Expression and comparative genomics of two serum response factor genes in zebrafish

JODY L. DAVIS1,#, XIAOCHUN LONG2,#, MARY A. GEORGER2, IAN C. SCOTT3, ADAM RICH1 and JOSEPH M. MIANO2,*

1Department of Biological Sciences, State University of New York College at Brockport, Brockport, NY, 2Aab Cardiovascular Research Institute, University of Rochester School of Medicine & Dentistry, Rochester, NY and 3Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, USA

ABSTRACT  Serum response factor (SRF) is a single copy, highly conserved transcription factor that governs the expression of hundreds of genes involved with actin cytoskeletal organization, cellular growth and signaling, neuronal circuitry and muscle differentiation. Zebrafish have emerged as a facile and inexpensive vertebrate model to delineate gene expression, regulation, and function, and yet the study of SRF in this animal has been virtually unexplored. Here, we report the existence of two srf genes in zebrafish, with partially overlapping patterns of expression in 3 and 7 day old developing animals. The mammalian ortholog (srf1) encodes for a 520 amino acid protein expressed in adult vascular and visceral smooth muscle cells, cardiac and skeletal muscle, as well as neuronal cells. The second zebrafish srf gene (srf2), encoding for a presumptive protein of only 314 amino acids, is transcribed at lower levels and appears to be less widely expressed across adult tissues. Both srf genes are induced by the SRF coactivator myocardin and attenuated with a short hairpin RNA to mammalian SRF. Promoter studies with srf1 reveal conserved CArG boxes that are the targets of SRF-myocardin in embryonic zebrafish cells. These results reveal that SRF was duplicated in the zebrafish genome and that its protein expression in all three muscle cell types is highly conserved across vertebrate animals suggesting an ancient code for transcriptional regulation of genes unique to muscle cell lineages.

KEY WORDS: SRF, myocardin, promoter, muscle

Introduction

Serum response factor (SRF) is a widely expressed and highly conserved transcription factor that binds to 1,216 permutations of a 10-base pair element known as the CArG box (Camoretti-Mercado et al., 2003; Miano 2003). A growing number of genes have been shown to contain functional SRF-binding CArG boxes, particularly those encoding for proteins involved with a cell’s cytoarchitecture (Minty and Kedes, 1986; Philippar et al., 2004; Zhang et al., 2005; Sun et al., 2006). Representative SRF orthologs have been discovered in all kingdoms except for Monera, and in virtually all cases where SRF has been genetically inactivated, defects in cytoskeletal organization and function have been defined (Miano et al., 2007). Thus, expression of SRF, which arises from a single copy gene in essentially all species, is vital for the mating phenotype in Saccharomyces cerevisae (Elble and Tye, 1991), cytoskeletal organization and motility in Dictyostelium discoideum (Escalante et al., 2004), animal locomotion in Caenorhabditis elegans (Fukushige et al., 2006), cellular migration and tracheal development in Drosophila melanogaster (Guillemin et al., 1996), and gastrulation in Mus musculus (Arsenian et al., 1998). Further, recent conditional knockout studies in mice have demonstrated a critical role for SRF in the normal organization of the cyto-contractile apparatus in cardiac, skeletal, and smooth muscle cells (SMC) as well as proper neuronal cell migration and cytoarchitecture (Miano et al., 2004; Li et al., 2005; Knöll et al., 2006). These studies all point to a conserved function of SRF in orchestrating programs of gene expression that primar-
rily coordinate a cell’s migratory behavior and its cytocontractile apparatus.

In addition to activating genes involved in appropriate cytocontractile organization and function, SRF is known to induce growth-related genes, including a number of immediate early genes such as c-fos (Norman et al., 1988; Johansen and Prywes, 1995). The ability of SRF to regulate such disparate gene sets as growth and muscle differentiation was an enigma for many years. In 2001, the laboratory of Eric Olson cloned an SRF-associated factor called myocardin (Myocd) (Wang et al., 2001). Myocd is most highly expressed in cardiac and SMC where its encoded protein functions, in conjunction with SRF bound CArG elements, as one of nature’s most potent coactivators of gene expression (Wang et al., 2001). Subsequent work revealed that Myocd is a component of a molecular switch for the SMC program of reduced growth potential and differentiation (Chen et al., 2002), findings that were confirmed by several other labs (Du et al., 2003; Wang et al., 2003; Yoshida et al., 2003; Milyavsky et al., 2007). Further work showed that SRF toggles from regulating growth-related genes to SMC differentiation genes through the mutually exclusive binding of Elk1 (pro-growth) and Myocd (pro-differentiation) to a common interface of SRF (pro-growth) and Myocd (pro-differentiation) to a common interface of SRF (Wang et al., 2004; Zaromytidou et al., 2006). Indeed, the ability of SRF to regulate these and other context-dependent gene sets is related to its association with more than 50 coregulators of gene expression (Miano et al., 2007).

