maf, and MafB). There are three large Maf proteins in chicken (L-Maf, c-Maf, and MafB), man (c-Maf, MafB, and Nrl), and mouse (c-Maf, MafB, and Nrl). Hence, the number of large Maf proteins may range between three to five. In this study, none of the large Maf genes was founded to be expressed in the retina of zebrafish. It has been reported that a mutation in Nrl is associated with human autosomal dominant retinitis pigmentosa (*5*). This indicates that Nrl is a key regulator of retinal development. Since Nrl has been cloned in the mouse, it would be interesting to check the existence of Nrl or another large Maf family member in the retina of zebrafish.

Analyses of both natural and gene-targeted mutations of mouse and zebrafish indicated that large Maf factors are vital for proper development of several cellular lineages. Hence, we anticipate that SMaf1 and SMaf2 will play functionally significant roles in the development of somites, which are the sites of prominent SMaf1 and SMaf2 expression. Further loss and/or gain of function studies will be required to answer this question.

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