Zebrafish (Danio rerio) has emerged as a popular model organism for the study of gene regulation and function. For example, the study of cardiovascular development and physiology is simplified in zebrafish owing to this animal’s external development, its rapid maturation (24 hr), as well as its highly conserved organization of blood vessels (Isogai et al., 2001). Surprisingly little information exists with respect to Srf in zebrafish. Early studies using the beta actin promoter, disclosed the essential nature of a conserved CArG element for optimal transegene expression, implying the existence of Srf in zebrafish (Moav et al., 1993). Subsequent work revealed the presence of a single copy srf allele with expression documented only in early embryonic skeletal muscle (Vogel and Gerster, 1999). We now report the presence of two paralogous srf genes in zebrafish and provide expression analysis of each paralog in larval and adult tissues. We also show that zebrafish Srf functionally associates with human myocardin to drive endogenous Srf-dependent target genes. Moreover, the promoter of zebrafish srf1 is shown to harbor three CArG elements, including two with high conservation, that appear to function as an ancient code for the autoregulation of this important transcription factor. The discovery of two srf alleles in zebrafish paves the way for functional and expression regulation analyses in this vertebrate animal model.

**Results and Discussion**

**Two paralogs of srf exist in zebrafish**

In a previous report (Vogel and Gerster, 1999), a single srf cDNA was cloned and found to encode a truncated protein of only 247 amino acids (mammalian SRF proteins are > 500 amino acids). We now report that the original srf (srf1) is actually a much larger protein of over 390 amino acids (mammalian SRF proteins are > 500 amino acids). We now report that the original srf (srf1) is actually a much larger protein of over 390 amino acids. Two paralogs of Srf exist in zebrafish as shown in a ClustalW alignment of the human SRF protein (HsaSRF) with the two zebrafish Srf proteins (DreSrf1 and DreSrf2). Dashes represent gaps, astersisks indicate identical amino acid residues and colons designate conservative amino acid substitutions. The bold amino acid residues represent the dynamically functional MADS domain (Shore and Sharrocks, 1995). The lines above the sequences, GTVQIPVSASV and LTELQ/VNLQ, indicate probable epitopes recognized by the Santa Cruz monoclonal SRF antibody used in studies shown here in Fig. 4.

**Fig. 1. Amino acid sequence alignment of human and zebrafish Srf proteins.** Shown is a ClustalW alignment of the human SRF protein (HsaSRF) with the two zebrafish Srf proteins (DreSrf1 and DreSrf2). Dashes represent gaps, asterisks indicate identical amino acid residues and colons designate conservative amino acid substitutions. The bold amino acid residues represent the dynamically functional MADS domain (Shore and Sharrocks, 1995). The lines above the sequences, GTVQIPVSASV and LTELQ/VNLQ, indicate probable epitopes recognized by the Santa Cruz monoclonal SRF antibody used in studies shown here in Fig. 4.
The zebrafish genome underwent duplication more than 100 million years ago and has retained some 25% of the original duplicated genes (Amores et al., 1998). During the sequence analysis of srf, we identified a second, distinct series of overlapping cDNA and EST clones and assembly of these sequences indicated a duplicated srf/srf2 encodes for a protein of only 314 amino acids and, as with Srf1, shares 100% amino acid identity across the MADS domain of human SRF (Fig. 1). Visual analysis reveals little homology between Srf1 and Srf2 outside the stretch of 95 amino acid identity shared with higher vertebrates (Fig. 1). Extensive database mining has failed to reveal any additional coding sequence to Srf2. The fact that both zebrafish Srf paralogs contain identical MADS domains suggests these proteins could carry out redundant functions. This could explain why there has yet to be any report of a mutant phenotype associated with zebrafish srf.

**Zebrafish srf mRNA and protein expression analysis**

To begin evaluating the expression of each zebrafish srf gene, we designed specific riboprobes to interrogate expression during zebrafish development. At three days of development, expression of each srf gene was present predominantly in the developing chevron-shaped somites and this staining persisted through day 7 of development (Fig. 2A-2D). Similar somitic staining was seen at day 7 for the mammalian SRF target gene, myh11 (Fig. 2H). We also examined sm22α staining as the mammalian ortholog is a known SRF target gene and shows expression in all three muscle lineages during development (Li et al., 1997). In contrast to mammalian studies, but congruent with a recent whole mount zebrafish study (Georgijevic et al., 2007), sm22α is not seen in developing somites despite the presence of both srf transcripts. On the other hand, abundant expression of sm22α mRNA (Fig. 2E) and myh11 (Fig. 2G) are observed in the gut, consistent with previous reports (Wallace et al., 2005; Georgijevic et al., 2007).

A number of papers have reported vascular SMC marker expression, including sm22α, in developing SMC of the zebrafish dorsal aorta (Pham et al., 2001; Roman et al., 2002; Yang et al., 2003). We see little evidence of either srf or sm22α in developing blood vessels of day 3 embryos or day 7 larvae (Fig. 2A-2F). The absence of these SMC markers within the vasculature of such early stage zebrafish is consistent with recent ultrastructural and expression analysis in the developing zebrafish aorta (Miano et al., 2006; Georgijevic et al., 2007). Up to 7 days of development very few SMC are recruited to the dorsal aorta and those present display little evidence of myofilaments or peripheral dense plaques (Miano et al., 2006). Thus, analysis of vascular SMC markers should be done in zebrafish beyond the larval stage of development and will yield the most pertinent information when differentiated vascular SMC fully invest the dorsal aorta by 3 months of age (Miano et al., 2006). At the latter stage of development, we can readily detect srf1 mRNA across multiple tissues by RT-PCR, including female germ cells (Fig. 3). Comparatively lower levels of srf2 mRNA are seen in such tissues as gut and liver with undetectable transcripts in other adult tissues (Fig. 3). Collect-
Fig. 4. Adult tissue expression of Srf1 protein. Adult (3 month) tissues were processed for immunohistochemistry as described in Materials and Methods. Arrows point to Srf1 nuclear staining (in red) in cardiomyocytes of the heart (A), skeletal muscle of posterior trunk (B), visceral SMC of gut (C), vascular SMC of dorsal aorta (D) and cortical neurons of brain (E). The asterisk in (A) indicates nucleated red blood cells within the ventricular chamber of the heart. Note the peri-aortic melanin (dark staining rim in panel D) that has recently been described and validated (Miano et al., 2006). Staining specificity was demonstrated with a competing peptide to human SRF (data not shown). Magnifications are 400x.

tively, these results reveal both similarities (early somitic) and differences (adult tissue level and distribution) in the mRNA expression of the two srf genes, suggesting perhaps tissue-specific functions for each zebrafish Srf.

We next evaluated Srf protein expression in adult tissues by immunohistochemistry. These results reveal high-level expression of Srf protein in trabecular cardiomyocytes of the heart, skeletal muscle in the trunk, visceral SMC of the gut, vascular SMC of dorsal aorta, and cortical neurons of the brain (Fig. 4). Since the antibody used was raised to the C-terminal 50 amino acids of human SRF and only Srf1 extends to this region of the protein (Fig. 1), we conclude that the immunoreactive protein observed here represents Srf1. It is important to point out that a previous report showing absence of Srf1 protein in cardiac and SMC was constrained to an analysis of only early zebrafish embryos (Vogel and Gerster, 1999). We now report that Srf1 protein is distributed across adult tissues containing each of the three muscle types (cardiac, skeletal and smooth), which is in good agreement with SRF expression in higher vertebrates (Croissant et al., 1996). The adult tissue expression of Srf2 protein will require further analysis with newly constructed antisera that can distinguish between the two paralogous proteins.

Regulation of srf1 and srf2 expression by SRF and myocardin

The mammalian SRF promoter contains tandem CArG elements that are the targets of SRF auto-regulation (Spencer and Misra, 1996; Belaguli et al., 1997) and induction by Myocd (Chow et al., 2007). To begin evaluating the expression regulation of each zebrafish srf gene, we used adenoviral gene transfer of human MYOCD and a short-hairpin RNA to mammalian Srf (shSrf) in cultured ZF4 cells, an immortalized zebrafish cell line (see Materials and Methods). Previous studies from this lab have established the utility of these reagents in modulating SMC marker expression (Streb and Miano, 2005; Chow et al., 2007). We note slight down-regulation of both srf mRNAs with shSrf, despite the presence of a 1 bp mismatch between human and zebrafish srf/mRNA target sequences. Conversely, increases in both srf transcripts are seen with human MYOCD transduction (Fig. 5). Importantly, we also observe MYOCD-mediated increases in the endogenous smooth muscle myosin heavy chain (myh11) mRNA consistent with this gene’s regulation by SRF in higher vertebrates (Manabe and Owens, 2001). These results suggest that both zebrafish Srf proteins can interact with human MYOCD to drive Srf-dependent gene expression and both are subject to silencing with shRNA targeted to mammalian SRF.

To determine whether functional CArG elements are present in srf, we cloned the srf1 promoter; extensive database mining failed to reveal reliable srf2 promoter sequence. Figure 6A shows that the 5’ promoter region of srf1 contains two CArG elements (C1-C2) separated by 13 base pairs, a distance that is very close to the 10 base pairs separating all known tandem CArGs within Srf promoters in higher animals. Moreover, the distance of the srf1 tandem CArG elements from the start site of transcription is in close agreement with that of other vertebrates. An analysis of Srf promoters from the genomes of cat, chicken, cow, dog, human, monkey, mouse, opossum, and rat reveals that C1 (cctataagg) has virtually 100% sequence identity; only chicken Srf, with its C1 element being ccttaaagg deviates from this consensus. The C2 CArG box diverges slightly more across the 10 vertebrates studied with base pair substitutions in 5/10 sequences across the CArG box. These comparative genomic data, summarized in Figure 6B as a sequence logo, indicate a high degree of CArG sequence conservation among Srf promoters. Note that the zebrafish srf1 promoter has a third CArG box (C3), which is not present in
other animal species (Fig. 6A).

To begin appraising the srf1 promoter in a functional assay, we transfected various cell lines with wildtype or CaRg mutant srf1 promoter constructs linked to a luciferase reporter gene in the presence or absence of Myocardin or an SRF-VP16 transactivator and measured the resultant luciferase activity. Results in mammalian cells reveal both SRF-VP16- and Myocd-dependent activation of the srf1 promoter. Mutations in each of the three CaRG elements reduced basal and induced promoter activities, with the most dramatic decreases seen when the two conserved CaRG boxes were mutated together with the third CaRG element (Fig. 6C-D). Mutations in all three CaRG boxes also blunted the presence or absence of basal and Myocd-activated promoter activities, with the most dramatic decreases seen when the two conserved CaRG elements reduced basal and induced promoter activities, with the most dramatic decreases seen when the two conserved CaRG elements were mutated together with the third CaRG element (Fig. 6C-D). Collectively, the results indicate that vertebrate Srf promoter CaRG elements (particularly C1) appear to function as an ancient code for SRF-mediated auto-regulation and SRF-dependent target gene expression control. An important future goal should be to ascertain the role of Srf1/2 in regulating expression of SMC markers expressed in zebrafish such as myh11 (Wallace et al., 2005), sm22α and the SMC isoforms of alpha actin (Georgievic et al., 2007) and calponin. In addition, it may prove effective to evaluate mammalian Srf regulatory elements in zebrafish given similar tissue expression profiles and the ease in which such analyses can be done in this model organism.

Materials and Methods

Aquaculture and cell lines

Zebrafish were obtained and cared for following methods described previously (Rich et al., 2007). Wild-type adult zebrafish were obtained from the Zebrafish International Resource Center and were maintained according to standard guidelines in accordance with IACUC guidelines (Westerfield 2000). Fish were maintained at 28ºC in deionized water containing 240 mg/L of Instant Ocean Salts and 75 mg/L NaHCO3 with 20% system water change each day (pH and conductivity were adjusted to approximately 7.2 and 450 ppm respectively). Zebrafish were fed three times daily, alternating Cyclopeeze (Argent, Redmond, WA) with live brine shrimp. The cycle of light and dark was 14 hr light followed by 10-hr dark. Embryos and larvae (3 and 7-day old) were obtained from Aquatica Tropicals (Plant City, FL) and were cared for in accordance with an approved IACUC protocol. Addition of 1.5 mM 1-phenyl-2-thiourea (PTU) to E3 media was used to prevent pigmentation of embryos from 24 hrs to the 7 day larval stage without obvious toxicity.

The ZF4 embryonic zebrafish cell line (Driever and Rangini, 1993) was used to test the presence or absence of Myocardin or an SRF-VP16 transactivator and measured the resultant luciferase activity. Results in mammalian cells reveal both SRF-VP16- and Myocd-dependent activation of the srf1 promoter. Mutations in each of the three CaRG elements reduced basal and induced promoter activities, with the most dramatic decreases seen when the two conserved CaRG elements were mutated together with the third CaRG element (Fig. 6C-D). Collectively, the results indicate that vertebrate Srf promoter CaRG elements (particularly C1) appear to function as an ancient code for SRF-mediated auto-regulation and SRF-dependent target gene expression control. An important future goal should be to ascertain the role of Srf1/2 in regulating expression of SMC markers expressed in zebrafish such as myh11 (Wallace et al., 2005), sm22α and the SMC isoforms of alpha actin (Georgievic et al., 2007) and calponin. In addition, it may prove effective to evaluate mammalian Srf regulatory elements in zebrafish given similar tissue expression profiles and the ease in which such analyses can be done in this model organism.

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Primers specific to zebrafish shSRF; target sequence is ac
transduced with adenovirus containing shRNA to mammalian SRF (Ad-
20C. Prepared embryos/larvae were re-hydrated, step-wise, and then
digested with Proteinase K to improve permeability for probe penetration.
The hybridization solution was added and incubated at 70°C for 1 hour
before addition of 150 ng probe and continued incubation overnight at
70°C. The probe was removed through several washes at 70°C and then
PBSW containing 5% heat inactivated sheep serum was added as a
blocking agent. Detection of probe was carried out by adding anti-
Digoxigenin (Alkaline Phosphatase conjugated) in PBStw/sheep serum
(1:5000) and incubation at 4°C. Removal of the antibody solution was
followed by washes in staining buffer and then staining at RT in the dark
in NBT/BCIP (Roche) as described (Scott et al., 2007). When staining
completed the embryos/larvae were washed in PBSW and then fixed in
4% para-formaldehyde. Newly washed and dehydrated embryos/larvae
were then cleared in benzyl benzote.benzyl alcohol (2:1) clearing agent
and photographed using a Leica MZ125 microscope equipped with a Spot
Insight digital camera (Bannockburn, IL).

Immunohistochemistry

Adult wild-type zebrafish tissues were fixed in 4% buffered para-
formaldehyde, paraffin embedded and cut at 5 micron thickness. All slides
were deparaffinized and rehydrated in PBS (pH, 7.4). Endogenous
peroxidase activity was quenched using 3% aqueous hydrogen peroxide
for 10 minutes and antigen retrieval was performed utilizing heat induced
epitope retrieval in 0.05% citraconic anhydride as described (Namimatsu
et al., 2005). Srf slides were blocked in 5% normal goat serum (Vector S-
100) for 30 minutes then incubated in SRF antibody 1:1200 (Santa Cruz
sc-335, Santa Cruz, CA) overnight at 4°C. This SRF polyclonal antibody
was raised to the C-terminal 50 amino acids of human SRF and therefore
only detects the Srf1 protein (see Figure 1). The secondary antibody,
biotinylated goat anti-rabbit (Vector BA-1000), was used at a dilution of
1:400 and applied for 30 minutes at RT. Alkaline phosphatase detection
system (Vector AK-5000) was applied for 30 minutes followed by Vector
Red chromagen (Vector SK-5100) in the dark for 30 minutes.

Cell transfections and luciferase assay

ZF4, PAC1, and Cos-7 cells were plated in 24-well plates and grown
to 80% confluency prior to co-transfection with wildtype or various
srf1 mutant promoter constructs in the absence or presence of expression
plasmids to either Myocd or SRF-VP16. A Renilla plasmid was included
in all transfections to normalize raw data for slight variations in cell
number, pipeting, and transfection efficiency. Triplicates for each condi-
tion were assayed 48 hours post transfection as previously described
(Miano et al., 2000) with a Dual Luciferase Assay kit (Promega, Madison,
WI). Normalized data were analyzed with GraphPad Prism Software (San
Diego, CA, version 4) using paired t-tests for statistical significance. All
experiments were repeated at least once in an independent study.

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